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Approche expérimentale de la pathogénie et du traitement de l'arthrose

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Introduction générale

L'arthrose est une maladie dont la prévalence est élevée et le retentissement socio-économique important. On situe habituellement l'arthrose au deuxième rang des maladies chroniques, après les affections cardiovasculaires (Reginster and Khaltbev, 2002). La fréquence de l'arthrose augmente avec l'âge, elle atteint près d'un tiers des sujets entre 45 et 64 ans et 60 à 70 % après 65 ans. Les démographes prévoient une lente augmentation de la population des sujets de plus de 65 ans jusqu'en 2010, puis une progression importante avec 28 % de la population âgée de 60 ans et plus en 2020! Il est donc primordial de comprendre les mécanismes pathogéniques qui sous-tendent cette maladie, d'identifier les bonnes cibles thérapeutiques et d'expérimenter de nouveaux médicaments anti-arthrosiques. Nos recherches s'inscrivent dans ce canevas.

Elles ont débuté en 1989 sous la direction du professeur P. Franchimont , pour aboutir en 1994 à la publication d'une thèse de doctorat en kinésithérapie (Henrotin *et al.*, 1994). Dans ce travail, nous avions développé un modèle de culture de chondrocytes en suspension en phase liquide, dit à "court terme". Dans ce modèle, les chondrocytes étaient isolés de la matrice tissulaire par une triple digestion enzymatique, et ensuite cultivés en suspension sous agitation constante durant des périodes s'étalant de 12 à 72 heures. Ce modèle nous a permis d'étudier les réponses précoces et non soutenues du chondrocyte à un stimulus extérieur, telle que la production de cytokines ou de formes activées de l'oxygène (FAO) (Henrotin *et al.*, 1992, 1993, 1996).

Dans ces conditions expérimentales, nous avions observé que les chondrocytes arthrosiques produisaient spontanément de l' interleukine (IL)-6 et -8, du leukemia inhibitory factor (LIF) et de la prostaglandine (PG) E_2. Ces facteurs sont tous impliqués dans l'initiation et le développement de la réaction inflammatoire. De plus, nous avions montré que la production de ces médiateurs était fortement modulée par l'interleukine (IL)-1ß et le Tumor Necrosis Factor (TNF)-α (Tableau A), deux médiateurs de l'inflammation et de la destruction tissulaire. Ces observations nous ont permis d'émettre l'hypothèse que le chondrocyte, par la sécrétion massive de ces médiateurs, contrôle l'inflammation de la membrane synoviale au cours de l'arthrose (Henrotin *et al.*, 1996).

Tableau A: Récapitulatif des effets de cytokines pro-inflammatoires sur le chondrocyte humain.

	Paramètres						
Cytokines ajoutées	ADN	IL-6	IL-8	LIF	IL-1ß	AGG	PGE_2
IL-1ß	O	↗	↗	↗	ND	↘	↗
TNFα	O	↗	↗	↗	ND	↘	↗
IL-6	O	ND	↘	↗	ND	O	O
IL-8	O	↗	ND	O	ND	O	O
LIF	O	↗	↗	ND	↗	↘	↗

Les chondrocytes humains fraîchement isolés de la matrice tissulaire étaient maintenus en culture durant 72 heures en l'absence ou en présence des différentes cytokines.
ADN = Acide déoxyribonucléique ; IL-1ß = Interleukine-1 bêta ; TNF-α = Tumor Necrosis Factor alpha ; IL-6 = interleukine-6 ; IL-8 = Interleukine-8 ; LIF = Leukemia Inhibitor Factor ; O = absence d'effet ; ↗= stimulation ; ↘ = inhibition. ND = non déterminé. AGG = agrécane; PGE_2 = prostaglandine E_2.

Notre travail a également contribué à une meilleure compréhension du rôle joué par le LIF dans la pathogénie de l'arthrose. Dans nos conditions expérimentales, le LIF stimulait la production de médiateurs de l'inflammation et inhibait la synthèse d'agrécane. A l'aide d'anticorps neutralisant l'activité de l'IL-1α et ß, nous avions observé que les effets du LIF étaient secondaires à la libération d'IL-1ß. Par contre, les effets de l'IL-1ß sur le chondrocyte, n'étaient pas modifiés par l'addition d'anticorps neutralisant le LIF (Figure A).

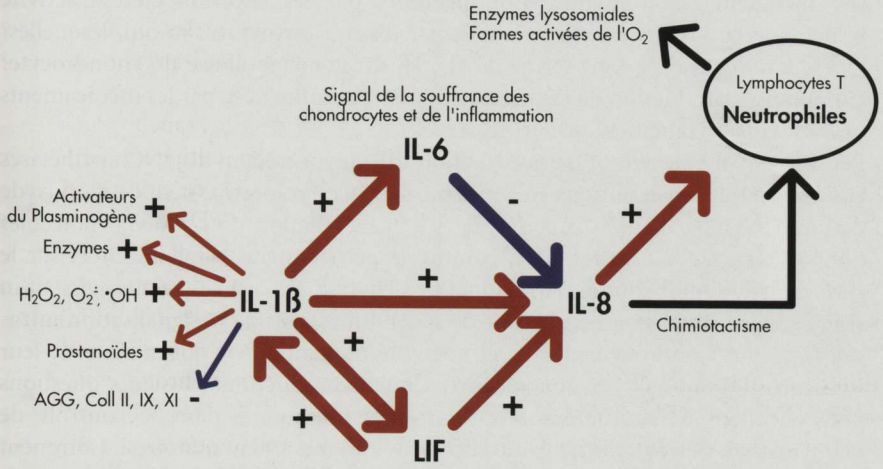


Figure A: *Représentation schématique des relations entre l'IL-1ß, l'IL-6, l'IL-8 et le LIF mises en évidence sur notre modèle expérimental. + = stimule; - = inhibe. (Henrotin, 1994)*

Un autre aspect très important de la pathogénie de l'arthrose que nous avions étudié dans notre thèse, était la production de formes activées de l'oxygène (FAO) par les chondrocytes. Rappelons que les FAO sont, avec les métalloprotéases, impliquées dans la dégradation du cartilage. Elles sont également présentées comme des acteurs de la signalisation intracellulaire et des médiateurs de l'inflammation. A l'époque de notre thèse, la production de FAO par les chondrocytes était méconnue. Notre travail démontrait pour la première fois que les chondrocytes étaient capables de produire du peroxyde d'hydrogène (H_2O_2) et du radical hydroxyle (•OH). La production de ces FAO était augmentée par le phorbol myristate acétate, l'oxygénation (5 minutes en présence de carbogène 95 % O_2-5 %CO_2), des cycles d'anoxie/réoxygénation ou encore des cytokines comme l'IL-1ß et le TNFα (Henrotin *et al.*, 1992 et 1993).

En résumé, cette première thèse montrait que les chondrocytes "activés" *in vitro* modifiaient leur phénotype, et produisaient en grande quantité une série de médiateurs de l'inflammation et de la dégradation tissulaire, dont les cytokines et les formes activées de l'oxygène.

Ces résultats ont permis d'élaborer des hypothèses, qui ont orienté la suite de nos travaux:

- Le chondrocyte "activé" occupe une position centrale dans la pathogénie de l'arthrose, et peut être considéré comme une cellule effectrice de la réaction inflammatoire qui accompagne cette maladie. De nombreuses questions subsistent quant aux mécanismes d'activation des chondrocytes. Quels sont les facteurs biochimiques responsables du dysfonctionnement des chondrocytes? Quels sont les médiateurs de l'inflammation produits par les chondrocytes? L'activité biologique de l'IL-1ß est-elle modulée par d'autres cytokines? Si oui, lesquelles? Quels sont les effets à long terme de l'IL-1ß sur le métabolisme du chondrocyte? Comment la production de ces médiateurs est-elle influencée par les médicaments utilisés dans le traitement de l'arthrose?
- Récemment, il a été démontré que les chondrocytes possèdent des •NO synthétases et une NADPH oxydase, deux enzymes productrices, respectivement de monoxyde d'azote (•NO) et d'anion superoxyde ($O_2^{\bullet-}$). La réaction de ces deux molécules donne naissance à d'autres FAO, comme le peroxynitrite, dont les effets sur le chondrocyte sont inconnus. Nous émettons l'hypothèse que ces agents agissent in situ, dans la cellule, et jouent le rôle de second messager de la signalisation intracellulaire du chondrocyte et que leur activité biologique est dépendante de leur structure chimique et des circonstances de leur production. Plusieurs questions nous viennent à l'esprit: Les FAO sont-elles impliquées dans le contrôle de l'homéostasie des cytokines? Sont-elles pro- ou anti-inflammatoires? Comment régulent-elles l'expression des gènes de l'inflammation? Leurs effets sont-ils généraux ou spécifiques?
- Durant de nombreuses décennies, la prise en charge pharmacologique de l'arthrose s'est surtout concentrée sur l'aspect symptomatique, sans chercher à interférer avec la progression à long terme de cette affection. A côté des anti-inflammatoires classiques, dont l'administration est souvent de courte durée en raison des effets secondaires qu'ils occasionnent, d'autres molécules sont utilisées dans le traitement de fond de la maladie. Ces agents sont classés comme "agents à action symptomatique lente" et caractérisés par une action lente et rémanente sur la symptomatologie, une période d'administration très longue (parfois plusieurs années), et leur quasi-innocuité. Actuellement, les mécanismes d'action de ces médicaments ne sont pas encore tous connus. De plus, les rares études *in vitro* réalisées à ce jour sont souvent limitées aux premiers jours qui suivent la mise en culture. Quels sont les effets de ces médicaments sur le chondrocyte? Quels sont leurs effets à long terme?

Dans le travail qui suit, nous avons tenté de répondre à ces questions et concentré notre attention sur les points suivants:

1) ***Mise au point d'un modèle de culture de chondrocytes en billes d'alginate.*** Nous avons développé un modèle de culture de chondrocytes humains en billes d'alginate. Dans ce modèle, les chondrocytes restent différenciés et continuent à exprimer le phénotype cartilagineux durant une longue période (12 jours au

moins). Nous avons étudié la formation de la matrice extracellulaire, mais également la distribution au sein de cette matrice de l'agrécane, de l'IL-6, de l'IL-8 et du Macrophage Inflammatory Protein-1ß (MIP-1ß).

2) *Etude des effets à long terme de l'IL-1ß.* L'IL-1ß induit un dysfonctionnement important du chondrocyte, caractérisé par une synthèse accrue d'enzymes protéolytiques et une inhibition de la synthèse de protéines matricielles. Ce dérèglement de l'activité synthétique du chondrocyte est un facteur déterminant de la dégradation du cartilage. Notre objectif est de reproduire en culture ces conditions pathologiques et d'étudier sur ce modèle l'influence de médicaments. Dans ce but, les chondrocytes cultivés en billes d'alginate ont été incubés durant douze jours en présence d'IL-1ß. De nombreux facteurs biochimiques impliqués dans la pathogénie de l'arthrose ont été quantifiés dans les différents compartiments (matrice péricellulaire et territoriale, matrice inter-territoriale et surnageants) de la culture en billes d'alginate. Parmi ceux-ci, citons la stromélysine-1 (MMP-3), le Tissue Inhibitor of Metalloproteases-1 (TIMP-1), les interleukines (IL)-1ß, -6, -8, le Macrophage Inflammatory Protein-1ß (MIP-1ß), le monoxyde d'azote (•NO) et la prostaglandine (PG)E_2.

3) *Etude de la régulation de l'activité biologique de l'IL-1ß par d'autres cytokines.* L'IL-1ß n'est pas la seule cytokine impliquée dans la pathogénie de l'arthrose. Il est probable que d'autres cytokines interagissent avec l'IL-1ß et modulent ses effets. Dans ce travail, nous avons exploré l'influence de l'interféron gamma (IFNγ), de l'Insulin-like Growth Factor-1 (IGF-1) et du Transforming Growth Factor (TGF) ß-1 sur les effets de l'IL-1ß.

4) *Etude de la régulation des effets de l'IL-1ß par les FAO.* Les FAO sont présentées comme des agents délétères responsables de la dégradation des tissus conjonctifs. Nous émettons l'hypothèse que les FAO sont aussi des modulateurs de la réponse du chondrocyte à l'IL-1ß et que l'action des FAO est variable selon le gène exploré et la voie de signalisation intracellulaire activée. Afin de vérifier cette hypothèse, les chondrocytes ont été cultivés en présence d'un antioxydant (N-acétyl-cystéine; NAC), d'un inhibiteur des •NO synthétases (N^G-monométhyl-L-arginine; L-NMMA), de formes activées de l'oxygène (H_2O_2, -ONOO) et soumis à des flux exogènes de FAO générés par des donneurs chimiques (SIN-1, SNAP).

5) *Etude des mécanismes d'action des médicaments utilisés dans le traitement de l'arthrose.* De nombreux médicaments sont actuellement utilisés en clinique humaine, alors que leurs mécanismes d'action ne sont pas encore connus. Dès lors, nous avons étudié de façon systématique les effets de huit anti-inflammatoires non-stéroïdiens (AINS), d'un mélange d'insaponifiables d'avocat et de soja (A1S2), et d'une anthraquinone (la rhéine) sur le métabolisme des chondrocytes. La recherche *in vitro* est aussi utilisée à un stade pré-clinique, lors de l'évaluation des propriétés pharmacologiques de nouvelles molécules. Afin d'illustrer cette application de nos modèles expérimentaux, nous avons testé le ranélate de strontium (S12911), une molécule développée pour le traitement de l'ostéoporose (Meunier *et al.*, 2002). Nous avons vérifié l'hypothèse selon laquelle ce médicament pourrait également interférer avec le métabolisme du chondrocyte.

Dans un souci de clarté, nous avons développé nos travaux de recherche dans qua-

tre chapitres dont les contenus sont définis ci-dessous.
Le *chapitre I* est l'exposé des connaissances actuelles de la pathogénie de l'arthrose. Les données de ce chapitre sous un prérequis à la compréhension des travaux développés dans les chapitres qui suivent.

Le *chapitre II* est consacré à la caractérisation d'un modèle de culture de chondrocytes humains arthrosiques en billes d'alginate. Nous y abordons l'effet de l'IL-1ß sur la production d'agrécane, de MMP-3, de TIMP-1, de cytokines pro-inflammatoires (MIP-1ß, IL-6, IL-8), de PGE_2 et de •NO. Ce modèle a également été utilisé pour l'expertise des effets de médicaments sur le métabolisme du chondrocyte (Chapitre IV).

Le *chapitre III* est dédié à notre contribution à l'identification des mécanismes physiopathologiques impliqués dans la genèse et le développement de l'arthrose. Nous exposons le rôle modulateur de l'IFNγ sur l'activité biologique de l'IL-1ß. Nous étudions également les effets des FAO sur l'homéostasie des cytokines, des métalloprotéases et de la prostaglandine E_2.

Enfin, le *chapitre IV* est dévolu aux effets des médicaments utilisés dans le traitement de l'arthrose sur le métabolisme du chondrocyte. Les mécanismes d'action qui sous-tendent l'efficacité clinique de ces médicaments y sont développés.
Avant d'entamer la lecture de ce travail, et afin de faciliter la compréhension du lecteur, nous souhaitons attirer son attention sur le fait que deux modèles de culture ont été utilisés dans ce travail. D'une part, un modèle dit à "court terme" qui consiste à cultiver des chondrocytes humains en suspension en phase liquide pendant trois jours, et d'autre part un modèle dit à "long terme" dans lequel les chondrocytes sont cultivés en suspension dans une matrice d'alginate pour une période de douze jours.

CHAPITRE I

Pathogénie de l'arthrose

Ce chapitre est consacré à la description des facteurs biochimiques impliqués dans la pathogénie de l'arthrose. Ces facteurs ont été l'objet de plusieurs de nos travaux (Henrotin *et al.*, 1993; 1996; 1998b; 1999a; 2000b; Ayache *et al.*, 2002; Mathy-Hartert *et al.*, 2001a et 2002) (Annexes 3, 4, 5, 6, 7).

1.1 Structure et fonction du cartilage normal

Le cartilage articulaire est un tissu conjonctif hautement spécialisé, ne contenant ni vaisseaux sanguins, ni vaisseaux lymphatiques, ni structures nerveuses. La fonction principale de ce tissu est d'assurer le glissement des extrémités osseuses formant l'articulation, tout en amortissant et en répartissant les pressions de contact. Le cartilage articulaire assure ces propriétés mécaniques grâce à une matrice extracellulaire abondante au sein de laquelle sont logées les cellules cartilagineuses, appelées aussi chondrocytes. Ces cellules contrôlent l'homéostasie du tissu, en assurant la synthèse et le renouvellement de la matrice extracellulaire. Sur une coupe transversale du cartilage normal, on peut distinguer quatre zones qui se différencient par l'organisation et la forme des cellules ainsi que par la composition de la matrice extracellulaire. La couche la plus superficielle est en contact avec le fluide synovial alors que la couche la plus profonde sert de zone d'encrage du cartilage à l'os sous-chondral. Cette zone est appelée zone intermédiaire ou calcifiée. Elle est séparée du cartilage non calcifié par une couche basophile dénommée " tidemark " (Figure I-1) (Thonar *et al.*, 1999).

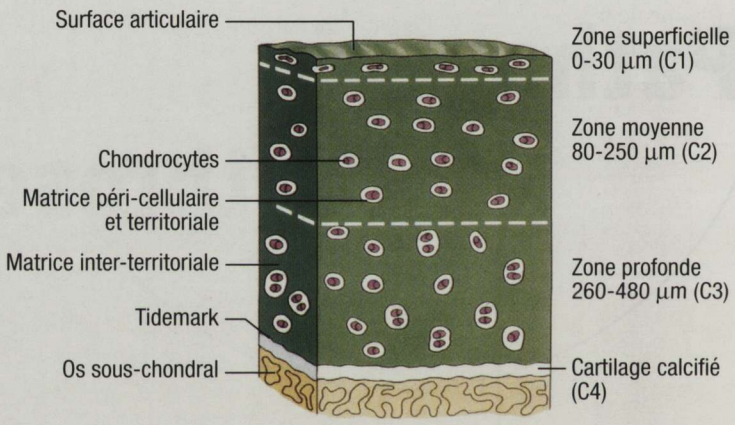


Figure I-1: *Illustration des différentes couches du cartilage adulte normal (coupe transversale). C1 = couche superficielle ; C2 = couche intermédiaire ; C3 = couche profonde ; C4 = cartilage calcifié.*

La matrice extracellulaire du cartilage adulte est formée de plusieurs compartiments chacun avec une apparence morphologique et une composition biochimique différentes. Il s'agit de la matrice péri-cellulaire, directement adjacente à la membrane du chondrocyte, la matrice territoriale, et la matrice inter-territoriale qui est la matrice la plus éloignée du chondrocyte (Figure I-2). Le chondrocyte exerce un contrôle actif sur le métabolisme des constituants de la matrice péri-cellulaire et ter-

ritoriale (unité fonctionnelle du cartilage) mais a peu d'influence sur la matrice inter-territoriale (Poole *et al.*, 1988 ; Thonar *et al.*, 1999).

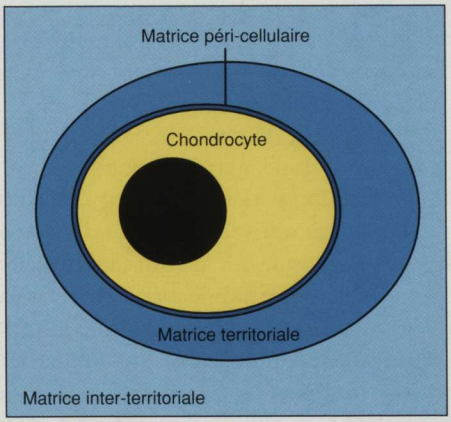


Figure I-2: *Compartimentation de la matrice extracellulaire du cartilage.*

La matrice extracellulaire du cartilage est principalement composée d'eau (65-70 % du poids frais), de molécules d'agrécane (10 % du poids frais ; 90 % des protéoglycanes du cartilage) et de collagène de type II (10-20 % du poids frais: 90 % des collagènes du cartilage). Les autres molécules sont minoritaires. Il s'agit principalement des collagènes VI, X, IX, XI, XII et XIV, et des petits protéoglycanes riches en leucine, le biglycane et la décorine (Eyre *et al.*, 1987 ; van der Rest *et al.*, 1988 ; Fischer *et al.*, 1989 ; Heineguard et Oldberg, 1989) (Figure I-3).

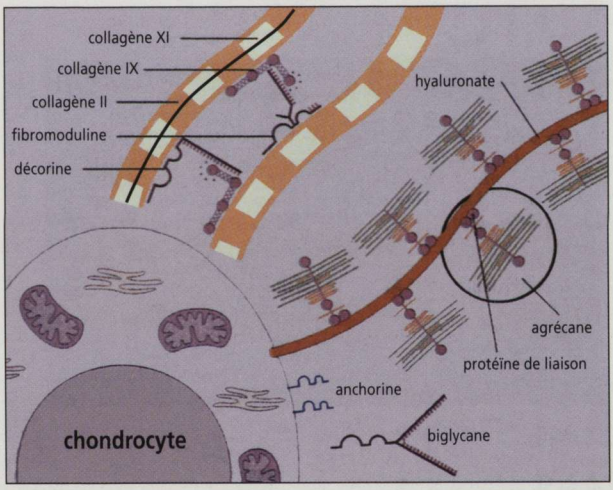


Figure I-3: *Organisation de la matrice extracellulaire du cartilage. Notons que les molécules d'agrécane se lient à l'acide hyaluronique pour former des complexes de haut poids moléculaire. Le collagène de type IX est attaché à la fibrille de collagène de type II alors que le type XI est localisé à l'intérieur de celle-ci. L'acide hyaluronique est lié à la membrane plasmique du chondrocyte par un récepteur de type CD44 (Thonar et al., 1999).*

1.2. Définition de l'arthrose

La définition de l'arthrose fait encore l'objet de nombreuses discussions. Plutôt que de parler d'arthrose au singulier, de nombreux auteurs préfèrent parler d'arthroses, voire de syndromes arthrosiques, dans la mesure où l'arthrose peut être idiopathique (primitive) ou secondaire à de nombreux facteurs (traumatiques, métaboliques, endocriniens, congénitaux, neurologiques).

Le terme arthrose désigne donc, un ensemble de maladies, d'étiologies variées mais dont la séméiologie est commune. Actuellement, l'arthrose humaine se définit comme une maladie de progression variable affectant l'ensemble des tissus articulaires et péri-articulaires, en ce y compris le cartilage, les ménisques, l'os sous-chondral, la membrane synoviale, la capsule, les tendons, les muscles et les terminaisons nerveuses. Ces tissus vont subir des modifications structurelles et fonctionnelles importantes conduisant à l'altération du statut algo-fonctionnel de l'articulation. La membrane synoviale est le siége d'une réaction inflammatoire (synovite) qui est essentiellement congestive, contingente et secondaire aux lésions du cartilage (Lindlad et Hedfors, 1987). L'os sous-chondral est l'objet d'un remaniement important caractérisé par une ostéophytose, une condensation osseuse des zones de pression et des géodes. Les muscles péri-articulaires sont contracturés, déficitaires et atrophiés (Madsen *et al.*, 1998; Crielaard et Henrotin 1999; Henrotin *et al.*, 1999; Croisier *et al.*, 2000; Henrotin *et al.*, 2002). Les tendons sont enflammés, la capsule rétractée et les ligaments sclérosés ou distendus (Nelissen *et al.*, 2001). A un stade avancé de la maladie, le cartilage articulaire dégénère en se fibrillant, se fissurant, s'ulcérant et finalement en disparaissant complètement. L'arthrose est donc la résultante clinique et morphologique d'une variété de pathologies contribuant à la faillite structurelle et fonctionnelle d'une ou plusieurs articulations (Reginster et Henrotin, 2001a et b) (Figure I-4).



Figure I-4: *Tête fémorale humaine normale (A) et arthrosique (B).*

L'arthrose peut être mono- ou polyarticulaire. La polyarthrose touche, en règle générale, la femme après la ménopause, au niveau des articulations interphalangiennes distales (IPD), des articulations trapezo-métacarpiennes, des articulations vertébrales postérieures et des articulations fémoro-patellaires. Certaines rares

polyarthroses familiales précoces, ont une origine génétique et sont liées à la mutation d'une base nucléique au niveau du gène COL2α1, convertissant le codon arginine en position 519 de la chaîne α1 du collagène de type II en codon cystéine, un acide aminé habituellement absent du collagène de type II (Knowlton *et al.*, 1990; Eyre *et al.*, 1991).

Tableau I-1: Les principales localisations arthrosiques chez les octogénaires*

Localisation	Prévalence
Interphalangiennes distales	40 à 80%
Interphalangiennes proximales	30 à 60%
Trapézo-métacarpiennes	30 à 60%
Interapophysaires cervicales et lombaires	70 à 80%
Genoux	30 à 50%
Hanches	10 à 20%

* Prévalence déterminée sur base de l'observation de lésions radiologiques. Deux localisations sont observées dans près d'un 1/3 des cas (le plus souvent genoux et mains), 10% des sujets présentent trois localisations. (Selon Tran et coll, 1994; Llach *et al.*, 1999)

La vitesse et l'importance de la dégradation du cartilage sont des paramètres très variables de l'arthrose. A titre d'exemple, des études radiologiques montrent que dans la gonarthrose fémoro-tibiale interne, le pincement de l'interligne est en moyenne voisin de 0.25 mm par an, mais peut atteindre 1 à 2 mm par an dans les formes destructives rapides (Dougados *et al.*, 1992). De plus, la destruction du cartilage n'est pas toujours linéaire, mais peut être entrecoupée de phases de restitution de l'interligne articulaire (Buckland-Wright, 1995). D'un point de vue clinique, l'arthrose peut être considérée comme évoluant progressivement d'une forme asymptomatique dite "préclinique" vers une forme symptomatique et morbide appelée aussi "forme clinique " (Figure I-5).

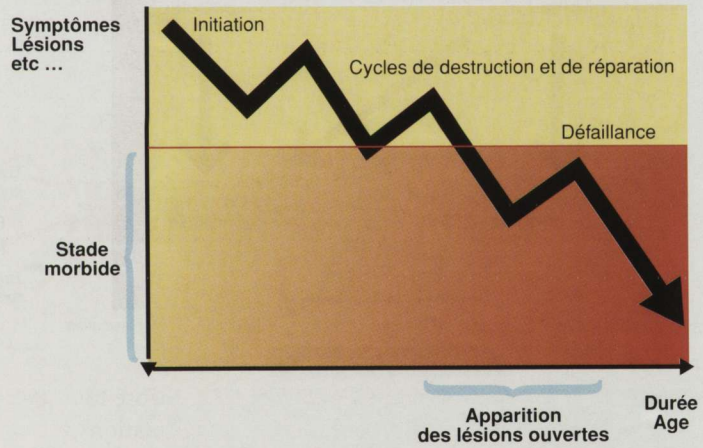


Figure I-5: *Représentation schématique de l'évolution radio-clinique de l'arthrose (Selon Lohmander et al., 1999).*

Le symptôme majeur de l'arthrose est la douleur. Cette douleur mécanique apparaît dès la mise en fonction (dérouillage articulaire) ou après un certain délai de fonction. Mais les symptômes de l'arthrose ne se limitent pas à une douleur, et le clinicien averti peut reconnaître d'autres signes cliniques. Il s'agit:

- d'une hypertrophie non liquidienne de la jointure, bien visible aux mains, pieds et genoux.
- d'un épanchement articulaire, qui peut se voir dans certaines arthroses (par ex. gonarthrose), et surtout pendant les poussées inflammatoires.
- d'une sensibilité anormale à la pression au niveau de l'interligne et des épiphyses osseuses adjacentes.
- d'une raideur articulaire douloureuse toujours présente, mais plus ou moins discrète et rarement importante, sauf à la hanche.
- de sensations de craquement, d'accrochages ou de dérobements articulaires.

1.3 Les facteurs biochimiques de l'arthrose

La faillite structurelle et fonctionnelle du cartilage articulaire résulte d'une perte d'équilibre entre, d'une part, la combinaison d'un stress mécanique et du dysfonctionnement métabolique des tissus articulaires, et d'autre part, la capacité de ces tissus articulaires à supporter une agression et à réparer les dommages constitués. D'une manière générale, on peut considérer que la destruction structurelle du cartilage articulaire et de l'os sous-chondral résulte soit d'un stress mécanique accentué dégradant un tissu normal ou de l'impossibilité pour un cartilage déficient d'assumer les sollicitations mécaniques normales de la vie quotidienne (Figure I-6)

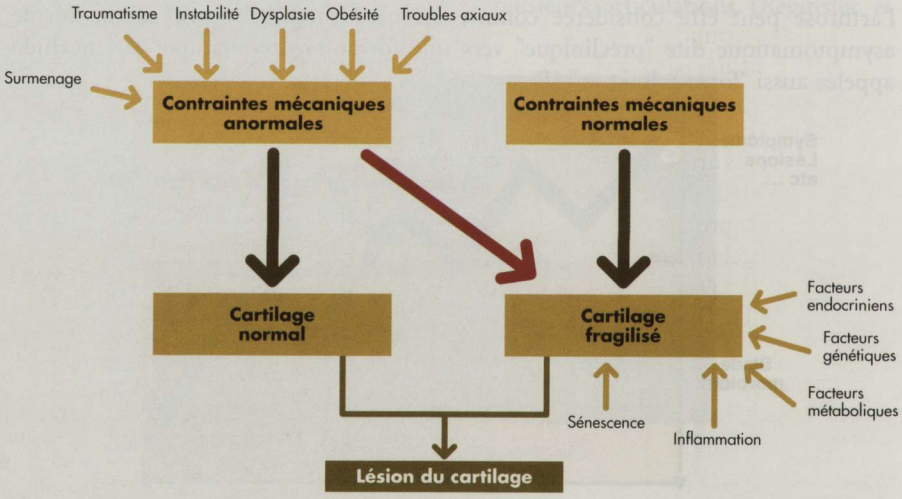


Figure I-6: *Physiopathologie de l'arthrose.*

Ces circonstances pathogéniques s'accompagnent de perturbations métaboliques importantes se traduisant par des synthèses anormales du chondrocyte et la libération excessive de médiateurs de l'inflammation et de la chondrolyse.

1.3.1. Les enzymes et leurs inhibiteurs

a) les métalloprotéases

Dans l'arthrose, les métalloprotéases sont les principaux agents de la dégradation du cartilage. Il s'agit essentiellement des métalloprotéases matricielles (MMPs) dont les collagénases (MMP-1, -8, -13), les agrécanases (ADAMTS-4 et -5), la stromélysine-1 (MMP-3), les gélatinases (MMP-2, -9) ainsi que les métalloprotéases membranaires (MT-1,-2,-3-MMPs) (Tableau I-2). Ces enzymes ont été localisées en quantités anormalement élevées dans les régions adjacentes aux lésions du cartilage et dans le fluide synovial des patients arthrosiques (Mannicourt *et al.*, 1994; Walter *et al.*, 1998; Ishiguro *et al.*, 1999; Naito *et al.*, 1999; Yoshihara *et al.*, 2000; Bluteau *et al.*, 2001; Tetlow *et al.*, 2001).

Dans le cartilage humain arthrosique, trois collagénases ont été identifiées: la collagénase-1 (MMP-1), la collagénase-2 (MMP-8) et la collagénase-3 (MMP-13). La distribution des collagénases dans le cartilage articulaire est spécifique pour chacune d'entre-elles. Ainsi, les collagénases -1 et -2 sont prédominantes dans la couche superficielle (C1) et la partie supérieure de la couche intermédiaire (C2) du cartilage, alors que la collagénase-3 est principalement présente dans la partie inférieure de la zone intermédiaire et dans la couche profonde (C3) (Fernandes *et al.*, 1998). La collagénase-3 (MMP-13) se distingue des autres collagénases par son large spectre d'activité et sa grande affinité (Km la plus faible des collagénases) pour le collagène de type II (Knauper *et al.*, 1996 et 1997).

Le second groupe de métalloprotéases impliquées dans la dégradation du cartilage est constitué par les stromélysines et les gélatinases. Actuellement, trois stromélysines ont été identifiées chez l'homme: la stromélysine-1 (MMP-3), -2 (MMP-10) et -3 (MMP-11). Seule la stromélysine-1 (MMP-3) semble impliquée dans le processus arthrosique (Hembry *et al.*, 1995). Elle dégrade l'agrécane mais est aussi un activateur des collagénases latentes, et donc participe à la cascade d'activation des métalloprotéases. Dans le cartilage humain, deux types de gélatinases ont été trouvés: la gélatinase 72 kDa (MMP-2) et 92 kDa (MMP-9)(Tanaka *et al.*, 2000). La gélatinase 92 kDa (MMP-9) est absente du cartilage normal, mais exprimée dans le cartilage arthrosique (Mohtai *et al.*, 1993; Tsuchiya *et al.*, 1997; Freemont *et al.*, 1997).

Finalement, trois métalloprotéases de type membranaire (MT-1,-2,-3-MMP) sont exprimées par les chondrocytes (Ohuchi *et al.*, 1997). Les MT-MMPs (MMP-14, -15, -16) possèdent les caractéristiques enzymatiques des collagénases, et seraient impliquées dans le phénomène de protéolyse focalisée, soit par une action directe sur la matrice péri-cellulaire, soit par l'activation de la pro-collagénase-3 (MMP-13) et de la pro-gélatinase 72 kDa (MMP-2) (Imai *et al.*, 1997; Buttner *et al.*, 1997).

Enfin, signalons la découverte récente des agrécanases-1 et -2 (ADAM-TS4 et -TS5) (Carterson *et al.*, 2000; Tortorella *et al.*, 2000; Kashiwagi *et al.*, 2001). Ces endopeptidases de la famille des ADAM-TS (A desintegrin/cystein rich metalloproteinase with thrombospondin motifs) dégradent l'agrécane au niveau du domaine inter-globulaire (G1-G2) NH_2-terminal (Glu^{373}-Ala^{374}), à un endroit différent du site de clivage de la stromélysine-1 (Sandy *et al.*, 1992, Sugimoto *et al.*, 1999). Les agrécanases clivent également la protéine centrale de l'agrécane aux sites Glu^{480}-Gly^{481}, Gly^{667}-Gly^{668} et Glu^{871}-Leu^{872} situés dans la région riche en chondroïtine sulfate (Tortorella *et al.*, 2001).

Tableau I-2: Les principales métalloprotéases impliquées dans la dégradation du cartilage.

Nomenclature	Abréviation	Substrats
Collagénases		
Collagénase -1	MMP-1	Collagènes I, II, III, VII, X , agrécane, gélatine, pro-MMP-2, -9
Collagénase-2	MMP-8	Collagènes I, II, III, VII, VIII, X, agrécane, gélatine
Collagénase-3	MMP-13	Collagènes I, II, III, gélatine, agrécane
Stromélysines		
Stromélysine-1	MMP-3	Collagènes II, III, IV, IX, X, XI, élastine, pro-MMP-1, 7, -8, -9, -13, agrécane, fibronectine, laminine
Gélatinases		
Gélatinase A (72 kDa)	MMP-2	Collagènes I, II, III, IV, V, VII, X, XI, XIV, gélatine, élastine, fibronectine, agrécane, pro-MMP-9, -13
Gélatinase B (92 kDa)	MMP-9	Collagènes IV, V, VII, X, XIV, gélatine, élastine, agrécane.
ADAM-TS		
Agrécanase-1	ADAM-TS4	Agrécane, brevicane, versicane
Agrécanase-2	ADAM-TS5	Agrécane
MMP Membranaires		
MT1-MMP	MMP-14	Collagènes I, II, III, gélatine, agrécane, laminine, fibronectine, Pro-MMP-2, -13
MT2-MMP	MMP-15	Gélatine, laminine, fibronectine, pro-MMP-2
MT3-MMP	MMP-16	Pro-MMP-2

Selon Henrotin *et al.*, 2002(annexe 9). ADAM-TS = A desintegrin/cystein rich metalloproteinase with thrombospondin motifs; MMP =Matrix Metalloproteinase; MT-MMP = Membrane type matrix metalloproteinase.

b) La cascade enzymatique d'activation des métalloprotéases

La transformation du zymogène en enzyme active, semble résulter d'une cascade protéolytique d'activation dans laquelle agissent en synergie des protéases à sérine,

des métalloprotéases et des formes activées de l'oxygène (Figure I-7). Les activateurs du plasminogène tissulaire (t-PA) et membranaire (urokinase ou u-PA) jouent un rôle prépondérant dans cette cascade d'activation (Martel-Pelletier *et al.*, 1999). Ils transforment le plasminogène en plasmine. La plasmine est une protéase à sérine active dans la dégradation des membranes basales, de la matrice extracellulaire et dans la transformation des pro-MMPs en MMPs actives (He *et al.*, 1989; Milner *et al.*, 2001). Les taux d'urokinase (uPA) et de plasmine sont anormalement élevés dans le cartilage arthrosique (Martel-Pelletier *et al.*, 1991; Belcher *et al.*, 1996). L'activité de u-PA et t-PA est contrôlée par des inhibiteurs de l'activateur du plasminogène-1 et -2 (PAI-1 et PAI-2) (Treadwell *et al.*, 1991; Campbell *et al.*, 1991). La synthèse de cet inhibiteur est moindre dans les tissus arthrosiques, favorisant l'activation des MMPs par l'urokinase (Blasi *et al.*, 1999).

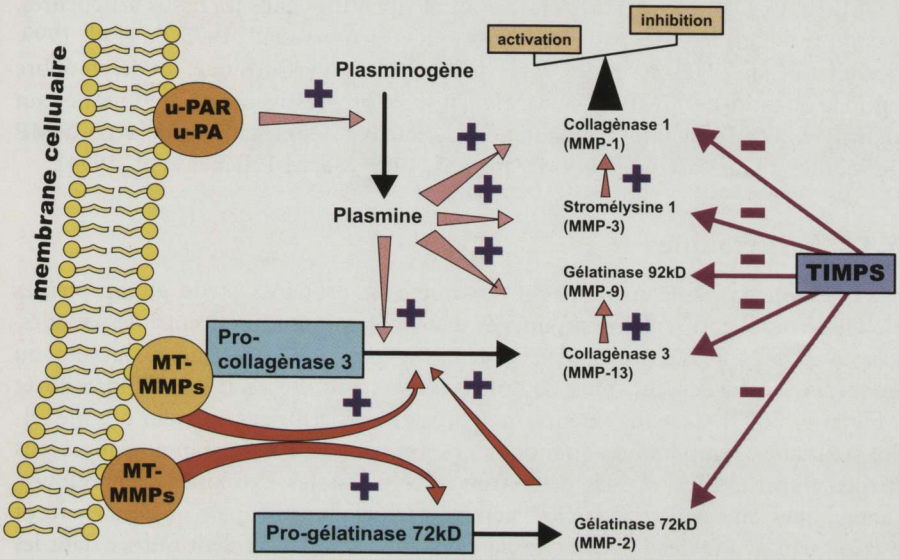


Figure I-7: *Cascade protéolytique d'activation des metalloproteinases (Selon Amersham Life Science en 1998 modifié par Martel-Pelletier en 1999 et Henrotin et al., 2001d)*

De nombreuses cathepsines (B, K L), sont également impliquées dans les processus de dégradation du cartilage. Dans le fluide synovial des patients arthrosiques, l'activité enzymatique de type cathepsine est anormalement élevée (Ikeda *et al.*, 2000). Le rôle de la cathepsine B dans la genèse des lésions arthrosiques est bien documenté. Elle apparaît comme la cathepsine la plus active dans ce processus. Son activité est augmentée dans le cartilage arthrosique, surtout dans les zones d'hypercellularité, aux endroits où les chondrocytes forment des clones (Baici *et al.*, 1995; Berardi *et al.*, 2001). La cathepsine B dégrade les molécules d'agrécane et active la pro-MMP-1 et la pro-MMP-3 (Mort *et al.*, 1998). Elle s'attaque également aux

TIMP-1 et -2, rendant ces inhibiteurs de MMPs inactifs. L'activité enzymatique des cathepsines K et L est principalement concentrée dans l'os sous-chondral, au niveau des ostéophytes et des zones de remodelage osseux (Lang *et al.*, 2000). Récemment, il a été démontré que la cathepsine K est synthétisée par les macrophages de la membrane synoviale arthrosique (Dodds *et al.*, 1999) et que cette enzyme est capable de dégrader le collagène de type I et II (Li *et al.*, 2000; Hou *et al.*, 2001). Le principal inhibiteur des cystéines peptidases est la cystatine C. Il est présent dans la membrane synoviale des articulations arthrosiques (Keyszer *et al.*, 1998; Hansen *et al.*, 2000).

c) Les inhibiteurs de métalloprotéases.

L'activité des métalloprotéases est contrôlée par des inhibiteurs tissulaires appelés dans la littérature anglo-saxone " Tissue Inhibitor of Metalloproteinases " (TIMPs). A ce jour, trois TIMPs (1-3) ont été identifiés dans les tissus articulaires, y compris le cartilage des patients arthrosiques (Hembry *et al.*, 1995; Su *et al.*, 1999; Yoshihara *et al.*, 2000). Dans les tissus de l'articulation arthrosique, un déséquilibre entre la quantité de TIMPs et de MMPs s'installe progressivement, conduisant à un déficit en TIMPs et une augmentation de l'activité enzymatique de type MMP au sein des tissus pathologiques (Dean *et al.*, 1989; Martel-Pelletier *et al.*, 1994).

1.3.2. Les cytokines

Le terme cytokine regroupe un ensemble de protéines et de glycoprotéines de faible poids moléculaire impliquées dans la communication entre les cellules. Elles exercent leur activité régulatrice de façon autocrine, paracrine, juxtacrine ou endocrine. Actuellement, plus de 150 cytokines ont été recensées (Dinarello et Moldawer, 2000). Certains auteurs ont tenté de les répertorier selon leur origine cellulaire (par ex. lymphokines ou monokines) ou selon leur activité biologique. Nous avons choisi de les classer dans trois catégories: les cytokines cataboliques, anaboliques ou régulatrices selon l'activité biologique principale qu'elles exercent sur le cartilage (Tableau I-3). Cette classification est régulièrement utilisée dans les articles de synthèse traitant ce sujet (van den Bergh *et al.*, 1999; Dinarello and Moldawer, 2000; Goldring, 2000). Au sein de l'articulation, ces cytokines sont produites par les chondrocytes, les cellules de la membrane synoviale et de l'os sous-chondral.

a) Les cytokines cataboliques.

Parmi les cytokines cataboliques impliquées dans la dégradation du cartilage, l'interleukine (IL)-1ß et le tumor necrosis factor (TNFα) jouent un rôle primordial. L'injection intra-articulaire de ces cytokines induit chez l'animal la dégradation du cartilage (Goldring, 1999). Le taux de ces deux cytokines est anormalement élevé dans le fluide synovial des patients arthrosiques (Khale *et al.*, 1992; Chikanza *et al.*, 1993). Dans le cartilage arthrosique, IL-1ß et le TNFα sont synthétisés principalement par les chondrocytes de la couche superficielle et plus précisément au niveau

Tableau I-3: Cytokines impliquées dans la régulation du métabolisme du cartilage

1. Cataboliques
Interleukin-1 (IL-1α et ß)
Tumor necrosis factor alpha (TNFα)
Interleukin-8 (IL-8)
Interleukin-17 (IL-17)
Interleukin-18 (IL-18)
Macrophage inflammatory protein-1 beta (MIP-1ß)
Leukemia inhibitory factor (LIF)
2. Régulatrices
Interleukin-6 (IL-6)
Interleukin-4 (IL-4)
Interleukin-10 (IL-10)
Interleukin-13 (IL-13)
Interleukin-1 receptor antagonist (IL-1Ra)
Interferon-γ (IFNγ)
Oncostatin M (OSM)
3. Anaboliques
Bone morphogenetic proteins (BMP-2, -4, -6, -7, -9,-13)
Fibroblast growth factor (FGF-2,-4,-8)
Growth differentiation factor (GDF-5)
Insulin-like growth factor 1 (IGF-1)
Platelet-derived growth factor (PDGF)
Transforming growth factor ß (ß-1, ß-2,ß-3)

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des lésions (fibrillations et clones cellulaires) (Tetlow *et al.*, 2001). Les chondrocytes possèdent des récepteurs membranaires spécifiques de l'IL-1ß et du TNFα (IL-1R et TNF-R) (Martel-Pelletier *et al.*, 1992; Westacott *et al.*, 1997; Webb *et al.*, 1997). Pour chacune de ces cytokines deux récepteurs ont été identifiés: le type I et le type II IL-1R et le TNFR55 et TNFR75 (Tartaglia et Goeddel, 1992; Slack *et al.*, 1993). La proportion de chondrocytes exprimant ces récepteurs est plus grande dans le cartilage arthrosique, que dans le cartilage normal. De plus, la densité des récepteurs est plus élevée sur les chondrocytes arthrosiques, que sur les cellules normales. Ce phénomène a pour effet d'augmenter la sensibilité des chondrocytes à l'IL-1ß et au TNFα (Martel-Pelletier *et al.*, 1992; Westacott *et al.*, 1994; Alsalameh *et al.*, 1999; Westacott *et al.*, 2000).

In vitro, l'IL-1ß et le TNFα ont des effets similaires et synergiques (Tableau I-4). Ces cytokines induisent un déséquilibre fonctionnel au sein de la matrice cartilagineuse, non seulement en modifiant le statut enzymatique du cartilage mais

également en réduisant la production de ses constituants. Elles stimulent la synthèse par les chondrocytes de la majorité des metalloprotéases, des cystéines et des sérines protéases et réduisent la production de TIMP-1 et d'inhibiteur de l'activateur du plasminogène (PAI) (Martel-Pelletier *et al.*, 1991; Martel-Pelletier *et al.*, 1994; Beekman *et al.*, 1998; Flannery *et al.*, 1999; Stove *et al.*, 2000; Hui *et al.*, 2001a). De plus, elles inhibent la synthèse des protéines matricielles, et s'opposent aux effets stimulant des facteurs de croissance (par ex. TGFß), réduisant la capacité de réparation du cartilage (Lefebvre *et al.*, 1990; Redini *et al.*, 1993; Demoor-Fossard *et al.*, 1998; van den Berg *et al.*, 1999). L'IL-1ß et le TNFα provoquent également la libération massive par les chondrocytes de chimiokines (Pulsatelli *et al.*, 1999), d'IL-6, de LIF (Henrotin *et al.*, 1996) mais aussi de formes activées de l'oxygène (FAO; •NO, H_2O_2, •OH, $O_2^{\bullet}$) (Tiku *et al.*, 1990; Henrotin *et al.*, 1992), de leucotriène B4 (LTB_4) et de prostaglandines (PG) E_2 (Tawara *et al.*, 1991).

Tableau I-4: Les principaux effets *in vitro* de l'IL-1ß et du TNFα sur le métabolisme des chondrocytes.

Diminution	Augmentation
Collagènes (II, IX, XI)	Métalloprotéases
Agrécane	Cathepsines
Biglycane, décorine	Gélatinases
TIMP-1	u-PA et t-PA
PAI-1	PGE_2
	LTB4
	Phospholipase A2
	FAO (•NO, H_2O_2, •OH, $O_2^{\bullet}$)
	IL-6, IL-8, MIP-1α, MIP-1ß, MCP-1, LIF, RANTES, GRO-α

TIMP-1 = Tissue inhibitor of metalloproteinases, PAI-1 = Plasminogen activator inhibitor-1, uPA = urokinase-plasminogen activator; t-PA=tissular-plasminogen activator; FAO = Formes activées de l'oxygène; IL-8 = Interleukine-8; IL-6 = Interleukine-6; GRO-α = Growth related gene product alpha; LIF = Leukemia inhibitory factor; MCP-1 = Monocyte chemoattractant protein-1; MIP-1α et ß = Macrophage inflammatory protein-1; RANTES = Regulated on activation, normal T cell expressed and secreted.

Chez les mammifères, l'IL-1ß est synthétisée sous la forme d'un précurseur de 31 kDa (pro-IL-1ß) et sécrétée sous une forme active de 17,5 kDa. L'activation de l'IL-1ß est réalisée par une cystéine aspartate protéase membranaire dénommée caspase-1 ou IL-1ß-converting enzyme (ICE) (Black *et al.*, 1988; Kronheim *et al.*, 1992; Wilson *et al.*, 1994). Quant au précurseur du TNFα (26 kDa), il est stocké au niveau de la membrane cellulaire. Sa libération dans le milieu extracellulaire et son activation nécessitent l'intervention d'une enzyme de la famille des adamalysines (ADAM) appelée TNFα-converting enzyme (TACE) (Aggarwal *et al.*, 1985; Gearing *et al.*, 1994; Black *et al.*, 1997). Les taux d' ARNm codant pour la caspase-1 et la TACE sont anormalement élevés dans le cartilage humain arthrosique (Amin *et al.*, 1997).

L'activité des cytokines cataboliques peut-être inhibée par des inhibiteurs naturels. Ceux-ci peuvent être classés en trois catégories selon leur mode d'action:

- Les antagonistes du récepteur qui entrent en compétition avec la cytokine catabolique pour la liaison avec son récepteur. A ce jour, seul un antagoniste du récepteur pour l'IL-1 (IL-1Ra) a été identifié. Les chondrocytes arthrosiques produisent spontanément de l'IL-1Ra et cette production est augmentée par l'IL-1ß (Pelletier *et al.*, 1996; Henrotin *et al.*, 2000). Dans un modèle d'arthrose induite chez le lapin par ménisectomie, l'injection intra-articulaire du plasmide de l'IL-1Ra réduit la sévérité des lésions du cartilage (Fernandes *et al.*, 1999). De même, chez le chien, l'injection intra-articulaire de cellules synoviales transgéniques exprimant le gène IL-1Ra ou d'IL-1Ra recombinant, ralentit la progression des lésions cartilagineuses secondaires à une rupture du ligament croisé antérieur (Caron *et al.*, 1996; Pelletier *et al.*, 1997b).
- Les récepteurs solubles, qui inhibent l'activité de la cytokine par liaison avec celle-ci. Des formes solubles des récepteurs de l'IL-1 et du TNF ont été identifiées dans le fluide synovial et le sérum des patients arthrosiques (Roux-Lombard *et al.*, 1993; Chikanza *et al.*, 1993). Il s'agit de formes tronquées des récepteurs IL-1RI (IL-1sRI), IL-1RII (IL-1sRII), TNF-R55 (TNFsR55) et TNF-R75 (TNFsR75).
- Enfin, les cytokines dites "anti-inflammatoires" qui sont capables d'inhiber la synthèse et/ou de s'opposer à l'activité des cytokines cataboliques. Il s'agit principalement des TGFß, de l'IL-4, de l'IL-10 et de l'IL-13. Les chondrocytes arthrosiques produisent ces cytokines (Tanabe *et al.*, 1996; Moos *et al.*, 1999). Leurs actions sur le chondrocyte seront décrites ultérieurement dans le paragraphe consacré aux cytokines régulatrices.

L'IL-1ß et le TNFα ne sont pas les deux seules cytokines impliquées dans la dégénérescence du cartilage. Les chimiokines sont également des médiateurs de ce processus. Les chondrocytes produisent de nombreuses chimiokines de la famille des C-C (C correspondant à cystéine)(RANTES, MIP-1α et ß, MCP-1) et des C-X-C (IL-8, GRO-α)(Tableau I-5) (Henrotin *et al.*, 1996; Borzi *et al.*, 1999; Pulsatelli *et al.*, 1999) et expriment un nombre important de récepteurs membranaires (CCR-1, CCR-2, CCR-3, CCR-5, CXCR-1, CXCR-2) de ces cytokines (Borzi *et al.*, 2000). L'IL-1ß et le TNFα augmentent fortement la production de chimiokines par les chondrocytes humains. L'interaction des chimiokines avec leurs récepteurs active les fonctions cataboliques du chondrocyte. MCP-1, MIP-1α et ß et RANTES diminuent la production de glycosaminoglycanes et augmentent la synthèse de stromélysine-1 par les chondrocytes humains (Borzi *et al.*, 2000; Yuan *et al.*, 2001). De plus, RANTES, GRO-α et IL-8 provoquent une libération massive de N-acétyl-ß-D-glucosaminidase, une enzyme impliquée dans la dégradation des glycosaminoglycanes et de l'acide hyaluronique (Borzi *et al.*, 2000).

Les interleukines (IL) -17 et -18 provoquent également le dérèglement du métabolisme des chondrocytes. Ces cytokines augmentent la production d'IL-1ß, d'IL-6, de MMP-3, d'agrécanases, de •NO synthétase (iNOS) et de cyclooxygénase-2 (COX-2), mais diminuent la production de glycosaminoglycanes par les chondrocytes (Shalom-Barak *et al.*, 1998; Olee *et al.*, 1999; Lubberts *et al.*, 2000; Cai *et al.*, 2001). IL-18 a été localisée par immunohistochimie dans les régions lésées du carti-

Tableau I-5: Les principales chimiokines impliquées dans la dégradation du cartilage

Chimiokines C-C
Regulated upon activation, normal T cell expressed and secreted (RANTES)
Macrophage inflammatory protein-1α et ß (MIP-1α et MIP-ß)
Monocytes chemoattracttant proteins-1 (MCP-1)
Chimiokines C-X-C
Interleukin-8 (IL-8)
Growth related oncogen α (GRO-α)

C = Cystéine

lage arthrosique (Saha *et al.*, 1999; Moldovan *et al.*, 2000). Dans la polyarthrite rhumatoïde, l'IL-17 est produite par les lymphocytes T, essentiellement de la sous-classe CD4+. Actuellement, l'implication de l'IL-17 dans la physiopathologie de l'arthrose reste hypothétique.

On connaît peu de chose sur le rôle joué par le leukemia inhibitory factor (LIF) dans la pathogénie de l'arthrose. Le LIF fait partie de la famille des cytokines dont les effets sont transmis via des récepteurs membranaires formés d'une sous-unité gp130 (Hui *et al.*, 2000b). Le LIF est synthétisé par les chondrocytes et sa production est fortement augmentée par l'IL-1ß et le TNFα (Henrotin *et al.*, 1996). Il stimule la production d'IL-1ß, d'IL-6 et d'IL-8 inhibe la synthèse d'agrécane par les chondrocytes (Henrotin *et al.*, 1994; Bell et Carroll, 1995) et induit la dégradation d'explants de cartilage (Bell *et al.*, 1997; Hui *et al.*, 1998).

b) Les cytokines régulatrices

De nombreuses études réalisées sur des cultures d'explants de cartilage ont montré que l'IL-1ß administrée seule était incapable d'induire la dégradation de la trame de collagène du cartilage. La présence d'autres cytokines, agissant en synergie avec l'IL-1ß, est nécessaire à cet effet (Cawston *et al.*, 1995). L'IL-6, en présence de son récepteur soluble, et l'oncostatine M (OSM) ajoutées conjointement avec l'IL-1α au milieu de culture, induisent *in vitro* la dégradation de la trame de collagène du cartilage, alors que l'administration séparée des ces agents ne s'accompagne d'aucun effet (Milner *et al.*, 2001; Rowan *et al.*, 2001). L'addition simultanée de ces cytokines provoque une augmentation importante de la synthèse de MMP-1, -3 et -13 par les chondrocytes (Milner *et al.*, 2001; Rowan *et al.*, 2001). Dans l'articulation arthrosique, l'IL-6 est produite par une grande variété de cellules dont les lymphocytes B et T, les macrophages, les synoviocytes et les chondrocytes, alors que l'OSM est principalement produite par les macrophages et les lymphocytes T (Horii *et al.*, 1988; Cawston *et al.*, 1998). L'IL-1ß et le TNFα, mais également d'autres agents prophlogistiques (neuropeptides, complexes immuns, anaphylatoxine), stimulent la production de ces cytokines (Guerne *et al.*, 1989; Henrotin *et al.*, 1996). Dans le fluide synovial, le taux d'IL-6 est moins élevé chez les gonarthrosiques que chez les patients souffrant de polyarthrite rhumatoïde. Le taux synovial d'IL-6 n'est pas en

relation avec celui du sulfate de kératane, de la D-pyridinoline ou de la pyridinoline, suggérant que cette cytokine joue un rôle secondaire dans la dégradation du cartilage au cours de l'arthrose (Manicourt D *et al.*, 2000). L'interféron gamma (IFNγ) est aussi impliqué dans la régulation des effets de l'IL-1ß et du TNFα. Il est présent dans le fluide synovial des patients arthrosiques et est essentiellement sécrété par les lymphocytes T (Khale *et al.*, 1992; Schlaak *et al.*, 1996; Sakkas *et al.*, 1998). Cependant, le rôle joué par cette cytokine reste obscur et ses effets sur le cartilage très peu explorés. *In vitro*, il induit la production de FAO et de PGE_2, ainsi que l'expression de l' "Intercellular adhesion molecule-1" (ICAM) et de l'antigène leucocytaire (HLA) de classe II par les chondrocytes (Tiku *et al.*, 1990; Iwasaki Y *et al.*, 1990; Quintavalla *et al.*, 1993; Bujia *et al.*, 1996). Cependant, l'IFNγ est surtout connu pour ses effets modulateurs de l'activité de l'IL-1ß et du TNFα. Il agit en synergie avec le TNFα pour inhiber la synthèse de l'agrécane et des collagènes de type II, IX et XI (Reginato *et al.*, 1993; Dodge *et al.*, 1998). Par contre, il s'oppose aux effets stimulants de l'IL-1ß et du TNFα sur la synthèse de métalloprotéases (Andrews *et al.*, 1990) et inhibe la dégradation de protéoglycanes induite *in vitro* par ces cytokines (Andrews *et al.*, 1989). Enfin, l'IFNγ et l'IL-1ß agissent en synergie pour stimuler la production de •NO, d'IL-6 et d'IL-1Ra et pour inhiber la synthèse d'agrécane. Par contre, l'IFNγ s'oppose à l'effet stimulant de l'IL-1ß sur la synthèse d'IL-8, d'IL-10 et de stromélysine-1 (Henrotin *et al.*, 2000b).

Il existe également des cytokines qui sont capables de s'opposer aux effets délétères de l'IL-1ß et du TNFα. Il s'agit des cytokines dites "anti-inflammatoires". Ce groupe de cytokines inclut l'IL-4, l'IL-10 et l'IL-13. Elles sont principalement synthétisées par les lymphocytes T (Th2) (McKenzie *et al.*, 1993) et exercent leurs effets anti-inflammatoires en bloquant la synthèse d'IL-1ß, de TNFα, d'IL-6 et d'IL-8 par les monocytes et les macrophages (Essner *et al.*, 1989; Hart *et al.*, 1989; De Waal *et al.*, 1991; Zurawski *et al.*, 1993). L'IL-4, l'IL-10 et l'IL-13 sont présentes à des taux anormalement élevés dans le fluide synovial de patients arthrosiques (Schlaak *et al.*, 1996; Leistad *et al.*, 1998). La présence de ces cytokines à été mise en évidence par immunohistochimie dans le cytoplasme des chondrocytes arthrosiques (Moos *et al.*, 1999; Iannone *et al.*, 2001). Les chondrocytes arthrosiques synthétisent plus d'IL-10 que les normaux. Par contre, l'expression du récepteur de l'IL-10 reste stable quel que soit le degré de sévérité de l'arthrose (Iannone *et al.*, 2001).

En présence de TNFα, l'IL-4, l'IL-10 et l'IL-13 inhibent la production de PGE_2 par des fibroblastes synoviaux arthrosiques cultivés en monocouche. Dans le cas de l'IL-4 et de l'IL-10, cet effet est associé à une diminution de la synthèse de COX-2 et de phospholipase A2 cytoplasmique (cPLA2). L'IL-13 diminue seulement la synthèse de COX-2 (Alaaeddine *et al.*, 1999; Lubberts *et al.*, 2000). Les fibroblastes synoviaux expriment les récepteurs R55 et R75 pour le TNF. L'IL-10 inhibe la liaison du TNFα à ces récepteurs membranaires, et augmente la libération dans le milieu extracellulaire du récepteur R75 (sTNF-R75) (Alaaeddine *et al.*, 1999). L'IL-4 et l'IL-13 s'opposent à la dégradation de la matrice extracellulaire d'explants de cartilage nasal de bœuf induite par l'association d'IL-1α et d'OSM (Cawston *et al.*, 1996; Cleaver *et al.*, 2001). Cet effet chondroprotecteur est le résultat d'une diminution

de la synthèse de MMP-3 et -13 et d'une augmentation de la production de TIMP-1 par les chondrocytes (Cawston *et al.*, 1996; Cleaver *et al.*, 2001).

c) Les cytokines anaboliques

De nombreuses cytokines anaboliques sont impliquées à différents stades de la pathogénie de l'arthrose. Les plus importantes sont les insulin-like growth factors (IGFs), les transforming growth factors (TGFßs), les fibroblast growth factors (FGFs) et le platelet-derived growth factor (PDGF). Il nous est impossible de commenter dans ce chapitre, les effets de l'ensemble de ces facteurs de croissance. Nous avons donc choisi de focaliser notre attention sur les effets des TGFßs et des IGFs, qui sont les deux facteurs les plus largement étudiés.

Les insulin-like growth factors (IGFs) ont des activités biologiques similaires à celles de l'insuline. Les chondrocytes synthétisent de l'IGF-1 et de l'IGF-2 (Keyszer *et al.*, 1995; Sonal, 2001; Nixon *et al.*, 2001). Le fluide synovial des patients arthrosiques contient plus d'IGF-1 que celui des sujets normaux alors que le taux d'IGF-2 est identique dans les deux groupes (Tavera *et al.*, 1996). Ceci suggère que l'IGF-1 soit un facteur actif dans la pathogénie de l'arthrose. L'IGF-1 est un puissant stimulateur de la synthèse d'agrécane et de collagène de type II par les chondrocytes et un inhibiteur de la dégradation du cartilage (Hui *et al.*, 2001). Dans le cartilage normal, l'IGF-1 n'a pas d'effet mitogénique. Par contre, il stimule la prolifération des chondrocytes des régions lésées et pourrait, par ce mécanisme, contribuer à la formation d'amas cellulaires (clusters) (Martel-Pelletier *et al.*, 1998). La sensibilité des chondrocytes à l'action de l'IGF-1 diminue avec l'âge et la sévérité des lésions arthrosiques (Dore *et al.*, 1995, Loeser *et al.*, 2000). A un stade avancé de la maladie, les chondrocytes arthrosiques deviennent même insensibles à l'IGF-1 (Doré S *et al.*, 1995). Ce phénomène peut s'expliquer par la présence dans l'environnement de la cellule d'IGF binding proteins (IGFBPs) qui en se fixant à l'IGF-1 modulent son activité biologique (Matsumoto *et al.*, 2000). Il existe six IGFBPs dont quatre ont été identifiées dans le fluide synovial de patients arthrosiques. Il s'agit des IGFBP-1, -2, -3 et -4. La concentration d'IGFBP-3, mais pas celle des autres IGFBPs, est augmentée dans le fluide synovial des patients arthrosiques (Tavara *et al.*, 1996). Selon des études récentes effectuées sur des souris déficientes en iNOS (iNOS knock-out), le •NO pourrait être l'agent responsable de l'hyposensibilité des chondrocytes à l'IGF-1 (Studer *et al.*, 2000). De plus, l'exposition des chondrocytes de lapin à des donneurs de •NO diminue l'effet stimulant de l'IGF-1 sur la synthèse de glycosaminoglycanes. Des chondrocytes génétiquement modifiés par l'introduction du transgène iNOS, sont également moins sensibles à l'action de l'IGF-1. L'ensemble de ces observations démontre le rôle inhibiteur du •NO sur les réponses du chondrocyte aux IGFs.

Il existe trois iso-formes de TGFß chez les mammifères. Ils ont une origine génétique différente, mais possèdent des structures relativement homologues ($\simeq$80 % d'homologie de la séquence en acides aminés) et des effets similaires sur de nombreux modèles expérimentaux. Les chondrocytes sécrètent les trois iso-formes de TGFß (TGFß-1, ß-2, ß-3) (Vivien *et al.*, 1992) et expriment au moins les trois prin-

cipaux récepteurs des TGFßs (TGFß-RI, -RII et -RII)(Wrana *et al.*, 1994). Cependant, le TGFß-1 est la forme majoritaire dans le cartilage (Moldovan *et al.*, 2000). Les TGFßs sont produits par les chondrocytes sous une forme latente associée à un peptide dénommé Latency Associated Peptide (LAP) (Fava *et al.*, 1989). L'activation des TGFßs nécessite l'intervention de protéases qui clivent le complexe TGFß-LAP. Plusieurs protéases à sérines, comme la plasmine et une furine convertase, semblent impliquées dans l'activation des TGFßs (Moldovan *et al.*, 2000). *In vitro*, le TGFß-1 inhibe la synthèse des MMP-1 et -3, mais augmente celle de TIMP-1 (Gunther *et al.*, 1994; Henrotin *et al.*, 2000a; Moldovan *et al.*, 2000). Il agit également sur la synthèse des protéines matricielles du cartilage. Il augmente, entre autre la synthèse d'agrécane et de petits protéoglycanes (biglycane et décorine) par les chondrocytes (van der Kraan *et al.*, 1992; Demoor-Fossard *et al.*, 1998; Hui *et al.*, 2000a). De plus, le TGFß-1 s'oppose à de nombreux effets délétères de l'IL-1ß, dont l'inhibition de la synthèse de protéines matricielles et la stimulation de MMPs. Il bloque également la dégradation du réseau de collagène induit *in vitro* par l'IL-1α et l'OSM (Hui *et al.*, 1999). Chez le lapin, dont l'arthrose était induite par la section des ligaments croisés du genou, l'expression du TGFß-1 et ß-2 était inchangée durant la phase précoce de la pathologie correspondant au stade de cartilage hypertrophique (3ème semaine après la lésion), mais ensuite diminuait considérablement au stade fibrillaire de la maladie (8ème semaine après la section). Sur ce même modèle, l'expression des récepteurs de type II des TGFßs, avait diminué dès les premiers stades de la pathologie, alors que l'expression du récepteur I restait inchangée (Boumediene *et al.*, 1998). Enfin, des souris n'exprimant pas le récepteur II des TGFßs, développent spontanément de l'arthrose, ce qui démontre l'importance de ce récepteur dans le contrôle de l'homéostasie du cartilage. Lorsque le TGFß-1 est injecté dans l'articulation, il induit la formation d'ostéophytes et l'épaississement des ligaments et des tendons (van Beuningen *et al.*, 1994; van den Berg *et al.*, 1999). Ces modifications contribuent à l'ankylose de l'articulation arthrosique.

1.3.3 Les formes activées de l'oxygène (FAO)

In vivo, il existe deux enzymes de base à l'origine de la cascade de formation des formes activées de l'oxygène (FAO). D'une part, la filière du monoxyde d'azote (•NO) qui est produit par les •NO synthétases, et d'autre part la filière de l'anion superoxyde ($O_2^{\bullet}$) qui découle, de l'activité de la NADPH oxydase. A partir de •NO et de $O_2^{\bullet}$, les autres FAO sont produites en cascade.

Les chondrocytes produisent des FAO (•NO, H_2O_2, •OH, $O_2^{\bullet}$) (Tiku *et al.*, 1990; Henrotin *et al.*, 1993; Rathakrishnan *et al.*, 1993; Hiran *et al.*, 1998; Tiku *et al.*, 1998) et possèdent une NADPH-oxydase (Hiran *et al.*, 1997; Moulton *et al.*, 1998). *In vitro*, la production de FAO par les chondrocytes est stimulée non seulement par des agents mitogènes tels que la concavaline A ou le calcium ionophore A23187, mais également par des activateurs de la protéine kinase C comme OAG (1-oleyl-2acétyl-sn-glycérol) et le TPA (12-0-tetradecanoylphorbolmyristate acétate), par des

cytokines (TNFα, l'IFNγ et l'IL-1α), des immunoglobulines (IgGs) ou encore des stimuli mécaniques (Henrotin *et al.*, 1992; Rathakrishnan *et al.*, 1992; Fermor *et al.*, 2001). En 1993, notre équipe a montré que des cycles d'anoxie-réoxygénation provoquaient une libération massive de FAO, probablement par l'altération du transport d'électrons au niveau de la chaîne mitochondriale (Henrotin *et al.*, 1993).

Les formes activées de l'oxygène sont des médiateurs de la dégradation du cartilage. Elles sont capables de réagir avec les protéines matricielles et d'altérer leur structure primaire, secondaire et tertiaire, au point de les dénaturer (Davies *et al.*, 1987). Dans le cartilage, l'agrécane, l'acide hyaluronique et les collagènes sont dégradées par oxydation, sous l'action des FAO (Roberts *et al.*, 1989; Bates *et al.*, 1984; Panasyuk *et al.*, 1994). Les FAO sont également capables d'activer les métalloprotéases latentes et d'inhiber l'activité de l'α1-inhibiteur de protéases. De cette façon, les FAO diminuent les mécanismes de défense de l'organisme contre l'action des protéases (Burkhard *et al.* 1986). L'exposition des chondrocytes à des concentrations élevées d'H_2O_2 (10^{-4} M) provoque une diminution de la synthèse des protéines matricielles du cartilage (Schalwijk *et al.*, 1985). Cette diminution est liée à une chute de la concentration intracellulaire en ATP due à l'activation de la glycéraldéhyde-3-phosphate déshydrogénase (Baker *et al.*, 1988). Les FAO sont aussi impliquées dans la transduction du signal intracellulaire. Elles modulent l'activation de certains facteurs de transcription (NF-κB, AP-1, etc.) et de certaines kinases (Li WQ *et al.*, 2000) en réponse, par exemple, à des cytokines (TNFα, IL-1ß) (Lo *et al.*, 1998).

Récemment, l'intérêt des chercheurs s'est porté sur les propriétés biologiques du monoxyde d'azote (•NO) et de ses dérivés. Le •NO est produit par les •NO synthétases, constitutive et inductible. La synthèse de iNOS est induite par l'IL-1ß, l'IL-17, le TNFα, l'IFNγ, mais réprimée par les TGFßs, l'IL-4 et l'IL-10 (Stadler *et al.*, 1993; Palmer *et al.*, 1993; Attur *et al.*, 1997; Hayashi *et al.*, 1997; Henrotin *et al.*, 2000b). Une forme chondrocytaire spécifique de la •NO synthétase (131 kDa) a été clonée et séquencée (Charles *et al.*, 1993). Elle présente 70 % d'homologie de séquence en acides aminés avec les formes endothéliale et neuronale. Le •NO est un composé instable (durée de vie < à 15 sec) mais très réactionnel. Il exerce ses activités biologiques en: 1) activant la guanylate cyclase et par conséquent, en augmentant le taux intracellulaire de guanosine-monophosphate cyclique (cGMP), 2) en réagissant avec les groupements thiols des protéines conduisant à la formation de composés stables et actifs, les S-nitrosothiols (durée de demi-vie > à 2 heures), 3) en réagissant avec l'oxygène pour donner une suite de dérivés nitrés aboutissant au nitrite et nitrate stables, 4) en formant, en présence d'anion superoxyde, le peroxynitrite (-ONOO) composé très oxydant, enfin, 5) par la nitrosation directe de protéines cibles. Le peroxynitrite est un intermédiaire azoté particulièrement intéressant, car il est impliqué dans l'oxydation et la nitration de protéines clés de la signalisation cellulaire (Go *et al.*, 1999; Valdez *et al.*, 2000).

En comparaison avec les chondrocytes normaux, les chondrocytes arthrosiques produisent une quantité plus élevée de •NO dans les conditions basales ou en réponse à IL-1 (Pelletier *et al.*, 1996). De plus, des taux anormalement élevés de

nitrite et de nitrate ont été mesurés dans le fluide synovial et le sérum des patients souffrant d'arthrose (Farrell *et al.*, 1992; Grabowski *et al.*, 1997).

Il est actuellement reconnu que la production locale de fortes concentrations de •NO dérègle les fonctions métaboliques du chondrocyte, dont l'inhibition de la synthèse de collagènes et de glycosaminoglycanes (Taskiran *et al.*, 1994), l'activation des métalloprotéases (Murrel *et al.*, 1995), la diminution de la synthèse d'IL-1Ra (Pelletier *et al.*, 1996), et l'induction de l'apoptose (Blanco *et al.*, 1995a). Le •NO est également un médiateur des effets de l'IL-1ß sur la synthèse de MMPs (Evans et Stefanovic-Racic, 1996; Lo *et al.*, 1998; Sasaki *et al.*, 1998). Il inhibe aussi la consommation d'oxygène, la production d'ATP, la prolifération et la migration des chondrocytes (Blanco *et al.*, 1995b; Frenkel *et al.*, 1996; Tomita *et al.*, 2001)

Dans l'organisme, la réactivité des FAO est limitée par des antioxydants naturels qui sont soit des piégeurs de radicaux libres (protection stoechiométrique), soit des piégeurs des métaux de transition (protection anticatalytique; par exemple les sidérophores comme la déferoxamine), soit des enzymes spécialisées dans la destruction des FAO (protection catalytique) comme les superoxydes dismutases (Cu/ZnSOD et MnSOD), la catalase et la glutathion peroxydase (Deby et Goutier, 1990; MacNee , 2001). Les chondrocytes synthétisent des superoxides dismutases, de la catalase et de la glutathione peroxydase (Bakker *et al.*, 1988; Deahl *et al.*, 1992; Graziolli *et al.*, 1998; Mazetti *et al.*, 2000). Le taux intracellulaire de Cu/ZnSOD et de MnSOD est fortement augmenté par l'IL-1ß et le TNFα (Mazzetti *et al.*, 2001). La formation de •NO peut être bloquée par des inhibiteurs compétitifs de la •NO synthétase tels que le N^G-monométhyl-L-arginine (L-NMMA) ou le L-iminoéthyl-L-lysine (L-NIL). L'injection intra-articulaire de ces inhibiteurs ralentit la progression des lésions du cartilage dans des modèles expérimentaux d'arthrite et d'arthrose (Pelletier *et al.*, 1999; Pelletier *et al.*, 2000a).

1.3.4. Les prostanoïdes et les leucotriènes

L'expression de COX-2 par les chondrocytes arthrosiques est accrue, et des taux anormalement élevés de prostaglandine (PG) E_2 sont produits par ces cellules (Amin *et al.*, 1997b). Les chondrocytes possèdent des récepteurs membranaires pour la PGE_2, et la fixation de ce récepteur s'accompagne d'une élévation du taux d'AMPc intracellulaire et d'une série de réponses divergentes (Amin *et al.*, 2000). La PGE_2 augmente la production d'IGF-1 par les chondrocytes, et par conséquent la production de protéines matricielles (Di Battista *et al.*, 1996). L'addition de PGE_2 au milieu de culture de chondrocytes induit l'apoptose des chondrocytes après 24 heures d'incubation (Miwa *et al.*, 2000).

Les chondrocytes arthrosiques sécrètent aussi des produits de la voie des lipoxygénases, comme le leucotriène B4 (LTB_4) (Tawara *et al.*, 1991; Fermor *et al.*, 2001). Le taux de LTB_4 est augmenté dans le fluide synovial des patients arthrosiques, suggérant que ce médiateur soit impliqué dans la pathogénie de l'arthrose (Atik *et al.*, 1990). *In vitro*, il stimule la libération d'IL-1ß par les cellules de la membrane synoviale (Rainsford *et al.*, 1996). Actuellement, le rôle joué par ce

médiateur dans la dégradation du cartilage est méconnu.

1.4. Les mécanismes pathogéniques de l'arthrose

Comme nous venons de le décrire, les facteurs biochimiques impliqués dans l'initiation et la progression de l'arthrose sont multiples. Ils exercent leur activité biologique au sein de trois boucles pathogéniques mettant en relation le cartilage, la membrane synoviale et l'os sous-chondral (figure I-8). Le chondrocyte occupe une position centrale et joue un rôle primordial dans l'ensemble de ces mécanismes physiopathologiques.

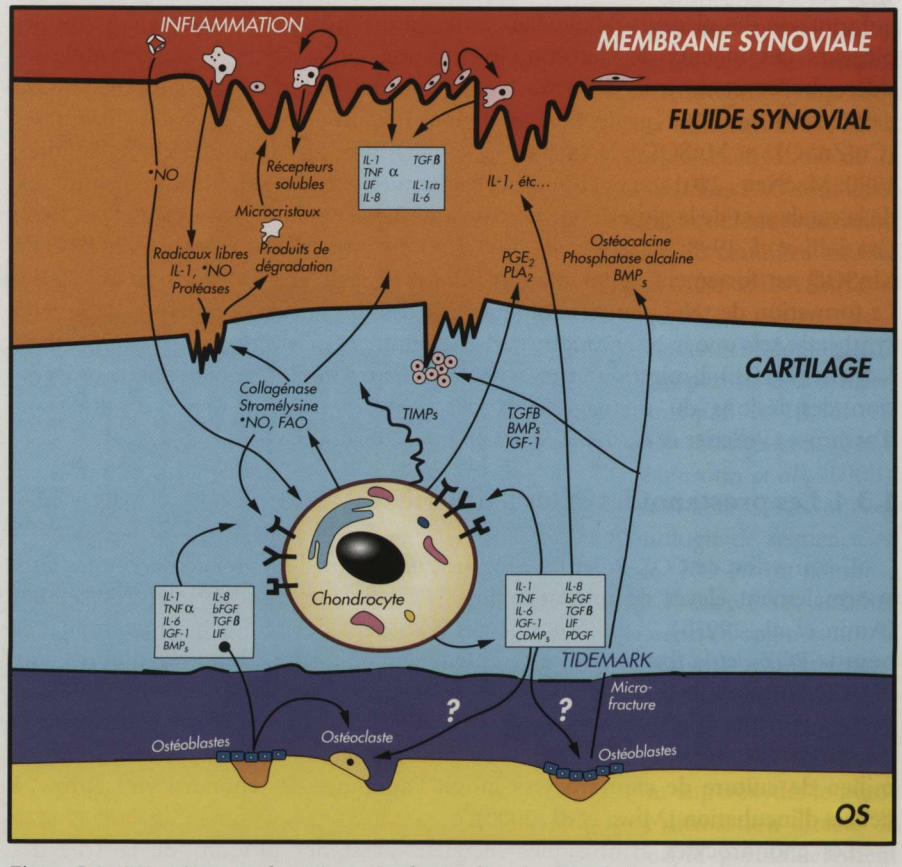


Figure I-8: *Mécanismes pathogéniques impliqués dans la dégradation du cartilage au cours de l'arthrose et organisation des facteurs biochimiques au sein de trois boucles.*

1.4.1. La boucle cartilagino-cartilagineuse

Le chondrocyte est l'acteur principal de la dégradation de la matrice du cartilage. Il sécrète dans son environnement immédiat des métalloprotéases et des FAO qui sont responsables de la chondrolyse péri-cellulaire. Il synthétise également des cytokines (IL-1ß, TNFα, TGFßs, IL-6, etc.) qui régulent de façon autocrine ses propres fonctions métaboliques (Henrotin et Reginster, 1999a; Tetlow *et al.*, 2001).

Chez l'animal, une lésion mécanique du cartilage induit d'importantes modifications métaboliques et morphologiques des chondrocytes disposés au voisinage de celle-ci (Adams et Brandt, 1991; van der Kraan *et al.*, 1992; Appleyard *et al.*, 1999) (Tableau I-6).

Tableau I-6: Caractéristiques du phénotype du chondrocyte arthrosique.

Fonctions métaboliques augmentées
Synthèse de collagène de type II et d'agrécane
Prolifération (clone) (stade précoce de la maladie)
Production de cytokines cataboliques (IL-1, TNF, etc.)
Protéases (MMPs, agrécanases, cathepsines, etc.)
IGFBP-3
Récepteur pour l'IL-1 de type I
Apoptose, iNOS, COX2
Synthèse de collagènes de type VI, III, X, IIA
Synthèse de fibronectine, tenascine, ostéonectine
Fonctions métaboliques diminuées
Synthèse de collagène de type II et d'agrécane (stade évolué de la maladie)
TIMPs
IL-1Ra
Sensibilité à l'IGF-1

MMPs= métalloprotéases; IGFBP-3 = insulin-like growth factor binding protein-3; NOS = NO synthétase; COX-2 = cyclooxygénase-2; IGF-1 = insulin-like growth factor-1; TIMPs = inhibiteurs tissulaires de métalloprotéases; IL-1Ra = Interleukin-1 receptor antagonist.

Rapidement, les chondrocytes se divisent, forment des agrégats "clusters" (Ghosh, 1999) et présentent une activité synthétique accrue (Kusuzaki *et al.*, 2001). Ils produisent du collagène de type III et X, normalement absent du cartilage, et des quantités importantes de fibronectine (Homandberg *et al.*, 1998; Yasuda et Poole, 2002). Cette phase est dite " hypertrophique " et s'accompagne d'un épaississement du cartilage. Elle est considérée comme une véritable tentative du cartilage à réparer la lésion. Cependant, cette phase est transitoire et est suivie par une accélération du turnover des constituants matriciels et d'une ossification endochondrale (Kirsch *et al.*, 2000). Les processus de chondrorésorption prennent progressivement le pas sur les processus de chondroformation et les chondrocytes meurent par apoptose ou nécrose (Figure I-9).

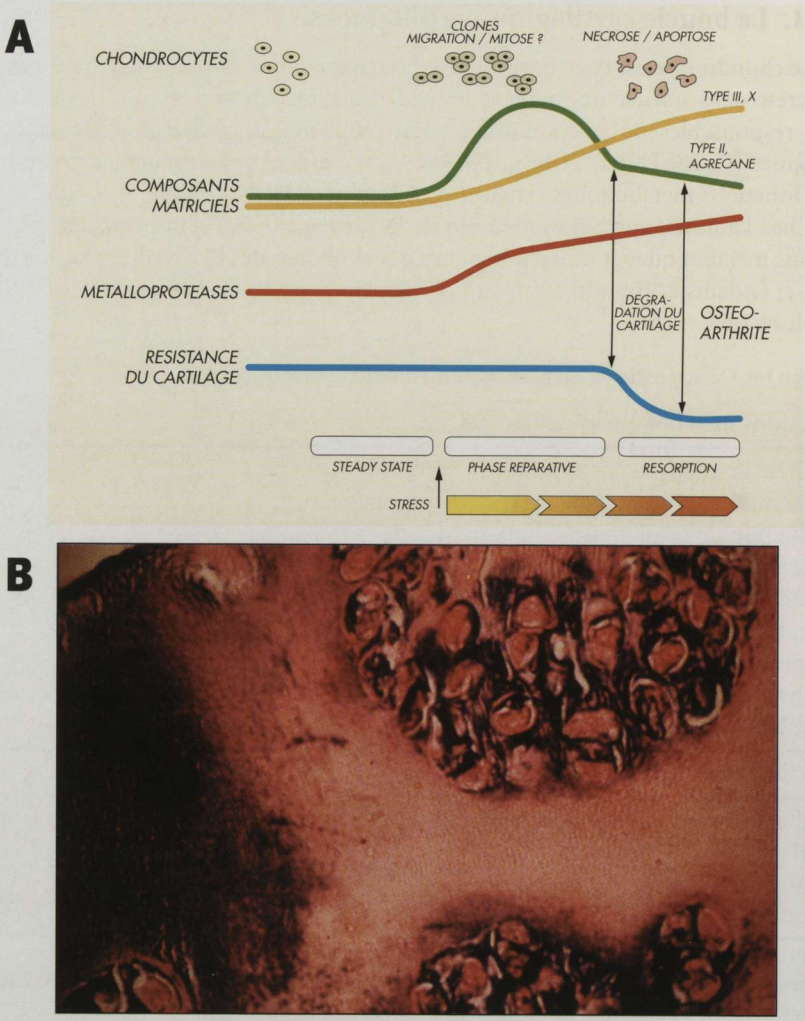


Figure I-9: *A) Comportement du chondrocyte dans les modèles animaux d'arthrose induite par rupture du ligament croisé ou ménisectomie (Selon Henrotin et al., 1999a, annexe 2), B) Vue microscopique de clones de chondrocytes chez l'homme.*

Chez l'homme, on peut également observer la formation de clones même aux stades les plus avancés de la maladie (Kusuzaki *et al.*, 2001). Des chondrocytes apoptotiques ou nécrotiques sont également visibles (Tew *et al.*, 2000). Le nombre de cellules en apoptose dans le cartilage arthrosique varie entre 0 et 6 % selon les auteurs et la sévérité des lésions dégénératives. Ces résultats suggèrent que l'apoptose des chondrocytes n'est pas un processus pathogénique important de cette maladie (Blanco *et al.*, 1998; Hashimoto *et al.*, 1998; Kouri *et al.*, 2000; Aigner *et al.*, 2001; Kim *et al.*, 2001).

1.4.2. La boucle synovio-cartilagineuse

Sur le plan histologique, l'arthrose s'accompagne toujours d'une inflammation de la membrane synoviale qui se limite à une congestion vasculaire, accompagnée dans les formes les plus sévères, d'une infiltration cellulaire (essentiellement des cellules mononucléées, macrophages, lymphocytes B et T) (Nakamura *et al.*, 1999; Krenn *et al.*, 1999). Cette réaction inflammatoire est secondaire à la libération dans le fluide synovial de débris ostéo-cartilagineux et/ou à la présence de microcristaux (pyrophosphate ou hydroxyapatite). Les chondrocytes jouent également un rôle déterminant dans l'initiation et la chronicité de la réaction inflammatoire. Les chondrocytes arthrosiques ont des similitudes avec les macrophages au niveau de leurs sécrétions (Figure I-10) (Henrotin *et al.*, 1996; Amin, 1999; Attur *et al.*, 2000; Tiku *et al.*, 2000; Sandell et Aigner, 2001). En effet, comme les macrophages, les chondrocytes activés produisent des quantités anormalement élevées de cytokines, prostanoïdes et formes activées de l'oxygène. Ces médiateurs de l'inflammation libérés par les chondrocytes régulent à leur tour l'activité synthétique des cellules de la membrane synoviale (synoviocytes et cellules de la réaction inflammatoire). En réponse à ces stimuli, les cellules de la membrane synoviale sécrètent des enzymes et des FAO qui s'attaquent à la couche la plus superficielle du cartilage. De plus, elles

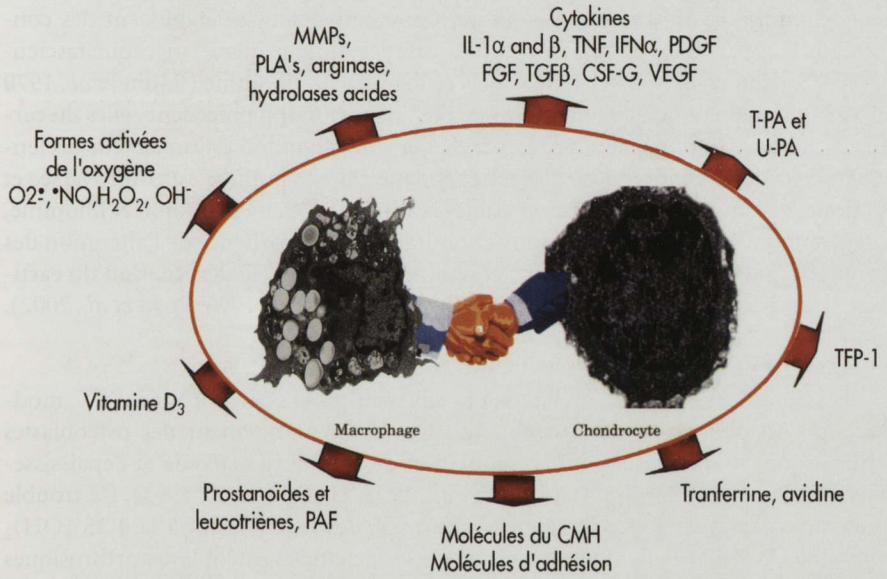


Figure I-10: *Médiateurs communs sécrétés par les macrophages et les chondrocytes arthrosiques (Selon Attur et al., 2002). COX = cyclooxygénase; FGF = Fibroblast growth factor; G -CSF = Granulocyte colony stimulating factor; IL-1 α et ß = interleukine-1 alpha et bêta; IFNα = interféron alpha; MIP-1ß = Macrophage inflammatory protein-1 beta; PAF = Platelet activating factor; PDGF = Platelet-derived growth factor; PLA = Phospholipase; TFPI = tissue factor pathway inhibitor; TNF-α = tumor necrosis factor alpha; u-PA = urokinase-plasminogen activator; u-PAR = urokinase plasminogen activator receptor; VEGF = Vascular endothelial growth factor; CMH = Complexe Majeur d'Histocompatibilité.*

libèrent des cytokines (IL-1ß, TNFα, etc.) qui stimulent les chondrocytes à produire davantage de facteurs cataboliques (MMPs et autres protéases, FAO). Ainsi s'installe un cercle vicieux entre le cartilage et la membrane synoviale responsable de la progression des lésions structurelles du cartilage arthrosique (Spector *et al.*, 1997; Sharif *et al.*, 2001).

1.4.3. La boucle ostéo-cartilagineuse

Dans l'articulation, l'os et le cartilage sont intiment liés. Ils forment une unité biomécanique responsable de l'amortissement de 35% des contraintes mécaniques exercées sur l'articulation. L'os sous-chondral en absorbe 30% et le cartilage seulement de 5%. Il en découle que toute modification des propriétés mécaniques de l'os sous-chondral se répercute sur les capacités d'amortissement de l'articulation. Deux théories tentent d'expliquer le rôle joué par l'os sous-chondral dans la dégradation du cartilage. Il s'agit de la théorie **mécanique** et de la théorie **biochimique**.

Théorie mécanique

L'épaississement de l'os sous-chondral, à cause de la rigidité qui en découle, contribue à l'augmentation des contraintes mécaniques exercées sur le cartilage. Ces agressions mécaniques peuvent provoquer des micro-fractures au niveau de l'os sous-chondral et du cartilage sus-jacent. Ces micro-fractures établissent des connexions entre ces deux tissus. Des études réalisées sur le primate (macaque fascicularis) (Carlson *et al.*, 1994 et 1996), mais également sur l'homme (Radin *et al.*, 1970 et 1986), indiquent que les altérations de l'os sous-chondral précèdent celles du cartilage, et que le durcissement progressif de l'os sous-chondral est un facteur déclenchant de la dégradation du cartilage. Cependant, cette hypothèse est controversée et toujours discutée. En effet, d'autres études réalisées sur le chien, le lapin et l'homme, suggèrent que la sclérose de l'os sous-chondral est la conséquence de l'altération des propriétés mécaniques du cartilage, et donc apparaît après la dégradation du cartilage (Dedricks *et al.*, 1993; Layton *et al.*, 1993; Watson *et al.*, 1996; Boyd *et al.*, 2002).

L'approche biochimique

Les modifications structurelles de l'os sous-chondral s'accompagne d'une modification du phénotype des ostéoblastes. Ce dysfonctionnement des ostéoblastes arthrosiques se manifeste par l'accumulation de substance ostéoïde et l'épaississement de l'os sous chondral (Grynpas *et al.*, 1991; Hanaan *et al.*, 1993). Ce trouble pourrait s'expliquer par une réponse anormale des ostéoblastes à la 1,25 $(OH)_2$ vitamine D3 (Hilal *et al.*, 1998). En réponse à ce facteur, les ostéoblastes arthrosiques produisent des quantités anormalement élevées d'ostéocalcine et de phosphatase alcaline. De plus, les ostéoblastes arthrosiques produisent beaucoup plus d'urokinase (u-PA), d'IGF-1 et de PGE_2 que les ostéoblastes normaux (Hilal *et al.*, 1998 et 2001). Ces facteurs, par des mécanismes de régulation autocrine et paracrine, influencent le remodelage de l'os sous chondral et contribuent à son épaississement.

Il est également suggéré qu'ils puissent, par les micro-fractures ostéo-chondrales, atteindre les chondrocytes de la couche profonde du cartilage sus-jacent, et influencer leur activité synthétique (Sokoloff, 1993). Récemment, des études *in vitro*, ont démontré que le milieu de culture conditionné par des ostéoblastes arthrosiques, provoquait la dégradation d'explants de cartilage (Westacott *et al.*,1997; Hilal *et al.*, 1998).

En conclusion, l'arthrose est une maladie multifactorielle touchant l'ensemble des tissus articulaires et péri-articulaires. Les tissus arthrosiques sont le siège de modifications morphologiques et structurelles, qui provoquent progressivement l'impotence de l'articulation. De nombreux facteurs biochimiques sont impliqués dans ces altérations tissulaires, et constituent des cibles potentielles pour les traitements actuels et futurs de l'arthrose.

CHAPITRE 2

Etude du métabolisme des chondrocytes arthrosiques articulaires humains en culture en billes d'alginate

Etude des effets de l'interleukine-1 bêta (IL-1ß) sur le métabolisme des chondrocytes.

2.1. Introduction

Durant la dernière décennie, de nombreux modèles de cultures de chondrocytes ont été proposés (voir article de revue, annexe 1, Henrotin et Reginster, 1999b). La problématique de la culture des chondrocytes est double: d'une part recréer *in vitro* l'environnement naturel de la cellule (PO_2, pression osmotique, pH du milieu, contraintes mécaniques, etc.) et d'autre part maintenir stable son phénotype. *In vivo*, le phénotype du chondrocyte se caractérise par des synthèses spécifiques (par ex. collagène de type II), une forme arrondie et une activité mitotique extrêmement limitée. Lorsqu'ils sont cultivés en monocouche, les chondrocytes se dédifférencient (Lemare *et al.*, 1998). Ils adoptent une forme allongée, se divisent intensément et modifient qualitativement et quantitativement leur activité synthétique. Les synthèses de collagènes de type II, IX et XI et d'agrécane diminuent alors que celles de versicane et de collagènes de type I et III, molécules qui sont absentes du cartilage normal, augmentent progressivement avec le processus de dédifférenciation (von der Mark *et al.*, 1977; Elima et Vuorio, 1989; Lefebvre *et al.*, 1990). Un moyen d'empêcher la dédifférenciation des chondrocytes en culture consiste à les placer soit en suspension en phase liquide, soit dans des matrices naturelles ou synthétiques de substitution (Bassleer *et al.*, 1986; Martinsen *et al.*, 1989; Sittinger *et al.*, 1993; Freed *et al.*, 1994; Bujia *et al.*, 1995; van Susante *et al.*, 1995; Fujisato *et al.*, 1996; Fortier *et al.*, 1997).

Dans ce travail, nous avons choisi de cultiver des chondrocytes humains en billes d'alginate. L'alginate est un polysaccharide linéaire isolé de l'algue brune. En présence de cations divalents comme le Ca^{++}, ce polymère se gélifie instantanément et forme une matrice poreuse autour des chondrocytes (Smidsrod et Skjak-Braek, 1990; Gombotz et Wee, 1998; Hedbom *et al.*, 2001). Dans ces conditions, les chondrocytes conservent leur phénotype au moins cinq semaines (Hauselmann *et al.*, 1992 et 1994; Binette *et al.*, 1998; Chubinskaya *et al.*, 2001).

Ce modèle offre de nombreux autres avantages:

- La matrice néo-synthétisée présente une compartimentation identique à celle retrouvée dans le cartilage hyalin *in vivo*. La microscopie électronique permet de distinguer très nettement deux compartiments. La matrice entourant la cellule ou "Cell-associated Matrix (CM)" qui correspond à la matrice péri-cellulaire et territoriale de la matrice du cartilage *in vivo*, et d'autre part la matrice contenue dans l'alginate ou "Further Removed Matrix (FRM)" qui correspond à la matrice inter-territoriale du cartilage (Figure II-1).
- Les cellules et la matrice extracellulaire néo-synthétisée peuvent être facilement récupérées, après dépolymérisation de l'alginate par addition d'un chélateur de calcium (citrate). Il est donc possible d'effectuer des analyses quantitatives et qualitatives des fractions CM et FRM.
- Les volumes occupés par les différents compartiments de la bille d'alginate correspondent à ceux observés dans le cartilage normal. Au terme de 13 jours de culture, les cellules, la CM, et la FRM représentent respectivement 3,5, 5,2 et 91, 3 % du volume de la bille (van Osch *et al.*, 1998).

- La demi-vie ($t_{1/2}$) de l'agrécane est plus longue que celle enregistrée dans d'autres modèles de culture, et se rapproche de la valeur observée *in vivo*. Dans la FRM, la $t_{1/2}$ de l'agrécane est de 95 jours (Mok *et al.*, 1994) alors qu'elle est de 40 jours dans les cultures d'explants et de 14 jours dans l'agarose (Campbell *et al.*, 1984; Aydelotte *et al.*, 1988; Barone-Valeras *et al.*, 1991).

Au début de notre travail, les effets d'une administration prolongée d'IL-1ß sur le métabolisme des chondrocytes articulaires humains cultivés en billes d'alginate n'avaient pas encore été investigués. Les objectifs de l'étude décrite dans ce chapitre sont:

- D'analyser les synthèses d'IL-6, d'IL-8, de MIP-1ß, d'agrécane, de stromélysine-1 (MMP-3), de TIMP-1, de PGE_2 et de •NO par les chondrocytes humains arthrosiques cultivés en bille d'alginate durant 12 jours;
- De connaître la distribution de ces molécules dans les différents compartiments de la culture en billes d'alginate (CM, FRM et milieu de culture conditionné);
- D'étudier les effets de l'administration répétée d'IL-1ß sur le métabolisme des chondrocytes;
- Et enfin, de valider un modèle de culture de chondrocytes qui sera ultérieurement utilisé pour l'étude du profil pharmacologique des médicaments utilisés dans le traitement de l'arthrose.

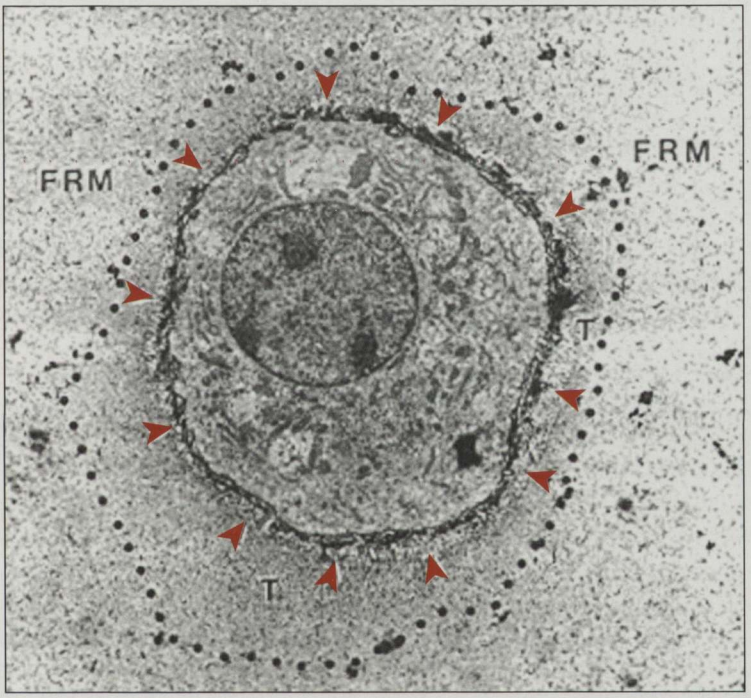


Figure II-1: *Micrographie électronique d'un chondrocyte cultivé en billes d'alginate durant 13 jours. T = matrice territoriale; FRM = Further Removed Matrix; ➤ = matrice péri-cellulaire ou CM. (Hauselmann et al.., 1996)*

2.2 Matériels et méthodes

Dans ce travail, nous avons utilisé des chondrocytes humains (voir annexes 16, 18 et 19) isolés à partir d'échantillons de cartilage arthrosique prélevés post mortem au niveau du genou. Les chondrocytes fraîchement isolés étaient enchâssés dans une matrice d'alginate (Macrocystis pyrifera; 1,2 %) et cultivés en billes d'alginate (~3 mm de diamètre) pendant 12 jours. Le milieu nutritif, contenant ou non de l'IL-1ß, était renouvelé tous les trois jours. Le milieu de culture était composé de Dulbecco's Modified Eagle's Medium (DMEM) contenant 10 mM d'HEPES, 1 % d'une solution d'antibiotiques et d'antimycotiques (10.000 U/ml de pénicilline, 10.000 mg/ml de streptomycine), 50 µg/ml d'acide ascorbique, 2 mM de proline, 200 µg/ml de glutamine et 1 % (v/v) d'ITS+. L'ITS+ contient dans un millilitre: 0,625 mg d'insuline, 0,625 mg de transferrine, 0, 625 µg de sélénium, 0,125 g d'albumine sérique de bœuf et 0,535 mg d'acide linoléique. Les résultats présentés dans ce chapitre sont les productions moyennes calculées à partir des valeurs de 10 cultures indépendantes réalisées avec des chondrocytes provenant de 10 donneurs arthrosiques différents. Les paramètres suivants ont été étudiés:

- L'ADN a été mesuré dans les extraits cellulaires par la technique de Labarca et Paigen (1984). Cette méthode est basée sur le principe de la fluorescence émise par un réactif, le bis-benzimidazole (Calbiochem-Behring, La Jolia, CA, composé Hoechst dye 33258);
- La synthèse d'ADN a été appréciée par l'incorporation de thymidine marquée au tritium (méthyl-^{3}H-thymidine, ICN biomedical, Asse-Relegem, Belgique) dans l'ADN néo-synthétisé. Dans ce protocole, la ^{3}H-thymidine était ajoutée au milieu de culture durant les 24 dernières heures de chaque période d'incubation;
- L'agrécane, l'IL-6, l'IL-8, le MIP-1ß, le TIMP-1, et la MMP-3 ont été quantifiés dans les milieux de culture conditionnés par les chondrocytes, mais également dans les fractions CM et FRM de la bille d'alginate, par des dosages immuno-enzymatiques (EASIA; Solid Phase Enzyme Amplified Sensitivity Immunoassay; Biosource Europe, Fleurus, Belgique).
- La production de monoxyde d'azote (•NO) a été appréciée par le dosage du NO_2^- par la méthode de spectrophotométrie basée sur la réaction de Griess (Green *et al.*, 1982). Dans cette méthode, le nitrate était préalablement converti en nitrite par l'addition de nitrate réductase dans le milieu réactionnel.
- Enfin, la quantité de prostaglandine (PG) E_2 produite par les chondrocytes a été mesurée par un dosage radio-immunologique spécifique (Serteyn *et al.*, 1988).

Notons que la production totale d'un de ces facteurs correspond à la somme des quantités dosées dans le surnageant de la culture (S), et les fractions FRM et CM.

Les cultures de chondrocytes ont été réalisées en présence ou en l'absence d'IL-1ß (10^{-10} M soit 85 U/ml). L'IL-1ß a été ajoutée dès le début de la culture et renouvelée tous les trois jours. Nous avons choisi de tester les effets de cette cytokine parce qu'elle joue un rôle important dans la pathogénie de l'arthrose, et qu'elle induit *in vitro* une série de changements métaboliques semblables à ceux observés *in vivo* dans le cartilage arthrosique.

2.3. Résultats

2.3.1 Etude histologique des billes d'alginate

L'étude histologique des billes d'alginate a été réalisée sur des coupes colorées au bleu/jaune alcian. Cette méthode permettait de distinguer les glycosaminoglycanes (GAG) hautement sulfatés (qui se colorent en bleu), comme l'agrécane, des polysaccharides faiblement sulfatés (qui se colorent en jaune), comme l'alginate (Figure II-2A). Elle mettait en évidence un halo bleu foncé autour des chondrocytes, témoignant de la forte concentration en GAG sulfatés de la matrice péri-cellulaire et territoriale (CM). L'étude cytologique montrait la présence dans la bille d'alginate de logettes formées dans la matrice et abritant un chondrocyte turgescent. Cette organisation était semblable à celle du cartilage *in vivo*.

L'utilisation d'anticorps spécifiques du collagène de type II, nous a permis de révéler la présence de collagène de type II dans la matrice extracellulaire. Le collagène de type II était présent dans les compartiments CM et la FRM de la bille d'alginate (Figure II-2B).

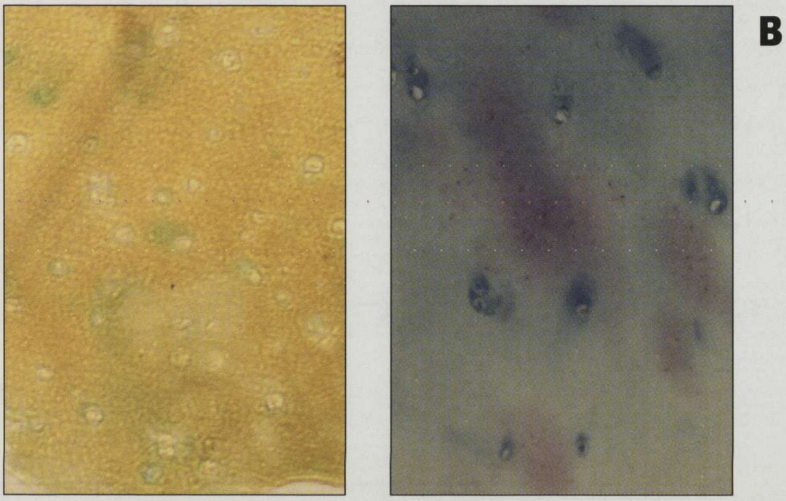


Figure II-2A et B: *Coupe réalisée dans une bille d'alginate de 12 jours et colorée au bleu/jaune alcian (x 300) (A); mise en évidence du collagène de type II par immunohistochimie (B).*

2.3.2. Etude de la synthèse d'acide désoxyribonucléique (ADN)

L'ADN à été mesuré dans la phase cellulaire de la culture au terme de chaque période de 3 jours de culture. Sur une période de 12 jours, le contenu en ADN augmentait de 38 % (ΔT12-T0; $p < 0,05$). L'augmentation la plus importante avait lieu durant les 3 premiers jours de la culture (+20 %, $p<0,05$, Δ T0-T3). L'IL-1ß (10^{-10} M) n'avait pas d'effet significatif sur le contenu en ADN de la culture.

Nous avons également étudié la synthèse d'ADN par l'incorporation de la thymidine marquée au tritium dans l'ADN néo-synthétisé. L'incorporation de ^{3}H-thymidine avait lieu essentiellement durant le premier jour de la culture. Ensuite, elle diminuait progressivement avec la durée de celle-ci (Figure II-3).

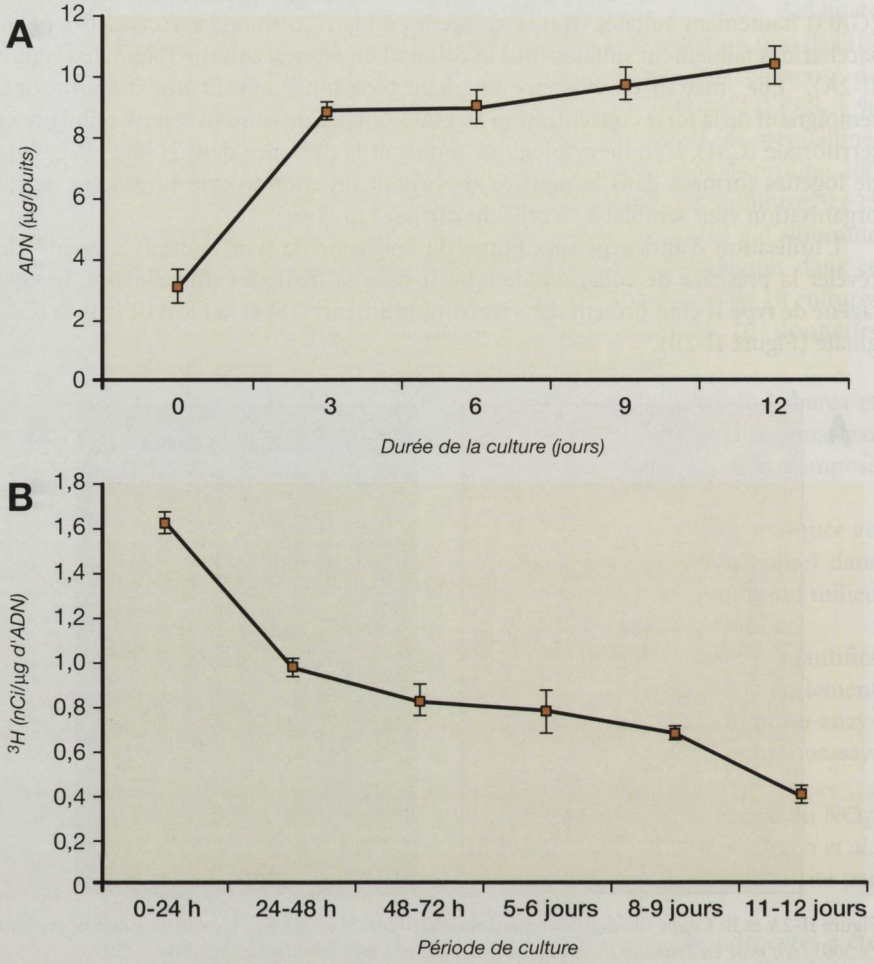


Figure II- 3: *A) Evolution du contenu en ADN des billes d'alginate en fonction de la durée de la culture. Les chondrocytes arthrosiques étaient cultivés pendant 12 jours et les mesures effectuées tous les trois jours. L'ADN était mesuré dans les extraits cellulaires obtenus après dissociation des chondrocytes par ultrasons. Les résultats étaient exprimés en µg d'ADN par puits de culture et représentés par la moyenne ± l'écart-type (n=10) des résultats de 10 cultures réalisées indépendamment avec des prélèvements de cartilage différents. B) Evolution de l'activité mitotique des cellules au cours de la culture. L'activité mitotique des cellules était appréciée par l'incorporation de ^{3}H-thymidine dans l'ADN nouvellement synthétisé. Les résultats étaient exprimés en nCi/µg d'ADN et représentaient la moyenne ± l'écart-type des résultats de 10 cultures réalisées indépendamment avec des prélèvements de cartilage différents.*

2.3.3. Etude de la production d'agrécane.

La quantité d'agrécane a été mesurée par un dosage immunologique spécifique dans les trois compartiments de la culture en billes d'alginate: la matrice péri-cellulaire et territoriale (CM), la matrice inter-territoriale (fraction recueillie après dépolymérisation de l'alginate par le citrate; FRM) et le milieu de culture conditionné par les chondrocytes (S). L'agrécane produit par les chondrocytes était immobilisée presque totalement (98 %) dans la bille d'alginate (CM et FRM). La production d'agrécane avait lieu principalement durant les six premiers jours de la culture (Figure II-4). Durant cette période, l'AGG se répartissait dans les compartiments CM et FRM selon un rapport 1/3, ce qui témoignait d'une accumulation préférentielle des molécules d'agrécane dans la matrice inter-territoriale. Après le 6ème jour de culture, la quantité d'agrécane contenue dans la fraction CM diminuait et celle contenue dans la fraction FRM augmentait, alors que le contenu total de la bille (CM + FRM) restait constant. Cette observation démontrait clairement, qu'après le 6ème jour de culture, l'agrécane contenue dans la fraction CM migrait vers le compartiment FRM. Au terme de 12 jours de culture, les chondrocytes arthrosiques avaient produit 4902 ± 630 ng d'agrécane par microgramme d'ADN dont 73,5 % étaient stockés dans la matrice inter-territoriale (FRM).

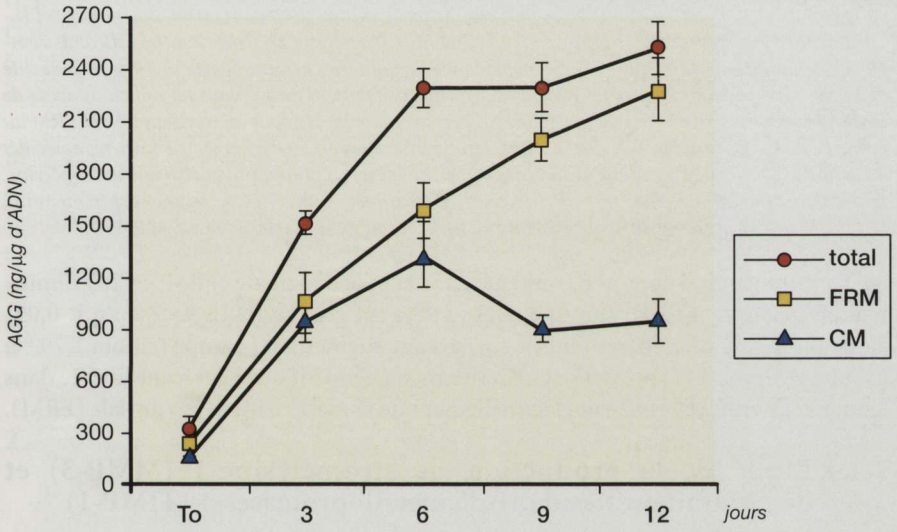


Figure II-4: *Distribution de l'agrécane néo-synthétisée dans les différents compartiments matriciels de la bille d'alginate en fonction de la durée de la culture. CM = Cell-associated Matrix qui correspond à la matrice péri-cellulaire et territoriale du cartilage; FRM = Further Removed Matrix qui correspond à la matrice inter-territoriale du cartilage. Le total est la somme des quantités d'agrécane accumulées dans la fraction FRM et CM. La quantité d'agrécane est exprimée en ng/µg d'ADN. Les valeurs représentées sont les moyennes ± l'écart-type de dix cultures réalisées avec des prélèvements de cartilage arthrosique provenant de 10 donneurs différents.*

L'addition d'IL-1ß dans le milieu de culture bloquait complètement la production d'agrécane par les chondrocytes et modifiait leur répartition dans les différents compartiments de la culture (Figure II-5 et 6).

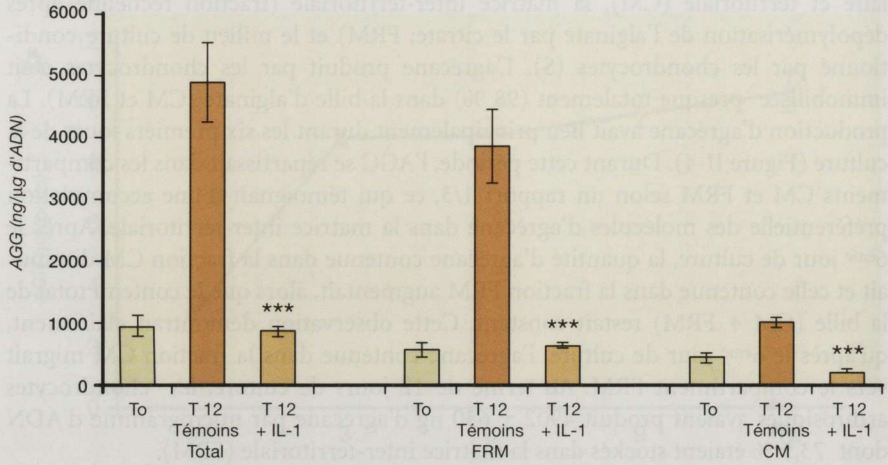


Figure II-5: *Effet de l'IL-1ß (10^{-10} M) sur la production d'agrécane par des chondrocytes arthrosiques cultivés durant 12 jours en billes d'alginate. Les milieux de culture contenant ou ne contenant pas l'IL-1ß étaient renouvelés tous les trois jours. T0 = Quantité d'agrécane présente dans les différents compartiments au début de la culture. A ce moment, les chondrocytes avaient bénéficié d'une période dite de "wash-out" pendant laquelle ils étaient maintenus pendant 48 heures dans un milieu composé de DMEM et d'Ultroser G 2 %. T12 = Quantité d'agrécane accumulée dans les fractions CM et FRM au terme de douze jours de culture. Les résultats sont représentés par les moyennes ± l'écart-type calculés à partir des valeurs de 10 cultures réalisées avec des chondrocytes provenant de 10 donneurs différents. Les astérisques indiquent les différences significatives entre les groupes expérimentaux (T12 + IL-1ß) et le groupe témoin (T12 sans IL-1ß) avec un ***p < 0,001 (Test d'ANOVA).*

La proportion d'agrécane contenue dans la bille d'alginate (FRM + CM) diminuait en présence d'IL-1ß (groupe témoin 98,3 %; groupe IL-1ß 93,7 %; $p < 0,05$) alors que la quantité libérée dans le surnageant augmentait (groupe témoin 1,79 %; groupe IL-1ß 6,31 %; $p < 0,05$). Ces résultats suggèraient que l'agrécane libérée dans le milieu de culture provenait essentiellement de la matrice inter-territoriale (FRM).

2.3.4. Etude de la production de stromélysine-1 (MMP-3) et de l'inhibiteur tissulaire de métalloprotéases-1 (TIMP-1)

Dans les conditions basales, la presque totalité (97 %) de la stromélysine-1 (MMP-3) produite par les chondrocytes arthrosiques était libérée dans le milieu de culture. La production de cette enzyme augmentait en fonction de la durée de la culture.

Ainsi, la quantité de MMP-3 sécrétée par les chondrocytes arthrosiques durant les trois premiers jours était de 728 ± 110 ng/µg d'ADN, mais atteignait la valeur de

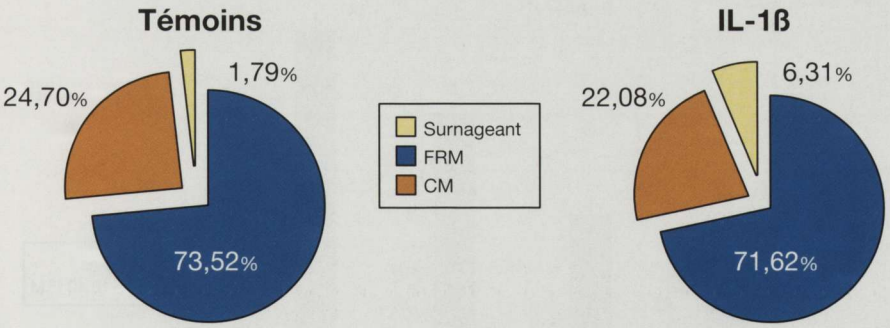


Figure II-6: *Distribution des molécules d'agrécane dans les différents compartiments au terme de 12 jours de culture en billes d'alginate en l'absence ou en présence d'IL-1ß (10^{-10} M). Les valeurs correspondent aux pourcentages moyens calculés à partir des résultats de 10 cultures indépendantes réalisées avec des chondrocytes d'origine différente.*

3500 ± 461 ng/µg d'ADN entre le 9ème et le 12ème jour de culture (Figure II-7A). A l'inverse, la production de TIMP-1 diminuait en fonction de la durée de la culture. Elle était de 110 ± 40 ng/µg d'ADN pendant les 3 premiers jours de culture, mais n'était plus que de 53 ± 6 ng/µg d'ADN entre le 9ème et le 12ème jour de culture (Figure II-7B). La conséquence de l'évolution divergente de ces deux paramètres était la croissance exponentielle du rapport MMP-3/TIMP-1. Ce rapport était égal à 6,6 après les 3 premiers jours de culture et égal à 66 entre le 9ème et le 12ème jour de la culture.

En présence d'IL-1ß, la production basale de MMP-3 était significativement augmentée après trois jours d'incubation ($p < 0,001$; test d'ANOVA). Ensuite, l'IL-1ß exercait un effet inhibiteur sur la synthèse de cette métalloprotéase. L'IL-1ß inhibait significativement la production de TIMP-1 et ce quels que soient la durée d'incubation et le nombre d'administrations ($p < 0,001$; test d'ANOVA). Le ratio MMP-3/TIMP-1 était significativement augmenté par l'addition d'IL-1ß ($0,01 < p < 0,001$). De plus, la valeur de ce rapport augmentait au fil des administrations d'IL-1ß. Après 3 jours de culture, ce rapport était égal à 32. Il atteignait la valeur de 145 au terme de 9 jours d'incubation avec l'IL-1ß.

2.3.5. Etude de la production de cytokines pro-inflammatoires

Les chondrocytes arthrosiques produisaient spontanément de l'IL-6, de l'IL-8 et du MIP-1ß. La production de ces trois cytokines augmentait avec la durée de la culture. Au terme de 12 jours de culture, les chondrocytes arthrosiques avaient produit une quantité totale d'IL-6, d'IL-8 et de MIP-1ß respectivement égale à 14 ± 1, 9, 20 ± 2,5 et 0,04 ± 0,001 ng/µg d'ADN (Figure II-8).

A la fin de la culture, plus de 95,4 % de l'IL-6 et du MIP-1ß synthétisés par les chondrocytes étaient libérés dans le milieu de culture. Par contre, 21,2 % de l'IL-8 produite étaient immobilisés dans la bille d'alginate (Figure II-9).

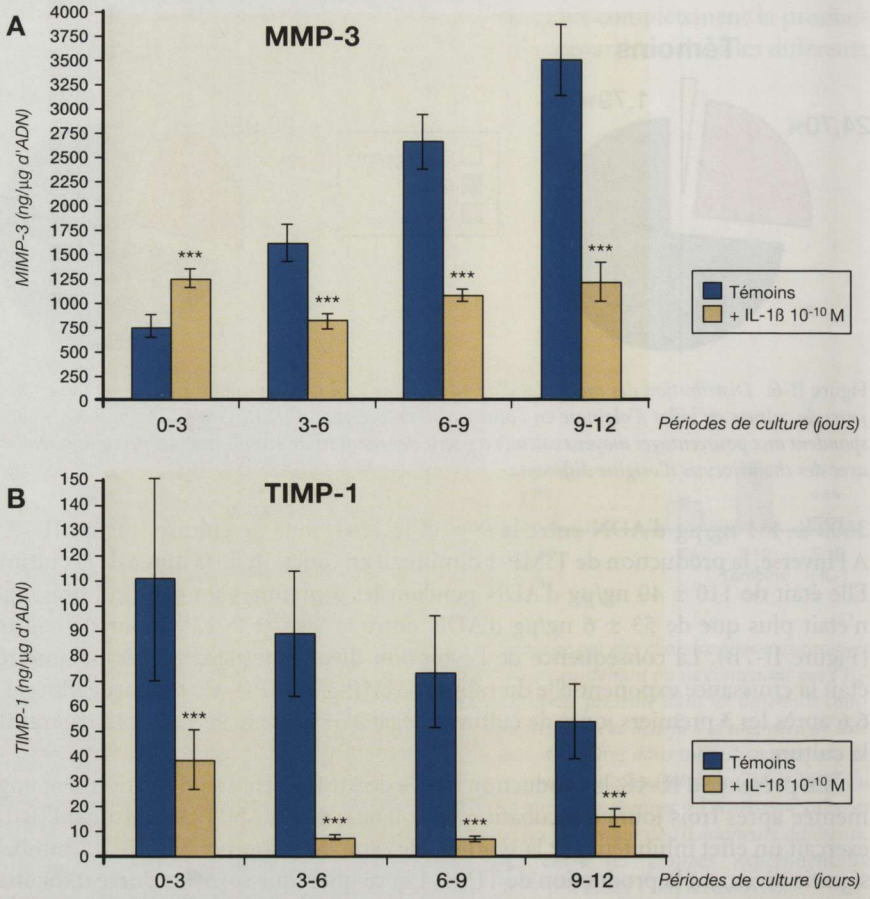


Figure II- 7: *Evolution de la production de stromélysine-1 (MMP-3) (A) et de l'inhibiteur tissulaire des metalloproteases-1 (TIMP-1) (B) en fonction de la durée de la culture. Les valeurs représentent les quantités de MMP-3 et de TIMP-1 mesurées dans les milieux de culture conditionnés par période de 3 jours de culture. Les valeurs sont les moyennes ± l'écart-type calculés à partir des résultats de 10 cultures indépendantes réalisées avec des chondrocytes isolés à partir de 10 prélèvements différents. Les astérisques indiquent les différences significatives entre les groupes témoins et les groupes traités à l'IL-1ß avec un ** $p < 0,01$ ou *** $p < 0,001$ (Test d'ANOVA).*

L'IL-1ß stimulait la production d'IL-6, d'IL-8 et de MIP-1ß par les chondrocytes arthrosiques. L'effet était déjà très significatif ($p < 0,001$) après trois jours d'incubation. Pendant cette période (0-3 j), l'IL-1ß augmentait 12 fois la production d'IL-6, 20 fois la production d'IL-8 et 60 fois la production basale de MIP-1ß. L'effet stimulant de l'IL-1ß perdurait au fil des administrations successives. L'IL-1ß augmentait également la quantité d'IL-8 immobilisée dans la bille d'alginate et plus particulièrement dans la matrice inter-territoriale (FRM). Dans les conditions basales, la quantité d'IL-8 retenue dans la FRM représentait 16,2 % de la production totale. En présence d'IL-1ß, cette proportion était de 23,8 % (Figure II-9).

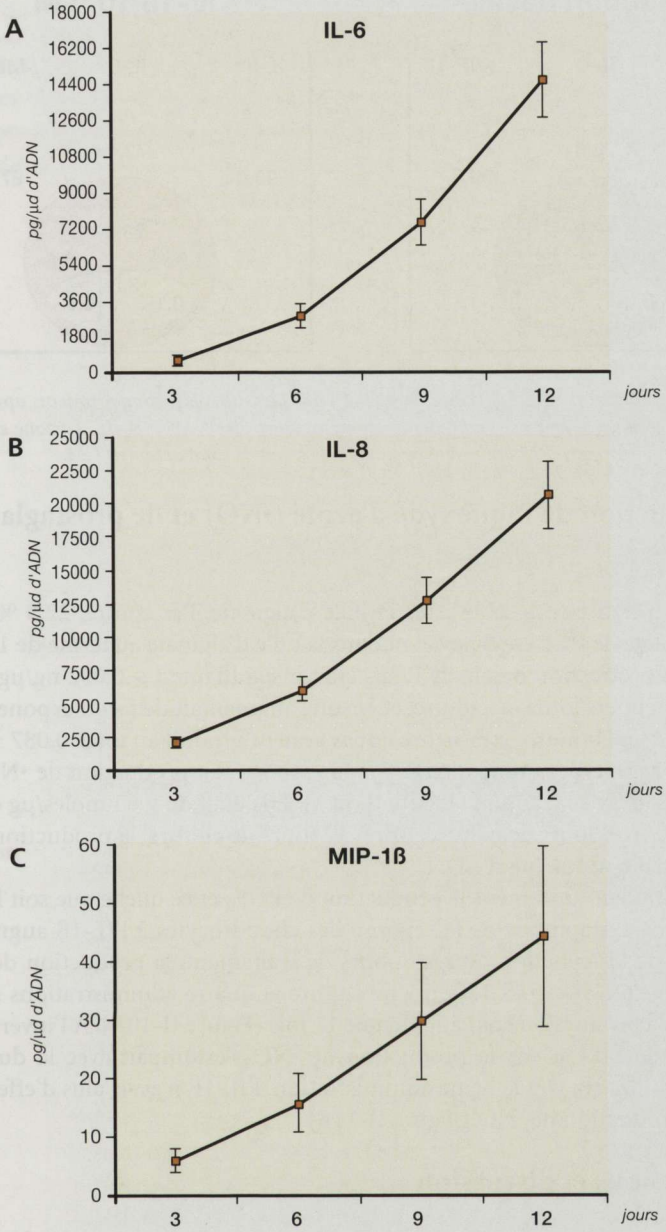


Figure II-8: *Production basale cumulée d'IL-6 (A), d'IL-8 (B) et de MIP-1ß (C) par les chondrocytes arthrosiques. Les valeurs sont les moyennes ± l'écart-type calculés à partir des résultats de 10 cultures réalisées avec des chondrocytes prélevés sur 10 donneurs différents.*

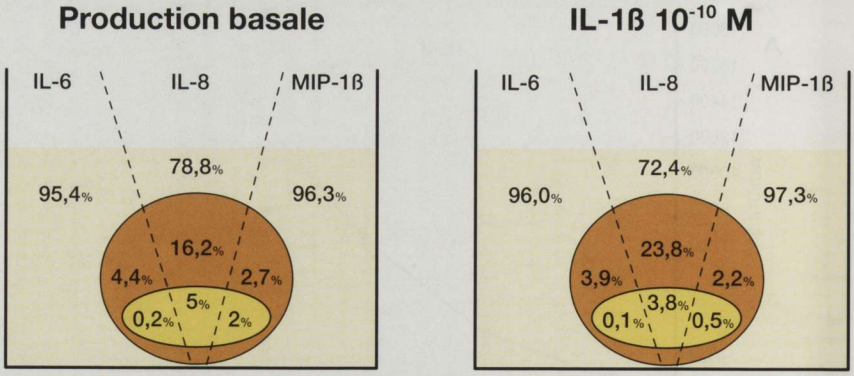


Figure II-9: *Distribution de l'IL-6, l'IL-8 et le MIP-1ß dans les différents compartiments après douze jours de culture en billes d'alginate en l'absence ou en présence (10^{-10} M) d'IL-1ß. La zone en orange représente le compartiment FRM et la zone en jaune représente le compartiment CM.*

2.3.6. Production de monoxyde d'azote (•NO) et de prostaglandine $(PG)E_2$

Le nitrite n'était pas dosable dans la bille d'alginate. Par contre, 22,5 % de la production totale de PGE_2 se trouvaient dans la bille d'alginate au terme de 12 jours de culture. La production basale de PGE_2 était insignifiante ($\simeq$ 0,002 ng/µg ADN) durant les 9 premiers jours de culture, et ensuite augmentait de façon exponentielle. Après 12 jours, les chondrocytes arthrosiques avaient produit au total 0,087 $\pm$ 0,025 ng de PGE_2 par microgramme d'ADN (Figure II-10). La production de •NO était constante au cours de la culture (Figure II-11A). Elle était de $\simeq$ 6 nmoles/µg d'ADN par période de trois jours de culture. Après 12 jours de culture, la production totale était de 25,8 $\pm$ 3,6 nmole/µg d'ADN.

L'IL-1ß stimulait fortement la production de PGE_2 et ce quelle que soit la période de culture. L'amplitude de la réponse des chondrocytes à l'IL-1ß augmentait avec la durée de l'incubation. Après 3 jours de traitement, la production de PGE_2 était augmentée 6 fois. Après 12 jours de culture et quatre administrations successives d'IL-1ß, la production était augmentée 17 fois (Figure II-10A). A l'inverse, l'effet stimulant de l'IL-1ß sur la production de •NO s'estompait avec la durée du traitement par l'IL-1ß. Dès la 3ème administration, l'IL-1ß n'avait plus d'effet stimulant sur la production de •NO (Figure II-11B).

2.4. Conclusions et discussion

Dans ce chapitre, nous avons analysé le comportement des chondrocytes humains arthrosiques cultivés en billes d'alginate, ainsi que leurs réponses à des traitements successifs par l'IL-1ß. Nos résultats contribuent à la validation du modèle de culture en billes d'alginate, et améliorent les connaissances sur l'activité biologique de l'IL-1ß. L'IL-1ß est une cytokine importante de la pathogénie de

l'arthrose. Elle constitue une cible privilégiée pour les nouveaux traitements de cette maladie. La plupart des travaux réalisés à ce jour sur le modèle en alginate, se sont concentrés sur la synthèse de protéines matricielles. Notre travail apporte de nouvelles informations sur la synthèse et la distribution dans la matrice cartilagineuse des médiateurs de l'inflammation. Nous avons isolé de notre travail trois observations majeures, que nous avons choisies de commenter et de discuter.

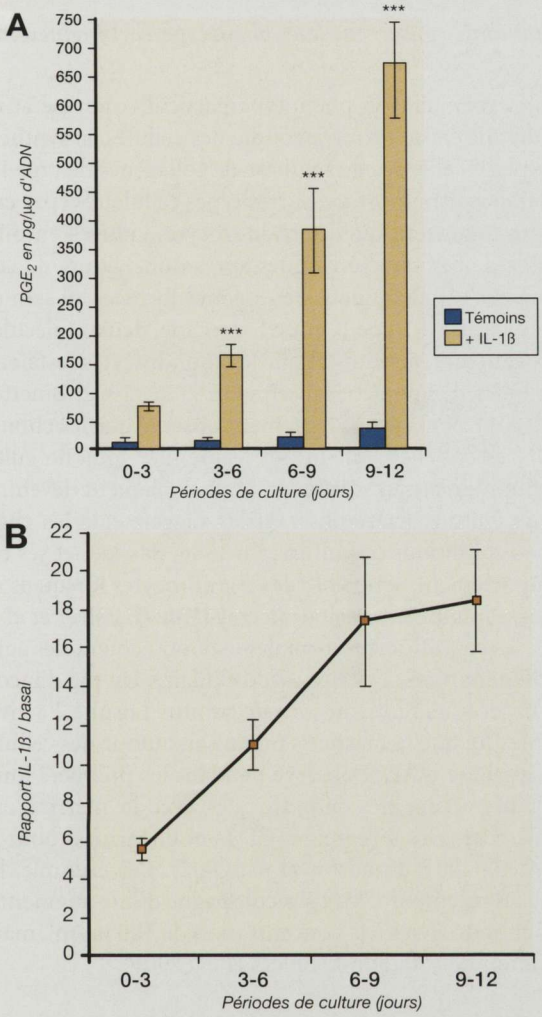


Figure II-10: A) *Production de PGE_2 par les chondrocytes arthrosiques en fonction de la durée de la culture en présence ou en l'absence d'IL-1ß (10^{-10} M). B) Effet de l'IL-1ß sur la production de PGE_2 exprimé par le rapport production avec IL-1ß/production basale. Les valeurs indiquées sur les graphiques sont les moyennes ± l'écart-type de 10 cultures indépendantes réalisées avec des chondrocytes isolés à partir de prélèvements de cartilage d'origine différente. Les astérisques indiquent les différences significatives entre les groupes témoins et les groupes traités à l'IL-1ß. *** = $p < 0,001$ (Test d'ANOVA).*

Lorsqu'ils sont cultivés en billes d'alginate,

- Les chondrocytes humains restent différenciés pendant une longue période;
- Les chondrocytes synthétisent une matrice dont l'organisation rappelle celle du cartilage *in vivo*;
- L'IL-1ß provoque une série de déséquilibres métaboliques favorables à la genèse de la réaction inflammatoire et à la dégradation de la matrice cartilagineuse.

Les chondrocytes humains conservent leur phénotype cartilagineux en culture en billes d'alginate

Les chondrocytes expriment un phénotype particulier appelé aussi phénotype cartilagineux. Il se définit par la forme arrondie des cellules, la synthèse d'agrécane et de collagène de type II, l'absence de synthèse de collagènes de type I et III, et une activité mitotique faible par rapport à d'autres types cellulaires (par ex. fibroblastes de peau). Nos travaux montrent que les chondrocytes cultivés en billes d'alginate conservent l'ensemble de ces caractéristiques après douze jours de culture. Ils ont une forme sphérique et s'installent dans des logettes formées au sein de la matrice. Ils synthétisent du collagène de type II et de l'agrécane, deux molécules spécifiques du cartilage. D'autres auteurs ont montré que les chondrocytes restaient différenciés pendant 8 mois en billes d'alginate (Hauselmann *et al.*, 1992, Binette *et al.*, 1998; Chubinskaya *et al.*, 2001). Nous avons également observé que les chondrocytes synthétisaient de l'ADN essentiellement durant le premier jour de culture. Ensuite, la synthèse d'ADN diminue progressivement, pour finalement devenir insignifiante après douze jours de culture. Cette observation suggère que les chondrocytes se divisent peu dans nos conditions de culture. En 1986, Bassleer et ses collaborateurs ont observé un comportement semblable des chondrocytes lorsqu'ils étaient placés en suspension en phase liquide sous agitation constante (Bassleer *et al.*, 1986, 1991). Dans ces conditions, les chondrocytes formaient spontanément des agrégats et s'entouraient progressivement d'une matrice extracellulaire. Un pic d'incorporation de ^{3}H-thymidine était observé au huitième jour de culture. Ensuite, l'activité mitotique diminuait au fur à mesure que la matrice s'organisait autour des cellules. Il est possible que ce pic de synthèse d'ADN observé pendant les premiers jours de culture soit le résultat de l'interaction des chondrocytes avec la matrice extracellulaire. Les chondrocytes possèdent des récepteurs (CD44 et intégrines) pour les molécules matricielles (Patti *et al.*, 2001; Knudson *et al.*, 2002). Par exemple, la fixation de l'acide hyaluronique au récepteur CD44 s'accompagne d'une augmentation de l'activité mitotique des chondrocytes à la concentration de 100 µg/ml, mais d'une inhibition à la concentration de 500 µg/ml (Ehlers *et al.*, 2001).

Une matrice extracellulaire s'organise autour du chondrocyte.

Les chondrocytes en culture en billes d'alginate produisent des molécules d'agrécane qui sont caractéristiques du cartilage hyalin. En comparaison avec la production de chondrocytes normaux (donneur sain de 18 ans), les chondrocytes arthrosiques produisent 4,5 fois moins d'agrécane (7035 ± 214 ng/µg ADN versus

1547 ± 96 ng/µg d'ADN) après 12 jours de culture. La synthèse a lieu essentiellement pendant les six premiers jours de culture. Notre étude montre que la majorité des molécules d'agrécane synthétisées par les chondrocytes est immobilisée dans la bille d'alginate. Après 12 jours de culture, seulement 2 % de la production d'agrécane sont libérés dans le milieu de culture. Au sein de la bille d'alginate, les molécules d'agrécane s'accumulent essentiellement dans la matrice inter-territoriale (fraction FRM; 73,5 % après 12 jours de culture). Cependant, l'analyse sectorielle du contenu

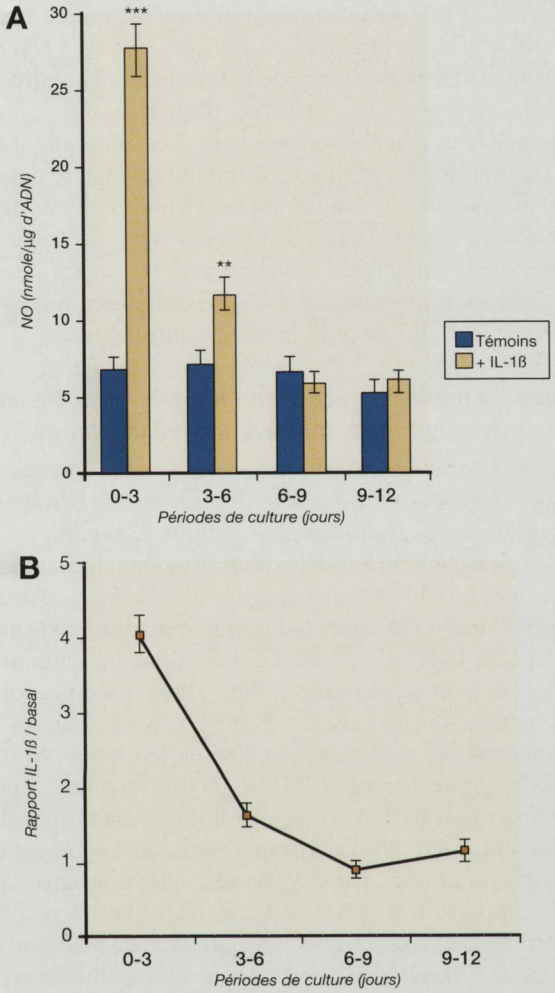


Figure II-11: *A) Effet de l'IL-1ß (10^{-10} M) sur la production de •NO par les chondrocytes arthrosiques cultivés 12 jours en billes d'alginate. Le milieu de culture contenant ou non l'IL-1ß était renouvelé tous les trois jours. Les valeurs correspondent à la production de NO_2^- pendant 3 jours. Les moyennes ± l'écart-type calculées à partir des valeurs de 10 cultures différentes ont été représentées sur les graphiques. B) L'effet de l'IL-1ß sur la production de •NO est exprimé par le rapport production avec IL-1ß/ production basale.*

en agrécane de la matrice extracellulaire suggère la présence d'un mouvement des molécules d'agrécane de la matrice peri-cellulaire (CM) vers la matrice inter-territoriale (FRM). Pendant les six premiers jours de culture, les quantités d'agrécane présentes dans les compartiments CM et FRM de la bille d'alginate augmentent de façon parallèle. Ensuite, le contenu de la fraction CM diminue et celui de fraction FRM augmente alors que le contenu total en agrécane de la bille d'alginate reste constant. Nous pouvons conclure de cette analyse qu'une partie des molécules d'agrécane produites par le chondrocyte, migre de la matrice péri-cellulaire vers la matrice inter-territoriale. Cette observation confirme l'hypothèse émise préalablement par d'autres auteurs (Hauselmann *et al.*, 1992; Mok *et al.*, 1994; Kikuchi *et al.*, 2001). Ce phénomène peut être expliqué par le fait que les chondrocytes en billes d'alginate synthétisent deux sortes de molécules d'agrécane qui se distinguent par leur affinité pour la molécule d'acide hyaluronique. Les molécules d'agrécane avec une affinité élevée pour l'acide hyaluronique forment rapidement des complexes de haut poids moléculaire avec l'acide hyaluronique et s'immobilisent dans la matrice péri-cellulaire. Par contre, les molécules d'agrécane avec une faible affinité pour l'acide hyaluronique, dites "immatures", migrent vers la matrice inter-territoriale. Au cours de cette migration, les molécules d' agrécane immatures subissent une maturation, qui accroît leur affinité pour l'acide hyaluronique.

L'IL-1ß provoque des déséquilibres métaboliques favorables à la dégradation du cartilage articulaire et au développement de la réaction inflammatoire.

Dans ce travail, nous avons également étudié le comportement des chondrocytes à des traitements successifs (4 administrations en 12 jours; 85 U/ml/3 J) par l'interleukine-1ß (IL-1ß). Nous avons réalisé les observations suivantes:

- L'IL-1ß ne modifie pas le contenu en ADN des cultures et n'induit pas la libération de lactate déshydrogénase (LDH), même après douze jours d'incubation. Ces observations suggèrent que le traitement à l'IL-1ß ne modifie pas le nombre de cellules contenues dans les billes d'alginate. Les variations de synthèse enregistrées en présence d'IL-1ß ne sont donc pas le résultat d'une modification du nombre de cellules.
- L'IL-1ß bloque la synthèse d'agrécane et augmente le rapport MMP-3/TIMP-1. Après douze jours de culture, le rapport MMP-3/TIMP-1, calculé à partir des productions cumulées, est égal à 25 dans les conditions basales mais il atteint une valeur de 63 en présence d'IL-1ß. Par comparaison, ce rapport est égal à 9 dans un milieu de culture conditionné par des chondrocytes normaux (donneur sain âgé de 18 ans). Un rapport MMP-3/TIMP-1 anormalement élevé a également été observé dans le cartilage arthrosique et est favorable à la dégradation de la matrice extracellulaire (Martel-Pelletier *et al.*, 1994). Dans nos conditions expérimentales, l'augmentation du rapport MMP-3/TIMP-1 est le résultat d'une forte inhibition de la synthèse de TIMP-1 par l'IL-1ß, et non la conséquence d'une augmentation de la synthèse de MMP-3. En effet, l'IL-1ß augmente la synthèse de MMP-3 uniquement pendant les trois premiers jours de traitement. Ensuite, l'IL-1ß inhibe la production de MMP-3. Au terme de 12 jours de culture et de quatre adminis-

trations successives d'IL-1ß, les chondrocytes produisent deux fois moins de MMP-3 que les chondrocytes non traités par l'IL-1ß. Nous n'avons pas d'explication à donner à ce phénomène et nous n'avons pas trouvé de publication évocatrice d'une inhibition de la synthèse de MMP-3 par l'IL-1ß, ni d'administration d'IL-1 sur une aussi longue période. Dès lors, nous devons nous contenter de quelques hypothèses basées sur des données de la littérature. Il est possible que l'inhibition de la synthèse de MMP-3, soit secondaire à l'action d'un autre facteur synthétisé par le chondrocyte en réponse à l'IL-1ß. Parmi les nombreux facteurs sécrétés par les chondrocytes activés, le TGFß-1 est un candidat potentiel à ce rôle. Le TGFß-1 est un inhibiteur de la synthèse de MMP-3 et sa production est augmentée par l'IL-1ß (Kerr *et al.*, 1990; Felisaz *et al.*, 1999; Felisaz *et al.*, 2001). De plus, il est capable de s'opposer à l'effet de l'IL-1ß sur la synthèse de MMP-3 (Henrotin *et al.*, 2000a). Le TGFß-1 est sécrété sous une forme latente par le chondrocyte. La stromélysine-1 (MMP-3) est capable d'activer le TGFß-1 latent, mais aussi de libérer le TGFß-1 complexé à la décorine (Maeda *et al.*, 2001; Imai *et al.*, 1997). Dès lors, il est possible que la stromélysine soit responsable de la libération et de l' activation du TGFß-1 stocké dans la matrice. Le TGFß-1 pourrait exercer un contrôle négatif sur la synthèse de MMP-3 par le chondrocyte.

Une autre explication trouve son origine dans le constat que l'IL-1ß perd son effet stimulant sur la synthèse de •NO après 6 jours d'incubation. Il a été démontré que le N^G-monométhyl-L-arginine (L-NMMA), un inhibiteur de l'activité des NO synthétases, inhibait l'effet stimulant de l'IL-1ß sur la synthèse de stromélysine-1, suggérant que le •NO est un médiateur des effets de l'IL-1ß sur la synthèse de MMP-3 (Murrell *et al.*, 1995). Nous pouvons émettre l'hypothèse que l'absence de production de •NO en réponse à l'IL-1ß, bloque les effets de cette cytokine sur la production de stromélysine.

- L'IL-1ß stimule la production par les chondrocytes d'IL-6, d'IL-8 et de MIP-1ß. Ces cytokines sont toutes impliquées dans le passage à la chronicité de la réaction inflammatoire de la membrane synoviale et sont capables seules ou en association avec d'autres cytokines, d'induire *in vitro* la dégradation de la matrice du cartilage. A titre indicatif, nous avons comparé la production de cytokines par les chondrocytes arthrosiques avec celle des chondrocytes normaux (sujet jeune âgé de 18 ans). Après 12 jours de culture en billes d'alginate, les chondrocytes arthrosiques produisent respectivement 27, 3,4 et 44 fois plus d'IL-6, d'IL-8 et de MIP-1ß que les chondrocytes normaux. Le MIP-1ß est la seule cytokine dont le taux est plus élevé dans le fluide synovial des patients arthrosiques que dans ceux des patients souffrant de polyarthrite rhumatoïde (Koch *et al.*, 1995). Ces deux observations nous permettent de penser que le MIP-1ß pourrait être un marqueur de l'état d'activation des chondrocytes au cours de l'arthrose. Un autre fait important mis en évidence par notre étude est l'accumulation d'IL-8 au sein de la matrice extracellulaire. L'IL-6 et le MIP-1ß présentent un profil sécrétoire totalement différent. Ces cytokines sont directement libérées dans le milieu de culture. Dans une étude réalisée sur des explants de cartilage humain, Recklies et Gold ont observé que l'IL-8 se fixait de façon transitoire à la matrice extracellulaire du cartilage, et

ensuite était libérée progressivement dans le milieu de culture (1992). Il est envisageable que ce mécanisme contribue à la formation d'un gradient d'IL-8 au sein du cartilage et soit responsable de l'attachement des neutrophiles et des lymphocytes T à la surface des tissus et de leur migration à travers ceux-ci. Les neutrophiles activés sécrètent des formes activées de l'oxygène et des enzymes lysosomiales qui sont capables de dégrader la matrice cartilagineuse (Elford *et al.*, 1991).

En conclusion, le modèle de culture en billes d'alginate est un modèle pertinent pour l'étude du métabolisme des chondrocytes. Il permet l'analyse qualitative et quantitative de la matrice extracellulaire. Ce modèle sera utilisé ultérieurement pour l'étude des effets à long terme de médicaments utilisés dans le traitement de l'arthrose.

CHAPITRE 3

Modulation de l'activité biologique de l'interleukine-1ß par d'autres cytokines et par les formes activées de l'oxygène.

3.1.Introduction

Dans le chapitre précédent nous avions décrit les effets de l'interleukine-1ß sur le métabolisme des chondrocytes arthrosiques. Il s'est avéré que cette cytokine altérait l'homéostasie de nombreux médiateurs, enzymes et constituants de la matrice du cartilage, et transformait le chondrocyte en cellule effectrice de la dégradation du cartilage et de l'inflammation de la membrane synoviale. Au début des années 90, nous avions également montré que les chondrocytes produisaient des formes activées de l'oxygène (FAO) en réponse à des cytokines, des cycles d'anoxie-réoxygénation ou encore du phorbol myristate acétate (Henrotin *et al.*, 1992, 1993 et 1994). Cependant, l'IL-1ß n'est pas la seule cytokine dont la production est augmentée au cours de l'arthrose et qui de surcroît est active sur le chondrocyte (Henrotin *et al.*, 1996). Notre hypothèse de travail est que l'activité biologique de IL-1ß est modulée par d'autres facteurs et que l'expression de ses effets est la résultante de nombreuses interactions, non seulement avec d'autres cytokines mais aussi avec des récepteurs solubles, des facteurs antagonistes ou encore des FAO.

Dans ce chapitre, nous étudierons le rôle régulateur exercé par l'IFNγ, l'IGF-1 et le TGFß-1 sur l'activité de l'IL-1ß. Ces trois facteurs sont présents dans le fluide synovial des patients arthrosiques et de nombreuses questions sont toujours en suspens concernant leur rôle dans l'arthrose. De plus, nous explorerons la régulation de l'activité de l'IL-1ß par les FAO. Les résultats de ces travaux ont fait l'objet de plusieurs publications que le lecteur peut trouver en annexe (Annexes 3, 4, 5, 6, 7)

3.2. Matériels et Méthodes

Dans cette partie de notre travail, nous avons utilisé des chondrocytes humains arthrosiques ou normaux primaires cultivés en suspension en phase liquide pour une courte période (entre 3 et 72 heures). Au terme de ces périodes d'incubation, les cellules et le milieu de culture étaient séparés par centrifugation et les analyses suivantes réalisées sur l'une ou l'autre de ces fractions:

- Les cytokines (IL-6, IL-8, IL-10), l'antagoniste du récepteur à l'interleukine-1 (IL-1Ra), la stromélysine-1 (MMP-3) et l'inhibiteur tissulaire des métalloprotéases (TIMP-1) ont été mesurés directement dans les milieux de cultures et dans les extraits cellulaires (obtenus par sonication) par des dosages immunologiques spécifiques (EASIA, Biosource, Fleurus, Belgique).
- La production de •NO était déterminée par la mesure dans le milieu de culture du nitrite, un produit final de la transformation du •NO. Le NO_2^- était mesuré par la méthode de spectrophotométrie basée sur la réaction de GRIESS (Green *et al.*, 1982). L'addition au milieu de culture d'une nitrate réductase permettait la conversion du nitrate contenu dans le milieu de culture en nitrite.
- L'activité enzymatique de type stromélysine était mesurée par une méthode fluorimétrique basée sur la digestion de la caséine marquée à la résorufine.
- Selon l'étude considérée, l'agrécane (AGG) était mesurée dans le milieu de culture soit par un dosage radioimmunologique (Gysen et Franchimont, 1984), soit par un dosage immunoenzymologique (EASIA, Biosource, Fleurus, Belgique).

- La prostaglandine (PG) E_2 était dosée uniquement dans le milieu de culture par un dosage radioimmunologique (Serteyn *et al.*, 1988).
- La synthèse de glycosaminoglycanes sulfatés était déterminée par l'incorporation de ^{35}S. Le $Na_2{}^{35}SO_4$ était introduit dans le milieu de culture 24 heures avant la fin de la période de culture.
- L'expression des gènes codant pour l'IL-1ß, l'IL-6, l'IL-8, l'iNOS, la COX-2 et l'agrécane était quantifiée par la méthode de réaction de polymérisation en chaîne des brins d'ADN en utilisant la technologie développée par la firme ROCHE (Light Cycler)(Voir annexe 7 et 8). Les ARN totaux étaient extraits selon le procédé "SV total RNA Isolation" commercialisé par la firme Promega (Leiden, Hollande). Ensuite, les ARN étaient convertis en ADN complémentaires (ADNc) par l'action enzymatique de la transcriptase inverse (Life Technologies, Merelbeke, Belgique). L'amplification des cDNA en temps réel était effectuée dans un "thermocycler" combiné avec un fluorimètre (LightCycler, Roche, Bruxelles, Belgique). Les réactions de polymérisation étaient réalisées en utilisant le kit "LightCycler-FastStart DNA Master SYBR Green I" (Roche, Bruxelles, Belgique). Le SYBR Green I est un fluorochrome se liant spécifiquement aux ADN double brin. La réaction en chaîne peut être quantifiée par une lecture de fluorescence. La fluorescence des ADN double brin était mesurée à la fin de chaque étape de duplication. Pour chaque amplification, une courbe standard était générée à partir d'ADN purifié dans une gamme allant de 10^6 copies à 10 copies par réaction. Afin de standardiser le nombre de copies d'ADNc dans les différents échantillons à analyser, un gène de référence, la glycéraldehyde-3-phosphate déshydrogénase (GAPDH), était amplifiée en guise de contrôle interne. L'expression standardisée d'un gène est calculée par le rapport entre le nombre de copies de ce gène et le nombre de copies de GAPDH.
- L'activation du complexe NF-κB était appréciée par la méthode de migration retardée sur gel d'électrophorèse (Electrophoretic Migration Shift Assay). Les chondrocytes (20 10^6 cellules) étaient lysés dans un tampon hypotonique (10 mM HEPES pH 7.9, 10 mM KCl, 1mM EDTA, 1mM EGTA, 1.5 mM $MgCl_2$, 0.1 % Nonidet NP-40, 0.5 mM PMSF, 0.5 mM DTT) contenant un cocktail d' inhibiteurs de protéases (Roche Diagnostics, Brussels, Belgique). Après une incubation de 15 min. à 4 °C, l'extrait était ensuite centrifugé (10 min, 10 000g, 4 °C) et le culot nucléaire remis en suspension dans 75 µl de tampon hypertonique contenant 450 mM NaCl, 20 mM HEPES pH 7.9, 25 % (v/v) glycérol, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT et un cocktail d'inhibiteurs de protéases (Roche Diagnostics, Brussels, Belgique). Les échantillons étaient placés sur un agitateur rotatoire à 4 °C pendant 90 min. Les extraits nucléaires étaient centrifugés (10 min, 10 000g, 4 °C) et le surnageant conservé en aliquotes à –70 °C. Les concentrations protéiques étaient estimées en utilisant le réactif Biod-Rad Protein Assay (Bio-Rad, Nazareth, Belgique). La séquence nucléaire consensus de NF-κB (AGTTGAGGGGACTTTCCCAGGC) a été utilisée. Cet oligonucléotide double brin est marqué au ^{32}P en utilisant la T4 polynucleotide kinase (Promega, Leiden, Hollande) en présence de [α-^{32}P]dATP (ICN, Asse-Relege, Belgique). Les extraits

nucléaires (10µg) étaient incubés avec la séquence consensus de NF-κB (20 fmoles) dans le milieu réactionnel suivant: (20 mM HEPES pH 7.5, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.05 % Nonidet NP-40, 20 % Glycerol, 1 mg/ml d'albumine, 1 µg of poly(dI.dC) (Amersham, Rosendaal, Hollande) (15 min, 20 °C). Les échantillons étaient séparés sur gel de polyacrylamide à 5 % dans le tampon TBE. Ils étaient finalement visualisés par une autoradiographie.

3.3. Les résultats

Dans ce chapitre, nous avons développé les résultats concernant la régulation de l'activité biologique de l'IL-1ß par d'autres cytokines (IFNγ, IGF-I, TGFß-1) ou par les formes activées de l'oxygène (FAO).

3.3.1. Régulation de l'activité biologique de l'IL-1ß par l'interféron γ.

L'IFNγ (1, 10 et 100 U/ml) augmentait de façon significative la production basale de •NO ($0,05 < p < 0,001$). Il stimulait également de façon dose-dépendante ($R = 0,85\text{-}0,95$; $p < 0,001$) la synthèse d'IL-6, d'IL-1Ra et de PGE_2, mais inhibait de façon dose-dépendante la production d'AGG ($r = 0,82$; $p < 0,01$)(Figure III-1) (Henrotin *et al.*, 2000b). Aux concentrations de 10 et 100 U/ml, la production d'IL-8 était respectivement réduite de 25 et 55 %. Quant à la production de MMP-3, elle était réduite par l'IFNγ uniquement à la dose de 100 U/ml ($p = 0,02$). Dans les conditions basales ou en présence d'IFNγ, l'IL-10 était indétectable dans les milieux de culture conditionnés par les chondrocytes normaux.

Lorsqu'ils étaient ajoutés conjointement, l'IFNγ (1, 10 et 100 U/ml) et l'IL-1ß (2.10^{-10} M) agissaient en synergie pour stimuler la production de •NO, d'IL-6 et d'IL-1Ra et pour inhiber la production d'AGG. Par contre, l'IFNγ s'opposait aux effets stimulateurs de l'IL-1ß sur la synthèse de MMP-3, d'IL-8 et d'IL-10 (Tableau III-1). Enfin, l'IFNγ ne modifiait pas significativement l'effet stimulant de l'IL-1ß sur la production de PGE_2.

3.3.2. Régulation de l'activité biologique de l'IL-1ß par l'IGF-1 et le TGFß-1

Le TGFß-1 et l'IGF-1 augmentaient la production de glycosaminoglycanes (GAGs) (mesurée par l'incorporation de ^{35}S) ($p < 0,01$) par les chondrocytes arthrosiques, alors que l'IL-1ß inhibait de façon dose-dépendante cette production ($r = 0,87$; $0,01 < p < 0,001$). Lorsque le TGFß-1 ou l'IGF-1 et l' IL-1ß étaient ajoutés simultanément, deux situations étaient observées (Figures III-2 et -3):

- En présence de faible quantité d'IL-1ß (0,1 U/ml soit $0,012\ 10^{-11}$ M soit 1,7 pg/ml), l'IGF-1 et le TGFß-1 conservaient leur effet stimulant sur la production de GAGs.
- En présence d'une concentration élevée d'IL-1ß (5 U/ml soit $0,6\ 10^{-11}$M), la production de GAGs était inhibée même en présence du TGFß-1 et de l'IGF-1.

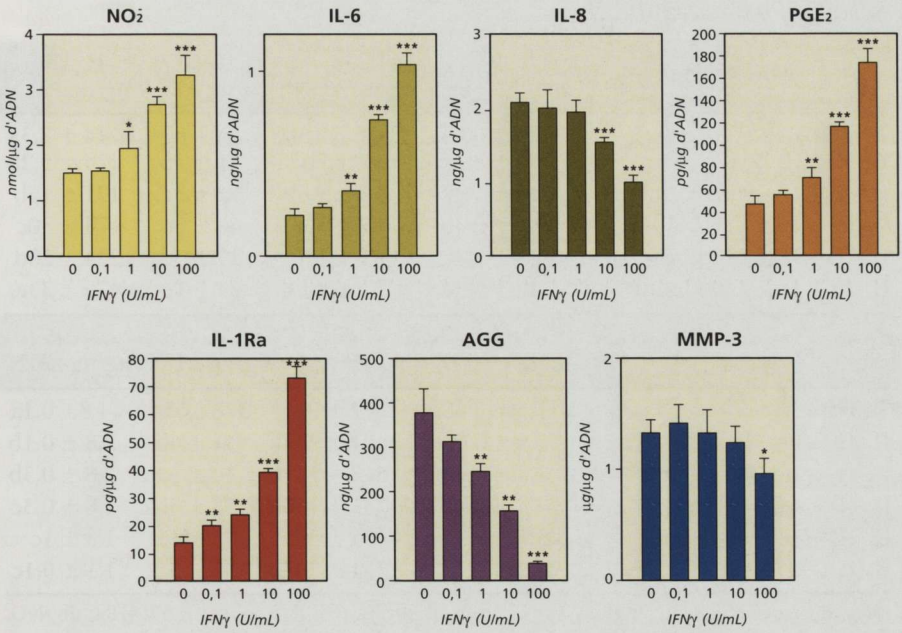


Figure III-1: *Effets de doses croissantes d'IFNγ humain recombinant sur la production de •NO, d'IL-6, d'IL-8, d'IL-1Ra, de PGE_2, et d'AGG par des chondrocytes sains cultivés durant trois jours en suspension en phase liquide. Les résultats sont exprimés par la moyenne et l'écart-type et sont représentatifs de trois expériences indépendantes réalisées avec des prélèvements de donneurs différents. Les astérisques indiquent les différences significatives entre le groupe témoin et les groupes traités par l'IFNγ avec un *p < 0,05, **p < 0,01 ou ***p < 0,001 (Test d'ANOVA)*

Cependant, le TGFß-1 et l'IGF-1 réduisaient de façon significative l'effet inhibiteur de l'IL-1ß.

Le TGFß-1 (2.10^{-5} M) diminuait de façon significative la production de MMP-3 ainsi que son activité enzymatique (test à la caséine marquée à la résorufine) mesurée dans les milieux de culture. De plus, le TGFß-1 augmentait la production de TIMP-1. A l'inverse, l'IL-1ß (1 et 5 U/ml), stimulait la synthèse de MMP-3 ainsi que l'activité enzymatique du milieu ($p < 0,01$, Test ANOVA) et à la concentration de 5 U/ml, inhibait la production de TIMP-1 ($p < 0,01$, test ANOVA). Lorsqu'il était ajouté simultanément avec l'IL-1ß, le TGFß-1 réduisait significativement l'effet de l'IL-1ß sur la synthèse de MMP-3 et ce quelle que soit la concentration d'IL-1ß. A l'inverse, le TGFß-1 diminuait de 50 % l'effet inhibiteur de l'IL-1ß sur la production de TIMP-1 (Figure III-4). Il en résultait que le rapport MMP-3/TIMP-1 était beaucoup plus faible en présence de TGFß-1 (Tableau III-2).

Tableau III-1: Effets de l'IFNγ et de l'IL-1ß sur la production d'IL-6, d'IL-8, d'IL-1Ra, d'IL-10, de PGE_2, de MMP-3 et d'AGG.

	IL-6 ng/µg ADN	IL-8 ng/µg ADN	IL-10 pg/µg ADN	IL-1Ra pg/µg ADN
Témoins	0,22 ± 0,04a	2,01 ± 0,14a	< 1 pg	14 ± 2,3a
IL-1ß 2.10^{-10}M	19 ± 2b	97 ± 7b	71 ± 3b	124 ± 5,2b
IL-1ß + IFNγ 0,1 U/ml	18 ±1,5b	96 ± 9,6b	62 ± 3c	131 ± 15b
IL-1ß + IFNγ 1U/ml	20,1 ± 2,3b	97 ± 8b	60 ± 5c	174 ± 30c
IL-1ß + IFNγ 10 U/ml	24 ± 1,4c	81 ± 11c	39 ± 3d	254 ± 20d
IL-1ß + IFNγ 100 U/ml	29 ± 5,3d	51 ± 8d	23 ± 1e	429 ± 33e

	NO_2^- nmole/µg ADN	PGE_2 pg/µg ADN	AGG ng/µg ADN	MMP-3 µg/µg ADN
Témoins	1,5 ± 0,08a	47 ± 7a	378 ±55a	1,3± 0,1a
IL-1ß 2.10^{-10}M	2,9 ± 0,09b	1401 ± 244b	41 ± 4b	2,8 ± 0,1b
IL-1ß + IFNγ 0,1 U/ml	2,8 ± 0,1b	1668 ± 341b	34 ± 3,8c	2,8 ± 0,3b
IL-1ß + IFNγ 1U/ml	3 ± 2b	1731 ± 160b	33 ± 3,2c	1,8 ± 0,3c
IL-1ß + IFNγ 10 U/ml	3,7 ± 0,3c	1454 ± 280b	35 ± 3c	1,3 ± 1c
IL-1ß + IFNγ100 U/ml	4,2 ± 0,1c	1534 ± 382b	32 ± 5c	1,1 ± 0,1c

Effets de doses croissantes d'IFNγ sur la production d'IL-6, d'IL-8, d'IL-10, d'IL-1Ra, de •NO, de PGE_2, d'AGG et de MMP-3 par des chondrocytes humains normaux primaires cultivés en l'absence ou en présence d'IL-1ß (10^{-10} M) durant 3 jours en suspension. Chaque condition de culture a été réalisée en triple et les valeurs représentées sont les moyennes et les écarts-types. Les lettres indiquent les différences significatives entre les groupes expérimentaux. $a \neq b \neq c \neq d \neq e$ avec un $p < 0,05$ (test d'ANOVA).

3.3.3. Régulation de l'activité biologique de l'IL-1ß par les formes activées de l'oxygène.

L'objectif de ce travail était de déterminer l'influence des FAO sur les synthèses de cytokines, de •NO, de PGE_2 et d'AGG par des chondrocytes humains traités par l'interleukine-1ß (IL-1ß) (Henrotin *et al.*, 1998; Mathy-Hartert *et al.*, 2001; Mathy-Hartert *et al.*, 2002). Dans ce but, nous avons utilisé deux protocoles expérimentaux:

Protocole 1: Les chondrocytes humains étaient cultivés en suspension en phase liquide pendant 48 heures en l'absence ou en présence d'IL-1ß humaine recombinante et d'un inhibiteur des •NO synthétases, le N^G-monométhyl-L-arginine (L-NMMA). Le milieu de culture était composé de DMEM sans rouge phénol enrichi par 1 % d'ITS+.

Protocole 2: Les chondrocytes humains étaient pré-incubés durant 3 heures dans du HBSS (Hank's Balanced Saline Solution) avec du S-nitroso-N-acétyl-D,L-penicillamine (SNAP considéré comme un donneur de •NO), du 3-morpholinisydnonimine (SIN-1, un donneur de •NO et de $O_2^{\cdot-}$), du peroxyde d'hydrogène (H_2O_2) ou du peroxynitrite ($ONOO^-$; solution préparée à partir de peroxynitrite pur solide

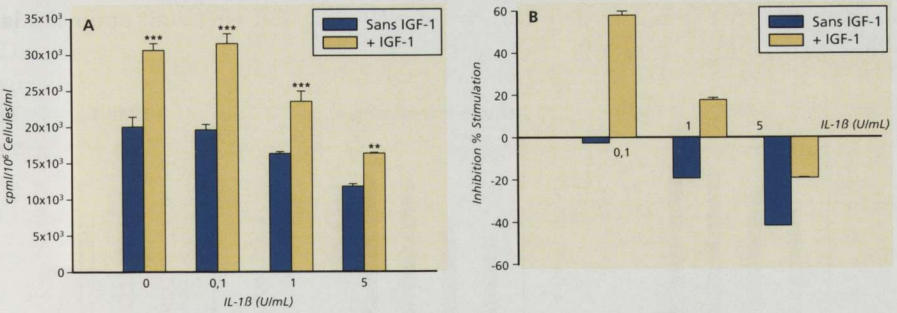


Figure III-2: *Effet de l'IGF-1 (10^{-8} M) sur la production totale de GAGs (déterminée par l'incorporation de ^{35}S) par des chondrocytes humains arthrosiques primaires cultivés 72 heures en l'absence ou en présence de doses croissantes d'IL-1ß (0,1-5 U/ml). Les résultats sont exprimés A) en valeurs absolues et B) en pourcentage de la valeur du groupe témoin (sans IL-1ß et sans IGF-1) et sont représentés par la moyenne et l'écart-type de trois cultures indépendantes réalisées avec des prélèvements d'origines différentes. Les astérisques indiquent les différences significatives entre le groupe témoin (IL-1ß seule) et les groupes traités par l'IGF-1: **= $p < 0,01$; ***=$p < 0,001$ (test ANOVA).*

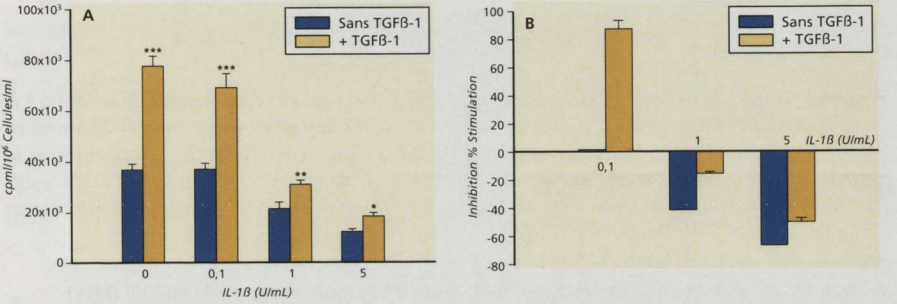


Figure III- 3: *Effet du TGFß-1 (2.10^{-5} M) sur la production totale de GAGs (déterminée par l'incorporation de ^{35}S) par des chondrocytes humains arthrosiques primaires cultivés 72 heures en l'absence ou en présence de doses croissantes d'IL-1ß (0,1-5 U/ml). Les résultats sont exprimés A) en valeurs absolues et B) en pourcentage de la valeur du groupe témoin (sans IL-1ß et sans TGFß-1) et sont représentés par la moyenne et l'écart-type de trois cultures indépendantes réalisées avec des prélèvements d'origines différentes. Les astérisques indiquent les différences significatives entre le groupe témoin (IL-1ß seule) et les groupes traités par le TGFß-1: *= $p < 0,05$; **= $p < 0,01$; ***=$p < 0,001$ (test ANOVA).*

synthétisé au laboratoire de Biochimie de l'Oxygène, Université de Liège). Après lavages, les cellules étaient cultivées durant 24 heures dans un milieu de culture (DMEM sans rouge phénol + 1 % ITS+) en l'absence ou en présence d'IL-1ß (10^{-11} M soit 170 pg/ml) (Figure III-5).

a) Effets du L-NMMA sur l'activité biologique de l'IL-1ß.

L'IL-1ß (10^{-12}-10^{-9} M) augmentait très significativement la production de •NO, appréciée par la mesure du taux de NO_2^- libéré par les chondrocytes dans le milieu de culture ($0,01 < p < 0,001$) (figure III- 6A). Notons, que l'effet stimulant de l'IL-1ß était maximal à la dose de 10^{-11} M. Le L-NMMA (0,5 - 2 mM) inhibait com-

plètement l'effet stimulant de l'IL-1ß (Figure III- 6B). Cet effet était optimal à la dose de 0,5 mM.

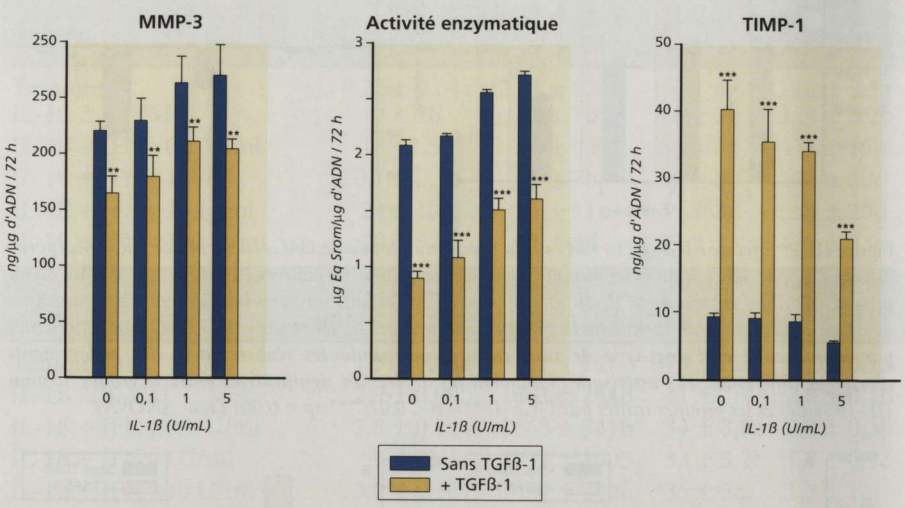


Figure III-4: *Effet du TGFß-1 (2.10^{-5} M) sur la production et l'activité enzymatique de la MMP-3 et la production de TIMP-1 par des chondrocytes humains arthrosiques primaires cultivés 72 heures en l'absence ou en présence de doses croissantes d'IL-1ß (0,1-5 U/ml). Les résultats sont représentés par la moyenne et l'écart-type de trois cultures indépendantes réalisées avec des prélèvements d'origines différentes. Les astérisques indiquent les différences significatives entre les groupes expérimentaux: **= p < 0,01; ***=p<0,001 (test d'ANOVA).*

Tableau III-2: Influence de l'IL-1ß et du TGFß-1 (2.10^{-5} M) sur le rapport MMP-3/TIMP-1

	Rapport MMP-3/TIMP-1			
	IL-1ß (U/ml)			
	0	0,1	1	5
- TGFß-1	21	22,5	32	55
+ TGFß-1	4	3,5	6,3	10

L'addition de L-NMMA (0,5 mM) au milieu de culture s'accompagnait d'une augmentation significative ($0,05 < p < 0,001$) de l'effet de l'IL-1ß sur la production d'IL-6, d'IL-8 et de PGE_2 (Figure III-7 A, B, C, D), suggérant que le •NO exercait un contrôle négatif sur la synthèse de ces médiateurs. Par contre, le L-NMMA ne modifiait pas significativement l'effet inhibiteur de l'IL-1ß sur la production d'agrécane (Figure III-7D).

Enfin, le L-NMMA (0,5 mM) ne modifiait pas le taux d' ARNm codant pour la COX-2, l'IL-6 et l'IL-8, indiquant que le •NO influençait les synthèses de PGE_2, d'IL-6 et d'IL-8 à un niveau post-transcriptionnel (résultats non illustrés).

b) Effets des FAO exogènes sur l'activité biologique de l'IL-1ß.

Dans nos conditions expérimentales, les chondrocytes arthrosiques exprimaient spontanément les gènes codant pour l'IL-6, l'IL-8, la COX-2, l' iNOS, la MMP-3 et la MMP-13. Le nombre de copies de cDNA a été quantifié par la technique de PCR en temps réel (Light cycler, Roche) et les valeurs répertoriées dans le tableau III-3.

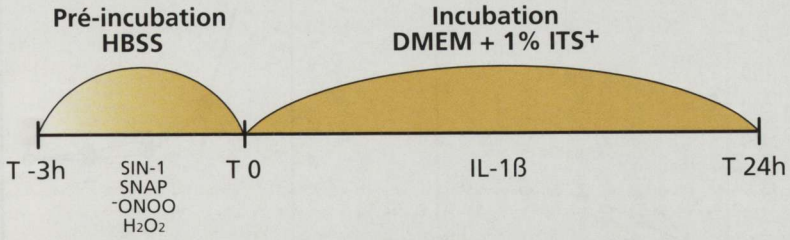


Figure III-5: *Représentation schématique du protocole d'incubation N° 2.*

L'addition d'IL-1ß au milieu de culture provoquait une forte augmentation du taux d'ARNm de l'IL-1ß, de l'IL-6 (42 x), de l'IL-8 (20 x), de l'iNOS (5,3 x), et de la COX-2 (8,8 x), et une diminution du taux d'ARNm de l'AGG (- 68 %) (Figure III-8 , 9 et 10). Le SNAP (10^{-4} M) ne modifiait pas l'effet stimulant de l'IL-1ß sur l'expression de ces gènes. Par contre, le SIN-1 (10^{-4} M), le ONOO- (10^{-5} M) et l'H_2O_2 (10^{-4} M) diminuaient significativement les taux d'ARNm de l' IL-1ß, de l'IL-6, de l'IL-8, de l' iNOS et de la COX-2 ($p < 0,001$). Notons que, l'H_2O_2 était significativement plus active que le SIN-1 ou le ONOO- sur l'expression de ces gènes ($0,05 < p < 0,01$; test d'ANOVA).

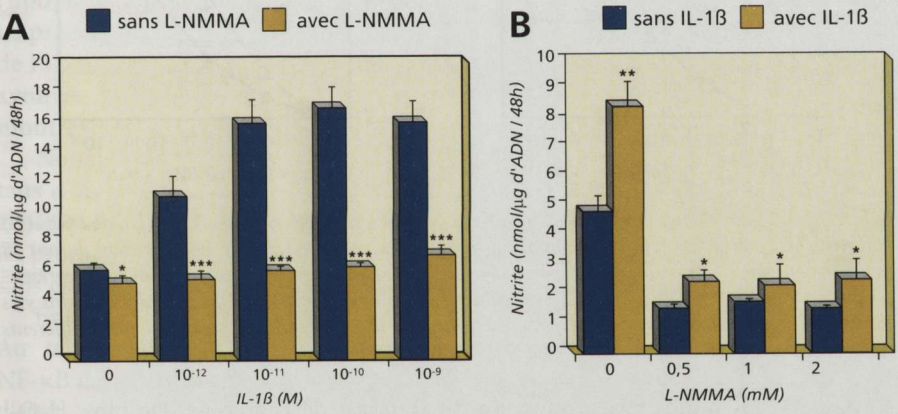


Figure III-6 A et B: *A) Production de NO_2^- (un des produits finaux du •NO) par les chondrocytes humains cultivés pendant 48 heures en l'absence ou en présence de doses croissantes d'IL-1ß, et avec ou sans L-NMMA à la concentration de 0.5 mM. B) Effet de doses croissantes de L-NMMA sur la production de NO_2^- par des chondrocytes humains cultivés en l'absence ou en présence d'IL-1ß 2.10^{-5} M. Chaque condition de culture a été réalisée en quadruple. Les résultats sont les moyennes et les écart-types de ces quadruples. Les astérisques indiquent les différences statistiquement significatives entre les groupes contrôles et les groupes traités: * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$ (test d'ANOVA).*

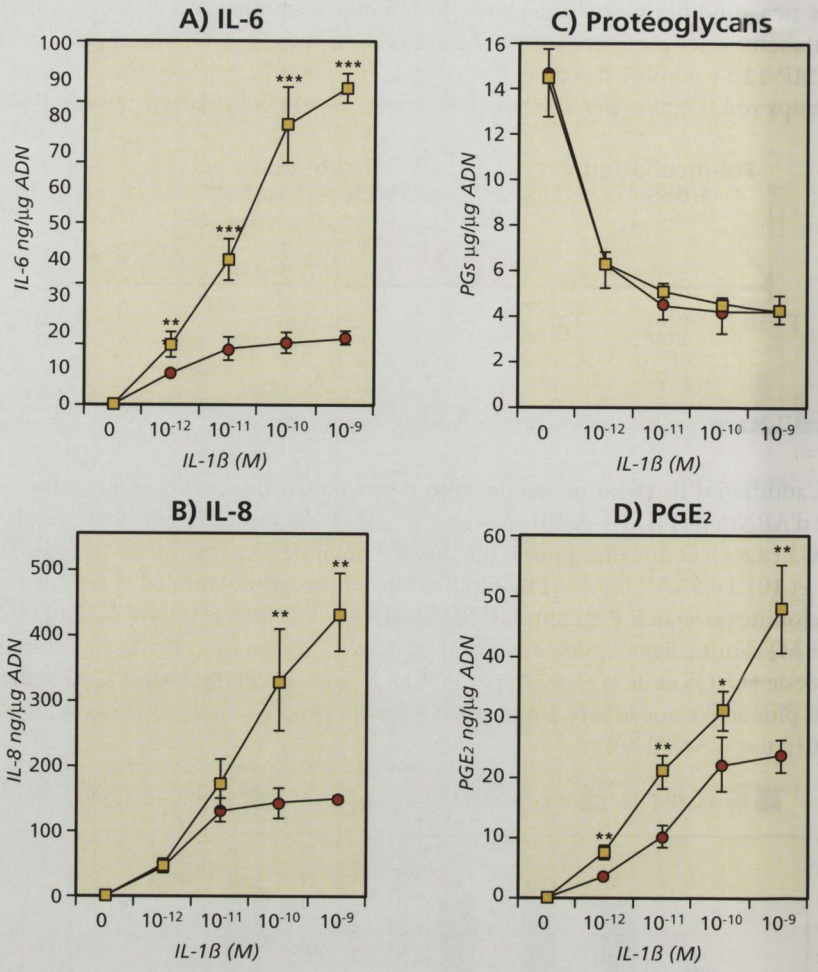


Figure III-7: *Effet de doses croissantes d'IL-1ß sur la production d'IL-6 (A), d'IL-8 (B), de PGE_2 (C) et d'AGG (D) par des chondrocytes humains primaires maintenus en culture en suspension durant 48 heures en l'absence (- ● -) ou en présence (- ■ -) de L-NMMA (0,5 mM). Les résultats sont représentés par la moyenne et l'écart-type de trois cultures réalisées indépendamment avec des chondrocytes provenant de donneurs différents. Les groupes traités par le L-NMMA sont statistiquement différents des groupes non traités avec un *p < 0,05; **p < 0,01 ou ***p < 0,001 (Test d'ANOVA).*

Seule H_2O_2 inhibait la production du transcrit de l'agrécane. De plus, H_2O_2 potentialisait l'effet inhibiteur de l'IL-1ß sur la production de cette molécule, alors que le SNAP et le SIN-1 étaient sans effet. Dans les conditions basales, le SNAP stimulait fortement l'expression des gènes codant pour la MMP-3 (x 95) et la MMP-13 (x 120). Par contre, SIN-1 et H_2O_2 n'avaient pas d'effets sur ces paramètres (Figure III-10).

Tableau III-3: Nombre de copies du cDNA exprimé par les chondrocytes arthrosiques dans les conditions basales ou en présence d'IL-1ß (10^{-11} M).

Gènes	Séquences	Nombre de copies de cDNA/ng d'ARN total	
		Basale n=3	+ IL-1ß n=3
hIL-1ß	5'-TCTCCGACCACCACTACAGCAA-3'		
	5'-GGGGAACTGGGCAGACTCAA-3'	ND	89 ± 9
hIL-6	5'-CCATGAGGAGACTTGCCTGG-3'		
	5'-GCACAGCTCTGGCTTGTTCC-3'	235 ± 16	9816 ± 678
hIL-8	5'-TGACTTCCAAGCTGGCCGTG-3'		
	5'-CGCAGTGTGGTCCACTCTCA-3'	6332 ± 92	127611± 1853
hiNOS	5'-CCATGGAACATCCCAAATAC-3'		
	5'-TCTGCATGTACTTCATGAAGG-3'	114 ± 13	610 ± 72
hCOX-2	5'-AACACCCTCTATCACTGGCATC-3'		
	5'-GCAGACATTTCCTTTTCTCCTG-3'	70 ± 2	612 ± 19
hAGG	5'-GCACCATGCCTTCTGCTTCCGAG-3'		
	5'-CTCCACTGCCTGTGAAGTCACCAC-3'	730± 61	235 ± 18

hIL-6 = interleukine-6 humaine; hIL-8 = interleukine-8 humaine; hiNOS = NO synthétase inductible humaine; hCOX-2 = cyclooxygénase-2 humaine; hAGG = agrécane humaine; ND = non détectable.

Nous avons réalisé des expériences similaires en utilisant une endotoxine (lipopolysaccharide, LPS, E. coli serotype O26:B6) à la place de l'IL-1ß. La pré-incubation avec le SNAP ou l'H_2O_2 inhibait l'expression des gènes de l'IL-1ß, de l'IL-6, de l'IL-8, de la COX-2 et de l'iNOS induite par le LPS, alors que le peroxynitrite et SIN-1 n'avaient pas d'effet significatif sur ces paramètres (résultats non montrés).

Nous avons également dosé, à l'aide de dosages immunologiques spécifiques, les taux d'IL-6, d'IL-8 et de PGE_2 dans les milieux de cultures collectés au terme de ces expériences. En présence d'IL-1ß, le SIN-1 et l'H_2O_2 diminuaient significativement les productions d'IL-6, d'IL-8 et de PGE_2 ($0,01 < p < 0,001$), alors que le SNAP était sans effet. (Figure III-11)

Enfin, nous avons recherché les effets des FAO sur la liaison du NF-κB à l'ADN. Au terme de trois heures d'incubation dans l'HBSS, seule l'H_2O_2 activait NF-κB dans les chondrocytes (Figure III-12). Par contre, SNAP et SIN-1 n'avaient pas d'effet. Nous avons également étudié l'activation de NF-κB dans les chondrocytes pré-incubés par les FAO et ensuite cultivés 24 heures en présence ou non d'IL-1ß. Il s'avérait que l'IL-1ß activait NF-κB et que H_2O_2 potentialisait cette activation. Par contre, le SNAP et le SIN-1 n'affectaient pas l'effet de l'IL-1ß.

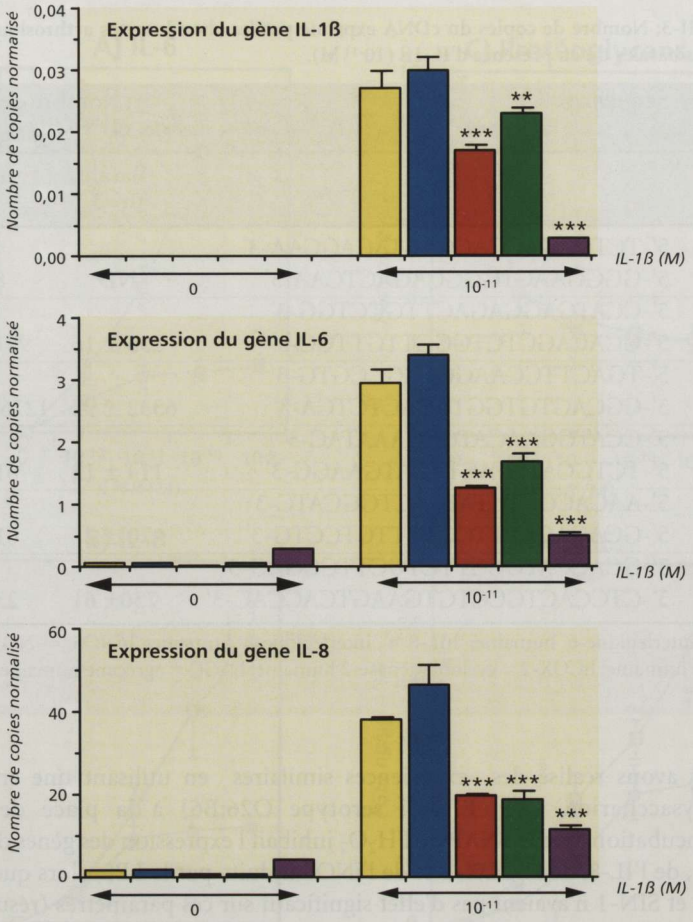


Figure III-8: *Effets des FAO sur l'expression des gènes codant pour l'IL-1ß, l'IL-6 et l'IL-8. Dans cette expérience, les chondrocytes étaient pré-incubés 3 heures en l'absence ( ) ou en présence de SNAP (10^{-4} M) ( ), de SIN-1 (10^{-4} M) ( ), de ONOO- (10^{-5} M) ( ), ou d'H_2O_2 (10^{-4} M) ( ). Après lavages, les cellules étaient mises en présence ou non d'IL-1ß (10^{-11} M) et incubées 24 heures. Les ARN totaux cellulaires étaient isolés et transformés en ADN complémentaires. Le nombre de copies d'ARN messager d'IL-1ß, d'IL-6 et d'IL-8 était mesuré par la technique de PCR en temps réel. Le nombre de copies des différents gènes était normalisé en fonction du nombre de copies du gène de référence GADPH. Les valeurs représentées sont la moyenne et l' écart-type de triple. Les groupes traités sont significativement différents du groupe contrôle (avec IL-1ß) avec un ** $p < 0,01$ ou ***$p< 0,001$ (Test d'ANOVA).*

3.4. Conclusions et discussion

Le chondrocyte joue un rôle prépondérant dans la physiopathologie de l'arthrose. Il synthétise les enzymes responsables de la chondrolyse et libère en grande quantité des médiateurs (cytokines, formes activées de l'oxygène, et

eicosanoïdes) impliqués dans le contrôle de la chondrolyse et de l'inflammation de la membrane synoviale. La régulation de la synthèse de ces médiateurs est un mécanisme physiopathologique important et constitue une cible thérapeutique pour les médicaments anti-arthrosiques. Dans ce travail, nous avons étudié l'influence de cytokines, telles que l'IGF-1, le TGFß-1 et l'IFNγ, ainsi que des FAO sur les

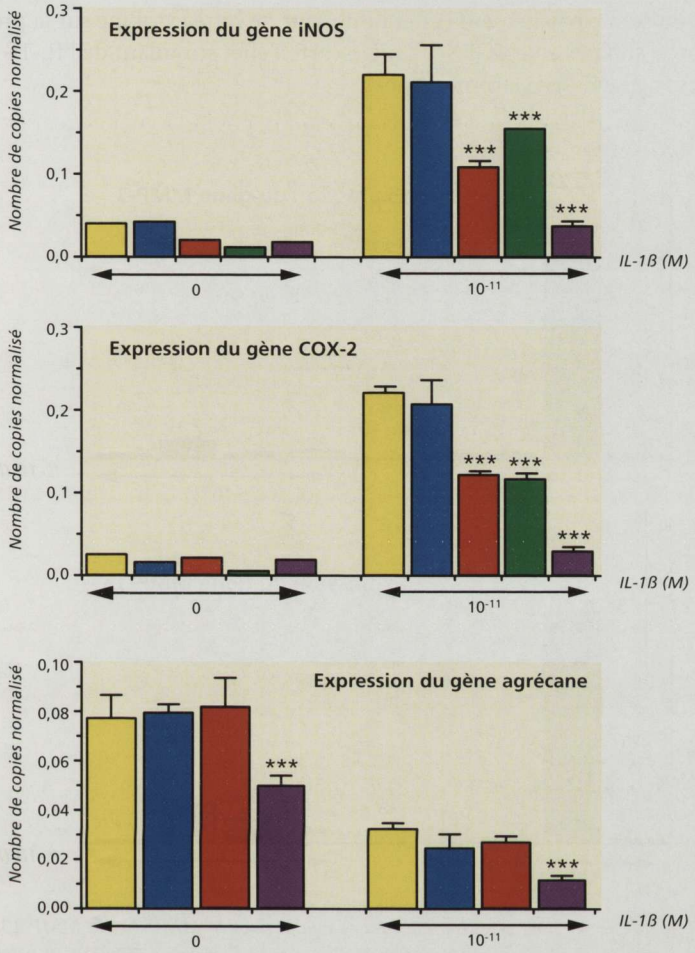


Figure III-9: *Effets des FAO sur l'expression des gènes codant pour l' iNOS, la COX-2 et l'AGG. Dans cette expérience, les chondrocytes étaient pré-incubés 3 heures en l'absence ( ) ou en présence de SNAP (10^{-4} M) ( ), de SIN-1 (10^{-4} M) ( ), de ONOO- (10^{-5} M) ( ), ou d'H_2O_2 (10^{-4} M) ( ). Après lavages, les cellules étaient mises en présence ou non d'IL-1ß (10^{-11} M) et incubées 24 heures. Les ARN totaux cellulaires étaient isolés et transformés en ADN complémentaires. Le nombre de copies d'ARN messager d'iNOS, COX-2 et d'AGG était mesuré par la technique de PCR en temps réel. Le nombre de copies des différents gènes était normalisé en fonction du nombre de copies du gène de référence GADPH. Les valeurs représentées sont la moyennes et l'écart-type d'expériences réalisées en triple. Les groupes traités sont significativement différents du groupe contrôle (avec IL-1ß) avec un ** $p < 0,01$ ou ***$p < 0,001$ (Test d'ANOVA).*

réponses du chondrocyte à l'IL-1ß. De notre travail, nous pouvons tirer les conclusions suivantes:

1) L'IFNγ agit en synergie avec l'IL-1ß pour stimuler la production de •NO, d'IL-6 et d'IL-1Ra et pour inhiber la production d'AGG. Par contre, il s'oppose aux effets de l'IL-1ß sur la synthèse de MMP-3, d'IL-8 et d'IL-10.
2) L'IGF-1 et le TGFß-1 s'opposent aux effets délétères de l'IL-1ß sur le cartilage. En effet, ils réduisent partiellement l'effet inhibiteur de cette cytokine sur la production de GAGs sulfatés et le TGFß-1 contre-carre l'effet stimulant de l'IL-1ß sur la synthèse et l'activité de la stromélysine-1.

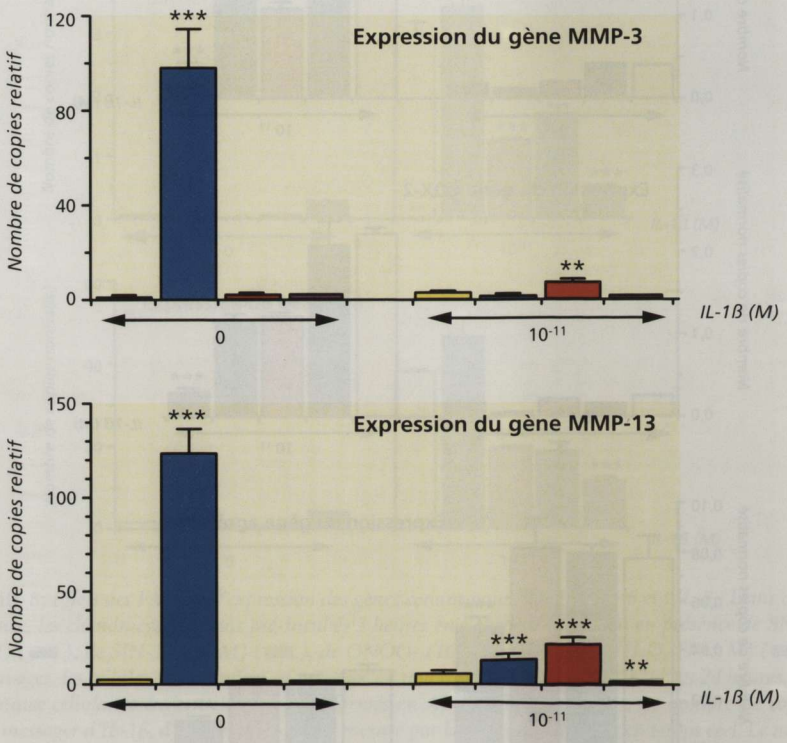


Figure III-10: *Effets des FAO sur l'expression des gènes codant pour la MMP-3 et la MMP-13. Dans cette expérience, les chondrocytes étaient pré-incubés 3 heures en l'absence ( ) ou en présence de SNAP (10^{-4} M) ( ), de SIN-1 (10^{-4} M) ( ), ou d'H_2O_2 (10^{-4} M) ( ). Après lavages, les cellules étaient mises en présence ou non d'IL-1ß (10-11 M) et incubées 24 heures. Les ARN totaux cellulaires étaient isolés et transformés en ADN complémentaires. Le nombre de copies d'ARN messager de MMP-3 et MMP-13 était mesuré par la technique de PCR en temps réel. Le nombre de copies des différents gènes était normalisé en fonction du nombre de copies du gène de référence GADPH. Les valeurs représentées sont la moyenne et l'écart-type d'expériences réalisées trois fois. Les groupes traités sont significativement différents du groupe contrôle (avec IL-1ß) avec un ** $p < 0,01$ ou ***$p< 0,001$ (Test d'ANOVA). Ce travail a été réalisé en collaboration avec le laboratoire du Prof P Bruckner (Université de Muenster).*

3) L'addition de L-NMMA, un inhibiteur des NO synthétases, amplifie l'effet stimulant de l'IL-1ß sur la synthèse d'IL-6, d'IL-8 et de PGE_2. Cette observation suggère que le •NO exerce un effet inhibiteur sur l'activité de l'IL-1ß sur la synthèse de cytokines inflammatoires.

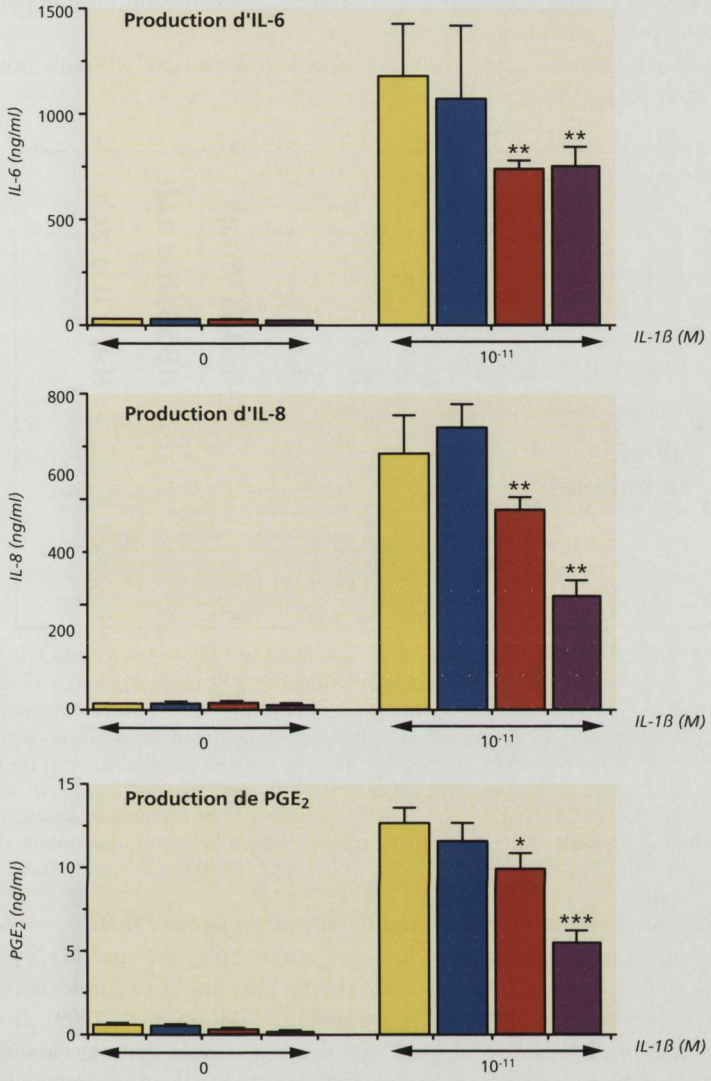


Figure III-11: *Effets des FAO sur la production d'IL-6, d'IL-8 et de PGE_2. Dans cette expérience, les chondrocytes étaient pré-incubés 3 heures en l'absence ( ) ou en présence de SNAP (10^{-4} M) ( ), de SIN-1 (10^{-4} M) ( ), ou d'H_2O_2 (10^{-4} M) ( ). Après lavages, les cellules étaient mises en présence ou non d'IL-1ß (10^{-11} M) et incubées 24 heures. L'IL-6, l'IL-8 et la PGE_2 étaient dosées dans les milieux de cultures conditionnés par les chondrocytes par des dosages immunologiques spécifiques de ces molécules. Les groupes traités étaient significativement différents du groupe contrôle (avec IL-1ß) avec un ** $p < 0,01$ ou ***$p < 0,001$ (Test d'ANOVA).*

4) Les effets de l'IL-1ß sur la quantité d'ARNm codant pour l'IL-6, l'IL-8, la COX-2, l'iNOS, l'AGG et MMP-3 ne sont pas modulés par le •NO. Par contre, dans les conditions basales, le •NO induit l'expression de MMP-3 et MMP-13, mais pas celle des autres gènes.
5) Le -ONOO et l'H_2O_2, mais pas le •NO, inhibent l'expression induite par l'IL-1ß des gènes inflammatoires.
6) Parmi les FAO testées, seule H_2O_2 est capable d'activer NF-κB dans nos conditions expérimentales.

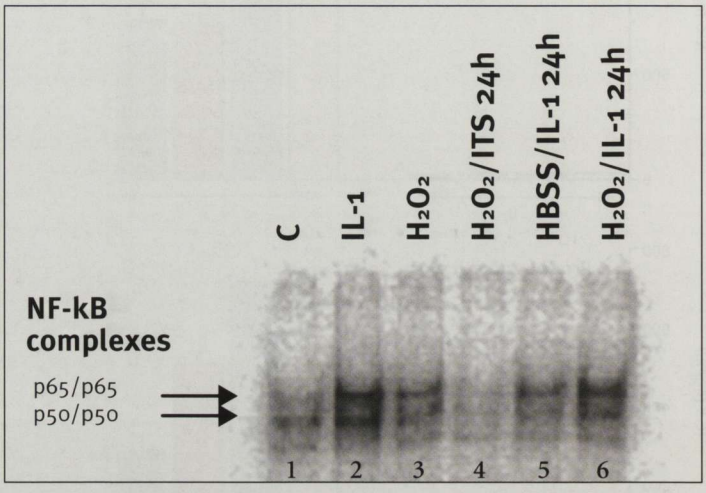


Figure III-12: *Effets des FAO sur l'activation de NF-κB. Dans cette expérience, les chondrocytes étaient pré-incubés 3 heures en l'absence ou en présence d'H_2O_2 (10^{-4} M). Après lavages, les cellules étaient mises en présence ou non d'IL-1ß (10^{-11} M) et incubées 24 heures. Au terme de cette période de pré-incubation de 3 heures (puits 1,2,3) et au terme de la période de culture de 24 heures en présence ou non d'IL-1ß (10^{-11} M) (puits 4,5,6), les protéines nucléaires étaient purifiées. Les extraits nucléaires (10 µg) étaient incubés en présence de la séquence nucléotidique consensus de NF-κB (AGTTGAGGGGACTTTCCCAGGC - 20 fmoles). Après liaison, les échantillons étaient fractionnés par électrophorèse en gel d'acrylamide (5 %). Ce travail a été réalisé avec la collaboration du laboratoire de Biochimie du Tissu Conjonctif du Professeur J-P Pujol (Université de Caen - France).*

L'interféron γ est une cytokine modulatrice des effets de l'IL-1ß

Actuellement, on connaît peu de chose sur le rôle joué par l'IFNγ dans la pathogénie de l'arthrose. L'IFNγ a été détecté par immunohistochimie dans le cytoplasme des chondrocytes de patients arthrosiques (Moos *et al.*, 1999). Il est synthétisé par les lymphocytes de la membrane synoviale et présent dans le fluide synovial des articulations arthrosiques à des concentrations biologiquement actives (20 - 100 U/ml)(Westacott *et al.*; 1990; Khale *et al.*, 1992; Schlaak *et al.*, 1996). Ces observations suggèrent que l'IFNγ est impliqué dans le développement de l'arthrose. Nos travaux supportent cette hypothèse et démontrent qu'à la concentration du fluide synovial de patients arthrosiques, l'IFNγ est actif sur le chondrocyte. Il augmente la production de •NO, d'IL-6 et d'IL-1Ra par les chondrocytes. Par contre,

il diminue la synthèse d'IL-8 et d'agrécane. Il est également un régulateur de l'activité biologique de l'IL-1ß. Il agit en synergie avec cette cytokine pour stimuler la production de •NO, d'IL-6 et d'IL-1Ra, mais s'oppose à l'effet stimulant de l'IL-1ß sur la production d'IL-8, d'IL-10 et de MMP-3. Il ressort de cette analyse que l'IFNγ est un agent frénateur du turnover des agrécanes. Des effets similaires ont été observés sur les synthèses de collagénases et de collagènes. L'IFNγ diminuait l'activité enzymatique de type collagénase mesurée dans des milieux de cultures conditionnés par des chondrocytes traités à l'IL-1ß (Andrews *et al.*, 1990) et inhibait en synergie avec l'IL-1ß et le TNFα l'expression des gènes codant pour les collagènes de type II, IX et XI (Goldring *et al.*, 1986 et 1994).

L'influence de l'IFNγ sur la libération de médiateurs de l'inflammation est différente selon le médiateur considéré. D'une part, il stimule la sécrétion par le chondrocyte de •NO, d'IL-6 et de PGE_2 et induit l'expression du complexe majeur d'histocompatibilité de classe II (CMH de classe 2) et de la molécule d'adhésion intercellular-adhesion-molecule-1 (ICAM-1) à la surface des chondrocytes (Quintavalla *et al.*, 1993; Bujia *et al.*, 1996). Par l'ensemble de ces actions, l'IFNγ contribue à la métamorphose du chondrocyte en cellule immuno-effectrice. Ces résultats plaident en faveur d'un effet pro-phlogistique de l'IFNγ et de sa contribution à l'exacerbation de la synovite de l'articulation arthrosique. D'autre part, l'IFNγ inhibe la production d'IL-8 et augmente la production d'IL-1Ra. Des effets similaires ont été observés par Seitz et ses collaborateurs (1994) sur des cultures de synoviocytes arthrosiques. Ces observations suggèrent que l'IFNγ puisse aussi exercer des effets anti-inflammatoires.

Cette dualité de l'IFNγ, nous encourage à émettre l'hypothèse selon laquelle l'IFNγ serait dans l'arthrose une cytokine régulatrice majeure de la réaction inflammatoire, responsable des caractéristiques particulières de la synovite du patient arthrosique. Celle-ci est caractérisée par l'absence de réaction systémique, la faible proportion de neutrophiles et une activité clinique modérée. Notre hypothèse repose sur la comparaison des rapports IL-1ß/IFNγ et TNFα/IFNγ des fluides synoviaux de patients souffrant d'arthrose ou de polyarthrite rhumatoïde. Dans les fluides synoviaux de patients souffrant de polyarthrite rhumatoïde (PAR) les rapports IL-1ß/IFNγ sont beaucoup plus élevés que dans les fluides de patients arthrosiques. Cette observation suggère que dans la PAR les effets de l'IL-1ß soient prépondérants sur ceux de l'IFNγ. Dans l'arthrose, le rôle modulateur de l'IFNγ sur l'IL-1ß serait beaucoup plus perceptible, limitant ainsi les effets inflammatoires de cette cytokine.

En conclusion, l'IFNγ est une cytokine régulatrice de l'expression des médiateurs de l'inflammation par le chondrocyte. La résultante de ses effets sur ces médiateurs pourrait déterminer le caractère particulier de la synovite arthrosique.

L'IGF-1 et le TGFß-1 s'opposent aux effets délétères de l'IL-1ß.

Le TGFß-1 et l'IGF-1 augmentent fortement la production d'agrécane par les chondrocytes et s'opposent à l'effet inhibiteur de l'IL-1ß sur cette production. Ces

effets ont déjà été décrits précédemment par plusieurs auteurs (Tyler *et al.*, 1989; Pujol *et al.*, 1991; Tesch *et al.*, 1992; Redini *et al.*, 1993; Lum *et al.*, 1996). A l'inverse, les effets de ces facteurs de croissance sur la synthèse de MMP-3 et de TIMP-1 par les chondrocytes restent peu explorés. Seules trois études on pu être recensées à ce jour (Su *et al.*, 1996; Hui *et al.*, 2001;Wang *et al.*, 2002). Nos travaux apportent de nouvelles informations sur le rôle antagoniste de l'IGF-1 et du TGFß-1 sur l'activité biologique de l'IL-1ß. Ils suggèrent que le résultat net de cet antagonisme sur la synthèse de GAGs sulfatés soit dépendant de la concentration de ces cytokines dans l'environnement de la cellule. Deux cas de figure ont été observés. En présence d'une faible concentration d'IL-1ß (< 1U/ml ou 20 pg/ml), l'effet stimulant de l'IGF-1 et du TGFß-1 sur la production d'AGG est prédominant. Par contre, lorsque la concentration d'IL-1ß est plus importante (≥ 1U ou 20 pg/ml), l'effet inhibiteur de l'IL-1ß prédomine même en présence d'une dose optimale de TGFß-1 ou d'IGF-1. Cette observation réalisée *in vitro*, apporte une explication rationnelle à l'échec des tentatives de réparation du cartilage observé *in vivo* au cours de l'arthrose. Cette tentative de réparation est caractérisée par la synthèse accrue des constituants de la matrice extracellulaire et un épaississement du cartilage. Nous émettons l'hypothèse que l'échec de cette tentative est secondaire à l'installation progressive d'un déséquilibre entre le taux et/ou l'activité de l'IL-1ß et des cytokines anabolisantes IGFs et TGFßs. Au début de la pathologie, l'activité du groupe TGFs/IGFs prédominerait sur celle de IL-1ß, expliquant l'hypertrophie du cartilage. Ensuite, l'inverse se produirait conduisant à l'accélération du turnover de la matrice extracellulaire et à la faillite de la tentative de réparation du cartilage. Ce déséquilibre pourrait résulter de l'association de plusieurs évènements tels que:

- L'augmentation de la synthèse d'IL-1ß par les chondrocytes ou les cellules de la membrane synoviale (Tetlow *et al.*, 2001);
- L'hypo-sensibilité acquise des cellules à l'action de l'IGF-1 et/ou du TGFß-1. Plusieurs études ont montré que les chondrocytes arthrosiques étaient moins sensibles à l'action de ces facteurs (Chevalier et Tyler, 1996; Loeser *et al.*, 2000; Boumédiène *et al.*, 2002). Ce comportement pourrait être secondaire à une augmentation de la concentration d'"IGF-1 binding proteins" (IGFBP) (Olney *et al.*, 1996;Sunic *et al.*, 1998; Loeser *et al.*, 2000) et à l'altération par les FAO des récepteurs de l'IGF-1 (Studer *et al.*, 2000) ou encore à la diminution de l'expression du récepteur TGFß-RII (Boumédiène *et al.*, 1998) au cours du développement de l'arthrose.

Nous avons également observé que le TGFß-1, mais pas l'IGF-1, réduisait l'activité enzymatique mesurée dans les milieux de culture conditionnés par les chondrocytes traités à l'IL-1ß. Cet effet est le résultat d'une diminution de la production de MMP-3, et d'une augmentation de la production de TIMP-1. En effet, il est intéressant de constater que le rapport MMP-3/TIMP-1 augmente avec la concentration d'IL-1ß même en présence de TGFß-1. Il en résulte une augmentation de l'activité enzymatique. Récemment, Hui *et al.*. (2001) ont montré sur des cultures primaires de chondrocytes humains que l'IGF-1 pouvait inhiber l'expression

induite par l'IL-1α des gènes de MMP-1, -3, -8 et -13, mais n'agissait pas sur l'expression du gène de TIMP-1. Ces résultats contradictoires peuvent s'expliquer par les méthodes utilisées et l'origine génétique de l'IL-1 administrée. L'IL-1α n'a pas les mêmes effets que l'IL-1ß sur les chondrocytes et l'expression d'un gène ne se traduit pas toujours par l'augmentation de la sécrétion de la protéine traduite. Par contre, nos résultats sur le TGFß-1 sont en accord avec ceux de la littérature. Sur des chondrocytes humains, Wang et ses collaborateurs (2002) ont montré que le TGFß-1 diminuait les taux intra-cellulaires de MMP-1 et -3, et augmentait ceux de TIMP-1 et -3.

En conclusion, le TGFß-1 et l'IGF-1 s'opposent à l'effet inhibiteur de l'IL-1ß sur la synthèse de GAGs sulfatés, mais seul le TGFß-1 semble actif sur la synthèse de MMP-3 et de TIMP-1. Il existe entre ces facteurs un équilibre précaire qui régit l'homéostasie des constituants de la matrice cartilagineuse.

Régulation de la production de médiateurs de l'inflammation par les formes activées de l'oxygène (FAO).

En 1993, nous avions démontré que les chondrocytes produisaient des FAO en réponse au phorbol myristate acétate (PMA), au TNFα ainsi qu'à des cycles d'anoxie-réoxygénation. Ensuite, d'autres auteurs ont décrit la présence d'une NADPH oxydase et de •NO synthétases dans le chondrocyte, suggérant que cette cellule est capable de produire de façon concomitante de l'anion superoxyde ($O_2^{\cdot -}$) et du monoxyde d'azote (•NO). A partir de $O_2^{\cdot -}$ et •NO, d'autres formes activées de l'oxygène et de l'azote (FAO) sont produites en cascade. De nombreux travaux suggèrent que les FAO agissent au sein de la cellule comme de véritables seconds messagers impliqués dans la régulation de la signalisation intracellulaire. Les effets enregistrés sont dépendants de la quantité de FAO produites, de la nature des FAO et de la voie de signalisation activée. Notre travail explore le rôle régulateur joué par les FAO sur l'expression de certains gènes inflammatoires par les chondrocytes. L'enjeu de cette recherche est important, car elle interroge un mécanisme physiopathologique responsable du passage à la chronicité de la réaction inflammatoire de la membrane synoviale. En effet, il est pour le moins étonnant d'observer que des souris déficientes en iNOS développent une arthrite plus sévère que celle observée sur des animaux exprimant cette enzyme (Veihelmann *et al.*, 2001).

Notre hypothèse de travail est que les FAO seraient des agents frénateurs de la synthèse de médiateurs de l'inflammation par le chondrocyte. Afin de la vérifier, nous avons utilisé deux protocoles expérimentaux différents:

- Dans le premier, les chondrocytes activés par le LPS ou l'IL-1ß, étaient cultivés en présence d'un inhibiteur des •NO synthétases, le L-NMMA;
- Dans le deuxième, les chondrocytes étaient exposés pendant trois heures à des flux exogènes de FAO, avant d'être activés par l'IL-1ß ou de LPS.

Ces expérimentations, nous ont permis de tirer les conclusions suivantes:

1) Le L-NMMA augmente très fortement l'effet stimulant de l'IL-1ß et du LPS sur la production de médiateurs de l'inflammation comme l'IL-6, l'IL-8 et la PGE_2 suggérant que le •NO ou une FAO dérivée, exerce un contrôle négatif sur la production de ces médiateurs de l'inflammation.
2) Le L-NMMA augmente fortement la production d'IL-6, d'IL-8 et de PGE_2, sans modifier le taux d'ARN messager codant pour ces médiateurs. Ces résultats suggèrent que le •NO exerce son action régulatrice à un niveau post-transcriptionnel.
3) Un traitement des chondrocytes par le SNAP, un donneur de •NO, inhibe l'effet stimulant du LPS sur l'expression des gènes inflammatoires, mais n'affecte pas l'effet de l'IL-1ß. A l'inverse, un traitement par le SIN-1, un donneur de •NO et d'$O_2^{\cdot-}$, ou par le peroxynitrite à l'état solide, inhibe l'effet stimulant de l'IL-1ß, mais pas celui du LPS. Il découle de ces observations que l'effet des FAO sur l'expression des gènes inflammatoires est tributaire de l'agent inducteur et par conséquent de la voie de signalisation activée.

Le •NO possède des propriétés anti-inflammatoires

De nombreux travaux s'accordent pour dire que le •NO est impliqué dans le contrôle de la réaction inflammatoire. Cependant, il existe de grandes divergences entre les auteurs sur le rôle joué par ce médiateur (Kolb et Kolb-Bachofen, 1998; St Clair, 1998). Certains auteurs lui attribuent des propriétés pro-inflammatoires (McCartney-Francis *et al.*, 1993; Stefanovic-Racic *et al.*, 1994) alors que d'autres lui confèrent des propriétés anti-inflammatoires (Veihelmann *et al.*, 2001). Nos résultats s'inscrivent dans ce second courant d'idée. En effet, nous avons clairement démontré que l'inhibition des •NO synthétases par le L-NMMA, augmente la libération de médiateurs de l'inflammation comme la PGE_2, l'IL-6 ou l'IL-8, suggérant que le •NO exerce un contrôle négatif sur la synthèse de ces médiateurs (Henrotin *et al.*, 1998). Nos travaux corroborent d'autres études qui décrivent le •NO comme un inhibiteur de la synthèse de PGE_2 par les macrophages de souris ou de rat (Habib A *et al.*, 1997; Patel *et al.*, 1999), d'IL-6 par les cellules de Kupffer (Stadler *et al.*, 1993), d'IL-1ß par les cellules endothéliales humaines ou encore d'anions superoxydes par les neutrophiles (Clancy *et al.*, 1992). L'ensemble de ces observations contribue à expliquer que des souris arthritiques déficientes en iNOS présentent une réaction inflammatoire exacerbée par rapport aux souris exprimant l' iNOS ou encore que le traitement de souris arthritiques par le L-NMMA aggrave la pathologie (Stefanovic-Racic *et al.*, 1995; Veihelmann *et al.*, 2001). Chez la souris iNOS déficiente, l' aggravation de la réaction inflammatoire s'accompagne d'une augmentation de l' expression des molécules d'adhésion P-selectine et VCAM-1 et de la fixation des leucocytes à l'endothélium vasculaire (Veihelmann *et al.*, 2001). Cette observation évoque que le •NO puisse agir comme un inhibiteur de l'expression de ces molécules, et par conséquent de l'adhésion des leucocytes à l'endothélium vasculaire. Une production accrue de cytokines inflammatoires par les cellules endothéliales et/ou leucocytaires pourrait aussi expliquer l'exacerbation de l'inflammation observée chez les animaux déficients en iNOS. Nos résultats appuient

cette hypothèse. En effet, dans nos conditions expérimentales, le •NO inhibe la libération par les chondrocytes d'IL-8 et de PGE_2, deux médiateurs de l'inflammation. L'IL-8 stimule le chimiotactisme des leucocytes vers le foyer inflammatoire, et la PGE_2 est une molécule vasodilatatrice impliquée dans la formation de l'œdème.

L'activité régulatrice des FAO n'est pas globale mais spécifique à certains gènes.

Les FAO sont considérées par de nombreux auteurs comme des acteurs de la signalisation intracellulaire. Elles modulent l'expression de nombreux gènes en agissant sur les récepteurs membranaires, les kinases cytoplasmiques ou encore les facteurs de transcriptions. Notre travail montre, que l'action des FAO n'est pas globale mais ciblée sur certains gènes, et que toutes les FAO n'ont pas les mêmes effets sur un même gène. Nous avons également montré que les FAO régulent différemment l'expression d'un même gène selon la voie de signalisation activée.

Ces conclusions émanent d'expérimentations réalisées sur des chondrocytes humains pré-incubés durant 3 heures avec des donneurs de FAO, (SIN-1 ou SNAP), du peroxynitrite à l'état solide ou du peroxyde d'hydrogène (H_2O_2) et ensuite, cultivés pendant 24 heures en présence ou non d'endotoxine (LPS) ou d'IL-1ß. Notons, que ni la quantité, ni l'intégrité de l'ADN (déterminée par le kit apoptotic DNA Ladder, Roche Diagnostic, Bruxelles, Belgique) n'étaient altérées par le traitement par les FAO. Le traitement des cellules par le SNAP, un donneur de •NO, inhibe l'expression des gènes de l'IL-1ß, de l'IL-6, de l'IL-8, de la COX-2 et de l' iNOS induite par le LPS, alors que l'expression des mêmes gènes par l'IL-1ß n'est pas modifiée par le SNAP. A l'inverse, l'induction de ces gènes par l'IL-1ß est diminuée par le SIN-1 et le peroxynitrite, alors que l'induction de ces gènes par le LPS n'est pas modifiée par le SIN-1. Ceci confirme la spécificité d'action des FAO.

Nous avons également observé que le SNAP induisait l'expression de MMP-3 et MMP-13, alors que le $ONOO^-$ et l'H_2O_2 n'avaient aucun effet. D'autres auteurs ont montré que l'incubation des chondrocytes avec le SNAP augmentait l'expression du gène de MMP-1 alors que l'addition de L-NMMA inhibait partiellement l'effet stimulant de l'IL-1ß sur l'expression de ce gène (Murrell *et al.*, 1995; Sasaki *et al.*, 1998). Ces observations suggèrent que le •NO agisse comme un second messager impliqué dans la transduction du signal intracellulaire. Son action semble spécifique de certains gènes. En effet, nous avons montré que le SNAP, dans les conditions basales induisait l'expression des gènes codant pour les MMPs, mais n'avait pas d'effet sur le gène de l'IL-6, de l'IL-8, de l'iNOS ou encore de la COX-2.

Nous avons également tenté de déterminer à quel niveau les FAO régulaient la transduction du signal. Nous avons étudié les effets du SNAP, du SIN-1 et d'H_2O_2 sur l'activation de NF-κB. Nous sommes conscients que les FAO peuvent contrôler la transduction du signal à de nombreux niveaux, et que le NF-κB n'est pas l'unique cible potentielle des FAO. Les données de la littérature de ces dix dernières années indiquent que les FAO peuvent aussi agir sur les récepteurs membranaires (Studer *et al.*, 2000), l'équilibre oxydo-réducteur de la cellule (Fialkow et Downey, 1997; Forman et Torres, 2001), les métabolites des phospholipides comme le diacylglycérol, les éléments de la voie du Ca^{2+} (Volk *et al.*, 1997; Karata et Hirata, 1997),

les phosphatases et les kinases (Pani *et al.*, 2000), d'autres facteurs de transcription nucléaire (c-jun, AP-1, p53, CREB, HIF1α, etc) (Schoonbroodt *et al.*, 1997; Piette *et al.*, 1997; Schoonbroodt et Piette, 2000), les mitochondries (Tomita *et al.*, 2001), la membrane et les protéines nucléaires (Leonarduzzi *et al.*, 2000), et l'ADN lui-même (Thannickal et Fanburg, 2000; Forman et Torres, 2001). Dès le début des années 1990, les FAO ont été considérées comme activatrices du NF-κB amenant sa fixation sur l'ADN (Shoonbroodt *et al.*, 1997: Piette *et al.*, 1997). Cependant, la nature des FAO impliquées reste imprécise et la répercussion de cette activation sur l'expression des gènes est toujours discutée (True *et al.*, 2000). Nos travaux apportent quelques données nouvelles sur ces points.

- Seule l'H_2O_2 parmi les FAO testées dans ce travail était capable d'activer le NF-κB. Cette activation était associée à une réduction du nombre de transcrits de l'IL-6, de IL-8, de COX-2, d'AGG et des MMPs.
- Le ONOO- réprimaient l'activation de certains gènes sans modifier l'activation du NF-κB, suggérant qu'il agisse via un autre mécanisme de la transduction du signal.
- Le •NO induit l'expression de MMP-3 et MMP-13, mais pas celle des autres gènes et ce, sans modifier l'activation du NF-κB.

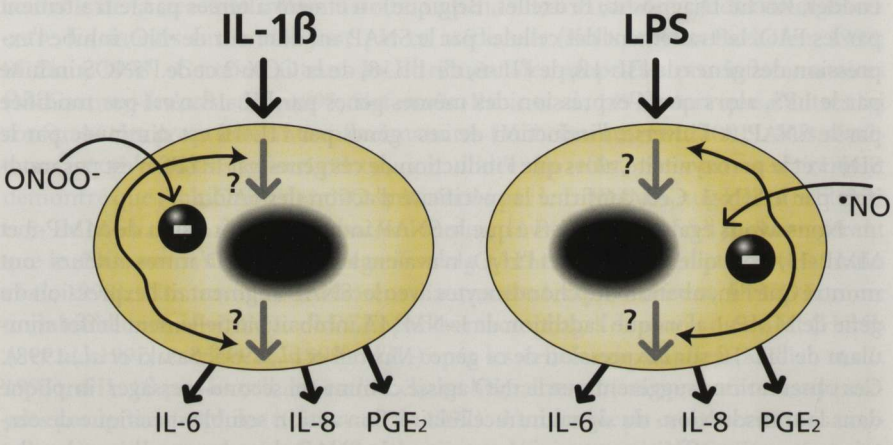


Figure III- 13: *Régulation de l'expression de gènes inflammatoires par différentes FAO. •NO = monoxyde d'azote; ONOO- = peroxynitrite; IL-6 = interleukine-6; IL-8 = interleukine-8; PGE_2 = Prostaglandine E_2.*

Il est donc plus que probable que d'autres facteurs de transcription ou d'autres cibles soient concernés dans la régulation, par les FAO, de l'expression des gènes du chondrocyte par l'IL-1ß. D'autres études réalisées sur le chondrocyte, ont montré que le NAC diminuait l'expression du gène codant pour c-fos et c-jun, alors que H_2O_2 augmentait l'expression de ces gènes (Lo *et al.*, 1996; Lo *et al.*, 1998a et b). Par contre, le SNAP et le L-NMMA n'exercent aucun effet significatif sur l'expression de c-fos et c-jun (Lo *et al.*, 1996; Lo *et al.*, 1998a et b). Les kinases, sont égale-

ment, la cible des FAO. Le traitement de chondrocytes par la N-acétyl-cystéine augmente la phosphorylation des protéines kinases de la famille des ERK-MAP et diminue celle de la jun-N-terminal kinase (JNK) (Li *et al.*, 2000). Par contre, le SNAP et l'H_2O_2 augmentent la phosphorylation de JNK. Enfin, le SNAP inhibe l'effet de IGF-I sur la synthèse de glycosaminoglycanes sulfatés en inhibant la phosphorylation de la tyrosine du récepteur IGF-IRß (Studer *et al.*, 2000).

Des études ultérieures seront nécessaires pour identifier les voies de signalisation régulées par les FAO. Quelques pistes seront explorées comme l'étude du statut oxydo-réducteur du chondrocyte, l'expression de gènes inhibiteurs, l'activation d'autres facteurs de transcription (AP-1) ou la genèse intracellulaire d'intermédiaire radicalaire. Les voies de recherche sont encore nombreuses. Cependant, notre travail ouvre de nouvelles pistes de réflexion sur la thérapie antioxydante dans le traitement de l'arthrose.

En conclusion, le •NO en inhibant la synthèse de médiateurs de l'inflammation par le chondrocyte pourrait exercer un effet anti-inflammatoire. Les FAO régulent l'expression de nombreux gènes impliqués dans pathogénie de l'arthrose. Leur action est spécifique de certains gènes et apparaît différente en fonction de la voie de signalisation intracellulaire activée.

CHAPITRE 4

Evaluation *in vitro* des médicaments à visée anti-arthrosique.

4.1. Les traitements médicamenteux de l'arthrose

Durant de nombreuses décennies, la prise en charge pharmacologique de l'arthrose s'est surtout concentrée sur l'aspect symptomatique de la maladie, sans chercher à interférer avec son évolution structurelle. Cependant, au cours de ces dernières années, tant les autorités réglementaires que des groupes d'experts ont défini les critères requis pour autoriser la mise sur le marché de médicaments dont l'indication est le traitement de l'arthrose (Dougados *et al.*, 1996, Altman *et al.*, 1996; Hochberg *et al.*, 1995a, b). Actuellement, les objectifs principaux de la prise en charge thérapeutique de l'arthrose sont d'améliorer le statut algo-fonctionnel des patients et de limiter la progression structurelle de la maladie. Les médicaments utilisés dans le traitement de l'arthrose sont administrés principalement par voie orale, par injection intra-articulaire ou par voie trans-cutanée locale (topique) (Tableau IV-1).

Tableau IV-1: Médicaments utilisés dans le traitement de l'arthrose.

Paracétamol
Anti-inflammatoires non stéroïdiens (AINS)
Salicylate non acétylé
Opioïdes
Tramadol
Sulfate de chondroïtine
Sulfate de glucosamine
Insaponifiables d'avocat et de soja (IAS)
Diacérhéine
Stéroïdes
Acide hyaluronique
Capsaicin
Méthylsalicylate

Selon l'American College of Rheumatology (2000)

Ces médicaments ont été classés en deux catégories principales selon la présence ou non d'un effet symptomatique et de la démonstration ou non d'une action sur la structure du cartilage (Reginster *et al.*, 1999; Altman *et al.*, 2000; Pendleton *et al.*, 2000) (Tableau IV-2).

Actuellement, seuls le sulfate de glucosamine, la diacérhéine (anthraquinone) et les insaponifibles d'avocat/soja ont montré des résultats encourageants sur la structure du cartilage dans des études randomisées, contrôlées contre placebo et de longue durée (2 à 3 ans) réalisées chez l'homme. Le paramètre utilisé était le pincement de l'interligne articulaire mesuré sur une radiographie standard (Reginster *et al.*, 2001; Dougados *et al.*, 2001; Lequesne *et al.*, 2002).

La stratégie thérapeutique utilisée dépendra de la localisation des plaintes, de l'intensité de la douleur, de la présence ou non d'une réaction inflammatoire sévère, de la réponse du patient à l'une ou l'autre de ces drogues et de la présence ou non

Tableau IV-2: Classification des spécialités pharmaceutiques selon leur pharmacodynamique

1. Médicaments à action symptomatique
a) à action rapide AINS, antalgiques
b) à action différée et rémanente (AASAL en français, SYSADOA en anglais) Sulfate de chondroïtine, sulfate de glucosamine, insaponifiables d'avocat/soja, diacérhéine et acide hyaluronique
2. Médicaments à action structurelle.
a) avec une action symptomatique* Sulfate de glucosamine, diacérhéine, insaponifiables d'avocat/soja
b) sans action symptomatique*

*Classification proposée par le Group for the Respect of Ethics and Excellence in Science (GRESS): osteoarthritis section. AASAL = anti-arthrosiques symptomatiques d'action lente; SYSADOA = Symptomatic Slow Acting Drugs for OsteoArthritis.

de facteurs de risque de co-morbidité (Reginster et Henrotin, 2001a,b).

Dans ce travail, nous avons étudié les effets des anti-inflammatoires non stéroïdiens (AINS), des insaponifiables d'avocat et de soja (IAS), de la rhéine et du ranélate de strontium (S12911) sur le métabolisme des chondrocytes. Les paragraphes qui suivent sont dévolus à la description des effets *in vitro* et *in vivo* de ces médicaments sur le cartilage articulaire.

4.1.1. Les anti-inflammatoires non-stéroïdiens (AINS)

L'utilisation des anti-inflammatoires non stéroïdiens (AINS) dans l'arthrose est justifiée par la présence d'une réaction inflammatoire siégeant au niveau de la membrane synoviale. Elle est modérée, souvent secondaire à la lésion du cartilage et limitée aux zones voisines de celle-ci (Lindblad et Hedfors, 1987; Amor, 1993). La réaction inflammatoire de la membrane synoviale est un élément pathogénique de l'arthrose. Elle est un facteur d'aggravation de la dégradation du cartilage et contribue à la détérioration du statut algo-fonctionnel du patient arthrosique.

Deux éléments essentiels doivent être pris en considération lors de la prescription d'un AINS chez un patient arthrosique: *la protection du cartilage* et *la bonne tolérance digestive.*

Protection du cartilage . Tout d'abord, l'action de ces molécules au niveau du cartilage est un critère de choix essentiel de la prescription d'un AINS pour traiter l'arthrose. De nombreuses études *in vitro*, ont montré que certains AINS étaient capables de modifier l'homéostasie des cytokines, des facteurs de croissance, des protéines matricielles ou des métalloprotéases, et de la sorte agir sur la structure du cartilage. Cependant, les preuves cliniques d'une interférence des AINS avec la progression structurelle des lésions d'arthrose sont rares. Nous n'avons recensé qu'une

seule étude prospective (n=819) multicentrique randomisée réalisée en double aveugle et contrôlée contre placebo, étudiant les effets de l'indométhacine (25 mg; 3 x/jour) et de l'acide tiaprofénique (300 mg, 2x/jour) sur l'évolution radiologique de l'arthrose du genou (Huskinsson *et al.*, 1995). Cette étude a montré qu'après 18 mois de traitement, 47 % des patients traités par l'indométhacine présentaient une aggravation des signes radiologiques, contre 22 % dans le groupe contrôle. Par rapport au contrôle, le risque relatif d'aggraver l'arthrose du genou après un an de traitement par l'indométhacine était de 2,1. Par contre, l'acide tiaprofénique était sans effet sur l'évolution radiologique de l'arthrose. Une autre étude a comparé les effets de l'indométhacine (50-75 mg/jour; n=56) et de l' azapropazone (600-900 mg/jour; n=49) sur l'évolution radiologique de la coxarthrose (Rashad *et al.*, 1989). Les patients inclus dans cette étude souffraient de coxarthrose sévère et étaient candidats à la pose d'une prothèse de hanche. Le pincement de l'interligne, mesuré tous les 3 mois, de la hanche affectée et de la hanche controlatérale progressait plus rapidement chez les patients traités à l'indométhacine que chez les patients sous azapropazone.

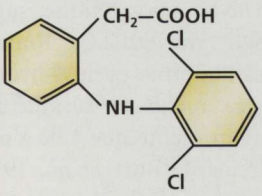
Bonne tolérance digestive. Le deuxième élément devant orienter le choix de prescription des AINS est, bien entendu, leur toxicité gastro-intestinale. Jusqu'il y a peu, la faible tolérance digestive, à long terme, des patients arthrosiques à la prescription d'AINS en limitait fortement l'utilisation (Griffin *et al.*, 1988; Griffin *et al.*, 1991; Smalley *et al.*, 1996). La genèse de nouvelles molécules, agissant préférentiellement sur l'isoenzyme II inductible de la cyclooxygénase, et épargnant aux doses thérapeutiques l'isoenzyme I constitutionnelle (COXIBs), a permis de réduire considérablement les effets secondaires (Emery *et al.*, 1999; Laine *et al.*, 1999; Hawkey *et al.*, 2000).

Les paragraphes qui suivent sont consacrés aux effets des AINS sur le statut algofonctionnel des patients arthrosiques et sur le métabolisme du cartilage. Nous avons concentré notre recherche bibliographique sur les AINS qui ont été testés sur nos modèles expérimentaux. Il s'agit de l'acéclofénac (ACECLO), du célécoxib (CELE), du diclofénac (DICLO), de l'indométhacine (INDO), de l'ibuprofène (IBU), du nimésulide (NIM), du piroxicam (PIROX) et du rofécoxib (ROFE) (Figure IV-1).

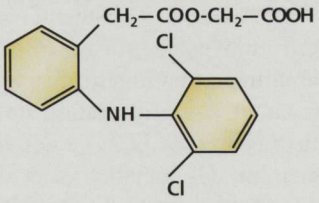
En clinique humaine

De nombreuses études contrôlées contre placebo ont étudié l'efficacité des AINS sur les symptômes du patient arthrosique. Tous les AINS que nous avons testés sur nos modèles expérimentaux ont montré un effet supérieur au placebo sur la douleur et la fonction articulaire des patients souffrant d'arthrose des membres inférieurs (Marcolongo *et al.*, 1997; Bocanegra *et al.*, 1998; Ravaud *et al.*, 1998). Le ROFE et le CELE, qui sont deux AINS dits COX-2 spécifique de référence, ont une efficacité clinique équivalente à celle d'un AINS classique (DICLO) sur la symptomatologie de la coxarthrose et de la gonarthrose, mais provoquent moins d'effets secondaires digestifs indésirables (Silverstein *et al.*, 2000; McKenna *et al.*, 2001).

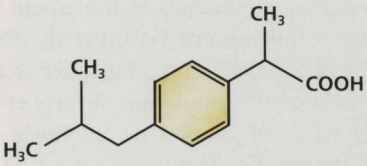
Diclofénac



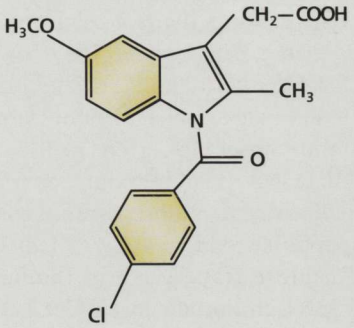
Acéclofénac



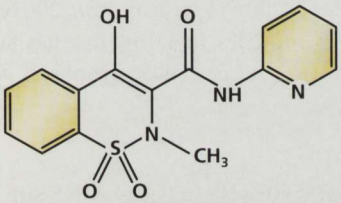
Ibuprofène



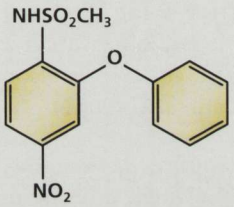
Indométhacine



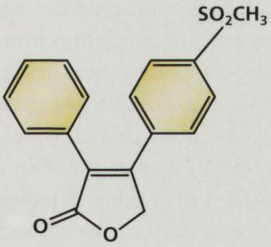
Piroxicam



Nimésulide



Rofécoxib



Célécoxib

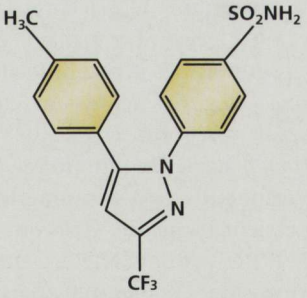


Figure IV-1: *Structure chimique des AINS testés dans notre étude.*

L'ACECLO est un AINS particulier qu'il est utile de traiter avec une attention toute particulière (Reginster et Henrotin, 2001c). En effet, la structure chimique de l'ACECLO, l'acide 2-[(2,6-dichlorophenyl)amino] phénylacétoxyacétique, suggère que cette substance puisse constituer un précurseur du DICLO. Toutefois, le métabolite plasmatique principal de l'ACECLO chez l'homme est le 4-hydroxy-acéclofénac et la concentration de diclofénac retrouvée au niveau sérique après l'administration d'ACECLO à des volontaires sains ne représente que 1 % à 6,7 % de l'ensemble des métabolites et de l'aceclofenac circulant (Bort *et al.*, 1996). L'ACECLO se caractérise, de manière constante dans les essais thérapeutiques l'ayant comparé aux AINS les plus fréquemment prescrits, par une efficacité similaire à celle de la molécule de référence et par un profil de tolérance dans de nombreux cas supérieurs à celui observé pour les autres AINS, dans le traitement de l'arthrose, de la polyarthrite et de la spondylarthrite ankylosante (Ward *et al.*, 1995; Diaz C *et al.*, 1996; Peris *et al.*, 1996; Kornasoff *et al.*, 1997; Perez-Busquier *et al.*, 1997; Yanagawa *et al.*, 1998). Dernièrement, une étude de population ouverte et de grande envergure, a été réalisée pour évaluer le profil général de tolérance de l'ACECLO (200 mg/jour) en pratique quotidienne. Le diclofénac retard (150 mg/jour) était utilisé comme comparateur de référence et l'étude a été revue et approuvée par la MCA (Medicine Control Agency): 10.142 patients souffrant d'arthrose, de polyarthrite rhumatoïde ou de spondylite ankylosante ont été inclus et suivis durant un an. L'ACECLO a engendré significativement moins de réactions indésirables, d'effets secondaires gastro-intestinaux et d'arrêt prématuré du traitement que le DICLO (Huskinsson *et al.*, 2000). Cette meilleure tolérance pourrait s'expliquer par la découverte récente de sa sélectivité d'inhibition pour la cyclooxygénase-2 (de Leval *et al.*, 2000; Lidbury *et al.*, 2000; Henrotin *et al.*, 2001c). Cependant, cette observation a été réalisée sur des modèles *in vitro* effectués sur sang complet, et devra être confirmée *in vivo.* (Warner *et al.*, 1999; Brooks *et al.*, 1999; Giuliano *et al.*, 2001)

Sur les cultures de chondrocytes

En 1999, Dingle a réalisé une vaste étude *in vitro* sur les effets des AINS sur la synthèse des GAGs par des explants de cartilage arthrosique prélevés au niveau de la tête fémorale. Dans cette étude, trois cent prélèvements provenant de donneurs différents ont été utilisés pour comparer les effets de dix AINS à la concentration plasmatique maximum (C_{max}) après la prise d'une dose thérapeutique de ces agents (Dingle, 1999 a et b). Les explants étaient maintenus en culture pendant 6 jours en présence ou non des drogues. L'analyse des résultats a permis de classer les AINS dans trois catégories selon leur effet sur la synthèse de GAGs. Dans le premier groupe étaient répertoriés les AINS qui augmentaient la synthèse de GAGs, tel que l'ACECLO. Dans le second groupe se trouvaient les AINS qui n'avaient aucun effet sur la synthèse de GAG, comme le DICLO ou le PIROX. Enfin dans le troisième groupe étaient inclus les AINS qui inhibaient la synthèse de GAGs. Il s'agissait du NIM, de l'IBUP, et de l'INDO. Cette étude a également démontré que les chondrocytes arthrosiques étaient plus sensibles aux AINS que les chondrocytes normaux.

Par exemple, l'ACECLO à la dose de 2 µg/ml n'avait pas d'effet sur la synthèse de GAGs lorsque les explants étaient normaux, mais stimulait la production de GAGs lorsqu'ils présentaient des lésions d'arthrose (Dingle, 1999a).

Tableau IV-3: Principaux effets des AINS sur le métabolisme des chondrocytes

Paramètres étudiés	AINS	Espèce	Modèle	Effet	Références
Synthèse de GAGs	INDO	Humain	OA Explants	*0 or ↘	McKenzie *et al.*, 1976
	IBUP				
	PIROX	Porc	N Explants	0	Herman *et al.*, 1986
	INDO			↘	
	INDO	Humain	N Explants	↘	Muir *et al.*, 1988
	NAP			0	
	DICLO	Porc	N Explants	↘	Dingle *et al.*,1991
	IBUP	Humain	N Monocouche	↗	Smith *et al.*, 1995
	DICLO			0	
	INDO	Humain	OA Explants	↘	Rainsford *et al.*, 1997
	PIROX			0	
	INDO	Porc	N Explants	↘	
	PIROX			0	
	NIM	Humain	OA Explants	↘	Dingle, 1999
	INDO			↘	
	IBU			↘	
	DICLO	Humain	OA Explants	0	Blot *et al.*, 2000
	ACECLO			↗	
Synthèse de collagènes	INDO	Poulet	Monocouche	↘	Fujii *et al.*, 1989
Effet de l'IL-1ß					
↘ synthèse GAG	ACECLO	Humain	OA Explants	><	Dingle, 1996
↗ Agrécane (dégradation)	ACECLO	Humain	OA Explants	><	Dingle, 1999
↗ MMPs activité	NIM	Humain	OA Explants	><	Pelletier *et al.*, 1993
	NAP			><	
	INDO	Bovin	N Alginate	><	Sadowski *et al.*, 2001
↗ Synthèse MMPs	INDO	Bovin	N Alginate	><	Sadowski *et al.*, 2001
	INDO	Humain	OA Monolayer	><	Yamada *et al.*, 1996
↗ IL-6 production	NIM	Humain	OA Explants	><	Pelletier *et al.*, 1993
	IBUP	Humain	N Monolayer	><	Smith *et al.*, 1995
	DICLO			0	
↘ TIMP-1 synthèse	INDO	Bovin	N Alginate	0	Sadowski *et al.*, 2001
	DICLO			0	
MMPs Activité	DICLO	Humain	OA Explants	↘	Vignon *et al.*, 1990
	INDO			↘	
	KETO			0	
TIMP-1 synthèse	INDO	Humain	N monocouche	↗	Yamada *et al.*, 1996
Expression du récepteur IL-1ß	INDO	Humain	N/OA monocouche	↘	Pelletier *et al.*, 1993

* Selon le prélèvement de cartilage; >< = s'oppose à l'effet de l'IL-1ß , ↘= diminue; ↗= augmente; 0 = sans effet significatif; N = Normal; OA = arthrosique; MMPs = métalloprotéinases; TIMPs= Tissue inhibitor of matrix métalloprotéinases; GAG = Glycosaminoglycanes; Il-6 = interleukine -6; IL-1ß = interleukine -1 bêta.

Les AINS s'opposent à certains effets de l'IL-1ß sur le métabolisme des chondrocytes. L'ACECLO réduit l'inhibition de la synthèse de GAGs induite par l'IL-1ß (Dingle, 1991 et 1996) et l'INDO contrecarre les effets néfastes de l'IL-1ß sur l'équilibre entre MMPs et TIMPs (Vignon *et al.*, 1990; Sadowski *et al.*, 2001). En effet, cet AINS diminue la synthèse et l'expression de MMP-1 et MMP-3 induite par l'IL-1ß, et s'oppose partiellement à l'inhibition de la synthèse du TIMP-1.

4.1.2. Les insaponifiables d'avocat et de soja (IAS)

Les insaponifiables d'avocat et de soja (IAS) utilisés dans le traitement de l'arthrose sont commercialisés sous le nom de Piasclédine 300® par le laboratoire Expanscience (Coubevoie, France). La posologie est d'une gélule à 300 mg par jour. La Piasclédine 300® contient 1/3 d'insaponifiable d'avocat et 2/3 d'insaponifiable de soja. La composition exacte de ces résidus n'est pas connue. Cependant, un certain nombre des composés du mélange ont été dosés par les laboratoires Expanscience (R&D, Epernon, France). Les résultats de ces analyses sont répertoriés dans le tableau IV-4.

Etudes cliniques

L'efficacité des insaponifiables d'avocat/soja sur la symptomatologie de la gonarthrose et de la coxarthrose a été rapportée par plusieurs auteurs (Blotman *et al.*, 1997; Maheu *et al.*, 1998; Appelboom *et al.*, 2001). Une étude prospective multicentrique, randomisée, contrôlée contre placebo et conduite en double aveugle, a étudié les effets des IAS (300 mg/jour en prise unique) sur la douleur et le handicap des patients coxarthrosiques et gonarthrosiques. La période d'administration du médicament ou du placebo était de 6 mois, et la durée du suivi clinique des patients de 8 mois. Cette étude a démontré que:

- Les IAS améliorent, dès le deuxième mois de traitement, le statut algo-fonctionnel (Index de Lequesne) et la douleur spontanée (mesurée à l'aide d'une échelle visuelle analogique) du patient arthrosique;
- L'effet des IAS sur la symptomatologie persistait 2 mois après l'interruption du traitement;
- Le nombre de patients ayant recours à un AINS durant la période de traitement était plus faible dans le groupe IAS que dans le groupe placebo (43 % versus 62 %). Cependant, la différence entre les deux groupes était à la limite de la sensibilité ($p = 0.054$);
- La prise d'IAS ne s'accompagnait pas d'effets secondaires indésirables importants.

Une autre étude prospective multicentrique randomisée, réalisée avec le concours de notre unité, incluant 260 patients souffrant de gonarthrose, a comparé les effets des IAS administrés aux doses journalières de 300 ou 600 mg et d'un placebo sur la symptomatologie (évaluée par l'indice algo-fonctionnel de Lequesne et une échelle visuelle analogique(EVA)) et la consommation d'AINS ou d'analgésiques (Appelboom *et al.*, 2001). Aux deux doses testées, les IAS diminuaient significativement la prise d'AINS/analgésiques (exprimée en mg/Equivalent de diclofénac par

Tableau IV- 4: Composition des insaponifiables d'huile d'avocat et de soja.

INSAPONIFIABLE D'HUILE D'AVOCAT		INSAPONIFIABLE D'HUILE DE SOJA	
FRACTION "H"	50 %	HYDROCARBURES	
SQUALENE	2 %	Hydrocarbures saturés	1 %
FRACTION "IA"	≈ 5 %	Hydrocarbures insaturés	≈ 4 %
FRACTION "I"	≈ 15 %	TOCOPHEROLS	
TOCOPHEROLS	traces	Teneur dans l'insaponifiable	≈ 10 %
STEROLS		(Teneur relative)	
Teneur dans l'insaponifiable	4 à 20 %	α-tocophérol	15 %
Teneur relative		γ-tocophérol	≈ 65 %
Cholestérol	traces	δ-tocophérol	≈ 20 %
Campestérol	10 %	STEROLS	
Stigmastérol	1 %	Teneur dans l' insaponifiable	≈ 45 à 65 %
ß-sitostérol	82 %	Teneur relative	
δ-5-avénasténol	7 %	Campestérol	15 à 30 %
δ-7-stigmasténol	traces	Stigmastérol	10 à 25 %
δ-7-avénasténol	traces	ß-sistostérol	40 à 70 %
ALCOOLS TERPENIQUES	traces	cholestérol	traces
METHYLSTEROLS	1 %	δ-5-avénasténol	traces
	≈	δ-7-stigmasténol	traces
		δ-7-avénasténol	traces
		ALCOOLS	
		Alcools terpéniques	1 à 10 %
		Alcools aliphatiques	traces
		METHYLSTEROLS	1 à 5 %

La fraction "H"= fraction contenant neuf constituants principaux non identifiés, fortement insaturés, peu polaires et migrant en chromatographie sur couche mince avec les hydrocarbures. La fraction "IA" = fraction composée de 3 constituants (IA1, IA2, IA3) répondant aux structures suivantes: IA_1 $CH_3(CH_2)_{25}CH_3$, IA_2 $CH_3(CH_2)_{27}CH_3$, IA_3 CH_3 $(CH_2)_{29}CH_3$. Fraction "I" = représente deux constituants principaux répondant aux formules: I_1 CH_2=CH-$(CH_2)_{10}$-CH_2-CHOH-CH_2-CHOH-CH_2OH, I_2 CH C-$(CH_2)_{10}$-CH_2-CHOH-CH_2-CHOH-CH_2OH.

jour) durant les trois mois de traitement par rapport au groupe placebo. Cependant, il n'y avait pas de différence significative entre la dose de 300 mg et 600 mg d'IAS. Cette étude confirme également le statut de médicament à action lente sur la symptomatologie de l'arthrose des IAS, et montre qu'une dose quotidienne de 600 mg n'a pas un effet supérieur à celui d'une dose de 300 mg. Récemment, l'effet structurel des IAS a été étudié sur une cohorte de 163 patients souffrant d'arthrose de la hanche au terme de 2 ans de traitement. Les IAS retardaient la progression du pincement de l'interligne articulaire mesuré sur radiographie standard, uniquement chez les patients souffrant de coxarthrose sévère (interligne inférieur à 2,5 mm à l'inclusion) (Lequesne *et al.*, 2002). Par contre, aucune différence significative entre les

groupes expérimentaux n'était observé lorsque l'analyse incluait l'ensemble des patients coxarthrosiques.

Chez l'animal

Sur un modèle d'arthrose induite chez le mouton par ménisectomie bilatérale, l'administration orale d'IAS (900 mg/jour) prévient la perte d'agrécane et augmente l'épaisseur du cartilage dans les zones voisines de la lésion chondrale. Par contre, le traitement par IAS ne prévient pas l'apparition de la lésion chondrale mais réduit significativement la sclérose de l'os sous chondral (Cake *et al.*, 2000).

Sur les chondrocytes en culture .

De nombreux effets *in vitro* des IAS ont été démontrés sur un modèle de chondrocytes de lapin cultivés en monocouche. Au terme de 7 jours de traitement, les IAS (10 µg/ml) augmentaient significativement la synthèse de collagènes (déterminée par l'incorporation de ^{3}H-proline) par les chondrocytes articulaires de lapin (Mauviel *et al.*, 1989). Aucun effet n'a pu être observé sur ce paramètre après 2 jours de traitement. Au terme de 2 jours d'incubation, les IAS (10 µg/ml) diminuaient également l'activité collagénolytique mesurée dans les milieux de culture de chondrocytes de lapin traités par l'IL-1ß recombinante humaine. Enfin, une pré-incubation de 5 jours avec les IAS, prévient l'augmentation de l' activité collagénolytique induite par l'IL-1ß (Mauviel *et al.*, 1991). Par ailleurs, les IAS (10 µg/ml) augmentent le taux d'ARN messager codant pour les transforming growth factors bêta (TGFß)-1 et -2 dans des chondrocytes bovins cultivés en monocouche (Boumédiène *et al.*, 1999). Cet effet apparaît après 24 heures d'incubation et s'accompagne d'une augmentation de la synthèse de TGFß-1 appréciée par un dosage immunologique. Par contre, les IAS n'exercent aucun effet sur l'expression des récepteurs pour les TGFßs, TGFßRI et TGFßRII. Ces éléments suggèrent que les IAS puissent avoir un effet bénéfique sur la matrice cartilagineuse, d'une part en bloquant l'activation des métalloprotéases et des cytokines cataboliques, mais également en contribuant à l'induction des mécanismes de réparation matricielle.

4.1.3. La Diacérhéine

La diacérhéine ou la diacétylrhéine est un dérivé de la famille des anthraquinones. Son métabolite actif est la rhéine (Figure IV-2). Cette molécule est commercialisée par la firme Negma (Courbevoie, France). Elle est administrée par voie orale sous la forme de gélules de 50 mg. La dose quotidienne recommandée est de 100 mg.

Chez l'homme

Chez des patients souffrant de coxarthrose (n=288), la diacérhéine (100 mg/jour) a été testée soit seule, soit en combinaison avec du tenoxicam (20 mg/jour), sur une période de 8 semaines. Dans cette étude, la diacérhéine s'est

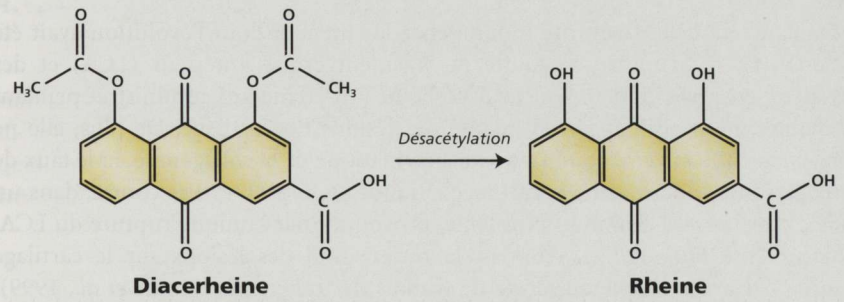


Figure IV-2: *Structure chimique de la diacérhéine et de la rhéine.*

révélée aussi efficace que l'anti-inflammatoire seul, sur la douleur (EVA) et le handicap (index de Lequesne) des patients. La combinaison des deux molécules a produit un effet supérieur à celui observé avec la diacérhéine ou le tenoxicam seuls (Nguyen *et al.*, 1994). Cependant, l'installation des effets sur la douleur était plus lente (6 semaines) dans le groupe diacérhéine que dans le groupe tenoxicam (2 semaines). De très récents résultats ont confirmé, chez 484 patients souffrant de gonarthrose, que la dose de 100 mg par jour de diacérhéine provoquait une diminution significative des phénomènes algiques au repos et au mouvement (appréciés par l'EVA et l'index algo-fonctionnel des "Western Ontario and Mac Master Universities" (WOMAC)), par rapport à un placebo, au terme de 16 semaines de traitement. La posologie de 100 mg correspond au meilleur rapport bénéfice/risque, par comparaison avec une posologie plus faible (50 mg/jour) ou plus élevée (150 mg /jour) (Pelletier *et al.*, 2000).

L'effet structurel de la diacérhéine sur l'arthrose de la hanche a été étudié dans une étude multicentrique en double-aveugle, incluant 507 patients. Dans cette étude, les patients recevaient pendant trois ans soit 100 mg de diacérhéine (2 x 50 mg/jour) soit un placebo. La progression de l'arthrose était évaluée par la mesure, sur une radiographie standard et à l'aide d'une loupe graduée, de la hauteur de l'interligne à l'endroit où il est le plus pincé. Bien que la variable principale qui était le pincement de l'interligne articulaire sur une période de 1 an ne soit pas affecté par le traitement à la diacérhéine, il apparaissait une diminution significative du nombre de patients présentant un réduction de l'interligne articulaire supérieur à 0,5 mm dans le groupe diacérhéine par rapport au groupe placebo. Par contre, aucun effet de la diacérhéine sur la symptomatologie n'a été observé dans cette étude. De plus, chez les patients qui avait terminé l'étude, la progression annuelle du pincement était plus lente dans le groupe traité à la diacérhéine que dans le groupe placebo (0,18 ± 0.25 mm/année versus 0,23 ± 0,23 mm/année; $p = 0.042$) (Dougados *et al.*, 2001). Cette étude avait également révélé que le nombre de patients souffrant d'effets secondaires indésirables était supérieur dans le groupe diacérhéine par rapport au groupe placebo. L'effet secondaire principal était de la diarrhée.

Chez l'animal

Dans un modèle d'arthrose induite chez le chien, et dont l'évolution avait été accélérée par la rupture conjointe du ligament croisé antérieur (LCA) et des afférences sensitives du membre, la diacérhéine (15-20 mg/kg) administrée pendant 8 semaines, ne modifiait pas la gravité des lésions du cartilage. De plus, elle ne changeait significativement, ni l'activité enzymatique de la collagénase, ni le taux de protéoglycanes mesuré dans le cartilage (Brandt *et al.*, 1997). Par contre, dans un modèle d'arthrose d'évolution plus lente, provoquée par l'unique rupture du LCA, la diacérhéine (40 mg/kg), retardait la progression des lésions sur le cartilage mesurée par arthroscopie au terme de 8 mois de traitement (Smith *et al.*, 1999). Cependant, au terme de 32 semaines de traitement, ni l'activité de type collagénase, ni le taux de protéoglycanes présents dans le cartilage arthrosique n'était modifié de façon significative par le médicament. Enfin, chez le mouton, présentant une arthrose secondaire à une ménisectomie, la diacérhéine (25 à 50 mg/kg/jour) administrée durant 9 mois, n'interférait pas avec les changements structuraux observés au niveau du cartilage et de l'os sous-chondral (Hwa *et al.*, 2001).

Sur les chondrocytes en culture

La diacérhéine et la rhéine à des concentrations comprises entre 10 et 20 µg/ml, inhibaient la production de monoxyde d'azote par les chondrocytes humains arthrosiques cultivés en monocouche (Pelletier *et al.*, 1998; Borderie *et al.*, 2001). Cet effet s'accompagnait d'une diminution du taux d'ARN messager codant pour la NO synthase ainsi que d'une diminution de la quantité d'enzyme. A l'inverse, la diacérhéine et la rhéine augmentaient l'effet stimulant de l'IL-1ß sur la production de cyclooxygénase de type 2 et de prostaglandine (PG)E_2 (Pelletier *et al.*, 1998).

L'incubation de chondrocytes de lapin cultivés en monocouche avec la diacérhéine (0,3 µg/ml) pendant 6 jours provoquait une stimulation de la synthèse du collagène (déterminée par l'incorporation de ^{3}H proline) et des glycosaminoglycanes (par incorporation de ^{35}S-sulfate) et une inhibition partielle de l'effet de l'IL-1ß sur ces synthèses. De plus, la diacérhéine (0,03 et 0,3 µg/ml) s'opposait à l'effet stimulateur de l' IL-1ß sur l'activité collagénolytique détectée dans le milieu de culture (Boittin *et al.*, 1993). La rhéine inhibait également de façon dose dépendante (0,03 -10 µg/ml), la production de métalloprotéase-1, -3, -9 et -13 et stimulait la synthèse de TIMP-1 par des chondrocytes de lapin en monocouche (Tamura *et al.*, 2001). Les effets bénéfiques de la diacérhéine sur le métabolisme du cartilage pourraient être secondaires à la sécrétion par les chondrocytes de TGFßs, et à l'inhibition de la production et/ou de l'activité de l'IL-1ß. En effet, la diacérhéine et la rhéine (20 µg/ml) inhibaient la synthèse de l'enzyme de conversion de l'IL-1ß (ICE) ainsi que la sécrétion d' IL-1ß et d'IL-18 par des explants de cartilage arthrosique (Moldovan *et al.*, 2000). De plus, ces drogues diminuaient de façon dose dépendante l'expression du récepteur de type I pour l'IL-1ß à la surface de chondrocytes humains arthrosiques. La diacérhéine (3 µg/ml) stimulait l'expression des gènes codant pour le TGFß-1 et -2 et la sécrétion de TGFßs actifs (déterminée par le test de croissance de cellules de poumon de vison CCL-64) (Félisaz *et al.*, 1999).

4.1.4. Le ranélate de Strontium

Le ranélate de strontium (S12911) est un sel de strontium présentant des effets sur le métabolisme osseux (Figure IV-3). Son utilisation est envisagée dans le traitement et la prévention de l'ostéoporose (Reginster, 1997). C'est dans cette optique que le S12911 a été développé par la firme Servier (Institut de Recherches Internationales, Servier, Courbevoie, France).

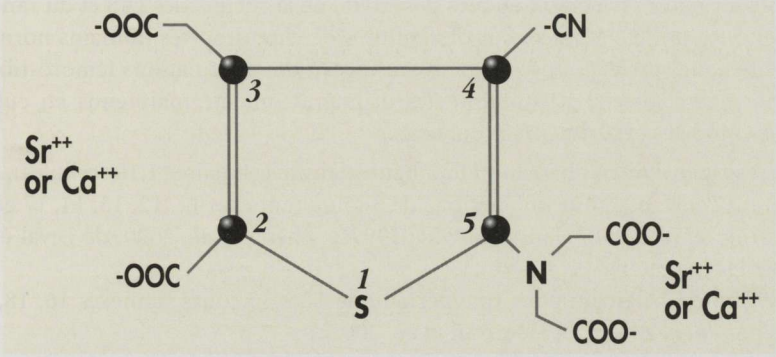


Figure IV-3: *Structure chimique du ranélate de strontium et de calcium (S12911 et S12911-0)*

Après deux ans d'administration, le ranélate de strontium (2 g/jour soit 517 mg de Sr^{2+}/jour) augmentait la densité osseuse lombaire chez des femmes souffrant d'ostéoporose post-ménopausique (Meunier *et al.*, 2002). Cette augmentation de la densité osseuse s'accompagnait d'une diminution de la résorption de l'os (évaluée par une diminution du taux urinaire de pyridinoline) et d'une augmentation de la formation osseuse (évaluée par une augmentation du taux sanguin de phosphatase-alcaline osseuse) (Reginster *et al.*, 1997). Ce médicament prévient également l'apparition de nouvelles fractures vertébrales durant la seconde année de traitement (Meunier *et al.*, 1996; 1997).

In vitro, le ranélate de strontium stimule la prolifération des cellules préostéoblastiques et la formation de matrice osseuse (Marie *et al.*, 1993; Canalis *et al.*, 1996) mais inhibe la résorption d'explants os et l'activité des ostéoclastes (Su Y *et al.*, 1992). Le S12911 (77-308 mg/kg/jour) administré durant 2 mois à des rates ayant subi une ovariectomie prévient l'ostéopénie par l'inhibition de la résorption osseuse, sans affecter l'ostéoformation (Marie *et al.*, 1993). Une autre étude a démontré que l'administration de sels de strontium chez le rat s'accompagne d'une augmentation du contenu en strontium non seulement de l'os, mais également du cartilage. De plus, l'administration de fortes concentrations de strontium (3 et 10 mM de $SrCl_2$) chez de jeunes rats, provoque des troubles de la croissance et une altération du métabolisme du chondrocyte (diminution de la consommation d'O_2, diminution de la synthèse de GAG et de collagènes, diminution de la capacité de fixation des agrécanes à l'acide hyaluronique) (Svensson *et al.*, 1985, Reinholt *et*

al., 1985). Ces résultats suggèrent que le strontium de ranélate, administré dans le traitement de l'ostéoporose, puisse également moduler le métabolisme des chondrocytes. Notre étude a été élaborée dans le but de vérifier cette hypothèse.

4.2. Matériels et méthodes

4.2.1. Les modèles de culture de chondrocytes.

Les propriétés pharmacologiques des AINS, de la rhéine, des IAS et du ranelate de strontium ont été étudiées sur des cultures de chondrocytes humains normaux ou arthrosiques prélevés post-mortem au niveau des articulations fémoro-tibiales et fémoro-patellaires. Les chondrocytes primaires ont été maintenus en culture selon les modèles expérimentaux suivants:

- soit en suspension en phase liquide, à haute densité cellulaire (1.10^6 cellules), sous agitation constante pour une période de 3 jours (annexes 11, 12, 13, 14, 17 et 20) (Henrotin *et al.*, 1998; Henrotin *et al.*, 1999c; Zheng *et al.*, 2000; de Leval *et al.*, 2000a; Henrotin *et al.*, 2001 b,c).
- soit en billes d'alginate pour une période de 12 à 15 jours (annexes 16, 18, 19) (Sanchez *et al.*, 2002 a, b; Henrotin *et al.*, 2002c).

Pour l'ensemble des expérimentations décrites dans ce chapitre, le milieu nutritif utilisé était composé de DMEM (Dubbelcco's Modified Eagle's Medium) enrichi par 1 % d'ITS+, 10 mM d'HEPES, de la glutamine 200 µg/ml, de la proline 2 mM, de la pénicilline (100 U/ml), de la streptomycine (100 µg/ml) et de la vitamine C 50 µg/ml. Dans les cultures réalisées pour déterminer le pouvoir antioxydant des molécules, le DMEM était remplacé par du HBSS (HANK'S Balanced Salt Solution, pH 7.4), afin d'éviter la présence dans le milieu réactionnel de molécules piégeuses des formes activées de l'oxygène.

4.2.2. Les traitements

Les AINS ont été testés à la concentration plasmatique maximale (C_{max}), obtenue chez l'adulte jeune après la prise orale d'une dose thérapeutique de la molécule. Le Tableau IV-5 reprend les valeurs des C_{max} pour les différents AINS testés dans cette étude.

Les insaponifiables d'avocat et de soja (A1S2; Piasclédine 300®) ont été testés à des concentrations comprises entre 0,625 et 40 µg/ml. Nous ne disposons pas d'information sur la pharmacocinétique des constituants des insaponifiables. Dès lors, nous avons choisi comme concentration de référence la dose de 10 µg/ml, qui est la dose couramment utilisée dans les études *in vitro*. De plus cette dose semble efficace sur plusieurs fonctions métaboliques du chondrocyte.

Tableau IV-5: Cmax des anti-inflammatoires nonstéroïdiens testés dans notre étude.

Nom	PM	Abréviation	Cmax mg/L	Cmax µM	Références
Aceclofénac	354	ACECLO	7,4	20,9	Brogden and Wiseman,1996
4-OH acéclofénac	370	4-OH ACECLO	2,6	6,9	Bort *et al.*, 1996
Diclofénac	318	DICLO	1,4	4,4	Todd *et al.*, 1988
Indométhacine	358	INDO	2	5,6	Oberbauer *et al.*, 1993
Nimésulide	308	NIM	3	9,7	Davis et Brogden, 1994
4-OH Nimésulide	328	4-OH NIM	1,4	4,2	Lucker PW, 1993
Rofécocib	314	ROFE	1	3,2	Depre *et al.*, 2000
Célécoxib	381	CELE	0,7	1,8	Clemett *et al.*, 2000
Piroxicam	331	PIROX	7	21,1	Hundal *et al.*, 1993
Ibuprofène	212	IBUP	25	118	Davies, 1998

PM = Poids moléculaire. Le 4-OH NIM est le métabolite principal du NIM dans le sang, et le 4-OH ACECLO le métabolite principal de l'acéclofénac.

La concentration plasmatique maximale de la rhéine (PM: 284) mesurée chez des volontaires sains après la prise orale d'une dose de 50 mg de diacérhéine est de 3 µg/ml. Dans le fluide synovial de patients arthritiques, le taux de rhéine mesuré au terme d'un mois de traitement à raison d'une capsule de 50 mg de diacérhéine toutes les 12 heures est compris entre 0,3 et 3 µg/ml (Spencer and Wilde, 1997; Nicolas *et al.*, 1998). Nous avons choisi d'évaluer les effets de la rhéine à des doses comprises entre 0,03 et 3 µg/ml. Les doses supérieures à 3 µg/ml étaient cytotoxiques.

Le ranélate de strontium (S12911; $C_{12}H_6N_2O_8S\ Sr_2$; PM = 665,2) a été testé aux concentrations de 10^{-4} M (0,066 mg/ml), 5.10^{-4} M (0,333 mg/ml) et 10^{-3} M (0,666 mg/ml) et comparé au ranélate de calcium (S12911-0; $C_{12}H_6N_2O_8S\ Ca_2$) ainsi qu' au chlorure de strontium ($SrCl_2 .6\ H_2O$; PM = 266,6). Afin de déterminer si le composé actif était le strontium ou le ranélate, nous avons comparé les effets du ranélate de strontium à ceux du ranélate de calcium et du chlorure de strontium. Le chlorure de strontium a été administré à la dose de $2 .10^{-3}$ M (0,530 mg/ml) afin d'obtenir une quantité de strontium équivalente à celle ajoutée avec le ranélate de strontium.

4.2.3. Les paramètres

De nombreux paramètres ont été mesurés afin de déterminer le profil pharmacologique de ces médicaments sur le métabolisme des chondrocytes articulaires normaux ou arthrosiques.

- L'agrécane a été dosée dans les différentes phases de la culture cellulaire par un dosage immunoenzymologique (EASIA, Enzyme Amplified Sensitivity Immunoassay; Biosource, Fleurus, Belgique) ou un dosage radioimmunologique (RIA) développé par Gysen et Franchimont en 1984.

- La production de glycosaminoglycanes sulfatés a été mesurée par l'incorporation de ^{35}S dans les chaînes polysacharridiques néo-synthétisées et le poids moléculaire des glycoprotéines déterminé par filtration sur Sépharose CL2B.
- L'activité enzymatique de type collagénase a été quantifiée dans le surnageant de culture par une méthode enzymatique basée sur la digestion de collagène de type I de rat marqué au tritium, et l'activité enzymatique de type stromélysine par la digestion de la caséine couplée à la résorufine (Cawston et Barret, 1979; Housley *et al.*, 1993).
- La stromélysine-1 (MMP-3) et l'inhibiteur tissulaire de métalloprotéases-1 (TIMP-1) ont été quantifiés dans les milieux de culture par un dosage immunoenzymologique (Biosource, Fleurus, Belgique).
- La quantité de prostaglandine (PG)E_2 libérée dans les milieux de culture a été mesurée à l'aide d'un dosage radioimmunologique spécifique mis au point par Serteyn et ses collaborateurs en 1988.
- Les interleukines-6 et -8, ainsi que le MIP-1ß (Macrophage Inflammatory Protein-1), ont été quantifiés dans le surnageant et la phase cellulaire des cultures par des dosages immunoenzymologiques (EASIA, Biosource Europe, Fleurus, Belgique).
- La production de monoxyde d'azote (•NO) était appréciée par la quantification du nitrite et du nitrate dans les surnageants de culture par une méthode de spectrophotométrie basée sur la réaction de Griess (Green *et al.*, 1982) .
- Dans cette étude, les formes activées de l'oxygène ont été détectées par la méthode de chimioluminescence amplifiée par le luminol.

4.3. Résultats

4.3.1. Effets des AINS sur le métabolisme des chondrocytes.

Les principaux objectifs de notre travail étaient de comparer les effets à long terme de huit AINS sur le métabolisme des chondrocytes arthrosiques et de contribuer à la compréhension des mécanismes qui sous-tendent leurs actions. Rappelons que les AINS étaient testés à la concentration qui correspondait au taux plasmatique maximum de la drogue atteint après la prise d'une dose thérapeutique de l'AINS (C_{max}). A cette concentration, tous les AINS rendaient indétectable la production de PGE_2 par les chondrocytes. Nous avons également tenté de démontrer que les AINS n'étaient pas que des inhibiteurs des cyclooxygénases mais qu'ils agissaient également sur d'autres mécanismes physiopathologiques comme la production de cytokines ou de formes activées de l'oxygène (FAO).

Ces travaux ont fait l'objet de six publications que le lecteur peut consulter en annexe (Annexe 11-16) (Henrotin *et al.*, 1999c; Mouithys-Mickalad *et al.*, 2000; Zheng *et al.*, 2000; Henrotin *et al.*, 2001; de Leval *et al.*, 2001a; Sanchez *et al.*, 2002a). Il ressort de ces publications les enseignements suivants:

a) Parmi les huit AINS que nous avons testés sur notre modèle en billes d'alginate, seuls l'ACECLO (+ 22 %) et l'INDO (+ 25,9 %) augmentaient de façon significative la production d'agrécane par les chondrocytes arthrosiques alors que le

NIM, le DICLO, le ROFE, le CELE, l'IBUP et le PIROX n'influencaient pas significativement ce paramètre (Figure IV-4). L'effet de l'ACECLO était optimal à la concentration de 7,4 µg/ml et celui de l'INDO à la concentration de 2 µg/ml (Figure IV-5). Ces concentrations correspondaient aux C_{max} de ces molécules. L'augmentation d'agrécane avait lieu essentiellement dans la matrice inter-territoriale (Further Removed Matrix, FRM), suggérant que les agrécanes synthétisées sous l'action de l'ACECLO et de l'INDO étaient fonctionnelles c'est-à-dire capables de s'intégrer à la matrice néo-synthétisée (Figure IV-6).

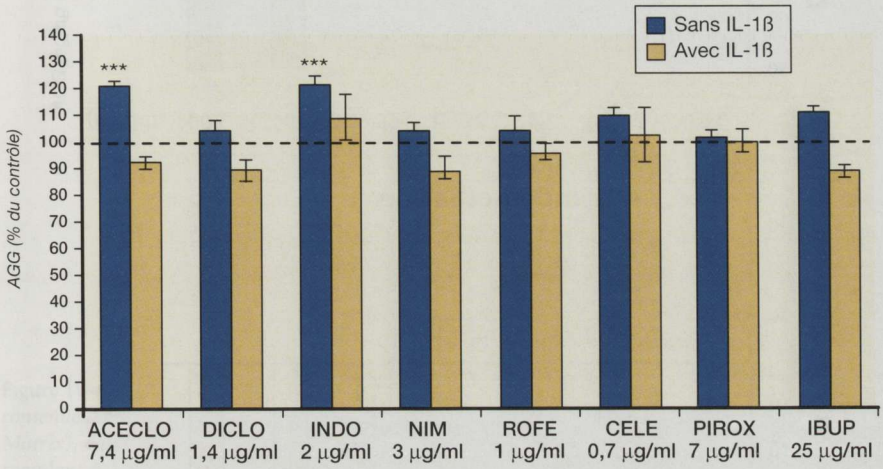


Figure IV-4: *Effets des AINS sur la production totale (CM + FRM + S) d'agrécane par des chondrocytes humains arthrosiques cultivés pendant 12 jours en billes d'alginate, en l'absence ou en présence d'IL-1ß 10^{-10} M. Les résultats sont exprimés en pourcentage du contrôle (avec ou sans IL-1ß) et correspondent aux moyennes ± 1 écart-type de trois expériences réalisées indépendamment avec du cartilage provenant de trois donneurs différents. ***p<0,001 (Test d'ANOVA).*

b) S'il apparaît clairement que certains AINS étaient capables d'augmenter la production d'agrécane par les chondrocytes, aucun n'avait démontré, dans nos conditions expérimentales, une capacité à réduire la synthèse ou l'activité de la stromélysine-1. Par contre, tous les AINS, à l'exception du DICLO et du NIM avaient une action stimulante sur la production basale de TIMP-1. En présence d'IL-1ß, la production de TIMP-1 était fortement inhibée. Tous les AINS, à l'exception du DICLO, s'opposaient partiellement à cet effet (Figure IV-7).

c) L'ACECLO, le DICLO, l'INDO et le NIM étaient des inhibiteurs de la production basale d'IL-6. Par contre, le ROFE, le CELE, le PIROX et l'IBUP n'avaient pas d'effet sur la production de cette cytokine (Figure IV-8). Il était également intéressant de constater que les AINS n'étaient pas actifs sur la synthèse de toutes les cytokines. En effet, à l'exception du CELE et de l'IBUP, aucun des AINS que nous avons étudiés n'avait d'effet sur la production d'IL-8. Le CELE et l'IBUP augmentaient significativement la production basale d'IL-8. Enfin, ils étaient tous inactifs sur la synthèse de MIP-1ß.

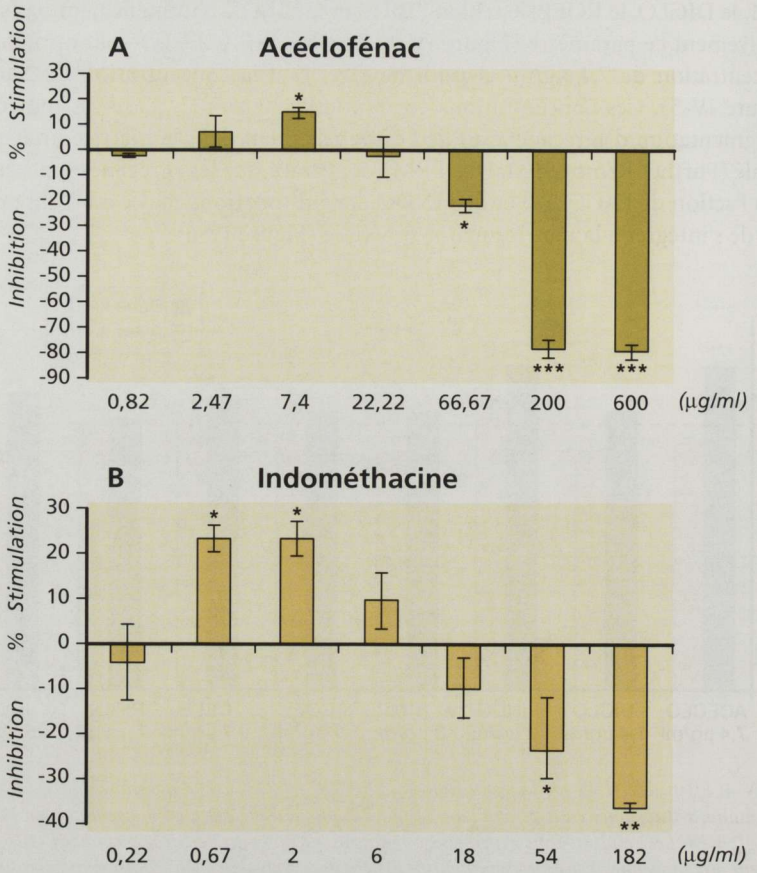


Figure IV-5: *Effet de doses croissantes d'ACECLO (A) et d'INDO (B) sur la production totale d'agrécane par les chondrocytes humains arthrosiques maintenus en culture durant 12 jours en billes d'alginate. Les résultats sont représentés par la moyenne ± 1 écart-type d'une culture de chondrocytes humains arthrosiques. Les groupes traités sont significativement différents du groupe contrôle avec un* $^{*}p < 0,05$, $^{**}p < 0,01$ *ou* $^{***}p < 0,0001$ *(Test d'ANOVA).*

d) Certains AINS possèdaient aussi des propriétés antioxydantes. En effet, l'ACECLO et le DICLO diminuaient significativement la production basale de •NO par les chondrocytes humains arthrosiques en billes d'alginate (Figure IV-9). En présence d'IL-1ß, la production de •NO était fortement augmentée. L'ACECLO, le DICLO, l'INDO et le NIM inhibaient significativement l'effet stimulant de l'IL-1ß, alors que le ROFE, le CELE, le PIROX et l'IBUP n'avaient pas d'effet.

Le NIM inhibait de façon temps et dose-dépendante la production spontanée de FAO, détectée par chimioluminescence (Figure IV-10), par les chondrocytes humains arthrosiques (voir aussi annexe 14) en culture en suspension en phase liquide.

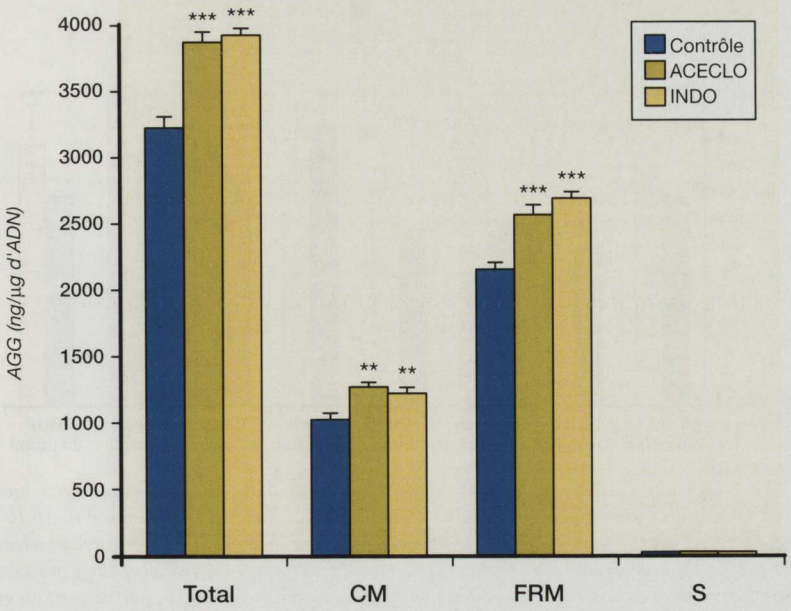


Figure IV-6: *Influence de l'ACECLO (7,4 µg/ml) et de l'INDO (2 µg/ml) sur la quantité d'agrécane contenue dans les différents compartiments de la culture en billes d'alginate. FRM (Further Removed Matrix), équivalant à la matrice inter-territoriale du cartilage; CM (Cell-associated matrix) correspondant à la matrice péri-cellulaire et territoriale du cartilage; S (milieu de culture). Les groupes traités sont différents du groupe contrôle avec ***p < 0,001 (Test de ANOVA).*

4.3.2 Effets des insaponifiables d'avocat et de soja (IAS) sur le métabolisme des chondrocytes.

Nous avons également déterminé le profil pharmacologique des IAS, un médicament formé d'un mélange d'insaponifiables d'avocat et de soja. La Piasclédine (A1S2) a été testée aux concentrations de 0,625, 2,5, 10 et 40 µg/ml. La dose de 10 µg/ml était la dose de référence utilisée dans la majorité des travaux de recherche *in vitro* réalisés sur cette molécule (annexes 17 et 18). Ils concluaient, notamment, que A1S2, aux doses de 10 et 40 µg/ml et après 12 jours d'incubation, augmentait de manière très significative (+ 40 %; $p < 0,001$) la production d'agrécane synthétisée par les chondrocytes arthrosiques cultivés en billes d'alginate (Figure IV-11) (Henrotin *et al.*, 1998; Henrotin *et al.*, 2002d). L'augmentation d'agrégane était observée essentiellement dans la zone inter-territoriale de la matrice extracellulaire (FRM).

Lorsque A1S2 et IL-1ß étaient ajoutées simultanément, A1S2 ne s'opposait pas à l'effet inhibiteur de l'IL-1ß sur la synthèse d'agrécane. Par contre, A1S2 restaurait la production d'agrécane, après que celle-ci ait été bloquée par l'IL-1ß (Figure IV-13).

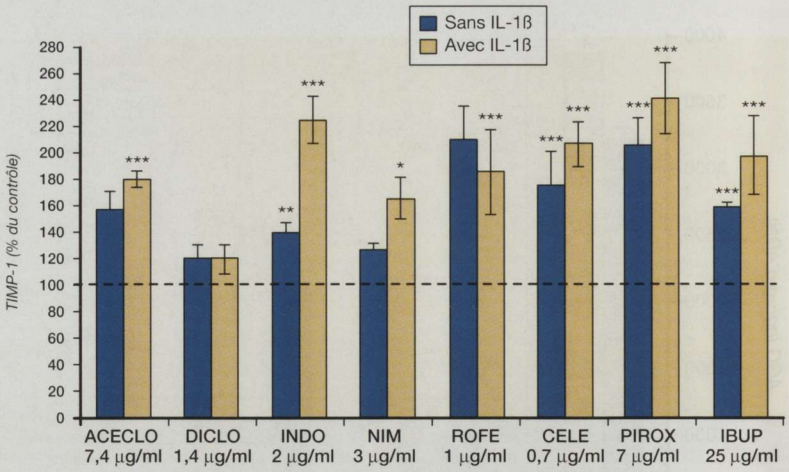


Figure IV-7: *Effets des AINS sur la quantité de TIMP-1 produite par les chondrocytes humains arthrosiques cultivés pendant 12 jours en billes d'alginate, en absence ou en présence d'IL-1ß 10^{-10} M. Les résultats sont exprimés en pourcentage du contrôle (avec ou sans IL-1ß) et correspondent aux moyennes ± l'écart-type de trois expériences réalisées indépendamment avec du cartilage provenant de trois donneurs différents. Les astérisques indiquent les variations significatives par rapport au groupe contrôle (avec ou sans IL-1ß): * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ (Test d'ANOVA).*

Contrairement aux AINS, A1S2 inhibait de façon dose-dépendante la production de stromélysine-1 (MMP-3) (Figure IV-13), mais n'avait pas d'effet sur la production basale de TIMP-1. Il en résultait une diminution du rapport MMP-3/TIMP-1. A la concentration de 10 µg/ml et après 12 jours d'incubation, A1S2 réduisait de 50 % le rapport MMP-3/TIMP-1.

A1S2 est aussi un inhibiteur de la synthèse de cytokines pro-inflammatoires. Il inhibait de façon dose- et temps-dépendante la production d'IL-6, d'IL-8 et de MIP-1ß par les chondrocytes humains arthrosiques. (Figure IV-13). De plus, il s'opposait partiellement à certains effets de l'IL-1ß. Il contrecarrait partiellement l'inhibition de TIMP-1 ainsi que la stimulation de PGE_2, d'IL-6, d'IL-8 ou de •NO (Résultats non illustrés).

4.3.3. Effets de la rhéine sur le métabolisme des chondrocytes.

La rhéine a été testée à des concentrations comprises entre 0,03 et 3 µg/ml. Chez des sujets sains, le taux plasmatique de rhéine mesuré après l'administration orale de 2 x 50 mg de diacérhéine est de 3 µg/ml.

La rhéine, aux concentrations comprises en 0,1 et 3 µg /ml, augmentait de façon significative la quantité d'agrécane contenue dans la bille d'alginate. A la dose de référence (C_{max}) et après 12 jours d'incubation, la rhéine augmentait de 46 % la quantité d'agrécane contenue dans la bille d'alginate (Sanchez C *et al.*, 2002b) (annexe 19) (Figure IV-14). L'augmentation était essentiellement observée dans la fraction FRM de la culture.

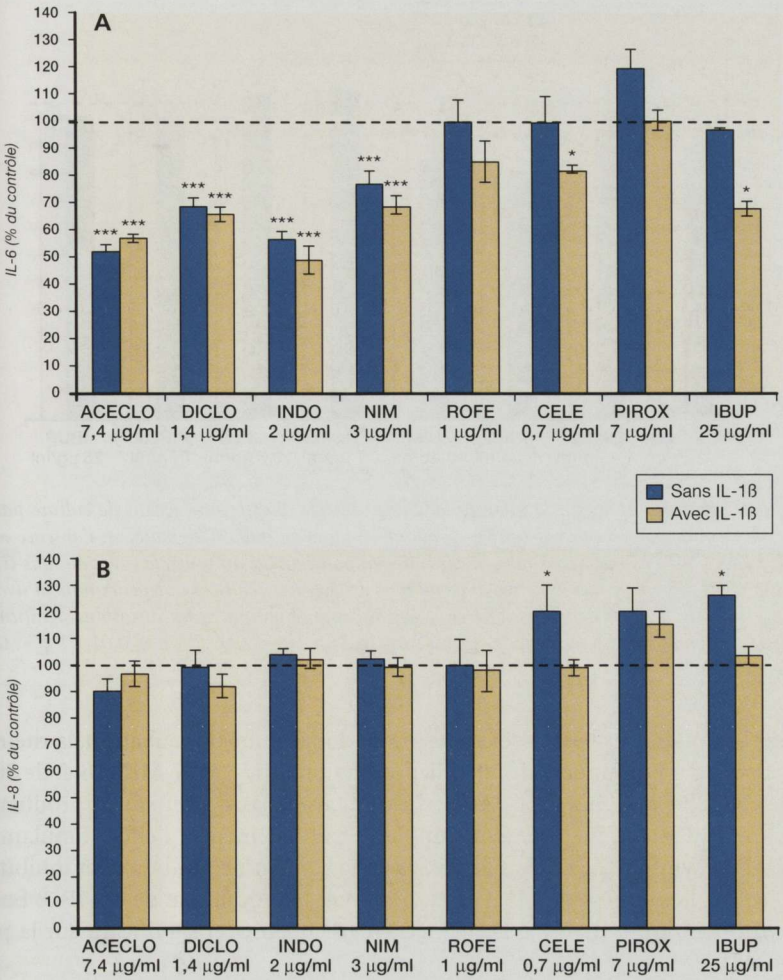


Figure IV-8: *Effets des AINS sur la quantité d'IL-6 (A) et d'IL-8 (B) produite par des chondrocytes humains arthrosiques cultivés pendant 12 jours en billes d'alginate, en l'absence ou en présence d'IL-1ß 10^{-10} M. Les résultats sont exprimés en pourcentage du contrôle (avec ou sans IL-1ß) et correspondent aux moyennes ± l'écart-type de trois expériences réalisées indépendamment avec du cartilage provenant de trois donneurs différents. Les astérisques indiquent les variations significatives par rapport au groupe contrôle (avec ou sans IL-1ß): * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ (Test d'ANOVA).*

La rhéine se distinguait des AINS classiques par son effet sur la production de PGE_2. En effet, la rheine augmentait la production basale de PGE_2 par les chondrocytes arthrosiques en culture en billes d'alginate et amplifiait de façon dose-dépendante l'effet stimulant de l'IL-1ß sur cette production (Figure IV-15). A la dose de 3 µg/ml, la rhéine multipliait par trois la production de PGE_2 en présence d'IL-1ß, sans modifier le taux d'ARN messager codant pour la cyclooxygénase-2.

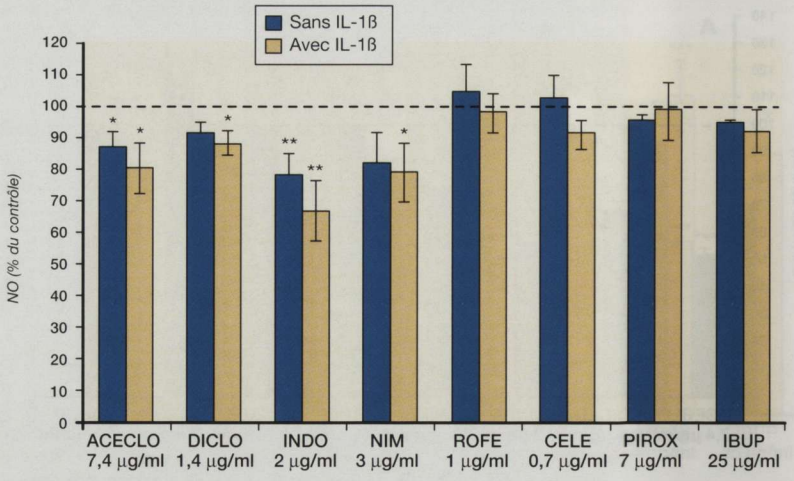


Figure IV-9: *Effets des AINS sur la quantité de NO_2^- libérée dans le surnageant de culture par des chondrocytes humains arthrosiques cultivés pendant 12 jours en billes d'alginate, en l'absence ou en présence d'IL-1ß 10^{-10} M. Les résultats sont exprimés en pourcentage du contrôle (avec ou sans IL-1ß) et correspondent aux moyennes ± l'écart-type de trois expériences réalisées indépendamment avec du cartilage provenant de trois donneurs différents. Les astérisques indiquent les variations significatives par rapport au groupe contrôle (avec IL-1ß ou sans IL-1ß): * $p < 0,05$, ** $p < 0,01$, ***$p < 0,001$ (Test d'ANOVA).*

Dans les conditions basales, et au terme de douze jours d'incubation, la rhéine à la dose de 3 µg/ml diminuait de 30 % la production d'IL-6 et de MIP-1ß et de 50 % celle de •NO. En présence d'IL-1ß, la rhéine n'avait pas d'effet sur la production d'IL-6. Cependant, ce médicament s'opposait partiellement à l'effet stimulant de l'IL-1ß sur la synthèse de •NO. Elle réduisait également de moitié l'effet inhibiteur de l'IL-1ß sur la synthèse de TIMP-1 et diminuait la production de MMP-3. Enfin, en présence ou non d'IL-1ß, la rhéine n'exercait aucun effet significatif sur la production d'IL-8.

4.3.4. Effets du ranélate de strontium (S12911) sur le métabolisme des chondrocytes.

Le ranélate de strontium est un médicament utilisé dans le traitement de l'ostéoporose. Chez l'animal et chez l'homme, il favorise la formation de la matrice osseuse. Notre hypothèse de travail était que le ranélate de strontium pourrait être également actif sur le cartilage. Avant la publication de nos travaux (Henrotin *et al.*, 2001b) (Annexe 20), les effets de ce médicament sur ce tissu étaient méconnus. Les expérimentations sur le ranélate de strontium ont été réalisées sur le modèle de culture de chondrocytes humains primaires en suspension en phase liquide. Après 3 jours d'incubation, le ranélate de strontium, aux doses de 5.10^{-4} et 10^{-3} M, augmentait (+ 20-35 %) la production d'agrécane par les chondrocytes. L'augmentation

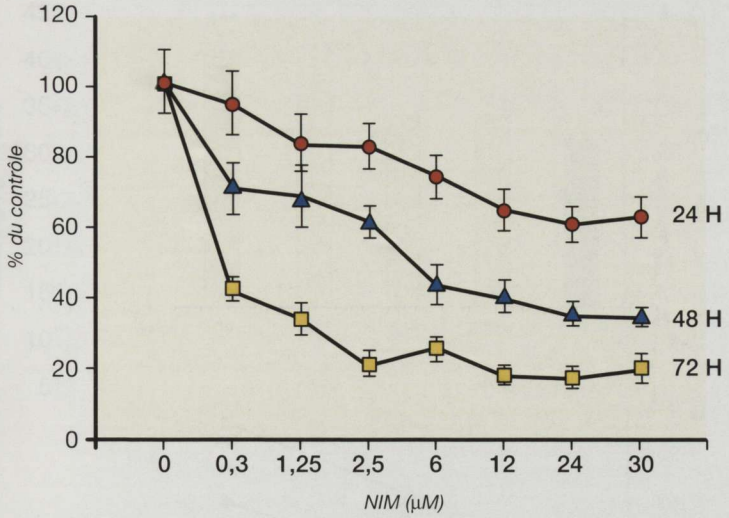


Figure IV-10: *Production de formes activées de l'oxygène par les chondrocytes humains arthrosiques pré-incubés pendant 24, 48 et 72 heures avec des doses croissantes de NIM. Dans cette expérience les chondrocytes ont été lavés à plusieurs reprises avec du HBSS avant la lecture de la chimioluminescence. Les résultats sont exprimés en pourcentage de la valeur de contrôle. Les valeurs sont les moyennes ± l'écart-type de 5 expériences réalisées séparément avec des prélèvements de cartilage provenant de donneurs différents. Après 48 et 72 heures d'incubation avec le NIM, l'effet est déjà significatif à la dose de 0,3 µM avec un $p < 0,05$ (Test d'ANOVA).*

avait lieu dans le milieu de culture ainsi que dans la phase cellulaire, suggérant que le ranélate de strontium favorisait la formation de la matrice cartilagineuse (Figure IV-16).

Le ranélate de strontium était actif sur les chondrocytes normaux, mais également sur les chondrocytes arthrosiques. Cependant, les chondrocytes normaux semblaient plus sensibles à l'action du ranélate de strontium que les chondrocytes arthrosiques. L'effet du ranélate de strontium sur la production totale d'agrécane par des chondrocytes normaux était significatif à la dose de 10^{-4} M alors que cette dose était inefficace sur des chondrocytes arthrosiques (Tableau IV-6).

Enfin, nous avons comparé les effets du ranélate de strontium (S12911) à ceux du chlorure de strontium ($SrCl_2$). L'objectif de cette expérience était de déterminer si le composé actif du ranélate de strontium est le strontium ou l'acide ranélique. Sur des chondrocytes jeunes ou arthrosiques, le chlorure de strontium (2.10^{-3}M) était aussi efficace que le ranélate de strontium sur la production totale d'agrécane. Il semble donc que l'élément actif soit le strontium. Cependant, une différence existait entre les deux molécules. Le ranélate de strontium favorisait l'accumulation des agrécanes dans la matrice extracellulaire, alors que le chlorure de strontium était sans effet sur le contenu en agrécane de la phase cellulaire.

Afin de déterminer si la nature du cation influence l'activité biologique du médicament, nous avons comparé le ranélate de strontium (S12911) et le ranélate

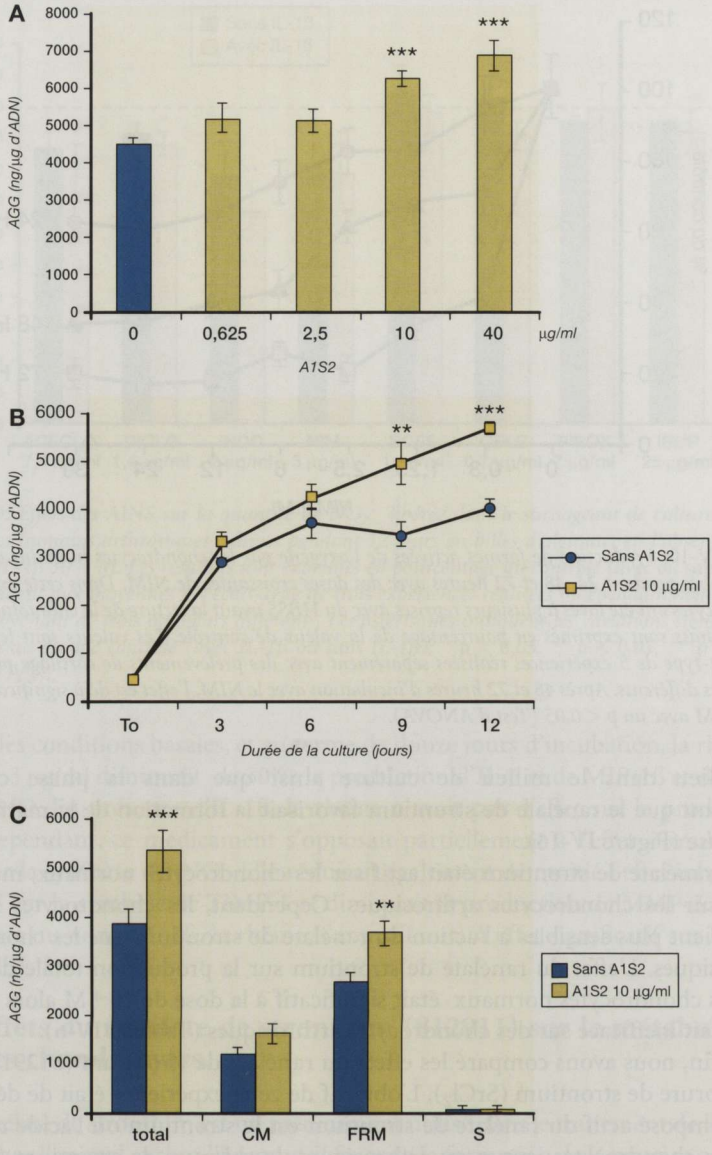


Figure IV-11: *(A) Effets de doses croissantes de A1S2 sur la production basale totale d'agrécane par des chondrocytes humains arthrosiques cultivés pendant douze jours en billes d'alginate. (B) Production cumulée d'agrécane en présence ou en l'absence d'A1S2 (10 µg/ml). Les milieux de culture sont renouvelés tous les trois jours. (C) Distribution de l'agrécane dans les différents compartiments de la culture au terme de 12 jours de culture en présence ou non d'A1S2 (10 µg/ml). Production totale est la somme des quantités d'agrécane présentes dans le CM, le FRM et le surnageant. FRM (Further Removed Matrix correspondant à la matrice inter-territoriale du cartilage); CM (Cell-associated Matrix correspondant à la matrice péri-cellulaire et territoriale du cartilage); S (milieu de culture). Les valeurs sont les moyennes ± l'écart-type de trois cultures réalisées à l'aide de trois prélèvements différents de cartilage. Les groupes traités sont significativement différents des témoins avec un $^{**}p < 0.01$ ou $^{***}p < 0.001$.*

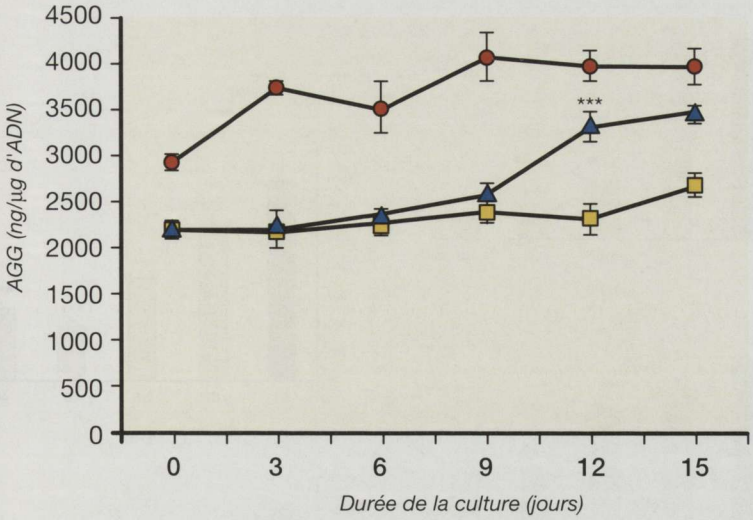


Figure IV-12: *Effet de A1S2 (10 µg/ml) sur la production d'agrécane après 3 jours de traitement par l'IL-1ß, un puissant inhibiteur de la synthèse d'agrécane. Dans cette expérience, les chondrocytes étaient pré-incubés durant 3 jours avec de l'IL-1ß (10^{-10} M). Ensuite, l'IL-1ß était éliminée et les chondrocytes maintenus en culture durant 15 jours avec ou sans A1S2. (-● -) chondrocytes non pré-traités par l'IL-1ß; (-▲ -) chondrocytes pré-traités par l'IL-1ß et ensuite cultivés 15 jours en présence de A1S2; (-□ -) chondrocytes pré-traités par l'IL-1ß puis cultivés 15 jours sans A1S2.*

de calcium (S12911-0). Le ranélate de strontium et le ranélate de calcium ont des effets identiques sur la production d'agrécane. Cependant, le ranélate de calcium, mais pas le ranélate de strontium, augmentait l'activité enzymatique de type stromélysine mesurée dans les milieux de culture. Par contre, les trois molécules testées n'influençaient pas la synthèse de stromélysine-1, suggérant que le calcium agissait sur l'activité et non la synthèse de l'enzyme.

4.4. Conclusions et discussion

4.4.1. Effets des anti-inflammatoires non stéroïdiens (AINS) sur le métabolisme des chondrocytes

De nombreux travaux ont été consacrés aux effets à court terme (2 à 7 jours) des AINS sur le métabolisme des chondrocytes, mais aucun n'a étudié les effets de cette classe pharmacologique sur une longue période. Ce paramètre est pourtant important à considérer lorsque l'on sait que les AINS sont les médicaments les plus souvent prescrits pour traiter l'arthrose, une maladie dont l'évolution s'étale sur plusieurs années. Notre étude est la première à comparer les effets de plusieurs AINS sur le métabolisme de chondrocytes arthrosiques cultivés en billes d'alginate. De nos travaux nous pouvons tirer les conclusions suivantes:

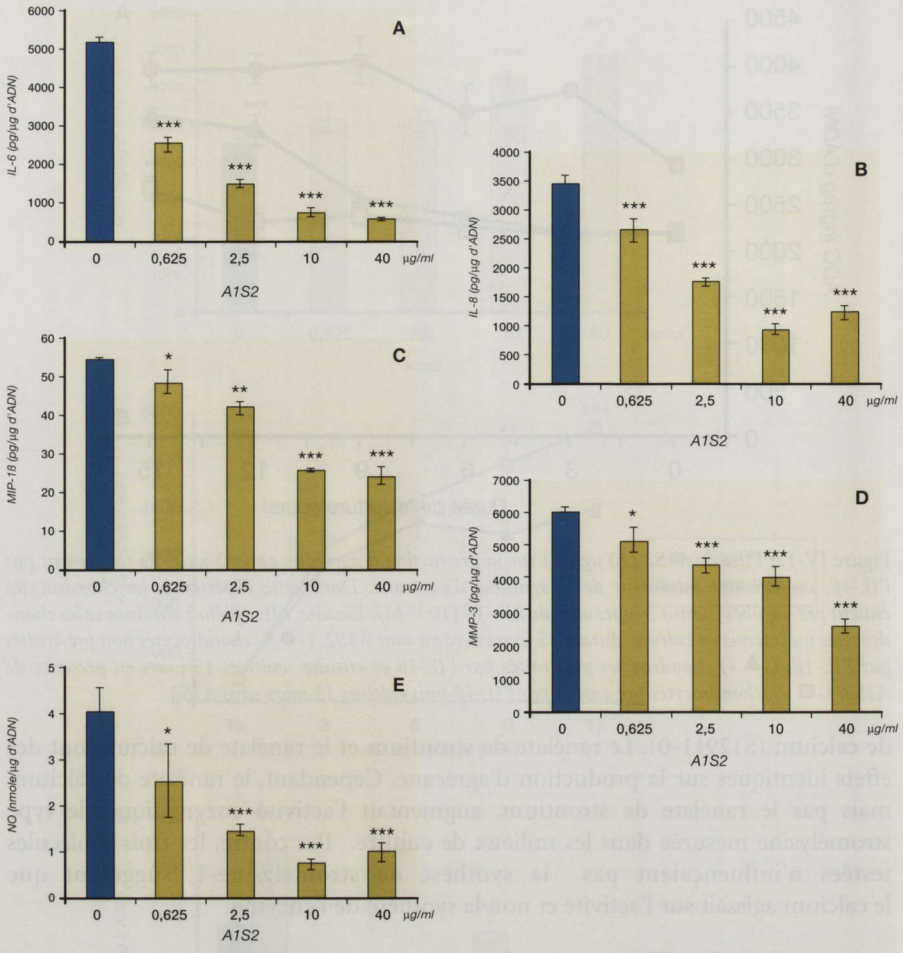


Figure IV- 13: *Effet de doses croissantes d'A1S2 sur la production basale d'IL-6(A), d'IL-8 (B), de MIP-1ß (C), de MMP-3 (D) et de NO_2^- (E) par des chondrocytes humains arthrosiques maintenus en culture pendant 12 jours en billes d'alginate. Les valeurs sont les moyennes ± l'écart-type de trois cultures indépendantes réalisées avec des chondrocytes d'origines différentes. Les groupes traités sont significativement différents du groupe contrôle avec un *p < 0,01, ** p < 0,01, ***p<0,001,*

- Les AINS sont des médicaments aux mécanismes d'actions multiples.
- Tous les AINS n'ont pas les mêmes effets sur le métabolisme des chondrocytes.
- Les AINS agissent sur plusieurs mécanismes pathogéniques de l'arthrose.

Nos travaux montrent que les AINS ne sont pas seulement des inhibiteurs des cyclooxygénases. Ils sont également antioxydants et interfèrent avec l'homéostasie des cytokines et des constituants matriciels du cartilage. D'emblée, indiquons que les AINS étudiés sur nos modèles expérimentaux, ne sont pas toxiques pour la cellule aux doses testées et qu'ils n'ont pas d'effet sur la prolifération cellulaire (Henrotin *et al.*, 1998; Henrotin *et al.*, 2001c; Sanchez *et al.*, 2000a). Dans le tableau IV-7, nous avons résumé les résultats découlant de nos travaux.

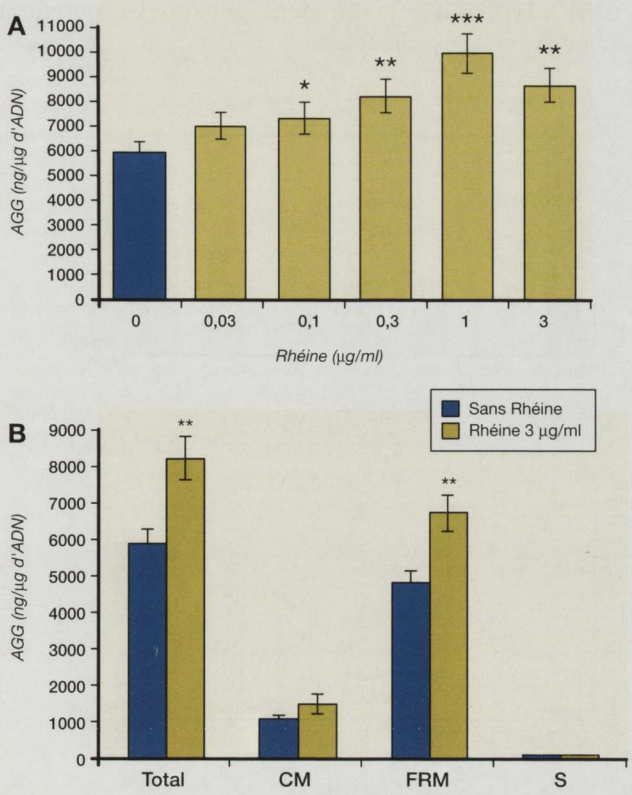


Figure IV- 14: *(A) Production totale d'agrécane par des chondrocytes arthrosiques maintenus en culture 12 jours en billes d'alginate en présence de doses croissantes de rhéine. (B) Effet de la rhéine 3 µg/ml sur la distribution de l'agrécane dans les différents compartiments de la culture en billes d'alginate (FRM, CM, S). Les cultures traitées par la rhéine sont significativement différentes des cultures contrôles: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

Tous les AINS testés inhibent totalement la production de PGE_2 par les chondrocytes.

Notre étude a démontré que tous les AINS, même les AINS dits "COX-2 sélectifs", à la dose thérapeutique et au terme de 12 jours de culture, inhibaient totalement la production de PGE_2. Cette observation est étonnante puisque les AINS dits "COX-2 sélectifs", ont été développés pour inhiber spécifiquement l'activité de l'isoenzyme II inductible de la cyclooxygénase, sans interférer avec l'isoenzyme I constitutionnelle. Or, les chondrocytes humains expriment les deux iso-formes de la cyclooxygénase, COX-1 et COX-2 (Blanco *et al.*, 1999). Nos travaux sur sang complet (annexe 9 et 13), ont mis en évidence que la sélectivité d'action d'une drogue envers COX-2 était dépendante de la concentration administrée (de Leval *et al.*, 2000a et b). En général, la sélectivité d'action disparaît lorsque la concentration en AINS testée augmente. A titre indicatif, nous avons calculé le pourcentage d'inhibition de l'activité de COX-1 et de COX-2 par les différents AINS

à la dose C_{max} (Tableau IV-8). Ces concentrations sont aussi les concentrations utilisées dans notre étude sur les chondrocytes.

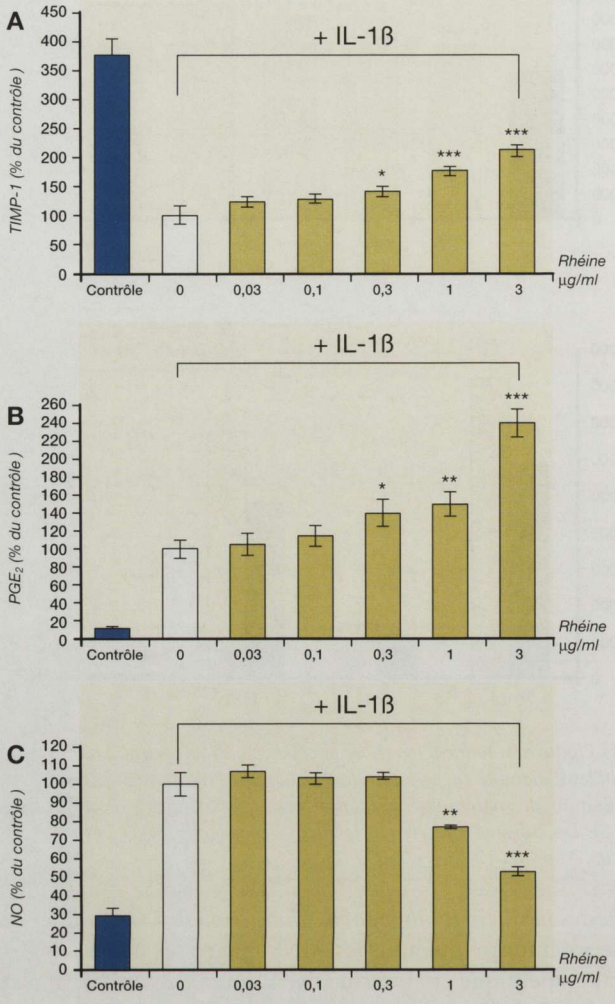


Figure IV- 15: *Effets de la rhéine sur la production de TIMP-1 (A), de PGE_2 (B), et de •NO (C) par des chondrocytes arthrosiques cultivés pendant 12 jours en présence de IL-1ß. Les résultats sont exprimés en pourcentage de la valeur du contrôle (en présence IL-1ß). Les valeurs sont les moyennes ± l'écart type de quatre expériences réalisées avec des donneurs différents. Les astérisques indiquent les effes significatifs avec un $*p < 0,05$, $**p < 0,01$ ou $***p < 0,001$.*

Nous pouvons observer qu'aucun des AINS testés sur nos modèles expérimentaux (sang complet et chondrocyte) n'est spécifique de COX-2 à la dose de référence C_{max}, mais que certains AINS comme l'ACECLO, le ROFE ou le CELE ont une action préférentielle sur COX-2. Cette observation permet d'expliquer, qu'au terme

de 12 jours d'incubation, la production de PGE_2 par les chondrocytes, soit totalement inhibée par les AINS. Ces résultats concordent avec les observations de Lidbury (2000a et b) et de Warner *et al.* (2001), qui confirment à l'aide d'un test similaire au nôtre que le ROFE, le CELE, le NIM ne sont pas spécifiques de COX-2.

Nous avons également étudié les effets de l'ACECLO et de ses principaux métabolites, le DICLO et le 4-OH ACECLO, sur l'expression du gène codant pour COX-2. Il apparaît qu'à la dose égale à C_{max}, ces molécules ne modifient pas significativement le taux d'ARN messager codant pour la COX-2 (Henrotin *et al.*, 2001c). Cette observation suggère que l'ACECLO et ses métabolites, agissent à un niveau post-transcriptionnel. Ces résultats sont en accord avec ceux publiés par Yamazaki et collaborateurs (1999). Ces auteurs ont démontré sur des cultures de synoviocytes humains, que ni l'ACECLO, ni ses métabolites n'avaient d'effet sur l'expression du gène COX-2 ou la synthèse de la protéine. Afin de déterminer si ces molécules sont capables d'interagir avec le site catalytique de l'enzyme, nous avons testé *in vitro* leurs effets sur l'activité des cyclooxygénases-1 et -2 ovines purifiées. Dans ces conditions expérimentales, l'ACECLO et le DICLO inhibaient l'activité de COX-1 et COX-2 avec une IC_{50} supérieure à 100 µM, alors que le 4-OH ACECLO n'avait pas d'effet (Henrotin *et al.*, 2001c; de Leval *et al.*, 2001a). Par comparaison, l'INDO inhibait de manière dose dépendante les deux iso-formes de l'enzyme, avec une valeur d'IC_{50} de 0,1 µM pour COX-1 et 0,5 µM pour COX-2. Ceci signifie qu'à l'échelle cellulaire, et à fortiori organique, l'action de l'ACECLO sur la production de PGE_2 n'est pas uniquement le résultat d'une action directe de la molécule avec le site catalytique de l'enzyme. D'autres mécanismes d'action peuvent être évoqués sur

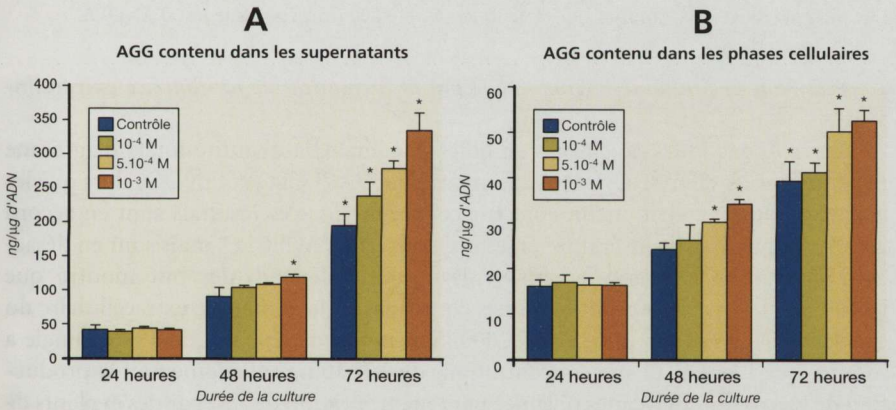


Figure IV-16: *Effet du strontium de ranélate sur la production d'agrécane par les chondrocytes normaux (cartilage sain prélévé sur des donneurs jeunes <35 ans) en fonction de la concentration et de la durée d'incubation. Les agrécanes ont été mesurées dans le milieu de culture (A) et les extraits cellulaires (B) par un dosage radioimmunologique (Gysen et Franchimont, 1984). Les résultats sont exprimés en nanogramme d'agrécane par µg d'ADN et sont représentés par la moyenne ± l'écart type de trois cultures réalisées indépendamment. Les groupes traités au ranélate de strontium sont significativement différents du groupe contrôle avec un $p < 0.05$ (Test d'ANOVA).*

base des données de la littérature. Les AINS peuvent également exercer leur action régulatrice sur la synthèse de PGE_2, en agissant sur l'activité de la phospholipase A_2 (Mitrovic *et al.*, 1981; Vignon *et al.*, 1989).

Tableau IV-6: Effet du S12911 sur la production d'AGG par les chondrocytes normaux ou arthrosiques.

	Témoins	S12911 10^{-4} M	S12911 5.10^{-4} M	S12911 10^{-3} M	S12911-0 10^{-3} M	SrCl2 2.10^{-3} M
Surnageants de culture						
Normal	622 ± 184	707 ± 146 p = 0,0012	814 ± 191 p = 0,0001	800 ± 176 p = 0,0001	752 ± 145	844 ± 206 p = 0,0001
OA	212 ± 68	216 ± 62 p = 0,86	267 ± 91 p = 0,03	292 ± 90 p = 0,0088	-	302 ± 12 p= 0,0006
Phases cellulaires						
Normal	68 ± 25	73 ± 24 p = 0,013	82 ± 20 p = 0,0003	85 ± 18 p = 0,0001	85 ± 26 p = 0,0001	71 ± 20 p = 0,3
OA	36 ± 15	36 ± 15 p = 0,9	41 ± 17 p = 0,13	45 ± 18 p = 0,27	-	40 ± 12 p = 0,24
Production totale						
Normal	686 ± 210	780 ± 167 p = 0,0005	894 ± 210 p = 0,0001	885 ± 194 p = 0,0001	833 ± 169 p = 0,0001	914 ± 223 p = 0,0001
OA	248 ± 81	253 ± 75 p = 0,85	309 ± 105 p = 0,02	362 ± 156 p = 0,002	-	340 ± 115 p = 0,0007

La production totale correspond à la somme du contenu de la phase cellulaire et du surnageant. Les différences entre les groupes expérimentaux ont été déterminées par le test d'ANOVA.

L'acéclofénac et l'indométhacine favorisent la formation de la matrice extracellulaire.

Après douze jours de culture en billes d'alginate, la quantité totale d'agrécane produite par les chondrocytes est augmentée par l'ACECLO et l'INDO, alors que les autres AINS n'exercent aucun effet sur ce paramètre. Ces résultats sont en accord avec les données de la littérature en ce qui concerne l'ACECLO, mais sont en désaccord pour l'INDO. Actuellement, trois études indépendantes ont montré que l'ACECLO favorisait la production des constituants de la matrice extracellulaire du cartilage (Dingle, 1996; Blot *et al.*, 2000; Sanchez *et al.*, 2002a). En 1996, Dingle a montré que l'ACECLO aux concentrations de 2 et 10 µg/ml augmentait la production de glycosaminoglycanes (évaluée par l'incorporation de ^{35}S) par des explants de cartilage arthrosique au terme de 6 jours d'incubation. En 2000, Blot *et al.* ont observé que l'ACECLO (1-3 µg/ml; 2 jours d'incubation) stimulait les productions de protéoglycanes et d'acide hyaluronique (déterminée par l'incorporation de ^{3}H-glucosamine) par des explants de cartilage humain arthrosique. Enfin, récemment, nous avons publié que l'ACECLO aux doses de 2 et 10 µg/ml accroissait la quantité d'agrécane contenue dans la bille d'alginate (Sanchez *et al.*, 2002a).

La majorité des travaux réalisés sur l'INDO ont conclu que cet AINS diminuait (Rainsfort *et al.*, 1997; Dingle *et al.*, 1999a et b) ou n'avait pas effet sur la synthèse de protéoglycanes (Herman *et al.*, 1986). Nos résultats sont donc divergents de ceux publiés précédemment. Cette divergence dans les résultats obtenus peut être expliquée par les concentrations en INDO utilisées dans les différentes études. Nous avons utilisé l'INDO à la dose de 2 µg/ml. Cette dose correspond à la dose plasmatique maximale mesurée dans le plasma de volontaires sains après la prise d'une dose thérapeutique d'INDO (Oberbauer *et al.*, 1993). Dans les autres études l'INDO était administrée à des doses comprises entre 20 et 50 µg/ml (Dingle, 1999; Rainsford, 1997), soit 10 à 25 fois la concentration utilisée dans notre étude. A ces concentrations, l'INDO a, dans nos conditions expérimentales, un effet toxique sur les chondrocytes (communication personnelle).

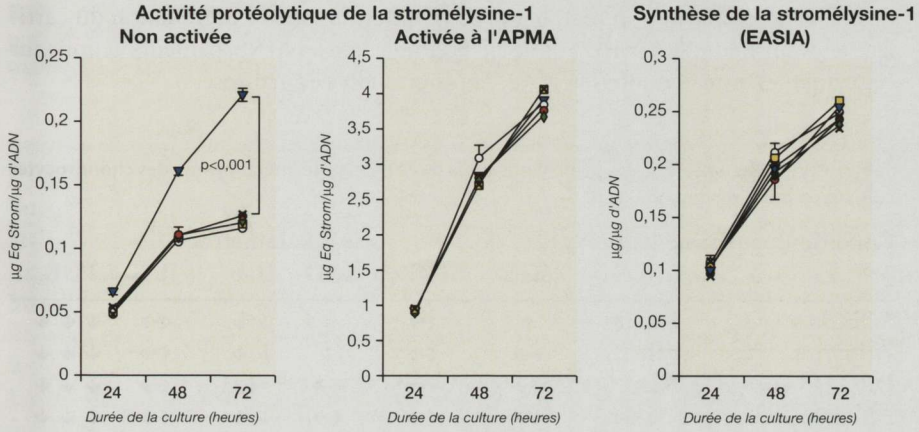


Figure III-17: *Effet de concentrations croissantes de S12911, S12911-0 et $SrCL_2$ sur la synthèse (mesurée par EASIA) et l'activité enzymatique (mesurée par le test à la caséine-résorufine) produite par des chondrocytes humains normaux (pas de lésion du cartilage; < 30 ans). Les chondrocytes primaires sont cultivés durant 24 à 72 heures en l'absence (- ○ -) ou en présence de strontium ranélate 10^{-4} M (- × -), 5.10^{-4} M (- ◆ -) ou 10^{-3} M (- □ -), de chlorure de strontium 2.10^{-3} M (- ● -), de ranélate de calcium 10^{-3} M (- ▼ -). Les résultats sont la moyenne et l'écart type d'une culture représentative. Les courbes ont été comparées entre elles par le test de Zerbe. (Zerbe, 1979)*

Certains AINS inhibent la synthèse et s'opposent à l'activité de l'IL-1ß.

Parmi les huit AINS testés dans notre étude, seuls l'ACECLO, le NIM, l'INDO, le DICLO, le CELE et l'IBUP inhibaient la synthèse d'IL-6. Par contre, le PIROX et le ROFE n'avaient aucun effet sur ce paramètre. Les AINS n'avaient pas d'effet sur la production d'IL-8 et de MIP-1ß, suggérant que l'action des AINS soit ciblée sur l'IL-6. Nous avons également démontré que l'ACECLO et le DICLO inhibaient partiellement l'expression du gène codant pour IL-1ß et s'opposaient à certains effets de cette cytokine (Henrotin *et al.*, 2001c). Par exemple, ils étaient antagonistes de l'effet stimulant de l'IL-1ß sur la production d'IL-6 et de PGE_2, mais étaient incapables de s'opposer à l'effet inhibiteur de l'IL-1ß sur la synthèse d'agrécane. Notons,

que dans une étude réalisée sur les explants de cartilage arthrosique, Dingle a observé que l'ACECLO s'opposait partiellement à l'effet inhibiteur de l'IL-1ß sur la synthèse de GAG (Dingle, 1999). D'autres effets antagonistes des AINS à l'action de l'IL-1ß ont été décrits dans la littérature. Par exemple, le DICLO et l'INDO s'opposent à l'effet stimulant de l'IL-1ß sur la synthèse de collagénase-1 (MMP-1) par des chondrocytes bovins en bille d'alginate (Sadowski et Steinmeyer, 2001).

Nos résultats montrent que par leurs actions sur la synthèse d'IL-1ß et d'IL-6, les AINS peuvent agir à différents niveaux de la pathogénie de l'arthrose. L'IL-1ß inhibe la synthèse des principaux constituants de la matrice extracellulaire et augmente la production de métalloprotéases. L'IL-6 est une cytokine impliquée dans l'initiation et le développement de la réaction inflammatoire de la membrane synoviale (de Hooge *et al.*, 2000). L'IL-6 agit en synergie avec l'IL-1ß pour stimuler la synthèse de MMPs et inhiber la synthèse de protéoglycanes (Nietfeld *et al.*, 1990). En présence, de la forme soluble de son récepteur, l'IL-6 induit *in vitro* la dégradation du cartilage (Rowan *et al.*, 2001). Il est donc envisageable que les AINS, par leurs actions sur ces cytokines, puissent interférer avec la progression de l'arthrose.

Tableau IV- 7: Récapitulatif des principaux effets des AINS sur le métabolisme des chondrocytes cultivés en billes d'alginate.

Conditions expérimentales		Paramètres					
		AGG	MMP-3	•NO	IL-6	IL-8	PGE_2
ACECLO	Basale	↑	↔	↓	↓ ↓	↔	↓ ↓ ↓
7,5 µg/ml	+ IL-1ß	↔	↔	↓	↓ ↓	↔	↓ ↓ ↓
DICLO	Basale	↔	↔	↔	↓	↔	↓ ↓ ↓
1,4 µg/ml	+ IL-1ß	↔	↔	↔	↓ ↓	↔	↓ ↓ ↓
INDO	Basale	↑	↔	↓ ↓	↓ ↓	↔	↓ ↓ ↓
2 µg/ml	+ IL-1ß	↔	↔	↓ ↓	↓ ↓	↔	↓ ↓ ↓
NIM	Basale	↔	↔	↔	↓	↔	↓ ↓ ↓
3 µg/ml	+ IL-1ß	↔	↔	↓	↓	↔	↓ ↓ ↓
ROFE	Basale	↔	↔	↔	↔	↔	↓ ↓ ↓
1 µg/ml	+ IL-1ß	↔	↔	↔	↔	↔	↓ ↓ ↓
CELE	Basale	↔	↔	↔	↔	↑	↓ ↓ ↓
0,7 µg/ml	IL-1ß	↔	↔	↔	↓	↔	↓ ↓ ↓
PIROX	Basale	↔	↔	↔	↔	↔	↓ ↓ ↓
7 µg/ml	IL-1ß	↔	↔	↔	↔	↔	↓ ↓ ↓
IBUP	Basale	↔	↔	↔	↔	↑ ↑	↓ ↓ ↓
25 µg/ml	IL-1ß	↔	↔	↔	↓ ↓	↔	↓ ↓ ↓

Les symboles indiquent les variations significatives de la production totale au terme de 12 jours d'incubation avec l'AINS. ↔ = pas d'effet significatif, augmentation significative = ↑ $p < 0.05$, ↑ ↑ $p < 0.01$; diminution significative: ↓ = $p < 0,05$, ↓ ↓ = $p < 0,01$, ↓ ↓ ↓ = $p < 0,001$.

Tableau IV- 8: Effets des AINS sur l'activité de COX-1 et de COX-2 sur sang complet

	C_{max} mg/L	COX-1 % d'inhibition	COX-2 % d'inhibition
ACECLOFENAC	7,4	3,2 %	98,2 %
CELECOXIB	0,7	13,5 %	65,9 %
ROFECOXIB	1	19,7 %	84 %
NIMESULIDE	3	52,2 %	99,1 %
DICLOFENAC	1,4	99,1 %	99,8 %
INDOMETHACINE	2	97, 3 %	100 %

Evaluation du pouvoir inhibiteur des AINS à la dose de référence (C_{max}) sur l'activité des cyclooxygénases sur sang total. L'activité de COX-1 était appréciée par la production de thromboxane (TXB_2) lors de la coagulation (1 h à 37 °C), et l'activité de la COX-2 par la production de PGE_2 après activation par un lipopolysaccharide (24 h à 37 °C). Les résultats sont exprimés en pourcentage d'inhibition de l'activité enzymatique des cyclooxygénases et sont représentatifs de trois expériences réalisées de façon indépendante.

Les AINS possèdent des propriétés antioxydantes

Les formes activées de l'oxygène (FAO) jouent un rôle important dans l'initiation et l'évolution de la réaction inflammatoire accompagnant les maladies ostéo-articulaires. Elles sont également impliquées dans la dégradation des protéines matricielles du cartilage et dans l'apoptose des chondrocytes (Burkhard *et al.*, 1986; Stevens *et al.*, 1989; Blanco *et al.*, 1999). Dès lors, elles constituent une cible privilégiée pour les médicaments à visée anti-arthrosique. Il existe très peu d'études sur le pouvoir antioxydant des AINS et aucune ne relate leurs effets sur la production de FAO par les chondrocytes. Nous avons démontré que le NIM et son métabolite, le 4-OHNIM, inhibaient de façon dose- et temps-dépendant la production de FAO par les chondrocytes arthrosiques (Zheng *et al.*, 2001). Dans cette expérience, les chondrocytes étaient pré-incubés durant 24 à 72 heures avec l'AINS, lavés, puis placés en présence de luminol, un amplificateur de la chimioluminescence. Nous

Tableau IV-9: Systèmes chimiques et enzymatiques utilisés pour déterminer le pouvoir antioxydant des AINS.

Système générateur	FAO étudiée	Système de détection des FAO
Réaction de Fenton	•OH	Résonance paramagnétique électronique (Spin trapper = DMPO)
xanthine/xanthine oxydase	$O_2^{\cdot-}$	Résonance paramagnétique électronique (Spin trapper = DMPO)
Fe^{2+}/acide linoléique-ascorbate	ROOH/Fe^{IV}-O	Résonance paramagnétique électronique (Spin trapper = 4-POBN)
Hypochloride de sodium	HOCl	Chimioluminescence
ONOO- décomposition	ONOO-	Spectrophotométrie

HOCl = acide hypochloreux; ONOO- = peroxynitrite; ROOH = peroxyde lipidique; FeIV-O = radical ferryl; •OH = radical hydroxyl; $O_2^{\cdot-}$ = anion superoxyde; 4-POBN = α-(4-pyridyl-N-oxide) -tert-butylnitrone); DMPO = 5,5-Diméthyl-1-pyrroline-N-oxide.

pouvons émettre l'hypothèse, selon laquelle le NIM exercerait son pouvoir antioxydant en agissant sur les systèmes enzymatiques intracellulaires générateurs de FAO (Zheng *et al.*, 2000). Un mécanisme d'action a été proposé par Bevilacqua et ses collaborateurs en 1994. Dans une étude réalisée sur des neutrophiles humains activés, ces auteurs ont montré que le NIM inhibait la production de FAO par un mécanisme impliquant la phosphodiestérase IV, une enzyme impliquée dans l'élimination de l'AMPc cytosolique. En inhibant l'activité de cette enzyme, le NIM provoquait une augmentation du taux d' AMPc cytosolique, et en conséquence une augmentation de l'activité de la protéine kinase A qui en phosphorylant successivement plusieurs substrats, inhibait l'assemblage des sous-unités de la NADPH oxydase (Bevilacqua *et al.*, 1994). Cependant, il est également concevable, à la vue de leur structure chimique, que les AINS puissent réagir directement avec les FAO. Cette propriété a été démontrée pour le NIM. Cet AINS est un piégeur de l'acide hypochloreux (Dallegri *et al.*, 1990) mais aussi des peroxydes lipidiques (radical peroxyl et alkoxyl) générés *in vitro* par l'hydroperoxyde de cumène (CuOOH) sur un modèle de membrane d'érythrocyte (Maffei-Facino *et al.*, 1993; Maffei *et al.*, 1995). Par contre, il n'existe pas à notre connaissance d'étude sur le pouvoir antioxydant d'AINS comme l'INDO, l'ACECLO ou le DICLO. Afin de compléter nos connaissances sur ce sujet, nous avons comparé la capacité de ces quatre AINS à piéger les FAO. Pour ce faire, nous avons utilisé des réactions chimiques et enzymatiques génératrices *in vitro* de FAO (les protocoles expérimentaux peuvent être consultés en annexe14 et 15; Zheng *et al.*, 2000; Mouithys *et al.*, 2000) (Tableau IV-9).

L'analyse du tableau IV-10 nous permet de tirer les enseignements suivants:

- Un AINS donné n'agit pas sur toutes les FAO. Il existe une spécificité d'action probablement liée à la structure chimique de la molécule. Par exemple, le NIM ne piège pas l'anion superoxyde, mais neutralise le radical hydroxyle, l'acide hypochloreux, les peroxydes lipidiques et le peroxynitrite. L'ACECLO n'a pas d'action sur les peroxydes lipidiques, mais piège le peroxynitrite. L'INDO est sans effet sur l'acide hypochloreux mais élimine le peroxynitrite et les peroxydes lipidiques.
- La forme hydroxylée du NIM, le 4-OHNIM, piège l'anion superoxide alors que le NIM est sans effet. Cette action particulière du métabolite hydroxylé peut s'expliquer par la présence d'un groupement phénol dans sa structure. Le radical phénol est très réactionnel avec l'anion superoxyde.
- Finalement, nous avons observé que tous les AINS testés accélèrent la décomposi-

Tableau IV-10: Classement des AINS selon leurs propriétés antioxydantes

Radical hydroxyle	NIM=4-OHNIM
Anion superoxyde	4-OHNIM > NIM (Pas d'effet)
Peroxydes lipidiques	NIM=4-OHNIM>4-OHACECLO>DICLO>INDO>ACECLO (pas d'effet)
Acide Hypochloreux	4-OHNIM>NIM=4-OHACE>ACECLO, DICLO, INDO (pas d'effet)
Peroxynitrite	NIM>INDO>ACECLO>4-OHNIM>4-OHACE

Classement des AINS en fonction de leur capacité à piéger les différentes FAO à la concentration de 10^{-5} M

tion du peroxynitrite. Une explication de ce phénomène serait que les AINS réagiraient avec les radicaux dérivés du peroxynitrite ($\cdot NO_2$ ou le $\cdot OH$), conduisant à la nitration ou l'hydroxylation de la structure cyclique de la molécule.

En conclusion, nos travaux ont montré que les AINS peuvent exercer leur action anti-inflammatoire par d'autres voies que l'inhibition de la cyclooxygénase-2. Ils inhibent également la synthèse des cytokines inflammatoires et sont antioxydants. Il apparaît également de grandes différences dans le profil d'activité des AINS.

4.4.2. Effets des insaponifiables d'avocat et de soja sur le métabolisme des chondrocytes.

Les insaponifiables d'avocat et de soja (A1S2), ont été utilisés dans le traitement de diverses pathologies du tissu conjonctif, depuis de nombreuses années (Thiers *et al.*, 1956; Dupurrat *et al.*, 1970; Roberts *et al.*, 1975). Ce médicament a également démontré son efficacité sur la symptomatologie de l'arthrose (Blotman *et al.*, 1997; Maheu *et al.*, 1998; Appelboom *et al.*, 2001). Actuellement, on connaît peu de choses sur les mécanismes d'action de ce médicament. Sur ce point, notre étude apporte des enseignements nouveaux et supporte l'hypothèse selon laquelle A1S2 exercerait une action structurelle sur le cartilage. Sur nos modèles expérimentaux, nous avons montré que A1S2 inhibe la synthèse de PGE_2, d'IL-6, d'IL-8, de MIP-1ß, de MMP-3 et augmente la synthèse d'agrécanes par les chondrocytes arthrosiques. De plus, A1S2 s'oppose à certains effets cataboliques et inflammatoires de l'IL-1ß comme l'augmentation de la synthèse de MMP-3 et de PGE_2 ou encore l'inhibition de TIMP-1. De plus, ce médicament favorise la restauration de la synthèse d'agrécane après un traitement à l'IL-1ß. Le profil pharmacologique de A1S2 est résumé par le Tableau IV-11.

Tableau IV-11: Récapitulatif des effets de A1S2 (Piasclédine) sur le métabolisme des chondrocytes humains en culture en bille d'alginate (12 jours).

	- IL-1ß	+IL-1ß
AGG	↗	↔
MMP-3	↘	↘
TIMP-1	↔	↗
IL-6	↘	↘
IL-8	↘	↘
MIP-1ß	↘	↔
$\cdot NO$	↘	↘
PGE_2	ND	↘

AGG = Agrécane; MMP-3 = Stromélysine-1; TIMP-1 = Tissue inhibitor of metalloproteinases-1; IL-6 = Interleukine-6; IL-8 = Interleukine-8; MIP-1ß = Macrophage inflammatory protein-1; $\cdot NO$ = Monoxyde d'azote; PGE_2 = Prostaglandine E_2. ↘ = diminue; ↗= augmente; ↔ = pas d'effet.

Ces observations permettent de mieux comprendre les mécanismes d'action de ce médicament en clinique humaine et apportent une série d'éléments nouveaux plaidant en faveur d'une action structurelle de cette drogue sur le cartilage. L'effet de A1S2 sur la synthèse d'agrécane trouve une explication dans les travaux de Boumédiène et collaborateurs (1999). Ces chercheurs ont montré que A1S2 stimulait la synthèse de TGFß-1 et -2 par des chondrocytes bovins cultivés en monocouche. Les TGFßs sont des facteurs de croissance très actifs sur la régulation du métabolisme des tissus conjonctifs. Au niveau du cartilage, ils stimulent la synthèse d'agrécane et diminuent la production de certaines MMPs par les chondrocytes. Ils sont également capables de s'opposer aux effets délétères de l'IL-1ß sur le cartilage (Galera *et al.*, 1992; Lum *et al.*, 1996; van Osch *et al.*, 1998).

Pour la première fois, nous avons démontré qu'A1S2 réduit la synthèse de cytokines pro-inflammatoires. L'inflammation de la membrane synoviale est un événement physiopathologique important de l'arthrose. Elle implique des cytokines inflammatoires (IL-6, LIF, MIP-1ß, ...) qui sont sécrétées par les chondrocytes voisins de la lésion chondrale. Le MIP-1ß est particulièrement actif dans le recrutement des monocytes/macrophages au niveau du foyer inflammatoire (Koch *et al.*, 1995; Borzi *et al.*, 1999). L'effet de A1S2 sur ce facteur, démontré pour la première fois dans notre étude, est particulièrement important pour la compréhension des propriétés anti-inflammatoires de A1S2 (Kerbel *et al.*., 1975; Khayyal et El-Ghazaly, 1998). A1S2 inhibe également la production d'IL-6 et d'IL-8 par les chondrocytes. Comme nous l'avons déjà souligné antérieurement, l'IL-6 est impliquée dans le développement de la synovite et des lésions du cartilage au cours de l'arthrose (de Hooge *et al.*, 2001). Par contre, le rôle joué par l'IL-8 reste obscur. Néanmoins, l'inhibition de la synthèse d'IL-8 par A1S2 permet d'expliquer son efficacité dans le traitement de maladies inflammatoires, telles les parodontites, ainsi que dans les modèles expérimentaux d'inflammation à la carragénine, qui impliquent une proportion importante de polynucléaires neutrophiles (Robert *et al.*, 1974 et 1975; Kurt *et al.*, 1998). Récemment, la présence de récepteurs pour les chimiokines de la

Tableau IV-12: Comparaison des effets de A1S2, A et S à la concentration de 10 µg/ml sur le métabolisme de chondrocytes.

Paramètres	Effets
AGG	A1S2=S=A
MMP-3	A1S2=S=A
TIMP-1	S > A et A1S2 (pas d'effet)
MIP-1ß	A1S2=S>A
•NO	A1S2>S=A
PGE_2	A1S2=A>S
IL-6	A1S2>A>S
IL-8	A1S2>A>S

AGG = Agrécane; MMP-3 = Stromélysine-1; TIMP-1 = Tissue inhibitor of metalloproteinases-1; IL-6 = Interleukine-6; IL-8 = Interleukine-8; MIP-1ß = Macrophage inflammatory protein-1; •NO = Monoxyde d'azote; PGE_2 = Prostaglandine E_2.

famille C-X-C, incluant l'IL-8, a été identifiée sur les chondrocytes. Il a été également observé que l'IL-8 augmentait la synthèse de N-acétyl-ß-D-glucosaminidase par les chondrocytes (Borzi *et al.*, 2000). Ces éléments suggèrent que l'IL-8 puisse être actifs dans les processus de dégradation du cartilage.

Nous avons également comparé les effets du mélange A1S2 à ceux de A ou S incubés séparément, afin de déterminer lequel des insaponifiables était actif sur le chondrocyte. Nous avons récapitulé les résultats dans le tableau IV-12.

Il ressort de cette étude que le mélange A1S2 présente une activité supérieure ou égale à celle de A ou de S sur tous les paramètres testés à l'exception de la synthèse de TIMP-1. Nous avons également comparé différents mélanges formés soit d'une part d'avocat et 2 parts de soja (A1S2), soit de 2 parts d'avocat et 1 part de soja (A2S1) ou soit de 2 parts d'avocat et 2 parts de soja (A2S2). Cette étude a démontré que seuls les mélanges composés de deux parts de S inhibaient la production de collagénases et de stromélysines. Ces actions contribuent à justifier l'utilisation du mélange A1S2 dans le traitement de l'arthrose. Afin de déterminer si A et S agissaient dans le mélange A1S2 de façon synergique ou non sur les différents paramètres expérimentaux, nous avons testé ces deux constituants aux concentrations présentes dans le mélange A1S2 (soit 3,3 µg/ml de A et 6,6 µg/ml de S). De cette façon, nous avons démontré que les effets de A et S sur la synthèse d'agrécane étaient additifs, mais que les deux constituants du mélange agissaient en synergie sur la production d'IL-6, d'IL-8, de PGE_2 et de •NO (Henrotin *et al.*, 1998; 2002d).

***In vitro*, les insaponifiables d'avocat et de soja créent des conditions métaboliques favorables à la formation de la matrice cartilagineuse. Ils stimulent la synthèse d'agrécane et diminuent le rapport MMP-3/TIMP-1.**

4.4.3. Effets de la rhéine sur le métabolisme des chondrocytes.

La diacérhéine est utilisée dans le traitement de fond de l'arthrose des membres inférieurs. Son administration diminue la douleur et améliore la fonction de l'articulation arthrosique (Nguyen *et al.*, 1994; Dougados *et al.*, 2001; Pelletier *et al.*, 2001). Dans notre étude, la rhéine (le métabolite actif de la diacérhéine) se distingue des autres drogues à visée anti-rhumatismale, par son action sur la synthèse de PGE_2. La rhéine stimule la production de PGE_2 dans les conditions basales et amplifie l'effet de l'IL-1ß sur ce paramètre. Parallèlement, la rhéine inhibe très significativement la production de •NO et de MMP-3, et augmente la production d'agrécane. L'effet de la rhéine sur la production de •NO a été confirmé par d'autres auteurs sur des macrophages et des chondrocytes en culture (Borderie *et al.*, 2001), et contribue à expliquer les propriétés anti-inflammatoires de cette substance observées sur des modèles animaux d'arthrite (Wang *et al.*, 2002; Tamura *et al.*, 2002) (Tableau IV-13). Ces effets pourraient être secondaires à la synthèse de TGFß et/ou à la diminution de la synthèse et de l'activité de l'IL-1ß. Felisaz et ses collaborateurs ont observé que la rhéine stimulait la synthèse de TGFß-1 et -2 par des chondrocytes de bœuf en monocouche (Felisaz *et al.*, 1999). La rhéine diminue également le nombre

de récepteurs de type I pour l'IL-1 exprimés par les chondrocytes humains arthrosiques et elle inhibe la synthèse d'IL-1ß par ces cellules (Martel-Pelletier *et al.*, 1998; Yaron *et al.*, 1998; Moldovan *et al.*, 2000).

Tableau IV-13: récapitulatif des effets de la rhéine sur le métabolisme des chondrocytes humains.

	- IL-1	+ IL-1
AGG	↗	↔
MMP-3	↘	↔
TIMP-1	↗	↗
•NO	↘	↔
PGE_2	ND	↗
IL-6	↘	↔
IL-8	↔	↔
MIP-1ß	↘	↔

Nous avons tenté d'expliquer l'effet stimulant de la rhéine sur la production de PGE_2. Comme le suggère l'absence d'effet de la rhéine sur le taux d'ARNm de COX-2, son action semble localisée à niveau post-transcriptionnel. D'autres études ont montré que la rhéine n'affecte pas la production de COX-2, de phospholipase A2 et de 5-lipoxygénase (Pomarelli *et al.*, 1980; Franchi-Michelli *et al.*, 1983; La Villa *et al.*, 1989). Il est donc probable que la rhéine agira directement sur l'activité de la cyclooxygénase.

Nous avançons deux hypothèses pour expliquer l'action de la rhéine sur l'activité de la cyclooxygénase-2. Premièrement, la rhéine agirait comme un antioxydant. En effet, la rhéine possède un noyau phénolique, capable de piéger des formes activées de l'oxygène. On sait qu' au cours de son fonctionnement, la cyclooxygénase produit un radical tyrosyl qui régule son activité catalytique. La neutralisation de ce radical par la rhéine pourrait prolonger son activité et donc la synthèse de PGE_2. La seconde hypothèse repose sur l'observation que la rhéine inhibe la production de •NO. Dans notre modèle expérimental, le •NO inhibe la production PGE_2 par les chondrocytes (Henrotin *et al.*, 1999). Nous pouvons donc supposer que l'inhibition de •NO par la rhéine contribue à accroître l'activité de la cyclooxygénase.

La rhéine se différencie des anti-inflammatoires non stéroïdiens conventionnels, par son action stimulante sur la production de prostaglandine E_2. Elle est également un inhibiteur de la synthèse de •NO, d'IL-6 et de MMP-3 et un stimulateur de la synthèse d'agrécane et de TIMP-1. La rhéine pourrait, par la correction des déséquilibres métaboliques liés au processus arthrosique, présenter des effets structuraux.

4.4.4. Effets du ranélate de strontium (S12911) sur le métabolisme des chondrocytes.

Le ranélate de strontium est un médicament actuellement à l'étude dans le

traitement de l'ostéoporose post-ménopausique (Reginster *et al.*, 1999a, 1999b, 2002). Des études *in vitro* ont montré que le ranélate de strontium stimulait la formation de la matrice osseuse et réduisait la résorption de l'os (Marie *et al.*, 1993; 2001). Dés lors, il est possible que le ranélate de strontium exerce un effet semblable sur la matrice du cartilage. Cette hypothèse nous a conduit à étudier les effets de ce médicament sur le métabolisme des chondrocytes. Après trois jours d'incubation, le ranélate de strontium augmentait de façon dose-dépendante la production de complexes de haut poids moléculaire d'agrécane (Henrotin *et al.*, 2000). Notre étude montrait également que ces complexes s'accumulaient dans la matrice néo-synthétisée. Sur base de ces deux observations, nous pouvons affirmer que le ranélate de strontium stimule la synthèse d'agrécane fonctionnelle, c'est-à-dire capable de former des complexes de haut poids moléculaire avec l'acide hyaluronique et de favoriser leur accumulation dans la matrice néo-synthétisée. L'élément responsable de l'augmentation de la synthèse d'agrécane est le strontium, et non l'acide ranélique, puisque le chlorure de strontium exerce un effet similaire. Cependant, une différence existe entre les effets de ces deux composés. Le ranélate de strontium, mais non le chlorure de strontium, augmente la quantité d'agrécane contenue dans la phase cellulaire des cultures. En d'autres termes, l'agrécane produite par les chondrocytes traités au chlorure de strontium ont une capacité moindre à s'accumuler dans la matrice extracellulaire. Une explication possible, est que le ranélate de strontium (Henrotin *et al.*, 1998c), et non le chlorure de strontium, stimule la synthèse de collagène de type II et favorise l'organisation d'un réseau de fibres de collagène dans lequel seraient retenues les molécules d'agrécane. Une autre explication serait que le chlorure de strontium, et non le ranélate de strontium, diminue l'affinité de la molécule d'agrécane pour l'acide hyaluronique et/ou la glycoprotéine de liaison.

Enfin, le ranélate de strontium et le ranélate de calcium ont des effets similaires sur la production d'agrécane. Cette observation suggère que l'effet du ranélate de strontium soit simplement la conséquence d'une modification de l'environnement cationique de la cellule. Cependant, un fait important distingue les deux composés. Le ranélate de calcium, mais non le ranélate de strontium, augmente l'activité enzymatique de type stromélysine contenue dans le milieu de culture. L'absence d'effet du ranélate de calcium sur la synthèse de stromélysine-1 mesurée par un dosage immunologique montre que cette augmentation est probablement le résultat de l'activation des pro-enzymes par la présence d'une quantité importante de calcium (Housley *et al.*, 1993). L'utilisation de ranélate de strontium semble donc préférable à celle du ranélate de calcium dans le cadre de pathologie dégénérative comme l'arthrose.

En conclusion, le ranélate de strontium favorise la formation de la matrice cartilagineuse sans modifier ni la synthèse, ni l'activité des métalloprotéases. Il pourrait donc s'avérer efficace dans le traitement de l'arthrose.

Conclusions et perspectives

1. Mise au point d'un modèle de culture de chondrocytes humains en billes d'alginate

Lorsqu'ils sont cultivés en monocouche, les chondrocytes perdent rapidement leurs caractéristiques morphologiques et synthétiques spécifiques, et se comportent comme des fibroblastes indifférenciés. Dans ces conditions, il est difficile de donner une interprétation réaliste des effets observés, tant le comportement du chondrocyte, cellule hautement spécialisée, est éloigné de celui du fibroblaste. Un objectif important de la culture des chondrocytes est de conserver stable leur phénotype cartilagineux durant une longue période. Dans ce but, les chondrocytes peuvent être cultivés en suspension sous agitation constante, dans des boîtes de culture à faible adhésion cellulaire, ou encore dans des matrices synthétiques ou naturelles. Dans ce travail, nous avons choisi de valider un modèle de culture de chondrocytes humains arthrosiques en billes d'alginate. L'alginate est un polysaccharide linéaire qui en présence de cations divalents comme le Ca^{++} se gélifie pour former une matrice poreuse autour des chondrocytes. Dans ces conditions, le chondrocytes synthétisent des constituants matriciels qui se répartissent dans des compartiments appelés par les Anglo-saxons "Cell-associated Matrix - CM" et "Further Removed Matrix - FRM". Ces compartiments correspondent respectivement, à la matrice péri-cellulaire et territoriale et à la matrice inter-territoriale du cartilage *in vivo*. Les connaissances du comportement du chondrocyte dans ces conditions de culture restent lacunaires. La plupart des travaux réalisés à ce jour sur ce modèle sont dévolus à l'étude de la synthèse des protéines matricielles. Notre travail s'est focalisé sur la production et la distribution dans la matrice cartilagineuse de certaines cytokines inflammatoires (IL-6, IL-8, MIP-1ß), de la stromélysine -1 et de la prostaglandine (PG) E_2. Jusqu'à présent, ces aspects étaient inexplorés. Il ressort de notre travail les observations suivantes:

- Les chondrocytes humains conservent leur phénotype cartilagineux après douze jours de culture en billes d'alginate. Ils ont une forme arrondie et sont localisés dans des logettes formées au sein de la matrice. Ils se multiplient peu et synthétisent du collagène de type II et de l'agrécane, deux molécules spécifiques du cartilage.
- Une matrice extracellulaire s'organise autour des chondrocytes. La presque totalité (98 %) des molécules d'agrécane synthétisées par le chondrocyte, reste immobilisée au sein de la bille d'alginate. Après 12 jours de culture, la majorité des molécules d'agrécane est localisée dans la matrice inter-territoriale. Cependant, nos observations montrent qu'une partie de l'agrécane néo-synthétisée migre de la matrice péri-cellulaire et territoriale (CM) vers la matrice inter-territoriale (FRM).
- La production basale de stromélysine par les chondrocytes arthrosiques augmente avec la durée de la culture, alors que la production de TIMP-1 diminue. Il en résulte un accroissement du rapport MMP-3/TIMP-1 avec la durée de la culture. Au terme de douze jours de culture, le rapport MMP-3/TIMP-1 est égal à 200, signifiant un large excès de MMP-3 par rapport à TIMP-1. Cette situation est favorable à la chondrolyse.

- Le chondrocyte arthrosique cultivé en billes d'alginate produit spontanément de l'IL-6, de l'IL-8 et du MIP-1ß, trois cytokines impliquées dans le développement des lésions du cartilage et de la réaction inflammatoire au cours de l'arthrose. La production de ces cytokines est soutenue pendant les douze jours de culture. Parmi ces cytokines, seule l'IL-8 s'accumule au sein de la matrice extracellulaire (22 %). Il est possible que ce mécanisme contribue à la formation d'un gradient d'IL-8 dans le cartilage, favorable à la migration des cellules immunitaires à travers le tissu.

Cependant, nous restons conscients que si ce modèle permet de maintenir stable le phénotype des chondrocytes, il est encore éloigné des conditions rencontrées *in vivo* par cette cellule. En effet, *in vivo*, le chondrocyte est soumis à des contraintes mécaniques et vit dans un environnement dont la pression partielle en oxygène (pO_2) est inférieure à celle de l'air ambiant. Dans les prochains mois, notre préoccupation majeure sera de reproduire en culture ces conditions particulières de vie. Nous cultiverons les chondrocytes en bille d'alginate à des tensions en oxygène de 1 et 5 %. Ces conditions correspondent à celles rencontrées par le chondrocyte *in vivo* dans le cartilage. Nous étudierons les réponses des chondrocytes à des cytokines et à des médicaments et les comparerons à celles obtenues à 21 % d'oxygène.

Nous utiliserons également la culture de chondrocytes en billes d'alginate en co-culture avec des ostéoblastes de l'os sous-chondral. La culture simultanée de ces deux types cellulaires nous permettra d'analyser la relation os-cartilage. Cette relation est impliquée dans la pathogénie de l'arthrose et reste à ce jours peu explorée. Grâce à ce modèle, nous tenterons d'identifier les médiateurs des ostéoblastes impliqués dans la dégradation du cartilage et d'évaluer les effets de médicaments sur ce mécanisme physiopathologique.

2. L'interleukine-1 est une cytokine majeure de la pathogénie de l'arthrose

Le chondrocyte joue un rôle prédominant dans la pathogénie de l'arthrose. Il libère dans l'environnement immédiat de la cellule des métalloprotéases et des formes activées de l'oxygène, deux médiateurs importants de la dégradation du cartilage. De plus, le chondrocyte participe au développement de la réaction inflammatoire en sécrétant des médiateurs de l'inflammation comme les cytokines inflammatoires ou encore la PGE_2. Parmi, ces facteurs, l'interleukine-1ß joue un rôle prépondérant car elle induit un dysfonctionnement important du chondrocyte. Nous avons observé une série de modifications de l'activité synthétique du chondrocyte en réponse à l'IL-1ß:

- L'IL-1ß bloque la synthèse d'agrécane durant toute la période d'incubation. De plus, son effet est rémanent et persiste au moins douze jours après l'élimination de la cytokine du milieu de culture.
- L'IL-1ß stimule de manière transitoire la production de MMP-3 et inhibe la synthèse de TIMP-1. La conjonction de ces effets provoque une forte augmentation

du rapport MMP-3/TIMP-1.

- IL-1ß augmente la synthèse d'IL-8, d'IL-6 et de MIP-1ß. Ces cytokines sont toutes impliquées dans le passage à la chronicité de la réaction inflammatoire de la membrane synoviale et sont capables seules ou en association avec d'autres, d'induire *in vitro* la dégradation de la matrice cartilagineuse. L'effet de l'IL-1ß sur la synthèse de MIP-1ß est particulièrement intéressant. En effet, cette cytokine est la seule dont le taux synovial est plus élevé dans l'arthrose que dans la polyarthrite rhumatoïde. Il est donc probable que cette cytokine joue un rôle particulier dans la pathogénie de l'arthrose.
- L'IL-1ß stimule la production de •NO. Cependant, notre étude montre que l'effet de l'IL-1ß s'estompe au fil des administrations. L'effet de l'IL-1ß sur ce médiateur est donc transitoire et pourrait être impliqué dans la transmission intracellulaire de certains effets de l'IL-1ß, comme la synthèse de MMP-3.

En résumé, ces résultats montrent que l'IL-1ß modifie l'activité synthètique des chondrocytes, perturbe l'homéostasie des cytokines, des métalloprotéases et des agrécanes, et crée une série de déséquilibres favorables à la dégradation du cartilage. Cette observation nous amène naturellement à discuter la régulation de l'activité biologique de l'IL-1ß par d'autres médiateurs solubles sécrétés par le chondrocytes. Nous émettons l'hypothèse que les réponses du chondrocyte à l'IL-1ß sont le résultat net de nombreuses interactions avec d'autres cytokines ou d'autres médiateurs. Nous avons étudié les effets de trois cytokines, l'interféron γ, l'Insuline-like Growth Factor (IGF-1) et le Transforming Growth Factor-1 (TGFß-1):

- L'IFNγ semble exercer une forte influence sur l'activité biologique de l'IL-1ß. Selon le paramètre étudié, il est frénateur ou activateur de l'activité biologique de l'IL-1ß. Il agit en synergie avec l'IL-1ß pour augmenter la production de •NO, d'IL-6 et d'IL-1Ra et pour inhiber la production d'agrécane. Par contre, il s'oppose aux effets stimulateurs de l'IL-1ß sur la production d'IL-8, d'IL-10 et de MMP-3. Cette dualité de l'IFNγ et le fait que le rapport IL-1/IFNγ semble moins élevé dans l'arthrose que dans la polyarthrite rhumatoïde, nous laisse penser que l'IFNγ pourrait être dans l'arthrose une cytokine régulatrice majeure de la réaction inflammatoire, responsable des caractéristiques particulières de la synovite du patient arthrosique.
- En l'absence d'IL-1ß, l'IGF-1 et le TGFß-1 augmentent la synthèse de glycosaminoglycanes sulfatés. Lorsque l'IL-1ß et l'IGF-1 ou le TGFß-1 sont administrés simultanément, deux situations peuvent être observées. En présence d'une faible concentration en IL-1ß (< 1 U/ml soit 18 pg/ml), l'IGF-1 et le TGFß-1 (à la concentration optimale) conservent leur effet stimulant sur la synthèse de GAG, mais cet effet est atténué par la présence d'IL-1ß. Par contre, en présence d'une dose plus élevée d'IL-1ß (> 1 U/ml ou 18 pg/ml), l'effet inhibiteur de l'IL-1ß prédomine même en présence d'une dose optimale d'IGF-1 ou de TGFß-1. Le TGFß-1, mais pas le l'IGF-1, diminue la synthèse de MMP-3 et augmente la synthèse de TIMP-1. De plus, il s'oppose aux effets de l'IL-1ß sur ces paramètres. En conclusion, il est probable que le comportement du chondrocyte au cours de l'arthrose soit dépendant de la quantité de ces facteurs de croissance dans l'envi-

ronnement de la cellule. Actuellement, on connaît peu de chose sur la régulation de la synthèse de ces cytokines par d'autres facteurs pathogéniques. Notre objectif futur sera d'étudier la synthèse de ces facteurs dans différentes conditions expérimentales (pO_2, cycles d'anoxie-réoxygénation, association de cytokines cataboliques, etc.) et d'étudier l'influence de nouveaux médicaments sur l'équilibre entre l'IL-1ß et le groupe IGF-1/TGFß-1. De plus, nous explorerons l'interaction de ces facteurs avec la matrice extracellulaire et leur activation par les formes activées de l'oxygène et les métalloprotéases.

Ces perspectives de recherche nous amènent à débattre du rôle joué par les FAO sur le contrôle de l'activité de l'IL-1ß. Deux observations majeures émanent de notre étude:

- Le N^G-monométhyl-L-arginine, un inhibiteur des •NO synthétases, amplifie l'effet stimulant de l'IL-1ß sur la synthèse d'IL-6, d'IL-8 et de PGE_2. Ceci suggère que le •NO, ou une FAO dérivée, exerce un contrôle négatif sur la production de ces médiateurs de l'inflammation.
- Le SNAP, un donneur de •NO, n'a pas d'effet sur l'expression induite par l'IL-1ß, des gènes codant pour l'IL-1ß, l'IL-6, l'IL-8, la COX-2 et l'iNOS, alors que le SIN-1, un donneur de •NO et de $O_2^{\cdot}$, et le -ONOO à l'état solide, inhibent l'expression de ces gènes. Par contre, le SNAP induit l'expression des gènes codant pour la MMP-3 et la MMP-13.

Nous pouvons conclure de ces observations que certaines FAO répriment l'expression de certains gènes inflammatoires, et par cette action pourraient atténuer la réaction inflammatoire. Il ressort également de cette étude que toutes les FAO n'exercent pas le même effet sur tous les gènes et qu'un même gène est régulé différemment selon la FAO impliquée. Il existe donc une spécificité d'action des FAO qui devrait être prise en compte dans le développement de nouvelles thérapies antioxydantes. Actuellement, nous savons peu de chose sur le pouvoir antioxydant du chondrocyte. La modification de ce pouvoir antioxydant au cours du développement de la maladie arthrosique est également méconnue. Notre recherche s'orientera dans cette voie. Nous évaluerons la production de superoxyde dismutases par les chondrocytes en culture et étudierons la régulation de cette production par des cytokines ou encore des médicaments à visée anti-arthrosique. Afin d'étudier le rôle joué par le •NO, dans la régulation du métabolisme du chondrocyte, nous développerons la technologie de l'antisense. Grâce à cette méthode, nous pourrons bloquer l'expression par le chondrocyte du gène codant pour la NO synthétase induite.

3. Approche *in vitro* du traitement de l'arthrose.

A côté des médicaments visant à réduire la symptomatologie (par ex. anti-inflammatoires non stéroïdiens, paracétamol) différentes molécules ont été développées, au cours de ces dernières années, dans le but d'adjoindre, à une amélioration de la douleur et de la fonction, une dimension de protection structurelle de l'articulation. L'objectif est, à long terme, de prévenir la dégradation des tissus articulaires et de réduire l'incidence des interventions chirurgicales d'arthroplastie de

remplacement. Actuellement, trois produits (insaponifiables d'avocat et de soja, sulfate de glucosamine et diacérhéine) ont montré, dans des études méthodologiquement acceptables un effet structurel à long terme sur le cartilage arthrosique. Cependant, les mécanismes d'action de ces médicaments ne sont pas encore tous connus, et les résultats cliniques doivent encore être confirmés dans des études de large échelle, méthodologiquement standardisées et réalisées indépendamment des laboratoires commercialisant ces produits. Notre objectif est de déterminer leurs effets à long terme sur le métabolisme du chondrocyte et d'identifier leurs mécanismes d'action. Dans ce travail, nous avons investigué les effets de trois spécialités pharmaceutiques utilisées dans le traitement de l'arthrose, les anti-inflammatoires non stéroïdiens, les insaponifiables d'avocat et de soja (IAS) et la rhéine (le métabolite actif de la diacérhéine). Ces agents ont tous été testés sur des chondrocytes arthrosiques cultivés en billes d'alginate durant 12 jours. Il ressort de cette étude les observations suivantes:

- Les AINS ont des mécanismes d'action multiples. La majorité des études réalisées sur ces molécules étaient focalisées sur leur action sur les cyclooxygénases. Notre étude montre que les AINS agissent aussi en inhibant la synthèse par le chondrocyte de cytokines dites "inflammatoires" en regard de leur implication dans la genèse de la réaction inflammatoire. Nous avons également observé que les AINS étaient antioxydants. Ils sont capables de piéger les FAO, mais aussi probablement d'interférer avec les systèmes enzymatiques intracellulaires générateurs de FAO.
- Tous le AINS n'ont pas les mêmes effets sur le métabolisme des chondrocytes. A ce sujet, notre étude est importante, car elle compare sur un même modèle expérimental les effets de huit anti-inflammatoires, présentant une structure chimique différente, administrés à la dose de référence C_{max}. Si tous les AINS sont capables de bloquer totalement la synthèse de PGE_2 à cette dose, deux seulement augmentaient la synthèse d'agrécane, trois inhibaient la synthèse de •NO et six la production d'IL-6. Par contre, aucun des AINS testés ne réduisait les productions de stromélysine-1 et d'IL-8. Il est donc indiscutable que tous les AINS n'exercent pas le même effet sur le chondrocyte. Ce point devra être pris en considération lors de la prescription d'un AINS dans le traitement de l'arthrose.
- Enfin, nous avons observé que certains AINS pouvaient également interférer avec certains mécanismes physiopathologiques de l'arthrose comme la synthèse d'IL-1ß ou encore la libération de formes activées de l'oxygène. Il est donc envisageable que certains AINS puissent interférer avec la progression de la maladie. Jusqu'il y a peu, la faible tolérance digestive, à long terme, des patients arthrosiques à la prescription d'AINS en limitait fortement l'utilisation prolongée dans l'arthrose. La genèse de nouvelles molécules, inhibant spécifiquement l'isoenzyme II inductible de la cyclooxygénase, sans interférer avec l'isoenzyme I constitutionnelle (COXIBs) ouvre de nouvelles perspectives qui permettront peut-être d'interférer avec les manifestations inflammatoires responsables de la dégradation de la matrice cartilagineuse sans engendrer d'effets secondaires digestifs supérieurs à ceux rencontrés chez les patients non traités. Dans ce contexte, l'acéclofénac présente un profil d'action intéressant. Il présente une excellente sélectivité d'ac-

tion vis-à-vis de l'isoenzyme II inductible, et de plus il stimule la synthèse d'AGG, inhibe la production d'IL-1ß et d'IL-6 et possède des propriétés antioxydantes.

Nous avons également testé deux autres médicaments utilisés dans le traitement de fond de l'arthrose. Il s'agit d'IAS (A1S2) et de la rhéine (le métabolite actif de la diacérhéine). Ces médicaments ont en commun d'être des médicaments à action symptomatique lente et rémanente et d'avoir démontré une action structurelle dans l'arthrose des membres inférieurs. Par contre, notre étude met en évidence de nombreuses divergences dans leurs mécanismes d'action (Tableau A). La rhéine et A1S2 se distinguent essentiellement par leurs effets sur la synthèse de PGE_2 et d'IL-8. La rhéine augmente la synthèse de PGE_2 alors que A1S2 inhibe cette production. Par ailleurs, A1S2 inhibe la production d'IL-8 alors que la rhéine n'a pas d'effet.

Tableau A: Récapitulatif des effets des AINS, de A1S2 et de la rhéine sur le métabolisme des chondrocytes en billes d'alginate.

	AINS classiques	A1S2	Rhéine
AGG	0 ou ↗	↗	↗
IL-6	0 ou ↘	↘	↘
IL-8	0	↘	0
MIP-1ß	0	↘	↘
•NO	0 ou ↘	↘	↘
PGE_2	↘	↘	↗
MMP-3	0	↘	↘
TIMP-1	↗	0	0

A1S2 et la rhéine se distinguent aussi des AINS conventionnels par leurs effets sur la MMP-3, la TIMP-1 et le MIP-1ß. Il est intéressant d'observer que les AINS classiques augmentent la production basale de TIMP-1 mais n'ont pas d'effet sur MMP-3. A l'inverse, A1S2 et la rhéine diminuent MMP-3, mais n'ont pas d'effet sur TIMP-1. Il s'avère donc évident que ces deux classes pharmaceutiques agissent sur le rapport MMP-3/TIMP-1 par des voies différentes.

Ceci nous encourage à évoquer la possibilité d'une complémentarité thérapeutique entre ces deux classes thérapeutiques. L'association d'un AINS présentant une bonne tolérance digestive avec la prise de A1S2 ou de rhéine pourrait s'avérer plus efficace sur l'évolution de la maladie que l'administration séparée de ces traitements. Dans un futur proche, nous envisageons d'étudier les effets d'une telle association sur le métabolisme des chondrocytes. Récemment, nous avons entrepris en collaboration avec le laboratoire de Chimie Pharmaceutiques du Professeur Delarge la conception, la synthèse et l'expérimentation *in vitro* de molécules originales. Ces molécules seront testées sur nos modèles expérimentaux, afin de déterminer leur action anti-IL-1ß. Nous étudierons l'effet de ces drogues sur l'expression du gène codant pour l'IL-1ß, l'activité de la caspase-1, la synthèse de la protéine mais aussi de son antagoniste. Ainsi, nous espérons identifier une structure active sur la synthèse et/ou l'activité de cette cytokine catabolique particulièrement active sur le chondrocyte.

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Annexes

Y. Henrotin

ANNEXE 1

In vitro models for the study of cartilage damage and repair.

Y. Henrotin and J-Y. Reginster

Osteoarthritis: Clinical and experimental aspects.
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1.1 *In vitro* Models For the Study of Cartilage Damage and Repair

1.1.1 Introduction

Cartilage is a very specialized tissue containing only one type of cell and characterized by the absence of blood and lymphatic vessels. Nutrition is mainly assumed by synovial fluid imbibition. Chondrocyte metabolism is regulated by soluble factors produced locally by chondrocytes themselves and also by neighboring tissues. Chondrocyte functions are also influenced by the composition of the extracellular environment (O_2 tension, ionic concentration, pH, etc), the extracellular matrix composition, the matrix-cell interactions and the physical signals. The first challenge of *in vitro* research is to reproduce in culture this complex extracellular environment without modifying the specific phenotype of mature chondrocytes. The second goal is to performed culture models to study the precocious, delayed, brief or sustained responses of chondrocyte to chemical and/or physical signals. *In vitro* culture also provides an opportunity to study chondrocytes behaviors during osteoarthritis (OA). The third aim is to develop co-culture systems that would allow investigation of the interactions between different tissues of the joint. The fourth goal is to prepare cartilage implants for further transplantation. Finally, *in vitro* research aims to screen growth factors, cytokines or therapeutic agents that could stimulate cartilage repair and/or reduce cartilage resorption. Over the last decade, different culture models have been developped. Monolayer culture, suspension culture, chondron culture, explant culture, co-culture systems and immortalized cell cultures will be described in this chapter. Each culture system presents advantages and disavantages and each is particularly suitable to explore one particular aspect of chondrocyte metabolism. Cartilage explant culture is an excellent model system for studying matrix turnover or mechanisms requiring original cell surface receptors and normal cell-matrix and matrix-matrix interactions. On the other hand, if the study aims to investigate matrix deposition or the regulatory pathways of chondrocyte metabolism, an isolated cell culture system is recommended to avoid the presence of a pre-existing matrix. Low density monolayer culture is appropriate to investigate cell dedifferentiation processes. Freshly isolated chondrocytes are recommended to investigate active oxygen species production and avoid the scavenging effect of matrix components. Suspended culture in a natural or synthetic matrix is a suitable model to analyze the adaptive responses of chondrocytes to mechanical stress. This chapter aims to review and analyze the culture models described in the literature.

1.1.2 Chondrocytes in Culture

1.1.2.1 Tissue Harvest

Several important considerations must be taken into account in the selection of cartilage tissue used for *in vitro* investigations. Matrix composition and metabolic activity vary within individual joints as well as among different joints. It is well

recognized that the metabolic activity of chondrocytes varies through the depth of the tissue. This fact has been reported in several studies where subpopulations of chondrocytes isolated from different depths of the cartilage were cultured. Some morphological and biochemical differences are clearly evident between superficial and deep chondrocytes cultured in agarose gel. Superficial cells synthesize a sparse, proteoglycan-poor fibrillar matrix whereas the deeper cells produce an abundant matrix rich in proteoglycan (Aydelotte and Kuettner, 1988a; Archer *et al.*, 1990). Moreover, superficial cells produce relatively more small non-aggregating proteoglycans and hyaluronic acid but less aggrecan and keratan sulfate than deep cells (Manicourt and Pita, 1988; Siczkowski and Watt, 1990; Korver *et al.*, 1992). Another important metabolic difference between chondrocytes isolated from different depths of the cartilage is their responsiveness to exogenous stimuli. Based on both their anabolic and catabolic responses, bovine chondrocytes derived from the superficial zone revealed a greater sensitivity to IL-1 than deep zone chondrocytes (Aydelotte *et al.*, 1988b).

Cell behavior also differs according to the localization of the tissue. Rib and ear chondrocytes isolated from the same animal respond differently to growth factors such as bFGF and TGFβ. bFGF increases thymidine, proline and leucine incorporation into rib, but not into ear, primary cultured chondrocytes. TGFβ enhances thymidine incorporation into both chondrocytes but does not affect proline or leucine incorporation into the ear cells (Lee *et al.*, 1997). Cartilage exised from high-load-bearing areas differs from that of low-load-bearing areas. In adult ovine stiffle joint, chondrocytes from the central region of the tibial plateau not covered by the meniscus, which is subject to high mechanical loads *in vivo*, synthesized less aggrecan but more decorin than cells from regions covered by the meniscus (Little and Ghosh, 1997). This study serves to emphasize the importance of using cartilage from identical joint areas when examining chondrocyte synthesis.

Chondrocytes metabolism and responsiveness to regulatory factors vary greatly according the age of the donor, the skeletal development and the pathological status of the joint from which the cartilage is harvested. Human chondrocytes show a continuous age-related decline in the proliferative response to serum. The greatest decrease is noted between cells of 40-50 year old donors and those from donors a decade older. Moreover, the magnitude of the proliferative response to growth factors (i.e. TGFβ or PDGF) decreased with the age of the donor. In addition to the quantitative changes in the proliferatives of chondrocytes, there is a qualitative change in the pattern of growth responsiveness during development. Cells from young donors (10-20 years of age) respond better to platelet derived growth factor (PDGF) than to transforming growth factor (TGFβ), while the inverse pattern is observed in cells from adult donors (Guerne *et al.*, 1995). Different mechanisms have been suggested to explain age-related change in the synthetic pattern and growth factor responsiveness of chondrocytes. Reductions of cell surface receptor number and affinity, changes in cytokine or growth factor synthesis and bioactivity, or modifications in post-receptor signaling events are possible explanations.

Osteoarthritis (OA) is a degenerative joint disease characterized by the destruc-

tion of the articular cartilage. The pathological status of the joint also alters chondrocyte morphology and metabolic activity. Recently, Kouri *et al.* (1996) have identified three distinct chondrocyte subpopulations in OA cartilage. Chondrocytes from the superficial and upper middle of fibrillated cartilage form clusters and synthetize higher levels of proteoglycan and collagen (Lafeber *et al.*, 1992a; Aigner *et al.*, 1997). Moreover, a focal onset of type III collagen expression was observed. TGFβ and IGF are able to stimulate proteoglycan synthesis by chondrocytes and partially reverse adverse effects of IL-1 and TNFα. Interestingly, osteoarthritic cartilage explants and isolated chondrocytes from osteoarthritic cartilage are much more sensitive to stimulation by TGFβ than healthy cartilage. This difference is largely attributed at phenotypically changed chondrocytes present in the damaged upper layer of osteoarthritic chondrocytes (Lafeber *et al.*, 1997).

1.1.2.2 Chondrocyte Isolation

Isolation of the individual chondrocyte is generally achieved through sequential proteolytic digestion of the extracellular matrix. After their release from the matrix, isolated cells are ideally suited for the study of *de novo* synthesis of matrix components. Some authors use only clostridial collagenase whereas others previously incubate cartilage dices with trypsin, pronase, DNAse and/or hyaluronidase. In this session, we report personal data on the effects of the enzymatic isolation method on the amount of matrix components contaminating the isolated cells. The residual amounts of protein and aggrecan contained in the cellular pellet after collagenase digestion (24 h; collagenase clostridial, type IA, 1 mg / 320 U / ml) and after successive enzymatic digestion with hyaluronidase (30 min.; Type IV S; 0.5 mg / 400 U / ml), pronase (1 h; 1 mg / 4000 U / ml) and collagenase (24 h; collagenase clostridial, type IA, 1 mg / 320 U / ml) are compared (figure 1). Chondrocyte clusters were obtained after collagenase digestion whereas chondrocytes were well isolated from each other after three successive enzymatic digestions with hyaluronidase, pronase and collagenase. Cell numbers isolated from 1 g of tissue were also widely varied. When collagenase was used alone, only 1.4 10^6 cells were isolated from 1 g of tissue whereas 4.3 10^6 cells were isolated after pronase, hyaluro nidase and collagenase treatments. Aggrecan, total proteins, IL-6 and IL-8 amounts found in the cell pellet obtained after collagenase digestion were significantly more elevated than in the cell pellet obtained after three successive enzymatic digestions (Table I). Forty-eight hours after isolation, proteoglycan and cytokine synthesis also fully differed according the isolation method used. Mono-digested chondrocytes produced more IL-6, PGE_2 and IL-8 but lower levels of aggrecans than tri-digested chondrocytes (Figure 2). Some information found in the literature contribute to the explanation of differences observed between the two isolation systems.

- Receptors may be down-regulated or damaged by enzymatic treatment (Loeser *et al.*, 1993). TGF-β inhibits DNA and proteoglycan synthesis of freshly isolated chondrocytes (first day) while the DNA and proteoglycan synthesis of chondrocytes cultured in monolayer (7 days) was stimulated by TGF-β (van der Kraan *et al.*, 1992). Therefore, adequate time for re-expression of such a membrane com-

ponent is essential before the initiation of experiment.

- The exogenously applied proteases can distrupt cell-matrix interactions mediated by integrins. The integrin family of transmembrane adhesion receptors mediates attachment of chondrocytes to extracellular matrix molecules (Shakibaei *et al.*, 1997). Disruption of normal integrin mediated cell-matrix interactions may influence gene expression (Hering *et al.*, 1994).

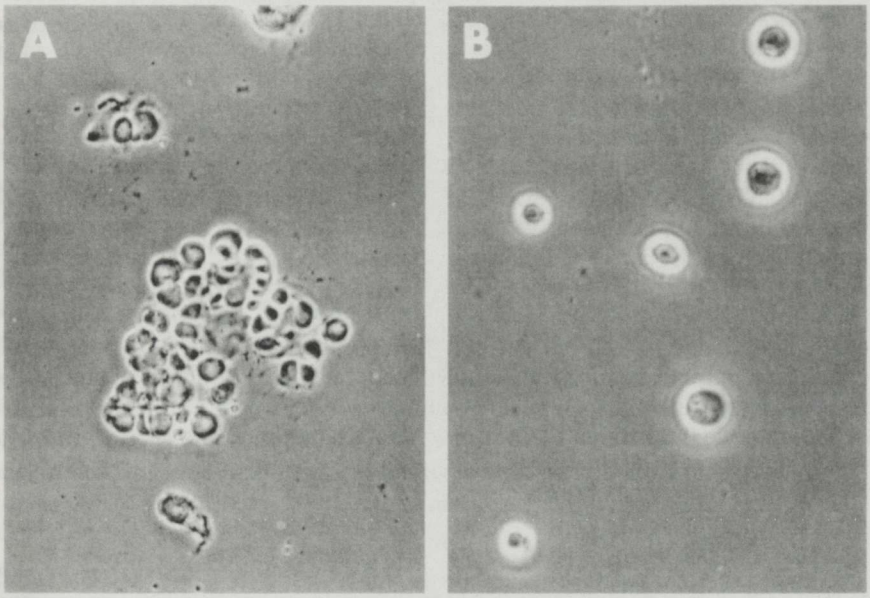


Figure 1: *Microscopic observation (magnification: X100) of human chondrocytes isolated from cartilage by a single digestion with collagenenase (A) or by a triple digestion with hyaluronidase, pronase and collagenase (B).*

Isolation method	Proteins μg/μg DNA	IL-8 pg/μg DNA	IL-6 pg/μg DNA	Proteoglycans ng/μg DNA
Mono-digestion	14 ± 0.07	58 ± 0.61	61 ± 5	210 ± 3.15
Tri-digestion	9.7 ± 0.04[a]	3.7 ± 0.93[b]	6.5 ± 0.91[b]	41 ± 3.75[b]

[a] $p<0.01$. [b] $p<0.001$.

Table 1: *Proteins, cytokines and proteoglycans contained in the cell pellets collected after digestion of the cartilage by collagenase alone (mono-digestion) or by three successive enzymatic treatments (tri-digestion) with hyaluronidase, pronase and collagenase. Before assays, cell pellets were homogenized by ultrasonic dissociation. IL-6 and IL-8 were measured by two specific EASIAs (Enzyme Amplified Sensitivity Immunoassays) and proteoglycans by a radioimmunoassay in which the antiserum is directed against the core protein of the aggrecan. The data are expressed as amounts of proteins, cytokines and proteoglycans found in the cell pellet per μg of DNA and presented as the mean and standard deviation of triplicate cultures. Comparison of mean values was performed using the unpaired Student's t-test.*

- Residual matrix components contaminating isolated cells may regulate chondrocyte synthesis. Integrins can recognize degradation products of the extracellular matrix, suggesting an important role in tissue repair following proteolytic matrix degradation. Larsson *et al.* (1989) reported that the addition of intact or fragmented proteoglycans to cell cultures stimulate the synthesis of proteins and proteoglycans. On the other hand, high level of hyaluronic acid (HA) was shown to cause an important reduction of the sulphate incorporation into proteoglycans synthesized by chick embryo chondrocytes, adult pig chondrocytes and Swarm rat chondrosarcoma cells (Nevo and Dorfman, 1972; Wiekin and Muir, 1973; Solursh *et al.*, 1974; Bansal *et al.*, 1986). Furthermore, HA is a potent inhibitor of the release of proteoglycans from the cell matrix layer into the medium even in the presence of IL-1β, TNFα, or FGF suggesting that HA can counteract the biological activity of cytokines and growth factors (Shimazu *et al.*, 1993). The precise mechanism underlying the action of HA remains unknown but chondrocytes contain a receptor for hyaluronate which is associated with cytosolic actin filaments and thereby influence its organization (Lacy and Underhill, 1987; Aruffo *et al.*, 1990). Binding of HA to receptors stimulates the tyrosine phosphorylation of proteins (Turley, 1989). Thus, all together, these findings demonstrate that fragmented or native cartilage matrix molecules may modulate chondrocytes metabolic functions via the activation of cell membrane receptors.
- The rapid up-regulation of chondrocyte matrix protein synthesis upon enzymatic resuspension of cells could also be a consequence of the change in chondrocyte shape and/or cytoskeletal reorganization.

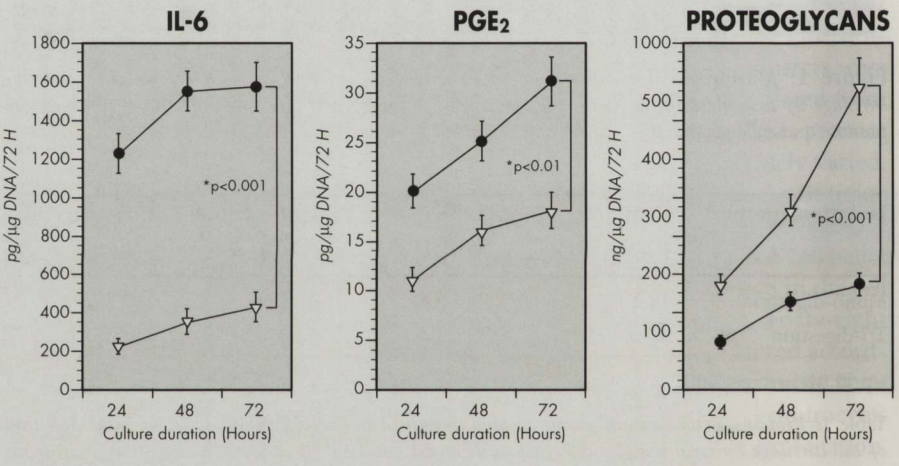


Figure 2: *Influence of the enzymatic isolation method on the synthesis of IL-6, PGE_2 and proteoglycans by human articular chondrocytes in vitro. Chondrocytes were isolated from the cartilage by a single enzymatic digestion (- ● -) or by triple digestion with hyaluronidase, pronase and collagenase (- ▽ -). Results are expressed as mean values of three cultures from the same donor. Comparison of mean values was performed using the unpaired Student's t-test.*

- Some cytokines (IL-8) and growth factors (i.e., IGF-1, TGFß) are trapped in the cartilaginous extracellular matrix (Luyten *et al.*, 1988; Morales *et al.*, 1991; Recklies and Gold, 1992). The best known example is the binding of TGFß by decorin. Indeed, it has been reported that the interaction between TGFß and decorin is partially responsible for the decreased ability of TGFß to induce cell growth in Chinese hamster ovary cells (Yamaguchi *et al.*, 1990). There is evidence that the decorin content in the cartilage increases with age suggesting that the bioavailability of TGFß could decrease with age (Roughley *et al.*, 1994). Growth factors and cytokines could be released from the residual matrix during the culture period and then directly modulate the chondrocyte functions.

1.1.3 Chondrocyte Culture Models

1.1.3.1 Culture in Monolayer

The differentiated phenotype of articular chondrocytes is primarily characterized by the synthesis of type II collagen and cartilage-specific proteoglycans and by the low level of mitotic activity. Many studies have shown that cultures in monolayer on plastic subtrata for prolonged periods or upon repeated passages lead to the loss of chondrocytes spherical shape and to the acquisition of an elongated fibroblast-like morphology (Von der Mark *et al.*, 1977). The synthesis pattern is fully modified by this dedifferentiation process. A progressive reduction in the synthesis of type II, IX and XI collagens and an increase in the synthesis of type I , III and V collagens were observed during fibroblastic metaplasia (Elima and Vuorio, 1989; Lefebvre *et al.*, 1990a). Small non-aggregating proteoglycans are synthesized at the expense of functional aggrecan (Okayama *et al.*, 1976; Kuettner *et al.*, 1982; Watt, 1988). The synthesis of cathepsin B and L are extremely low in differentiated cells, but are soon increased in parallel with the loss of the differentiated state. Inversely, collagenase-1 is strongly expressed by differentiated chondrocytes and declines rapidly with successive subcultures (Kostoulas *et al.*, 1997) while TIMP production progressively increases (Lefebvre *et al.*, 1990a).

Interestingly, dedifferentiated chondrocytes re-express the differentiated collagen phenotype when they are transferred from monolayer to suspension culture (Desmukh and Kline, 1976; Norby *et al.*, 1977; Benya and Schaffer, 1982; Elima and Vuorio, 1989). Differentiation process is not yet clearly identified but seems to be related to the shape of the cells (Loty *et al.*, 1995). This characteristic is regularly used by researchers investigating cartilage defect grafting with autologous chondrocytes. A small number of cells obtained from the biopsy specimen may be amplified in monolayer and therefore reinvested in a three-dimensional matrix before transplantation. The re-expression of cartilage specific phenotype by dedifferentiated chondrocytes transferred in agarose culture may be promoted by TGFβ, osteogenin and vitamin C (Harrison *et al.*, 1992).

Chondrocyte responses to growth factors and cytokines are modified during the dedifferentiation process. Cellular responses to cytokines and growth factors differ between primary chondrocytes and dedifferentiated cells. IL-1 stimulates fibroblasts

proliferation, whereas primary chondrocytes growth are inhibited by IL-1 (Guerne *et al.*, 1994). DNA synthesis is stimulated by IGF-1 in flattened chondrocytes but not in rounded cells (Lee *et al.*, 1993). IL-1β and TNFα stimulating effect on procollagenase production is more pronounced on dedifferentiated chondrocytes than on differentiated cells (Lefebvre *et al.*, 1990b).

1.1.3.2 Culture in Suspension

Maintenance of chondrocytes in suspension in a liquid medium or in a natural or synthetic three-dimensional matrix stabilizes the chondrocyte phenotype. Chondrocytes conserved their spherical shape and continue to produced cartilage-specific proteins. Suspension culture is particularly recommended to study new pericellular matrix formation. Culture of chondrocytes within synthetic or natural absorbable polymers have also been developed for implantation of cells into cartilage defects to promote regeneration of the articular joint surface. Cell scaffolds for cartilage engineering should meet several criteria: (1) implants should provide a pore structure that allows cell adhesion and growth, (2) neither the polymer nor its degradation products should provoke inflammation or toxicity when implanted *in vivo*, (3) transplantation vehicles should also provide binding capacity to adjacent cartilage or subchondral bone, (4) the scaffold should be absorbable and the degradation should match the rate of tissue regeneration, (5) to facilitate cartilage repair, the chemical structure and pore architecture of the matrix should allow the seeded cells to maintain the chondrocytes phenotype and the synthesis of cartilage-specific proteins, (6) the scaffolds need to be mechanically studied enough at the time of *in vivo* implantation.

Suspension in a liquid phase. Cellular attachment to the supporting plastic culture flask may be prevented by maintaining chondrocytes under constant agitation (Kuettner *et al.*, 1982; Bujia *et al.*, 1993) or by coating the plastic dishes with a viscous solution of methyl cellulose, agarose, hydrogel (poly-(2-hydroxyethyl methacrylate) or a composite collagen-agarose (Reginato *et al.*, 1994). In these conditions, chondrocytes form clusters and synthesize mainly aggrecan and cartilage-specific collagens (II, IX, XI collagens). Nevertheless, two types of cells are usually observed. The cells in the center of the nodules remains spherical and are surrounded by an abundant cartilaginous extracellular matrix, as evidenced by histochemical and ultrastructural examinations. The cells in the periphery have a discoid morphology and are surrounded by a sparse extracellular matrix. Little is known about the synthetic and functional characteristics of the flattened surface cells. On the other hand, plates coated with fibronectin and type I and type II collagens allow chondrocyte attachment and promote direct motility of cells in a haptotatic and chemotatic fashion (Shimizu *et al.*, 1997).

Chondrocytes can also be cultured on microcarriers maintained in suspension by constant agitation in a siliconized spinner flask (Freed *et al.*, 1993; Frondroza *et al.*, 1996). Different types of microcarriers, including dextran beads (Cytodex), dextran beads collagen coated (Cytodex III) and non-porous type I collagen

microspheres (Cellagen), have been tested for chondrocytes suspension culture. In these culture conditions, chondrocytes attach to the microcarrier surface and keep their spherical shape and produce a matrix-like material. Moreover, Cellagen culture promotes chondrocytes proliferation and re-expression of chondrocytic phenotype (Frondoza *et al.*, 1996). Therefore, Cellagen culture could be used for bulk production of chondrocytes that continue to express their original phenotype before transplantation.

Another method consists of culture chondrocyte as a pellet of high density cells obtained by centrifugation. The high density pellet culture system (0.5 to 1.10^6 cells) has been widely used to study epiphyseal and growth plate chondrocyte differentiation and hypertrophy (Ronzière *et al.*, 1997). Recently, it was also applied to the culture of adult bovine nasal and articular chondrocytes (Xu *et al.*, 1996). In this method, chondrocyte suspension is centrifuged and cultured for at least 3 weeks in polypropylene tubes. This study showed that adult chondrocytes cultured as pellets deposit a cartilage-like matrix that contains abundant proteoglycan and type II collagen but not type I as judged by histological, immunohitochemical and quantitative methods.

Suspension in natural extracellular matrix. Chondrocytes may also be cultured in suspension within a three-dimensional matrix such as soft agar, agarose, collagen gel and sponge, hyaluronan, fibrin glue or alginate beads.

Chondrocytes cultured in agarose are known to maintain their cartilage phenotype and synthesized type II collagen and tissue-specific aggregated aggrecan (Benya and Shaffer, 1982). In the agarose culture, most of the proteoglycan remains in the agar but is continuously released into the medium for more than 50 days. In the monolayer culture, by comparison, the cellular phase becomes saturated with GAG after 5-6 days whereas in the culture medium an increase initially occurs and is followed by a time-dependent decrease after the first 8-10 days (Spirito *et al.*, 1993). Nevertheless, chondrocytes in agarose do not have exactly the same behavior than *in vivo*. In agarose, the majority of the synthesized aggrecan aggregates contain less than 20 aggrecan molecules and are smaller than *in vivo* (Cornelissen *et al.*, 1993). TGFßs increase proteoglycan synthesis in explant culture (Morales and Roberts, 1988; Pujol *et al.*, 1991) but down-regulate aggrecan by the same cells in agarose (Skantze *et al.*, 1985).

Alginate is a linear polysaccharide isolated from brown algae and composed by two uronic acids, L-guluronic and D-manuronic acid linked by ß1,4 and α1, 4 glucoside binds. In the presence of divalent cations such as Ca^{++}, the polymer undergoes instant ionotrophic gelation (Guo *et al.*, 1989). Each chondrocyte entrapped in alginate is surrounded by a negatively charged polysaccharide matrix that has a pore size comparable to that of hyaline articular cartilage. The matrix formed by adult human chondrocytes in alginate beads is composed in two compartments; a thin rim of cell-associated matrix that corresponds to the pericellular and territorial matrix of articular cartilage and a more abundant further removed matrix, the equivalent of the interritorial matrix in the tissue. On day 30 of culture, the relative and absolute volumes occupied by the cells and each of the

two compartiments in the beads are nearly identical to those in native cartilage (Hauselmann *et al.*, 1996; Petit *et al.*, 1996). The chondrocytes maintain a spherical appearance for at least 30 days and mainly produce aggrecan of similar hydrodynamic size to aggrecan molecules present in the matrix of articular cartilage as well as cartilage characteristic collagens including type II, IX and XI (Mok *et al.*, 1994; Petit *et al.*, 1996; Platt *et al.*, 1997). Meanwhile, as observed in other supension culture models, flattened cells are present at the surface of the beads. These flattened cells produce small amounts of type I collagen molecules which are directly released in the culture medium and do not become incorporated in the extracellular matrix (Petit *et al.*, 1996). Chondrocytes proliferate moderately in the alginate beads. After 8 months in alginate gel, adult chondrocytes are still metabolically active and continue to synthesize cartilage specific type II collagen and aggrecan (Hauselmann *et al.*, 1994). Tanaka *et al.* (1984) investigated the diffusion characteristics of a variety of naturally occurring molecules into alginate and found that the diffusion of molecules larger than 70 Kda was excluded out of the matrix. This culture system is very well suited for the investigation of the regulation of matrix biosynthesis and extracellular matrix organization. Additionally, culture in alginate has the advantage that rapid depolymerization of the matrix can be achieved in the presence of a calcium chelating agent that allows the cells and matrix to be harvested for further analysis. The accessibility of the cells allows further investigations of the mode of action of regulatory peptides and pharmacological agents at the transcriptional, post-transcriptional and translational levels.

Chondrocytes are also cultured within type I and type II collagen matrices (Schuman, 1995; Fujisato *et al.*, 1996). Recently, Nehrer *et al.* (1997) compared the behaviors of canine chondrocytes in a porous collagen-glycosaminoglycan copolymer matrix comprising different collagen types. They observed an important difference in the morphology and biosynthetic activities of the cells in the type I and type II collagen matrices. The cells in the type II collagen matrix retained their spherical shape, while in the type I matrix chondrocytes displayed a fibroblastic morphology. Moreover, chondrocytes cultured in type II matrix produced higher amounts of GAG than in type I sponge. In addition, cell numbers increased with culture time in all collagen matrices. Van Susante *et al.* (1995) compared viability, phenotype, proliferation and sulfate incorporation of bovine chondrocytes cultured in alginate or type I collagen gel. The authors observed a significant increase in cell number in collagen gel, but the chondrocytes dedifferentiated into fibroblast-like cells from day 6. In alginate gel, initial cell loss occurred but the cells maintained their typical chondrocyte phenotype. The total quantity of proteoglycans initially synthesized per cell in collagen gel was significantly higher. However, in collagen gel, matrix production decreased from day 6, whereas in alginate gel it continued to increase. Subsequently, proteoglycan synthesis in alginate gel, expressed per chondrocytes, surpassed that in collagen gel on day 6.

Solid three-dimensional fibrin matrices represents a natural substance which also sustains suspended chondrocytes in a differentiated phenotype. Three-dimensional fibrin matrix was also tested as a vehicle in chondrocyte transplantation (Homminga *et al.*, 1993). The benefits of fibrin include its absence of cytotoxicity,

space-filling capabilities and adhesive capacity (Hendrickson *et al.*, 1994; Fortier *et al.*, 1997). Histology, autoradiography, electron microscopy and biochemical investigations revealed that chondrocytes seeded in fibrin glue kept their morphology, multiplied and produced matrix even after 14 days of culture (Fortier *et al.*, 1997). Nevertheless, Homminga *et al.* (1993) reported that glue desintegration began after 3 days and cell dedifferentiation progressed.

Suspension in a synthetic matrix. Cartilage implants for potential use in reconstructive or orthopedic surgery can be created by growing isolated chondrocytes *in vitro* into a synthetic, biocompatible and biodegradable scaffold.

Chondrocytes cultures in polyglycolic acid (PGA) scaffolds proliferate and keep their morphology and phenotype for 8 weeks. Chondrocyte-PGA constructs consisted of cells, GAG and collagen, and had an outer collagenous capsule. Nevertheless, both type I and type II collagen were present in the cell-PGA implants. Moreover, the authors reported that implants based on dedifferentiated chondrocytes by serial passages had similar cellularities, higher GAG and collagen contents than those based on primary chondrocytes. These findings indicate that tissue engineering can be amplified by a previous serial passage prior to seeding chondrocytes on PGA scaffolds (Freed *et al.*, 1994b). Freed and collaborators (1993) also compared the behaviors of human and bovine chondrocytes cultured on fibrous polyglycolic acid (PGA) and porous poly(L)lactil acid (PLLA). Over 6-8 weeks of *in vitro* culture, bovine chondrocytes cultured on PGA or PLLA proliferated and regenerated cartilaginous matrix. On PGA, chondrocytes appeared rounded and within lacunae surrounded by cartilaginous matrix. After 8 weeks of *in vitro* culture, regenerated tissue accounted for 50 % of the construct dry weight (4 % cell mass, 15 % GAG and 31 % collagen) (Freed *et al.*, 1994a). In contrast, on PLLA the cells appeared spindle-shaped and minimal matrix staining for GAG and collagen was observed. Moreover, the cell growth rate was approximately twice as high on PGA as it was on PLLA (Freed *et al.*, 1993a). *In vivo*, chondrocytes grown on both PGA and PLLA for 1-6 months produced tissue appearing glistening white macroscopically and resembling cartilage histologically. At this time, implants contained GAG, type I and type II collagen as shown by immunostaining (Freed *et al.*, 1993a).

Porous high density hydrophobic or hydrophilic polyethylene (HDPE) substrates have also been tested to culture fetal bovine chondrocytes. After 7 days of incubation, the cells within both subtrates remained spherical and contained mainly type II collagen. After 21 days, the majority of the cells had spread. The hydrophilic matrices contained significantly more type II collagen than the hydrophobic matrices (Livecchi *et al.*, 1994).

Bioresorbable co-polymer fleeces of vicryl and polydioxanon (Ethicon) or polylactic acid soaked with poly-L-lysine or type II collagen were tested for cartilage tissue engineering in perfusion culture (Sittinger *et al.*, 1993; Bujia *et al.*, 1995). The biomaterial containing cells were encapsulated in agarose gel to improve retention and accumulation of extracellular matrix component synthesized by the chondrocyte. The fleece organization offers a maximun of available internal polymer

surface and a minimun of solid polymer volume and therefore allows attachment of a great number of cells. Culture perfusion avoids repetitive culture manipulation, decreases the risk of bacterial contamination and achieves a constant supply of nutrient by diffusion. Moreover, perfusion not only stabilizes the components provided by the culture medium, it also stabilizes secreted autocrine factors at a constant level. For example, it was demonstrated that glucose concentrations fluctuate at each culture medium change when chondrocytes are cultured in monolayer in a stagnant medium environment (Bujia *et al.*, 1995). Using a perfusion culture system, a constant concentration of nutrients like glucose is provided. However, the measured concentrations of lactate are unstable and significantly increase during the perfusion culture of cell-Ethicon system tissue due to the hydrolytic degradation of the poly-a-hydroxy acids (Sittinger *et al.*, 1997). In this culture model, chondrocytes rapidly adhered onto bioresorbable carrier fleece but conserved their rounded morphology. The condrocytes in cell-polymer cultures continued and maintained synthesis of type II collagen and proteoglycan as analysed by immunohistochemistry.

Cartilaginous tissue may also be obtained by culturing chondrocytes as monolayer on Millicell-CM filters. Precoating the filters with collagen was necessary for attachment of the chondrocytes. Histological examination of the culture showed that the chondrocytes over time accumulated an extracellular matrix which contained proteoglycans and type II collagen and formed a continuous layers of cartilaginous tissue. No type I collagen was detected. Chondrocytes within the tissue were spherical but appeared flattened on the surface. The thickness of the tissue generated increased over time and was dependent on the initial plating density. Under optimal culture conditions, the cartilaginous tissue attained a thickness of 110 μm and showed organization of cells and collagen into superficial and deeper layers similar to that previously described for articular cartilage. The extracellular matrix contained approximately three-fold more collagen than proteoglycan, which was similar to *in vivo* cartilage. After 2 weeks, there was sufficient matrix accumulation that the tissue could be handled and removed easily from the supporting filter and used for transplantation. *In vivo*, engineered tissue did not integrate to the host cartilage and tissue fixation was needed. Moreover, fibrovascular tissue was observed in some transplants (Kandel *et al.*, 1995; Boyle *et al.*, 1995).

More recently, Sims *et al.* (1996) investigated the utilization of polyethylene oxide gels as encapsulating polymer scaffolds for delivering large numbers of isolated chondrocytes via injection. As early as 6 weeks after injection into the subcutaneous tissue of athymic mice, new cartilage was formed exhibiting a white opalescence similar to that of hyaline cartilage. Histologic and biochemical analysis showed the presence of actively proliferating chondrocytes with production of a well-formed cartilaginous tissue in the transplant.

Culture chondrocytes in a three-dimensional synthetic matrix seems to be suitable for long-term study of cartilage formation. Nevertheless, little information is available concerning the utilization of these systems for cartilage resorption investigation. One major disavantage of suspension cultures utilizing gel matrices is

the difficulty of harvesting the cells once they have been encapsulated within the artificial matrix.

1.1.3.3 Explant Culture

Cartilage explant culture has been used by many investigators to study various aspects of the anabolic and catabolic metabolism of articular cartilage (Campbell *et al.*, 1989; Sandy *et al.*, 1991). Articular explant cultures offer a panel of advantages for the *in vitro* study of matrix homeostasis, resorption and repair. The chondrocytes in cartilage explants maintain their phenotype and the extracellular matrix is similar to that observed *in vivo*. After 5 days in culture in the presence of serum, steady-state rates of synthesis and turnover are reached (Hascall *et al.*, 1983; Campbell *et al.*, 1984). Resorption may be accelerated in both basal and serum-supplemen-ted culture by several agents including IL-1β, TNFα, bacterial lipopolysaccharides, retinoic acid derivative or active oxygen species flux. Repair capacity of the cartilage may also be investigated. Initial injury to the cartilage is usually induced by soluble mediators of inflammation (H_2O_2, IL-1, TNFα) or by physical disruption of the matrix. Thereafter, healing of the tissue may be easily studied.

Organotypic culture is also an adequate *in vitro* model to study the effects of isolated environmental factors on chondrocytes and the surrounding matrix. *In vivo*, chondrocytes are sparsely distributed in the extracellular matrix and do not have contact between one another. Cartilage explant cultures conserve these structural organizations and the multiple interactions between chondrocytes and their direct extracellular environment.

This model is also used to test the effects of mechanical stress, pharmacological agents, growth factors, cytokines or hormones on the cartilage metabolism. The system yields some information on the possible interactions of the tested compounds with the matrix and their accessibility to the chondrocytes.

Another advantage is that chondrocytes are not damaged by enzymatic or mechanical stress occurring during cell isolation. Receptors and other membrane proteins and glycoproteins are protected from damaging stress. Removal and dicing of full-thickness cartilage to radomize the influence of geographic effects are recommended. On the other hand, there are no clear guidelines on the surface and the volume of the dice. Too large an explant may induce central necrosis. The shape of the explant may influence the size of the exchange surface between explant and culture medium. It is well known that the alteration in surface area of the explant cartilage may produce an initial increase in the rate of proteoglycan loss from the tissue.

1.1.3.4 Chondron Culture

Chondron is a morphological entity in articular cartilage composed of chondrocyte, its pericellular matrix and a compacted filamentous capsule (Poole *et al.*, 1984, 1985, 1986, 1987). It is considered as the primary structural, functional and metabolic unit in hyaline cartilage responsible for matrix homeostasis. Chondrons

are mechanically extracted from cartilage and collected by serially low-speed homogenisation. Chondrons isolated from full-deep articular cartilage are classified in four broad categories; single chondrons, double chondrons, multiple linear column (three or more chondrons) and chondron clusters/matrix chips. Single chondrons represent the smallest chondron unit identified and are commonly found in the middle layers of intact cartilage. Double chondrons are typically identified in the middle-to-deep layers of intact tissue. Multiple chondron columns consist of three or more chondron units organized in a linear array typical of those seen in the deep layers of intact cartilage. Finally, clusters consist of randomly organized groups of single and double chondrons that remain aggregated during the homogenisation procedure. Chips are described as large cartilage fragments usually containing several chondrons aligned and spatially organized in parallel with radial collagen fibres typical of the deep layer matrix (Poole *et al.*, 1988c). Techniques have now been introduced to immobilize chondrons in transparent agarose allowing a range of structural, molecular and metabolic investigations. This chondron agarose system is considered as a micro-cartilage explant model and differs from traditional chondrocyte-agarose cultures in that the natural microenvironment is retained and need not be resynthesized and assembled (Poole, 1997). Microscopy and immunohistochemistry analysis have ushered in a clearer understanding of the composition of the pericellular environment of the chondrocytes as well as the cell matrix interaction (Poole *et al.*, 1988a, 1988b, 1988c, 1990, 1992, 1993; Chang *et al.*, 1997). Chondrons are presented as a model system to study cell-matrix interaction in articular cartilage health and disease. Nevertheless, little information is available on the maintenance of chondrons in culture.

1.1.3.5 Immortalized Chondrocytes

Recombinant DNA or virus containing oncogene with an "immortalizing" function has been used with various cell types to establish permanent cell lines (Horton *et al.*, 1988; Thenet and Adolphe, 1992; Oxford *et al.*, 1994; Mallein-Gerin *et al.*, 1995). Immortalization of chondrocytes in culture, displaying both infinite proliferation capacity and stable phenotype, could be of interest because chondrocytes lose their phenotype when grown in monolayer. Mallein-Gerin *et al.* (1995) showed that SV40 large T oncogene is able to induce mouse chondrocyte proliferation without loss of expression of type II, IX and XI collagens, as well as cartilage aggrecan and link protein. Nevertheless, the cell line obtained also synthesized type I collagen when cultured in monolayer or in agarose gel. Horton *et al.* (1998) described an immortalized cell line having a low level of type II collagen mRNA expression. These cells were created by transformation with a murine retrovirus carrying the v-myc and v-raf oncogenes. This cell type represents a unique model to study cartilage matrix interactions in the absence of type II collagen and also the type II collagen synthesis regulatory pathway (Oxford *et al.*, 1994). Finally, it was shown that rat chondrocytes immortalized by myc express mRNA transcript coding for the proα2 chain of type I collagen when cultured in monolayer, but not in suspension as normal chondrocytes.

Chondrocytes with mutated or knock-out genes important for cartilage physiology can also be maintained in culture. This approach is of particular interest to investigate the role of specific molecules in the organization of cartilage matrix or the effect of regulatory factors on the cartilage metabolism. Chondrocytes knock-out for type IX collagen gene synthesize type II collagen fibrils larger than normal type II collagen fibrils suggesting that type IX collagen acts as a fibril diameter regulator (Mallein-Gerin *et al.*, 1995). Recently, molecular and genetic analyses have demonstrated the presence of mutations in COL2A1, the gene coding for type II collagen, in family with primary generalized osteoarthritis. To examine the influence of mutant type II collagen molecules on cartilage matrix, Dharmaravan *et al.* (1997) performed stable transfection of COL2A1 construct containing the Arg^{519} - Cys mutation into human fetal chondrocytes *in vitro.*

1.1.3.6 Co-Culture System

In joint, the articular cartilage develops a relationship with other cell types contained in synovial membrane, synovial fluid, ligament and subchondral bone. Chondrocytes metabolism may be influenced by soluble factors synthesized by these cells. In arthritis, cartilage is directly degraded by enzymes and free radicals produced by synovial cells. Many reseachers have developed culture models studying the interactions between cartilage and neighboring tissues.

Lacombe-Gleize *et al.* (1995), cultured rabbit chondrocytes and osteoblasts in a co-culture system (COSTAR) in which cells were separated by a microporous membrane (0.4 μm) that permitted exchanges between the two cell types without any direct contact. This study demonstrated that osteoblasts are able to stimulate growth of chondrocytes by way of soluble mediators. This model should be of interest to understand interaction between chondrocytes and osteoblasts.

Another model described by Malfait and collaborators (1994), studied the relation between peripheral blood mononuclear cells and chondrocytes. This model was particularly useful to investigate cytokine-mediated events at the cartilage-synovial pannus junction in destructive arthropathies. In this model, agarose cultures were performed in tissue culture inserts consisting of a low-protein binding membrane with 0.4 μm pores. Chondrocytes cultured in agarose were then placed in a petri dish on the top of mononuclear cells. This study showed that mononuclear cells stimulated by LPS produced IL-1 and TNFα which thereby depressed chondrocyte aggrecan synthesis and provoked the breakdown of newly synthesized aggrecan aggregates.

Tada *et al.* (1994) established an *in vitro* model in which vascular endothelial cells on type I collagen in an inner chamber, which featured a 0.4 μm millipore filter, are co-cultured with chondrocytes in an outer chamber. In this model, human endothelial cells formed tubes in the collagen gel in the presence of epidermal growth factor (EGF) or transforming growth factor-α. Interestingly, TGF-dependent tube formation by endothelial cells was inhibited when human chondrocytes were co-cultured in the outer chamber. This chondrocyte-induced inhibition

is partly abrogated by anti-TGFβ antibodies suggesting that avascularity of cartilage is partly due to TGFβ produced by chondrocytes themselves.

Groot *et al.* (1994) co-cultured chondrocytes from the hypertrophic and proliferative zones of 16-day-old fetal murine bone with pieces of cerebral tissue. After 4 days of co-culture, some chondrocytes had transdifferentiated into osteoblasts and started to form osteoids. After 11 days, part of the cartilage was replaced by bone and the bone matrix was partially calcified. The nervous system produces neuropeptides and neurotransmitters, of which some are known to affect osteoblast metabolism or have receptors on osteoblastic cells, such as norepinephrine (Kumagai *et al.*, 1989), vasoactive intestinal peptide (Bjurholm *et al*;, 1988), calcitonin gene-related peptide (Michelangeli *et al.*, 1989; Bjurholm *et al.*, 1990), substance P (Bjurholm *et al.*, 1990) and somatostatin (Mackie *et al.*, 1990). The co-cultured pieces of cerebral tissue may produce some of these factors capable of inducing transdifferentiation process of chondrocytes into osteoblasts.

1.1.4 Chondrocyte culture environment

1.1.4.1 Effects of oxygen tension on chondrocyte metabolism

Most chondrocyte cultures are developed within an atmospheric oxygen tension. Nevertheless, it is widely assumed that chondrocytes live in a hypoxic environment and that oxygen tension may vary in pathological situations. Morever, during maturational process, there are marked changes in the vascular supply to epiphysis. In concert with the observation that the vascular supply varies from zone to zone in the growth plate, sharp differences in the oxygen tension accross the plate have been noted. Brighton and Heppenstall (1971), demonstrated that in the tibial plate of the rabbit, the oxygen tension is lower in the hypertrophic zone than in surrounding cartilage. Measurement of a number of metabolic parameters indicated that chondrocytes are able to rapidly respond to local alterations in the oxygen concentration. Firstly, oxygen consumption by chondrocytes decreases at low oxygen tension (Haselgrove *et al.*, 1993). Secondly, glucose utilization, glycolytic enzyme activity and lactate synthesis progressively increase when the oxygen tension is lowered from 21 % to 0.04 %. Thirdly, the absolute levels of ATP, ADP or AMP remain stable even at very low oxygen tension (Rajpurohit *et al.*, 1996). These data demonstrate that chondrocytes exhibit metabolic characteristics directed at preserving maximun energy levels for vital processes. Nevertheless, synthetic activity, and thus repair processes, is altered by hypoxic conditions. Low oxygen tension inhibits sulfate incorporation and DNA synthesis (Lane *et al.*, 1997).

High oxygen tension may also induce adverse effects on chondrocyte metabolism including reduction of the proteoglycan and DNA synthesis (Lemperg *et al.*, 1975; Lane *et al.* 1977) or cartilage matrix degradation (Sledge and Dingle, 1965). These effects may be conducted by active oxygen species production (Henrotin *et al.*, 1993a, 1993b).

1.1.4.2 Effects of ionic and osmotic environment on chondrocyte function

In cartilage, the extracellular ionic environment is different from that of most cells, extracellular Na^+ being 250-350 mM and extracellular osmolality 350-450 mOsm. When chondrocytes are isolated from the matrix and incubated in a standard culture medium (DMEM; osmolality 250-280 mOsm), their extracellular environment changes sharply. Furthermore, Ca^{++} and K^+ concentrations in the medium are considerably lower than in the tissue, and anion concentrations are higher.

Hyperosmotic stimulus produced by addition of sucrose concentration, induces a transient intracellular elevation of proton and calcium anions in the cytosol (Dascalu *et al.*, 1996). This intracellular change may affect several cell processes such as chondrocyte differentiation and metabolic activity. Urban *et al.* (1995) demonstrated that ^{35}S-sulphate and ^{3}H-proline incorporation rates by isolated cells incubated in DMEM for 2-4 h was only 10 % of that in the tissue. Synthesis reached a maximun when the extracellular osmolality was 350-400 mOsm for both freshly isolated and for cartilage explant. Moreover, the cell volume of chondrocytes increased by 30-40 % when the cells were removed from their *in situ* environment into DMEM. If, however, chondrocytes were maintained for 12-16 h in non-physiological osmolalities, they appeared to adapt to some extent to their new extracellular environment so that as before, synthesis rates decreased in proportion to any further change in extracellular osmolality. Another study reported the effect of osmolarity on pig chondrocyte growth, synthesis and morphology when cultured at high density. This study showed a similar biochemical and morphological behavior of chondrocytes cultured at 0.28 and 0.38 Osm. At 0.48 Osm, cell proliferation and protein synthesis were reduced during the first 4-6 h of culture and then progressively recovered their rate and nearly reached the control value. In contrast, cells cultured in 0.58 Osm medium were unable to sustain their proliferative rate and after 6-days of exposure cell number drastically declined indicating cell detachment or death. At 0.58 Osm, protein synthesis was highly inhibited and protein synthesis did not restore to the control rate level during the subsequent period of observation. Furthermore, while chondrocytes cultured in 0.28-0.38 Osm medium maintained phenotypic characteristic in culture, the higher osmolarities (0.48-0.58) caused morphological change resulting in loss of phenotypic stability as demonstrated by their taking on a fibroblast-like shape as well as a lack of ability to assemble matrix proteoglycans (Borghetti *et al.*, 1995). These findings suggest that chondrocytes possess adaptative mechanisms capable of responding quickly at limited osmolarity variation. The change in concentration of other ionic species as the result of cell isolation may also affect matrix synthesis. Incorporation rates of ^{35}S-sulphate increased by 50 % as the medium concentration of K^+ was raised from 5 mM (DMEM concentration) to 10 mM (extracellular concentration in cartilage). Extracellular HCO_3 had no effect on the synthesis rate over the physiological range. In addition, Ca^{++} had no effect on tracer incorporation rates in cells or cartilage slices over 0-10 mM (Urban *et al.*, 1993; Koyano *et al.*, 1996). Nevertheless, $CaCl_2$

concentration below 0.5 mM to the selectively promoted the production of collagen by adult bovine chondrocytes whereas the lowest levels of collagen synthesis were reached at calcium concentrations of 1-2 mM (which corresponded at the amount found in DMEM). A moderate increase occurred at high levels of calcium (2-10 mM) (Koyano *et al.*, 1996). SO_4 became rate-limiting at 0.3 mM, but had no significant effect above this concentration. Moreover, cations were required for chondrocytes attachment and provided a mechanism to differentially regulate attachment to ECM proteins. Mg^{2+} and Mn^{2+} supported attachment to fibronectin and type II collagen while Ca^{2+} did not support adhesion to collagen (Loeser, 1994). These data indicate that synthesis rates in chondrocytes incubated in DMEM after isolation are affected by changes in extracellular K^+, Na^+, Ca^{++} and osmolality.

1.1.4.3 Effects of Mechanical Stress on Chondrocyte Metabolism

Joint immobilization causes reversible cartilage arthrophy suggesting that mechanical stimuli are required for physiologic turnover of the extracelular matrix. The majority of culture models reported in the literature is realised at normal atmospheric pressure and fails to expose chondrocytes at tensile or compressive strength. However, it has been clearly demonstrated that chondrocyte metabolism is influenced by the mechanical environment, with the level of response dependent on both the strain and frequency of the compressive load (Wright *et al.*, 1996). Loading experiments using intact articular cartilage explants *in vitro* have indicated that static loading reduced PG and protein synthesis, while dynamic loading might stimulate synthesis in a frequency dependent manner (Saamanen *et al.*, 1990; Korver *et al.*, 1992; Parkkinen *et al.*, 1992). The exact signaling pathways are complex and believed to involve cell deformation (Lee and Bader, 1995), hydrostatic pressure (Parkkinen *et al.*, 1993), osmotic pressure (Urban *et al.*, 1993), streaming potentials (Frank and Grodzinsky, 1987), or cell surface receptors for matrix molecule (Holmvall *et al.*, 1995). To assess the exact influence of each of the parameter, it is necessary to develop systems in which one parameter can be varied independently. For example, explant culture is not adapted to study cell deformation but may be used to investigate the general effect of pressurization on chondrocyte metabolic activity. Indeed, compression of cartilage results in deformation of cells but it is also accompanied by hydrostatic pressure gradients, fluid flow, streaming potentials and currents as well as physiochemical changes such as altered matrix water content, fixed charge density and modifications in osmotic pressure (Sah *et al.* 1992). Cell deformation can be studied using isolated cells embedded in agarose gel or collagen gel (Lee and Bader, 1995).

A variety of experimental systems have been developped to investigate the effects of mechanical stimulation on cultured chondrocytes. Some groups used an experimental system where pressure is applied to the cultured chondrocytes via a gas phase. Using pressure of 13 kPa above atmospheric at a low frequency of 0.3 Hz for 15 min, Veldhuijzen *et al.* (1979) demonstrated an increase in cytoplasmic cyclic AMP and proteoglycan synthesis and a reduction in DNA synthesis.

Complementarily, Smith el al. (1996) showed that primary bovine chondrocytes cultured at high density in the presence of fetal bovine serum and exposed to hydrostatic pressure (10 MPA) applied intermittently at 1 Hz for 4 h increased aggrecan and type II collagen mRNA signals whereas constant pressure had no effect on either mRNA. In a similar system, Wright *et al.* (1996) reported that cyclical pressurization was associated with hyperpolarization of chondrocyte cell membranes and activation of Ca^{2+}-dependent K-ion channels. This effect was associated with changes in microstrain applied on the base of the culture plate and subsequent stretching of the attached chondrocytes suggesting that effect observed after intermittent pressurization was mediated by stretch-activated ion channels in chondrocytes plasma membrane. The chondrocyte reaction to hydrostatic pressure depends on culture conditions and testing regimens. Cyclic hydrostatic pressure (5 MPa) decreases sulfate incorporation in chondrocyte monolayers when applied at 0.05, 0.25 and 0.5 Hz but stimulates sulfate incorporation when applied to cartilage explants at 0.5 Hz (Parkkinen *et al.*, 1993).

Fluid-induced shear can be applied on chondrocytes monolayer culture using a cone viscometer rotating within the cell culture medium at a constant speed. Chondrocytes submitted at fluid-induced shear adopted an elongate shape and aligned tangentially to the direction of the cone rotation. Fluid induced shear stimulated glycosaminolycan and increased the length of newly synthesized chain in human and bovine chondrocytes (Smith *et al.*, 1995). Moreover, the release of prostaglandin E_2 and mRNA signal for tissue inhibitor of metalloproteinases were enhanced by fluid-induced shear. In contrast, mRNA signals for the neutral metalloproteinases did not show major changes.

Recently, Buschmann *et al.* (1995) reported that chondrocytes in agarose gel respond biosynthetically to static and dynamic mechanical load in a manner similar to that of intact organ culture. However after the level of matrix development was more advanced, the response to compression was more pronounced in culture. This method has also demonstrated that mechanical loads generated a hyperosmotic stimulus and subsequent pH decrease at the level of the chondrocytes.

The effect of mechanical tensile may be studied within a gel embedded cell system or on adherent support cells. Tensile strain may be determinated with a system in a computer-controlled vaccum unit and a base plate to hold the culture dishes. When a precise vaccum level is applied to the system, the culture plate bottom is deformed to a known percentage elongation which is maximal at the edge and decrease at the center. The strain is translated to the cells cultured. By this way, Holmval *et al.* (1995) showed that chondrosarcoma cells cultured on collagen type II-coated dishes increased the mRNA expression of $\alpha 2$- but not $\alpha 1$-integrin. The integrin $\alpha 2\beta 1$ is able to bind type II collagen. Therefore, it is an excellent candidate for mechanoreceptors since it interacts with actin-binding proteins and thereby links the extracellular matrix with the cytoskeleton.

1.1.4.4. Effect of pH on Chondrocyte Metabolism

Chondrocytes exist in an extracellular environment where the pH of the interstitial fluid is more acidic than that of other tissues and which changes with tissue loa-ding. Values of pH 6.9 have been measured for the matrix of articular cartilage (Maroudas, 1980) and there are reports of values as low as 5.5 for diseased tissue (Diamant *et al.*, 1968). Chondrocytes live at low pO_2 implicating that metabolism is largely by glycolysis (up to 95 % of all glucose metabolism), with consequent production of significant quantities of lactic acid (Stefanovic-Racic, 1994). In addition to this acidification which arises from metabolism, there is also a contribution from the components of the matrix itself. The large numbers of fixed negative charges on proteoglycans modify the extracellular ionic composition. There are high levels of free cations (e.g., H^+, Na^+, K^+) and low concentration of anions (e.g., Cl^-, HCO_3^-) (Lesperance, 1992). Furthermore, the water present in the matrix can be expressed during tissue loading, resulting in a higher concentration of fixed negative charges, and so attracting more positive ions into the matrix. This includes a decrease of the extracellular pH, which is known to affect the intracellular pH, thereby modifying intracellular metabolism. Recently, Wilkins and Hall (1995) have studied the effect of extracellular and intracellular pH on matrix synthesis by isolated bovine chondrocytes. They have observed that matrix synthesis exhibited a bimodal relation with decreased pH. Slight reductions ($7.4 < pH < 7.1$) increased $^{35}SO_4$ and ^{3}H-proline incorporation by up to 50 % whereas profound acidification of the media ($pH < 7.1$) inhibited syntheses by up to 75 % of the control value. On the other hand, direct imposition of a sustained intracellular acidosis ($pH = 6.65$) using ammonium prepulse with amiloride inhibited matrix synthesis by only 20 %. These findings suggest that the modification of the matrix synthesis by extracellular pH, could not be entirely explained by changes of the intracellular pH. Moreover, chondrocytes possess a powerful regulating system of intracellular pH involving a Na^+-H^+ exchanger, a Na^+-dependent Cl^-HCO_3 transporter and H^+-ATPase pump (Dascalu *et al.*, 1993).

1.1.4.5. Culture medium composition

The culture medium must be adapted according the experimental requirement. Fetal calf serum has often been used in the past decade to optimize culture condition, but several problems with the use of serum should be noted: (1) outgrowth of cells from the periphery of tissue in organ cultures; (2) variability between the composition of different batches of serum (Morales *et al.*, 1991); (3) presence of an unknown amount of components; (4) elevated risks of interferences with the results of studies on the influence of various biologic factors on metabolic activities of cells. A example of interference was recently related to the effect of EGF on rat articular chondrocytes. EGF stimulated ^{3}H-thymidine incorporation and increased DNA content of cultures. The effect was strongest when serum concentration was low ($\leq$ 1 %) and was lost at high ($\geq$ 7.5 %) concentration (Ribault *et al.*, 1997). Moreover, it is well known that levels of synthesis and degradation in DMEM sup-

plemented with fetal calf serum are greatly elevated compared with *in vivo*. The differences between metabolism *in vivo* and *vitro* may be caused by differences between the synovial fluid and medium in which cells are batched. Therefore, Lee *et al.* (1997) cultured steer chondrocytes into agarose using a nutrient medium containing Dulbecco's Minimal Essential Medium supplemented with 20 % FCS and an increased amount of normal allogenic synovial fluid. Dilution of medium with synovial fluid induced an increase in proteoglycan amounts to levels of 80 % synovial fluid. On the other hand, tritiated thymidine uptake decreased with increasing concentrations of synovial fluid. These results suggest that culture in synovial fluid induces a metabolic state similar to that seen *in vivo*, with high levels of glycosaminoglycan synthesis and low levels of cell division.

Some substitutes of serum with a standardized composition are available commercially. The most currently used are ultroser G and ITS+ (Insulin, transferrin, selenium, BSA and linoleic acid). In order to avoid interferences, a completely defined serum-free culture media can be prepared in a composition adapted at the goals of the *in vitro* study. In a very interesting study, Verbruggen *et al.* (1995) demonstrated that ^{35}S-aggrecan synthesis by human chondrocytes cultured in agarose in serum-free DMEM was between 20 and 30 % of the value observed in DMEM supplemented with 10 % fetal calf serum. They determined the extent to which, IGF-1, IGF-2, TGF-β or insulin were able to restore aggrecan production in serum-free medium. The authors concluded that 100 ng/ml of insulin, IGF-1 or IGF-2 partly restored aggrecan synthesis to 39-53 % of the control level. No cumulative or synergic activities were observed when these factors were combined. Interestingly, 10 ng/ml of TGFß, in the presence of 100 ng/ml of insulin, stimulated aggrecan synthesis to more than 90 % of the reference level. Finally, human serum transferrin, alone or in combination with insulin, had no effects on aggrecan synthesis. When FCS was replaced by BSA, the proportion of aggrecan aggregates decreased dramatically. Supplementing the culture media with IGF, insulin or TGFß partly restored the ability of the cells to produce aggrecan-aggregates. On the other hand, IGF-1 and insulin have been shown to maintain homeostasis in organ cultures. After 40 days of culture, 10 to 20 ng/ml IGF-1 maintained proteoglycan synthesis at the same or higher levels than in a medium containing 20 % fetal calf serum. Catabolic rates were slower in IGF-1 medium than in medium with only 0.1 % albumin, but somewhat faster than for culture in medium with 20 % FCS. In long-term cultures, 20 ng/ml IGF-1 maintained a steady-state condition (Luyten *et al.*, 1988).

Lee *et al.* (1993) compared the effect of DMEM + 20 % FCS, DMEM and DMEM + 20 ng of IGF-1 on DNA synthesis in explant culture, monolayer culture or in suspension culture over agarose. When cultured over agarose in the presence of serum, there was a tendency for chondrocytes to clump together, forming large clusters. Cells cultured without serum or with IGF-1 remained rounded over agarose, clumping in small groups, but failed to form large aggregates. In monolayer, DNA synthesis was significantly greater in serum containing cultures than in cultures supplemented with IGF-1, which in turn, was significantly greater than in serum free cultures. In contrast, when chondrocytes were cultured in suspension

over agarose, no difference in DNA synthesis was observed between serum-free culture and culture supplemented with IGF-1. However, serum containing cultures incorporated significantly more radionucleotide (^{3}H-TdR) than in the other treatments.

Vitamin C is required for the activity of prolyl hydroxylases, RER enzymes that hydroxylate proline residues in the nascent procollagen chains and allow folding of the chains into a stable triple helix. Scorbutic chondrocytes synthesized underhydroxylated non-helical precursors of collagens that were secreted very slowly and accumulated in the RER. At a cute ascorbic acid treatment (50 μg/ml), type II and IX underwent hydroxylation and were secreted at normal high rates. On the other hand, proteoglycans synthesis and secretion were unchanged by acute acorbic acid treatment. These findings suggest that secretion of collagens is regulated independently of proteoglycan secretion and that RER must possess mechanisms able to discriminate between proteoglycans core protein and procollagens (Pacifici, 1990).

1.1.5 Concluding Remarks

Maintenance of chondrocytes in culture is challenging because chondrocyte phenotype and metabolism are strongly regulated by their close environment. Chondrocyte isolation from the extracellular matrix drastically modifies its extracellular environment and deeply alters its behavior. Chondrocytes are suddenly exposed to abnormal oxygen tension, ionic and osmotic environment and are released from cell-matrix interactions. Moreover, chondrocyte phenotype is not stable. When chondrocytes are seeded in a support plastic they lose their cartilage-specific phenotype and show a fibroblastic-like morphology. Monolayer culture does not allow a long-term study of chondrocyte metabolism but is particularly recommended to study cell dedifferenciation. Since the discovery that chondrocytes re-express the differentiated phenotype when they are transferred from monolayer to suspension, some tri-dimensional culture models have been developped. Chondrocytes were embedded in natural or synthetic matrix or maintained in suspension by constant agitation. In these culture conditions, chondrocytes conserve their phenotype for several weeks and are suitable to study matrix formation. Morever, cultures of chondrocytes in a tridimensional matrix allow tissue engineering for transplantion in cartilage defects. This culture method granted the study of matrix homeostasis, resorption and repair in environmental conditions close to that observed *in vivo*.

We can conclude that *in vitro* studies on cartilage explants or isolated chondrocytes provide important information on normal and pathological chondrocyte metabolic functions and may help to define the mode of action of regulatory factors and drugs. Nevertheless, some discrepancies in the conclusion can appear according the origin of the cartilage, the culture model and the experimental conditions used.

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ANNEXE 2

Anabolic Events in Osteoarthritis

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Homeostasis of normal cartilage in adults represents a delicate balance between the degradation and the synthesis of extra-cellular matrix components to maintain the functional integrity of the joint. Under the influence of mechanical stress or joint inflammation, this dynamic equilibrium is broken and catabolic events progressively become prevalent leading to cartilage destruction.

Animal models in the study of the development of osteoarthritis have identified two types of repair reaction that correspond to the depth of the injury. The reparative process of osteochondral defects involves the release of the issue of mesenchymal cells from subchondral bone. The extent of the repair is influenced by the width, direction and site of the injury. Better repair is observed when the injury is parallel to the direction of joint motion [1]. However, the histologic, biochemical and biomechanical characteristics of repair tissue are different from normal cartilage. The repair of chondral defects occur early following the induced cartilage lesion. This repair tissue is characterized by chondrocytes clustering (mitosis and/or migration), cartilage hypertrophy, an increase in glycosaminoglycan (GAG), fibronectin, and water cartilage content, and an enhancement of GAG and collagen (types II and III but not type I) synthesis by chondrocytes [2].

Unfortunately, the repair tissue ultimately fails and cartilage destruction ensues. Chondrocytes progressively decompensate, start secreting more proteolytic enzymes and catabolic cytokines and finally die probably by apoptosis (Fig. 1).

Questions remain concerning the repair process. It is unknown at what point the repair process ends and cartilage damage becomes irreversible. Many of the circumstances leading to aborted repair reaction remain unknown. Several factors could be involved including alteration in the macromolecule structure, chondrocyte phenotypic change, IL-1 overproduction, increase susceptibility of chondrocytes to cytokine-induced degradation, focal over-expression of IL-1 and tumor necrosis factor (TNF)α receptors, growth factor synthesis and bioactivity down-regulation, reduction of chondrocyte sensitivity to growth factors, chondrocytes apoptosis, etc. [3-6].

The cartilage repair reaction is regulated by soluble factors produced locally by chondrocytes and neighboring tissues, mainly synovial membrane and bone. The most important factors involved in the control of the repair reaction are transforming growth factor (TGF)-β, basic fibroblast derived growth (bFGF), insulin like growth factor (IGF)-1, interleukin (IL)-1 (α and β) and TNFα. Nevertheless, factors including platelet derived growth factor (PDGF), bone morphogenic proteins (BMPs) or cartilage-derived morphogenetic proteins (CDMPs) could also have an important regulatory function. TGF-β and IGF-1 play a significant role in promoting chondrocyte anabolism and inhibiting chondrocyte catabolism whereas IL-1β and TNFα inhibit cartilage matrix component synthesis and promote cartilage destruction by increasing metalloproteinase synthesis by chondrocytes. TGF-β and IGF-I may partially reverse IL-1β-mediated degradation of articular cartilage [7, 8].

The imbalance between anabolic growth factors and catabolic cytokine synthesis and/or bioactivity could be the key of cartilage repair failure. Several mechanisms can control growth factors and cytokine bioactivity during THO develop-

ment of osteoarthritis. Interactions with binding proteins, soluble receptors, matrix components, (e.g. decorin), or with other growth factors or cytokines may modulate their effects on chondrocyte metabolism.

On the basis of these experimental considerations, therapeutic intervention to promote the cartilage reparative process in osteoarthritis becomes more evident. Intraarticular injection of growth factors, interleukin-1 receptor antagonist (IL-1ra), docyxycline or nitric-oxyde (NO) synthase inhibitors has been tested successfully on experimental models of osteoarthritis (OA) [9, 10]. These substances can prevent the development of osteoarthritis or reduce the severity of the lesions. Gene therapy offers an elegant therapeutic alternative. Transfer of an appropriate gene(s) into chondrocytes or synoviocytes prior transplantation with a vehicle, may allow a high level of expression of growth factors *in vivo* for an extended period of time [11, 12].

However, prolonged high expression of growth factor transgene could lead to focal cartilage hyperplasia with loss of articular surface congruity, promoting osteophyte formation. Drugs which inhibit metalloproteinases activity/synthesis or IL-1 synthesis could also prolong the reparative response. Finally, if administrated during the early period of osteoarthritis, drugs stimulating cartilage matrix synthesis by chondrocytes could also be valuable tools to sustain repair reaction.

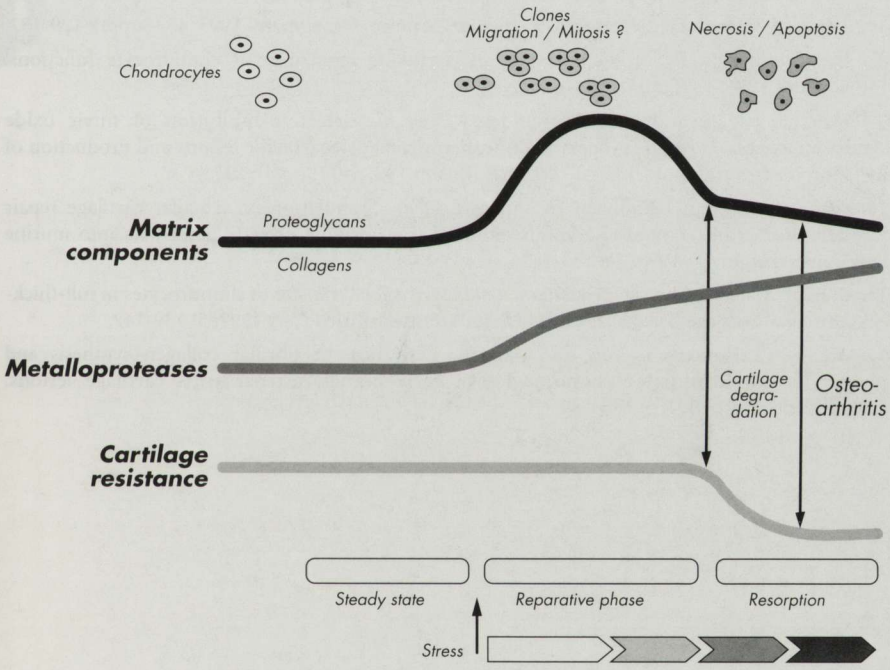


Figure 1: *The reparative attempt of chondral defect in which injury is strickly limited to cartilage.*

In conclusion, it is important to consider that the chondrocyte metabolic activity at the early stage of the osteoarthritis is different from that associated with the progression of the disease. Therefore, the possibility must be considered that therapeutic agents or growth factors, which are effective in the initial stage , may lose their effectiveness at the later stages of the disease and vice versa. This possibility must be taken in account in the design of experimental protocol studying the effect of potential disease modifying osteoarthritis drugs (DMOAD).

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ANNEXE 3

Effects of exogenous IL-ß, TNFα, IL-6, IL-8 and LIF on cytokine production by human articular chondrocytes

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Summary

Cytokines are potent regulators of the chondrocyte functions. Some of them are produced by chondrocytes and interact to regulate cartilage metabolism. In this study, we investigated the production of interleukin-l ß (IL-1ß), IL-6, IL-8 and leukemia inhibitory factor (LIF) by human chondrocytes and examined the modulation of their secretion by exogenous cytokines. Human articular chondrocytes were isolated from their extracellular matrix by a triple successive enzymatic digestion of the cartilage. Subsequently, chondrocytes were stimulated by increased amounts of human recombinant cytokines (IL-1ß, tumour necrosis factor α (TNFα), IL-8, LIF, IL-6). IL-1ß, IL-6, IL-8 and LIF were assayed into culture media and inside cell extracts by specific enzyme amplified sensitivity inimunoassays (EASIAs). Under these experimental conditions, we have identified various interactions between cytokines. IL-1ß and TNFα highly stimulated IL-6, LIF and IL-8 productions. IL-6 decreased IL-8 synthesis and increased LIF production. IL-8 slightly enhanced IL-6 production. Finally, LIF stimulated IL-1ß, IL-6 and IL-8 productions. Using neutralizing antibodies against IL-1, we demonstrated that the effects of LIF were secondary to the stimulation by LIF of IL-1ß production by the chondrocytes. In conclusion, chondrocytes secrete a variety of immunocompetent cytokines including IL-1ß, IL-6, IL-8 and LIF that can interact to regulate chondrocytes metabolism. These results also define new biological activities of LIF and IL-6, and raise questions concerning their role in the pathogenesis of joint diseases.

Key words: Chondrocytes, Arthritis, Leukemia inhibitory factor, Cytokines.

Introduction

In View of their presence in synovial fluid, various cytokines such as interleukin (IL-1ß), tumour necrosis factor (TNFα), IL-6, IL-8 and leukemia inhibitory factor (LIF) [1, 2, 3] are likely to be involved in the pathogenesis of destructive joint disorders. Cytokines are produced transiently in response to an activating signal and all cells, including chondrocytes, can produce some cytokines. These cytokines produced by chondrocytes may exert their effects on neighbouring cells or tissues, as a paracrine manner or on producing cells themselves, as an autocrine manner. TNFα and IL-1ß are identified as potent activators of chondrocytes playing a pivotal role in the etiology of cartilage degeneration. In response to these cytokines, chondrocytes start secreting neutral metalloproteinases [4, 5] and active oxygen species [6, 7] which are efficient factors in cartilage matrix destruction. Moreover, these cytokines inhibit proteoglycan [8] and collagen syntheses [9], markedly reducing cartilage repair capacities. Both cytokines may also stimulate bone resorption [10] and induce a transient inflammation and leukocyte infiltration of the synovial membrane [11]. Currently, it is suggested that TNF and IL-1 do not act in isolation and that their effects are often the result of an interaction of various cytokines including IL-6, IL-8 and LIF [12].

IL-6 is a potent B-cell growth factor and differentiation factor [13]. Therefore, through augmentation of rheumatoid factor and other autoantibodies in the synovium, it could indirectly exacerbate inflammation. Moreover, it also induces acute phase proteins synthesis such as C-reactive protein (CRP) and serum amyloid A (SAA) [14]. The effects of IL-6 on chondrocytes are still unclear. No effect [15], slight enhancement (16) as well as depression [17] of proteoglyean synthesis has been reported. Production of tissue inhibitor of metalloproteinases (TIMP) [18] and transforming growth factor (TGFß) [19] is stimulated by IL-6. In this role, IL-6 could be a moderator of the cartilage resorption processes. Because chondrocytes have been shown to proliferate in response to IL-6 [20], IL-6 could also be involved in cartilage early repair reaction occurring in osteoarthritis.

IL-8 is one of the most important chemotactic factors for neutrophils and T lymphocytes [21]. IL-8 also induces neutrophil degranulation, and thus may participate in cartilage degradation [22]. No direct effects of IL-8 on chondrocytes metabolism have been described.

Currently, the role played by LIF in the pathogenesis of joint diseases is not well understood. LIF was initially described on the basis of its ability to induce differentiation of murine myeloid leukemic cells [23]. It inhibits differentiation of pluripotent embryonic stem cells [24], induces the synthesis of hepatic acute phase proteins [25], promotes neuronal differentiation [26], inhibits lipoprotein lipase [27], increases neuronal substance P expression [28] and regulates bone metabolism [29, 30]. The role played by LIF in the homeostasis of cartilage remains unclear.

It is now recognized that cytokines exhibit both pleiotropy and redundancy and that differing cytokines exhibit both synergically and antagonistically. Some recent papers have reported that IL-1ß stimulates IL-6, IL-8, and LIF productions [31, 32].

Whether these cytokines are capable of feed back control of cytokines released remains largely uninvesti-gated. Therefore, we decided to systematically research interactions existing between those cytokines produced by chondrocytes themselves.

Materials and methods

Short-term suspension culture of human chondrocytes

Chondrocytes were cultured for a short period in order to maintain their phenotype. In this method, we used cartilage obtained from the knee joints of normal young adults shortly after death. Avoiding the calcified layer, cartilage slices were excised from the superficial and medium layers of cartilage, cut into small fragments and submitted to enzymatic digestion. Cartilage fragments were sequentially treated by hyaluronidase, pronase and collagenase. Cartilage pieces were incubated with hyaluronidase dissolved in Dulbecco's Modified Eagle's Medium (DMEM, ICN Biomedical) (0.5 mg/ml-3 g cartilage per 10 ml enzyme solution) for 30 min at 37 °C and constantly agitated (200 rpm). Cartilage fragments were then put into pronase solution (1mg/mI in DMEM-3g per 10ml enzymatic solution) and incubated 1 h at 37 °C and subsequently re-incubated, under constant agitation (200 rpm), with collagenase (1 mg/ml-3 g per 10 ml enzyme solution) dissolved in DMEM containing 1% Ultroser G (Gibco, Gent, Belgium) for 20 h at 37 °C. Ultroser G is a serum substitute which replaces fetal calf serum in *in vitro* culture. The biological activity of the reconstituted solution is fivefold that of fetal calf serum. Cells were then filtered through 25 µm nylon mesh, washed three times, counted (ranged among 1-1.5 10^6 cells/ml) and seeded in 10 ml polypropylene Falcon flasks. Chondrocytes were cultured in DMEM containing 1% Ultroser G. Cells were maintained in suspension by constant agitation on a giratory shaker (100 rpm) in an atmosphere of 95% air 5 % CO_2. Cells and supernatant were separated by centrifugation (1000 rpm, 5 min). The cell viability, estimated by trypan blue incorporation, was 95 %. Culture media were directly assayed for cytokines. Cell pellets were washed twice with HBSS (Hank's Buffer Saline Solution, Gibco, Merelbeeke, Belgium) and then homogenized in PBS pH 7.5 by ultrasonic dissociation (10 s pulses, power 50 W/cm^2) at 4 °C.

Treatments

Human recombinant IL-1ß was purchased from Boehringer (Boehringer Mannheim, Mannheim, Germany) and used at concentrations ranging from 50 to 2500 units per ml (specific activity about 5 x 10^7 U/mg) corresponding respectively to 0.58 x 10^{-10}-2.9 x 10^{-9} M. According to the manufacturer specifications, IL-1ß preparation was free of endotoxin.

Human recombinant TNFα was purchased from Boehringer (Boehringer Mannheim, Mannheim, Germany) and tested at concentrations from 2-100 ng/ml corresponding to 1.17 x 10^{-10} -5.9 x 10^{-9} M. The specific activity was superior at 1 x 10^8. One unit was defined as the amount of TNFα that was required to mediate

half-maximal cytoxicity with WEHI 164 cells in the presence of actinomycin. Endotoxin, evaluated by Limulus Amebocyte Lysate test (LAL-test) was < 10 EU/ml.

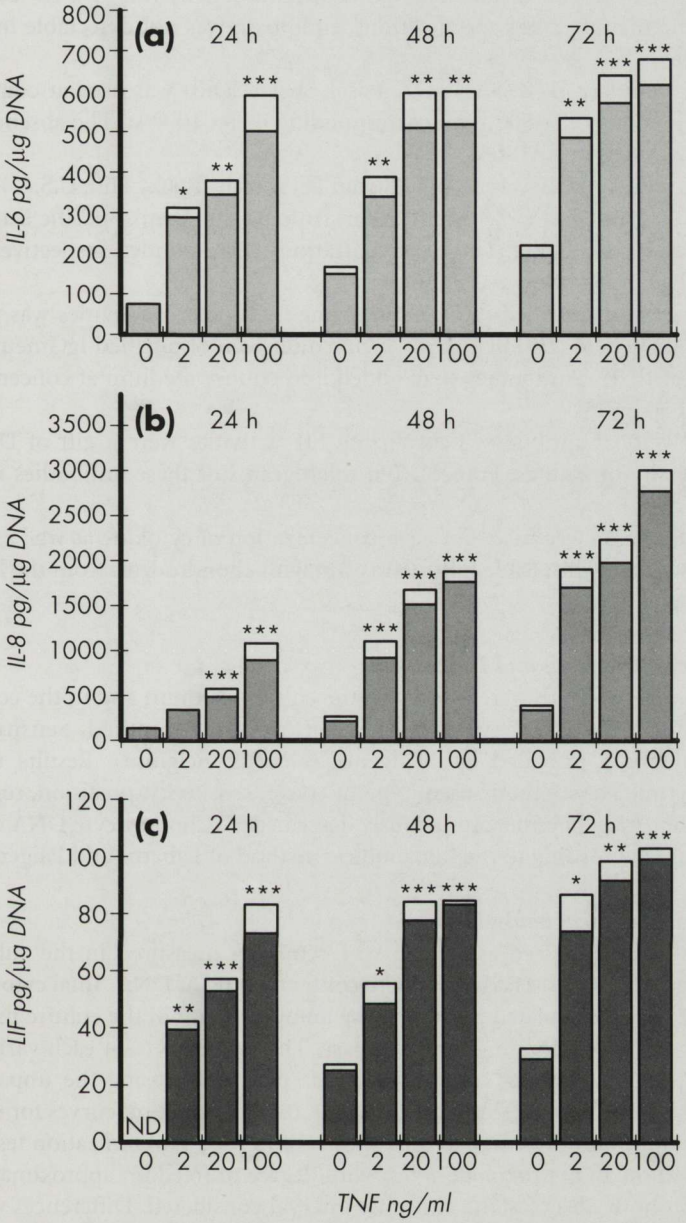


Fig. 1: *Effect of human recombinant tumour necrosis factor-α (TNFα) on IL-6 (a), IL-8 (b) and LIF (c) amounts produced and released into culture medium by human chondrocytes. Cytokines levels were measured after 24, 48 and 72 h of treatment with increased amounts of TNFα. The data are expressed as amounts of cytokines found into culture per µg of DNA and presented as the mean and standard deviation (S.D.) of triplicate cultures. Statistical significance: *P < 0.05; **P < 0.01; ***P < 0.001.*

Human recombinant IL-6 was kindly provided by SANDOZ (Basel, Switzerland) and used at concentrations of 100, 1000 and 10 000 U/mI which corresponded, respectively, to 2, 20 and 200 ng/ml. Specific activity was 52 x 10^6 U/mg. According to the manufacturer specifications, endotoxin was not detectable in the IL-6 preparation.

Human recombinant IL-8 (SANDOZ, Basel, Switzerland) was used at concentrations ranged among 0.8 - 80 ng/ml corresponding to 1 x 10^{-10} M. The absence of endotoxin was verified by LAL-test.

Human recombinant LIF was obtained from PeProtech (Rocky Hill, U.S.A.) and added to the culture medium at concentrations from 8 - 800 U/ml. Specific activity corresponded at 4 x 10^7 U/mg. These concentrations corresponded respectively at 10^{-11} - 10^{-9} M. The preparation was endotoxin free.

Rabbit antisecrum (purified IgGs) neutralizing IL-1ß and α activities was purchased from Calbiochem (La Jolia, U.S.A.). One milligram of purified IgG neutralized 10 000 U of IL-1ß. Antibodies were added into culture medium at concentrations of 10 and 50 µg/ml.

Purified polyclonal antibodies neutralizing LIF activities were a gift of Dr A. Godard (University of Nantes, France). Ten micrograms of these antibodies neutralized 2.5 ng of LIF.

Three culture flasks were used for each concentration of cytokine as well as for the corresponding controls. Each culture was run with chondrocytes from the knee joint of a single patient.

Immunoassays for cytokines and DNA assay

IL-1ß, IL-6, IL-8 and LIF were assayed in the culture medium and in the corresponding cellular phase by specific EASIA (Enzyme Amplified Sensitivity Immunoassay) kindly provided by Medgenix (Fleurus, Belgium). Results were expressed in pg/ml. The methods used, type of tracer, sensitivity, precision, reproducibility and accuracy have been previously described [33]. Chondrocyte DNA content was measured according to the fluorimetric method of Labarca and Paigen [34].

Calculation and statistical analysis

The results were expressed as amounts of cytokines measured in the culture medium per µg of DNA and as cellular phase content per µg of DNA. Total cytokine productions were also calculated by adding the amount found in the culture medium and within the corresponding cellular phase. The mean ± S.D. of each variable was calculated. Comparison of mean values was performed using the unpaired Student's t-test, with a limit of signification at $P < 0.05$. Production curves for each experimental condition were compared by means of Zerbe's randomization test [35]. This test is based on an approximate *F* test with degree of freedom approximation that depends on both observations and time interval considered. Differences were considered statistically significant when $P < 0.05$.

Results

Effect of TNFα on cytokine production

Cytokines released into culture medium

IL-6, IL-8 and LIF amounts released in the culture medium by unstimulated chondrocytes increased progressively as a function of the culture period. Nevertheless, analysis of the time courses suggested that a high ratio of the IL-6 and IL-8 production occurred in first 24h of the culture. When chondrocytes were cultured in the presence of TNFα, regardless of the TNFα dose (2, 20 and 100 ng/ml) and culture period (24, 48 or 72 h) [Fig. 1(a), (b), (c)] cytokine amounts measured in culture media were significantly higher than control values ($0.05 < P < 0.001$). This stimulating effect of TNFα was dose-dependent for IL-8 production ($0.95 < P < 0.98$; $P < 0.01$) whichever the culture period considered (Fig. 1b). Concerning IL-6 production, the stimulation was only dose-dependent after 24 and 48 h of culture ($0.65 < r < 0.84$; $0.05 < P < 0.001$). As shown by Fig. 1(c), unstimulated chondrocytes did not produce detectable amounts of LIF during the first 24 h of culture. Nevertheless, LIF production was significantly increased by TNFα.

Table I: IL-6 and IL-8 levels measured inside chondrocytes after 24, 48 and 72 h of culture with or without increased amounts of TNFα.

Culture duration and parameters study	Control	TNFα 2 ng/ml	TNFα 20 ng/ml	TNFα 100 ng/ml
IL-6 pg/μg DNA				
24 h	12.1 ± 0.85	12.6 ± 0.85	16.4 ± 2.64	21.3 ± 3.05*
48 h	4.35 ± 0.57	7.91 ± 1.12*	11.4 ± 1.92*	10.2 ± 1.2*
72 h	3.03 ± 0.27	4.91 ± 0.66*	5.63 ± 0.53*	5.81 ± 0.3
IL-8 pg/μg DNA				
24 h	17.9 ± 3.9	42 ± 3.5**	65 ± 11**	114 ± 14**
48 h	15.6 ± 2.0	39 ± 5.0**	62.5 ± 5.8***	110 ± 7.6**
72 h	14 ± 1.0	35 ± 1.4***	60 ± 8.6**	76 ± 10**

Values are the means of three separate determinations and are expressed as amounts of cytokines present inside cells per μg of DNA. *Statistically significant difference compared with the controls *P < 0.05; **P < 0.01; ***P < 0.001.

Cytokines content inside cell pellets

Table I shows amounts of cytokine measured in the cellular phase as a function of the culture time. It appears that cytokine levels present in the cellular phase reached a maximum after 24h and then decreased as a function of culture period. We observed also that cytokine levels in the cellular phase were lower than amounts found in the culture medium. TNFα significantly increased the level of IL-8 content in the cellular phase. Responses were dose-dependent at each culture time studied ($0.95 < r < 0.98$; $0.01 < P < 0.001$). IL-6 content was also enhanced by TNFα ($0.01 < P < 0.05$). In our culture and assay conditions, LIF was not detectable in the cellular phase.

Effect of IL-1ß on cytokine production

Fig. 2 shows the total IL-6, IL-8 and LIF production (sum of cytokine amounts released in the culture medium and within the corresponding cellular phase) after 72 h of culture with or without IL-1ß. Total cytokine productions were nearly equal

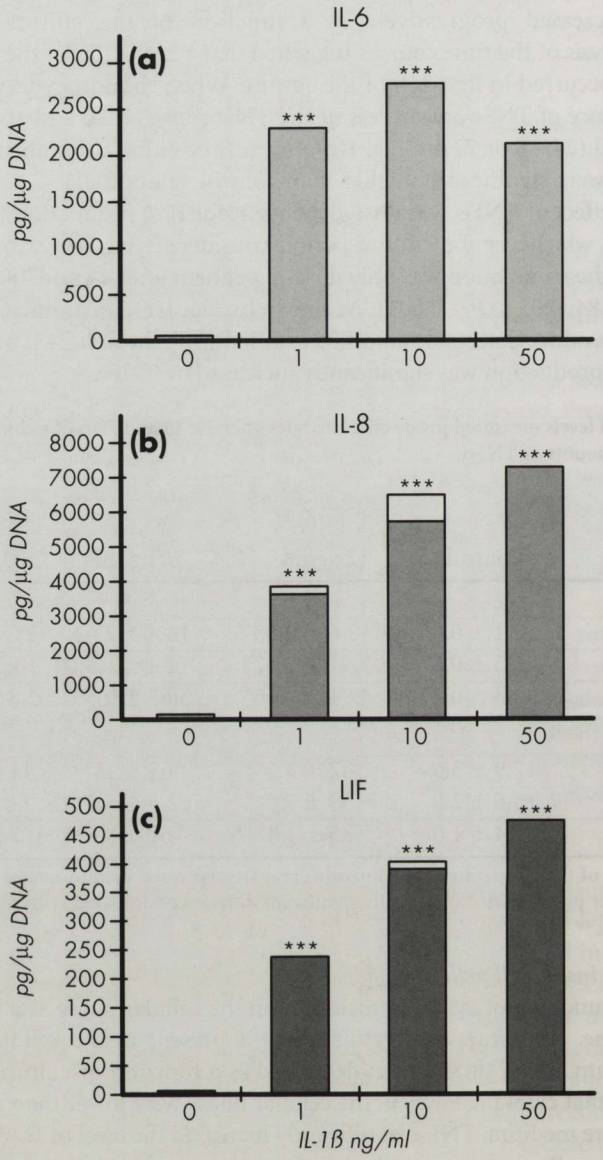


Fig. 2: *Total amounts (sum of the amount found within culture medium and inside cellular phase) of (a) IL-6, (b) IL-8 and (c) LIF produced by human chondrocytes cultured for 72 h in the absence or presence of increased doses of IL-1ß. Results are expressed as mean values of three cultures from the same donor. Responses are significant* $***P < 0.001$.

to the cytokine amounts released into culture medium after 72 h. Less than 5 % of the total cytokine production was present in the cellular phase at day 3. IL-1p induced a highly significant increase of the total IL-6, IL-8 and LIF production ($P < 0.001$; Fig. 2). Responses were dose-dependent for IL-8 ($r = 0.90$) and LIF ($r = 0.71$) productions.

Effect of IL-6 on cytokine production

During the first 48 h of incubation, IL-6 did not significantly affect the total IL-8 production by human chondrocytes. When culture time was prolonged until 72 h, IL-6 decreased IL-8 production whatever the concentration studied (2, 20 or 200 ng/ml; $0.001 < P < 0.01$; Fig. 3). IL-6 also enhanced LIF amounts secreted and released into culture medium by chondrocytes (Fig. 4). After 72 h of culture, the effect was significant at each concentration of IL-6 tested ($0.01 < P < 0.05$).

Table II: IL-6 amounts found in the cultured medium and inside chondrocytes cultures in the absence or in the presence of increase concentrations of IL-8

Culture duration	Control	IL-8 2 ng/ml	IL-8 20 ng/ml	IL-8 100 ng/ml
In culture medium				
24 h	18.2 ± 1.15	14.2 ± 3.2	15.6 ± 5.6	18.6 ± 1.15
48 h	29 ± 2.4	29 ± 1.8	31.6 ± 6	37 ± 1*
72 h	35.3 ± 2.5	33.3 ± 4.16	45 ± 4.2	53.3 ± 7.5*
Inside cell extracts				
24 h	5.1 ± 1	4.9 ± 0.1	6.2 ± 0.9	6.2 ± 0.4
48 h	3.6 ± 0.2	4.4 ± 0.4	4.3 ± 0.7	5.4 ± 0.9
72 h	1.03 ± 0.03	0.99 ± 0.004	2 ± 0.5	1.4 ± 0.19

Data are the mean ± S.D. and are expressed in pg per μg of DNA. N= 3 in each group. *Significantly different from the control with a $P < 0.05$.

Effect of IL-8 on cytokine production

At the concentration of 80 ng/ml, IL-8 slightly increased the amount of IL-6 released in the culture medium ($P < 0.05$; Table II). The effect was present after 48 and 72 h of culture. IL-8 had no significant effect on IL-6 content in the cellular phase. However, IL-8 had no effect on LIF production either in the cellular phase or in the culture medium (data not shown).

Effect of LIF on cytokine production

The time course analysis of IL-6 production shows that the IL-6 released in the culture medium continuously increased as a function of the culture period with or without the addition of LIF (Fig. 5). However, the curves obtained for the highest concentrations of LIF (2 and 20 ng/ml) were significantly higher than the control

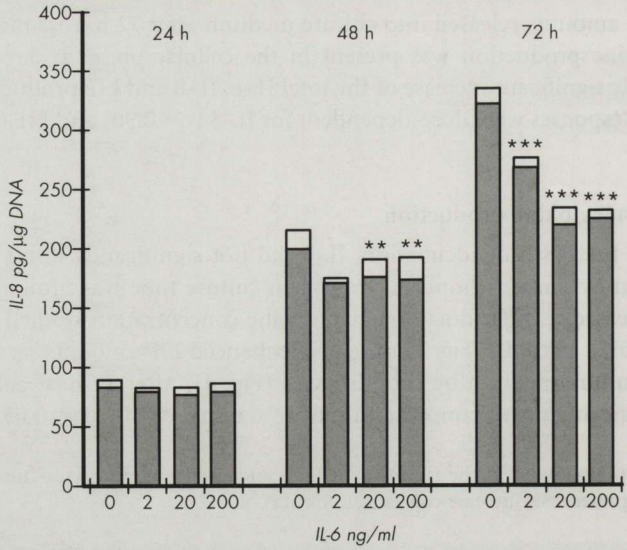


Fig. 3: *Human chondrocytes were incubated for the time specified in the absence or presence of varying concentrations of human recombinant interleukin(IL)-6. At the end of the period, supernatant and cells were separated by centrifugation and were assayed for interleukin (IL)-8 by a specific EASIA. The values correspond to the total amount of IL-8 (sum of the amount found inside cellular phase and within culture medium) produced by chondrocytes. After 72 h of incubation, IL-6 significantly decreased IL-8 production as compared with the control values. Responses are significant with a* ***$P < 0.001$.

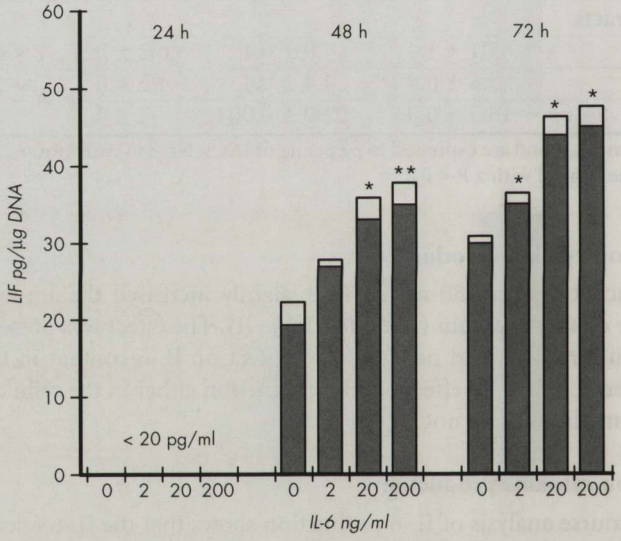


Fig. 4: *Leukemia inhibitory factor (LIF) amount released within culture medium as a function of culture duration. Interleukin-6 (IL-6) added into culture medium at concentrations ranging from 2-200 ng/ml increased LIF amount produced by human chondrocytes. Significant effects were observed after 48 h of culture at the concentrations of 20 and 200 ng/ml and for each concentration used after 72 h of culture. Statistical significance:* *$P < 0.05$; **$P < 0.01$.

curve showing a stimulation. No effect was observed at the concentration of 0.2 ng/ml. The amount of IL-6 found in cellular phase rapidly increased during the first 24 h of culture and progressively decreased as a function of culture duration. LIF increased significantly the IL-6 content at the doses of 2 and 20 ng/ml (Fig. 5).

LIF also stimulated total IL-8 production (Fig. 6). In the presence of 20 ng/ml. of LIF, stimulation was already significant after 12 h. In our culture and assay conditions, unstimulated chondrocytes did not spontaneously produce IL-1ß. Interestingly, the production of IL-1ß rapidly increased with the addition of LIF. These results suggested that LIF effects on IL-6 and IL-8 productions could be secondarily mediated by IL-1ß. To verify this hypothesis, we simultaneously cultured chondrocytes with LIF and antibodies neutralizing IL-1α and ß. Surprisingly, 5 and 10 µg of neutralizing antibodies added into culture medium inhibited the spontaneous production of IL-6. Neutralizing IL-1α and ß activities antibodies, totally inhibited the stimulating effect of LIF on IL-6 production (Fig. 7). Similar results were obtained on IL-8 production (data not shown).

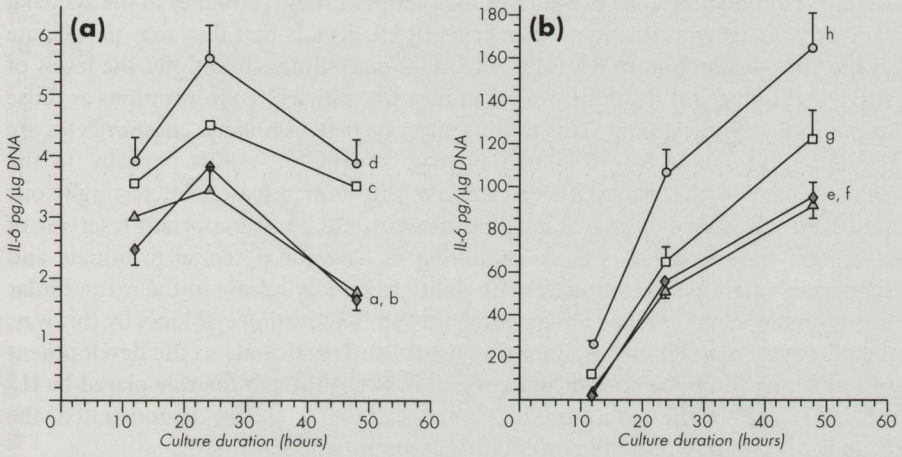


Fig. 5: *Time course of interleukin (IL)-6 production by human chondrocytes. Primary chondrocytes were cultured in the absence (-◆-) or presence of 0.2 nglml (-△-), 2 ng/ml (-□-) or 20 nglml (-○-) of LIF. (a) IL-6 present inside cellular phase. (b) IL-6 released into culture medium. Results are the mean values of triplicate of primary chondrocytes culture in a representative experiment and error bars represent* S.D. *Differences between curves are tested by Zerbe test. a,b ≠ c: $P < 0.025$; a,b ≠d: $P < 0.01$; c ≠ d: $P < 0.05$; e, f ≠ g; $P < 0.05$); e,f ≠ h: $P < 0.01$; g ≠ h: $P < 0.01$.*

Effect of polyclonal antibodies (PAB LIF) neutralizing LIF activity on cytokine production

When human articular chondrocytes were cultured for 72 h in the presence of antibodies neutralizing LIF activity, IL-6 production was significantly decreased (Table III). On the other hand, IL-8 produced by chondrocytes was slightly decreased as compared with the controls. In the presence of IL-1ß (17 ng/ml), the cytokines production was markedly enhanced ($0.01 < P < 0.001$). When IL-1ß and

LIF Pab were simultaneously added in the culture medium, the stimulating effect of IL-1ß was not significantly modified. These data suggest that LIF had no effect upon the IL-1ß activity.

Discussion

The data presented in this paper show that freshly isolated human chondrocytes can spontaneously secrete IL-6, IL-8 and LIF. It is now accepted that these cytokines mediate a variety of activities on immunocompetent cells and connective tissues. Nevertheless, regulator effects of these cytokines on the other cytokine production are still obscure and not well documented. In the present work, we have identified some regulator pathways existing between IL-1ß, IL-6, IL-8 and LIF. These regulator pathways may play an important part in the maintenance of the dynamic equilibrium between synthesis and degradation in cartilage as well as in the pathogenesis of joint disease.

We have demonstrated that IL-1ß and TNFα are potent stimulators of IL-6, IL-8 and LIF production. The presence of high levels of these cytokines in the synovial fluid of patients with rheumatoid arthritis (RA) suggests that they may participate in the joint destruction in RA (1, 2, 3). Under our culture conditions, the levels of IL-1ß that induced IL-6 and IL-8 were in the same range of concentrations as those found in RA synovial fluid. This finding suggests that stimulated chondrocytes are an important source of cytokines that may contribute, at least partially, to the enhancement of the cytokine levels observed in joint diseases. Interestingly, our results also provide evidence that chondrocytes could be an important reservoir of IL-8. This finding suggests those chondrocytes have the potential to initiate and propagate inflammation through their ability to rapidly release in the extracellular environment a high level of the potent neutrophils activating cytokine. By this way, chondrocytes may act on neighbouring tissues and participate in the development of synoviotis and bone remodelling present in RA. Although the role played by IL-1ß and TNFα in the pathogenesis of joint diseases is clearly demonstrated, the actions of IL-6, IL-8 and LIF require further examination.

Table III: Quantitation of cytokines (IL-6, IL-8, LIF) present in free cell supernatants after 48 h of culture with culture medium, purified antibodies neutralizing LIF activities (Pab LIF), IL-1ß only or simultaneously with Pab LIF and IL-1ß

Parameters studied	Control	Pab LIF 50 µg/ml	IL-Iß 17 ng/ml	IL-1ß 17ng/ml Pab LIF 50 µg/ml
IL-6 pg/µg DNA	509±47a	234 ± 5b*	7774 ± 1499c***	8138± 272c***
IL-8 pg/µg DNA	2909±113a	2748 ± 137a	11093 ± 129b**	13881±1100c***
LIF pg/µg DNA	890 ± 17.6a	Not detectable	2987 ± 234b**	Not detectable

Letters indicate differences between treated and untreated groups. *P < 0.05; **P < 0.01; ***P < 0.001.

Because equally high amounts were found in the supernatant of chondrocytes culture stimulated with IL-1ß, we tested human recombinant IL-6 at concentrations ranging from 2-200 ng/ml. In this concentration range, IL-6 induced a depression of IL-8 production and a stimulation of LIF synthesis. In light of the biological functions of IL-8, the inhibition of this cytokine suggests a negative feedback regulation to limit inflammatory reaction and joint invasion by neutrophils and T lymphocytes. Indeed, it is now well established that IL-8 is one of the most potent chemoattractant factors for these cells [21]. This cytokine also induces neutrophil degranulation and the release of lysosomial enzymes and active oxygen species in the joint cavity [22]. In this way IL-8 may contribute to cartilage degradation. This inhibitory effect of IL-6 on IL-8 production can be included in the series of protective actions recently described for IL-6. In fact, IL-6 stimulates TIMP production by chondrocyte without affecting collagenase activity [36]. Moreover, IL-6 increases TGFß [19] synthesis and stimulates chondrocytes proliferation [20] but does not modify prostaglandin E_2 (PGE_2), collagen or proteoglycan syntheses [15, 18]. On the other hand, together with the secretion of IL-6, chondrocytes may contribute to the development of autoimmunity directed toward cartilage antigens such as type II collagen. In fact, IL-6 is required for T-cell activation, B-cell maturation and antibody production, including the rheumatoid factor [13, 37]. IL-6 may also contribute to the development of the inflammatory reaction accompanying RA. IL-6 induces acute phase production by hepatocytes [25]. Nevertheless, a recent *in vivo*, study failed to demonstrate the efficacy of anti-IL-6 treatment in experimental arthritis [38].

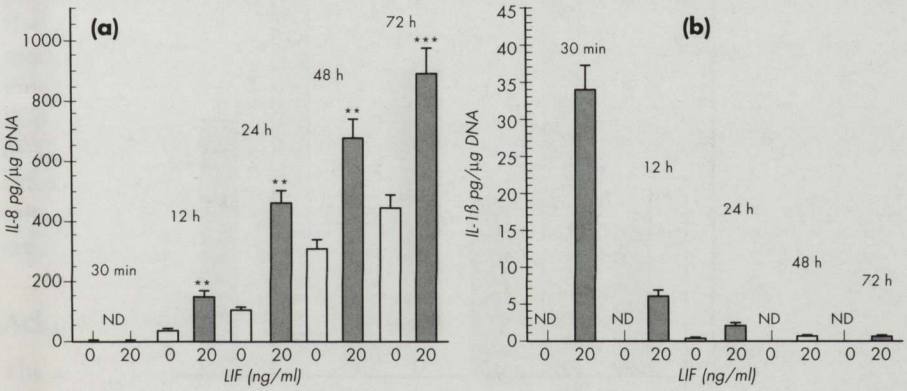


Fig. 6: *Effect of leukemia inhibitory factor (LIF) on (a) interleukin (IL-8) and (b) IL-1ß released into culture by human chondrocytes. Cytokine levels were assayed by enzyme amplified sensitivity immunoassays at each time point. Values are the mean and* S.D. *(N=3). Statistical analysis is performed with unpaired Student's t-test for assessing significant differences between controls and treated groups:* $^{**}P < 0.01$; $^{***}P < 0.001$.

In the present study, we have also demonstrated that chondrocytes produced LIF. This production was highly increased by pro-inflammatory cytokines such as IL-1ß, TNFα and IL-6. These results are in agreement with previous studies that showed that LIF production was induced not only by pro-inflarmnatory cytokines but also by growth factors such as TGFß, platelet-derived growth factor (PDGF),

Table IV: The negative and positive effects of the different cytokines on each other

	Cytokines assayed			
Cytokines added	**IL-6**	**IL-8**	**LIF**	**IL-1**
IL-1ß	+	+	+	ND
TNFα	+	+	+	ND
IL-6	ND	-	+	ND
IL-8	+	ND	0	ND
LIF	+	+	ND	+

+ = stimulation; inhibition; 0 = no effect; ND = Not determined.

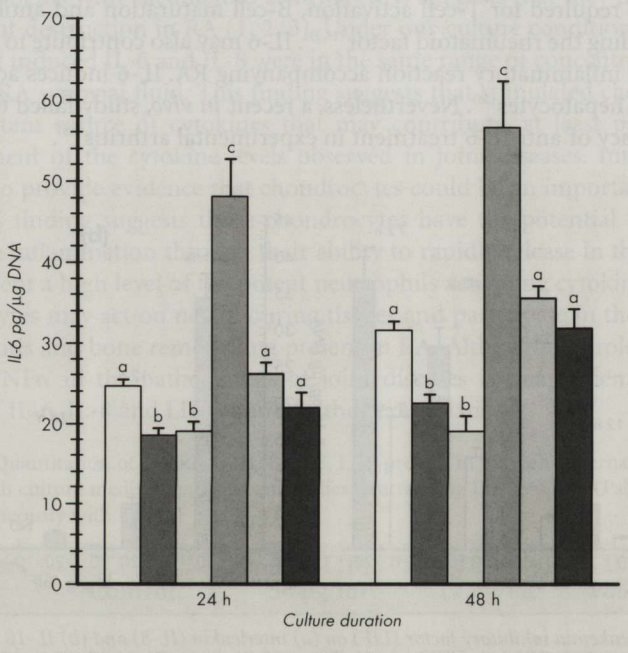


Fig. 7: *Leukemia inhibitory factor (LIF) induction of interleukin (IL)-6 is mediated by IL-1. During 48 h, primary chondrocytes cultures were simultaneously incubated with LIF (20 ng/ml) and specific antibodies neutralizing IL-1α and ß activities (Pab IL-1), or with LIF (20 ng/ml) or specific neutralizing antibodies only. IL-6 was directly assayed in the culture medium by specific enzyme amplified sensitivity immunoassay. Results are expressed as mean values from one representative experiment performed in triplicate. Statistical significance: a ≠ b ≠ c P < 0.05.* □ *= controls;* ■ *= Pab 5 µg/ml;* □ *= Pab 10 µg/ml;* ■ *= LIF 20 ng/ml;* □ *= LIF 20 ng/ml + Pab 5 µg/ml;* ■ *= LIF 20 ng/mI + Pab 10 µg/ml.*

insulin-like growth factor (IGF) and basic fibroblast growth factor (bFGF)[39]. The role played by LIF in the development of articular diseases is still obscure. We have previously described that LIF decreased proteoglycans production[40]. Together with the observations of Lotz *et al.*[39] that LIF can induce expression of collagenase and stromelysin by human articular chondrocytes without affecting TIMP production, LIF appears to be a potent mediator of cartilage resorption[32]. Furthermore, we have shown that LIF stimulated production of pro-inflammatory cytokines including IL-6, IL-8 and IL-1ß. These observations support the notion that LIF may be directly or indirectly involved in the development of cartilage destruction and the process of joint inflammatory events. The finding that LIF induced IL-1ß production by chondrocytes suggested that the LIF effect could be mediated indirectly by IL-1. To verify this hypothesis, chondrocytes were cultured in the presence of antibodies neutralizing IL-1α and ß activities. We observed that LIF-induced IL-6 and IL-8 productions are totally inhibited by antibodies neutralizing IL-1α and IL-1ß activities. This finding provides evidence that LIF effects are secondary at the release of IL-1. This hypothesis is supported by the observation that LIF induced a rapid secretion of IL-1ß which occurred within 30 min, before the induction of IL-8 and IL-6. Interestingly, the addition of antibodies neutralizing LIF activity in the culture medium decreased significantly the spontaneous IL-6 production but did not modify the effect of IL-1ß on this parameter. These results suggest that LIF and IL-1ß are not synergic.

In summary (Table IV), IL-1ß and TNFα are potent inducers of the production of pro-inflammatory cytokines. These latter cytokines may interact to regulate their productions by chondrocytes. We have clearly identified the following interactions. IL-6 decreases IL-8 production and slightly increases LIF synthesis. IL-8 have a moderate stimulatory effect on IL-6 production but does not affect LIF production. Finally, LIF is a potent stimulant to induce cytokines production, such as IL-1ß, IL-6 and IL-8. Nevertheless, these effects are likely to be mediated by, and secondary to, IL-1 secretion. In conclusion, our study suggests that through the production of cytokines, chondrocytes may act on neighbouring connective tissue metabolism and initiate and modulate the inflammation and immune responses. These productions are regulated by a complex interactive network of mediators.

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ANNEXE 4

Modulation of human chondrocyte metabolism by recombinant human interferon

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Abstract

Objectives: Interferon gamma (IFNγ) is found to be elevated in the synovial fluid of patients with rheumatoid arthritis and osteoarthritis suggesting its implication in joint disease pathogenesis. In this study, we investigated the effects of IFNγ on the production of cytokines (IL-6, IL-8, IL-10), prostaglandin E_2 (PGE_2), proteoglycans (PG), nitric oxide (NO), interleukin-1 receptor antagonist (IL-1ra) and stromelysin by non-stimulated and IL-1β-treated human chondrocytes. The role played by NO in the responses of chondrocytes to IFNγ was also examined by incubation of chondrocytes with N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NO synthase.

Methods: Enzymatically isolated human chondrocytes were cultured for 48h in the absence or presence of IL-1β, IFNγ or N^G-monomethyl-L-arginine (L-NMMA) added solely or in combination. The productions of IL-6, IL-8, IL-10, IL-1ra and stromelysin were measured by enzyme amplified sensitivity immunoassays (EASIA). PG and PGE_2 were quantified by specific radioimmunoassays (RIA). Nitrite concentrations in the culture supernatants were determined by a spectrophotometric method based upon the Griess reaction.

Results: As expected, IL-1β highly stimulated NO, IL-6, IL-8, IL-10, IL-1ra, PGE_2 and stromelysin synthesis but dramatically decreased PG production. NO, IL-6, IL-1ra and PGE_2 production by non stimulated chondrocytes was dose-dependently increased by IFNγ while PG production was inhibited. In the absence of IL-1β, IL-10 was undetectable in the culture supernatants. At the doses of 10 and 100 U/ml, IFNγ markedly inhibited the constitutive and IL-1β-stimulated IL-8, IL-10 and stromelysin productions. Interestingly, IFNγ synergized with IL-1β to increase NO, IL-6, IL-1ra and to depress PG production. As previously reported, the inhibition of NO synthesis by the competitive inhibitor L-NMMA led to enhancement of IL-6, IL-8 and PGE_2 production by IL-1β treated chondrocytes but did not significantly modify IL-10, PG and MMP-3 productions. Inhibition of NO synthase significantly inhibited the stimulating effect of IFNγ on IL-6 and IL-1ra but did not affect the inhibitory effect of IFNγ on IL-8, PG or stromelysin production.

Conclusions: These findings suggest that IFNγ and IL-1 synergistically stimulate the production of IL-6, IL-1ra, NO and PGE_2 and inhibit PG synthesis. By contrast, IL-1β and IFNγ have opposite effects on IL-8, IL-10 and stromelysin productions. These effects are not reversed by L-NMMA, suggesting that NO is not the principal mediator involved in responses of chondrocytes to IFNγ. ©2000 Osteoarthritis Research Society International

Key Words: Interferon, Cytokines, Chondrocytes, Osteoarthritis.

Introduction

The role of interferon gamma (IFNγ) in the pathogenesis of inflammatory joint diseases and the accompanying destruction of cartilage and other joint tissues remains poorly defined. IFNγ was found to be elevated in synovial fluid and sera of patients with rheumatoid arthritis and osteoarthritis (OA), suggesting a direct implication of this cytokine in joint disease pathophysiology[1,2]. Moreover, the presence of activated T-cells and TH1 cytokine transcripts in the synovial membrane of patients with osteoarthritis (OA) suggests that T-cells and thus, IFNγ, contribute to chronic inflammation in a large proportion of these patients[3].

At this time, divergent *in vitro* and *in vivo* results have been reported in the literature. When administered by systemic injection in a polyarthritis model induced by injection of peptidoglycan-polysaccharide (PG-APS), IFNγ suppressed the severity of acute and chronic erosive polyarthritis[4,5]. This effect was associa-ted with a marked reduction in the number of neutrophils and monocytes infiltra-ting the tissue, and correlated with a decrease in C5a receptors in the inflammatory cells. Conversely, intra-articular injection of IFNγ exacerbated arthritis in the joint[5]. This enhancement of arthritis could reflect the capacity of IFNγ to activate the production of pro-inflammatory cytokines such as IL-1 and TNFα by monocytes already recruited into the synovial tissue. Furthermore, the effects of IFNγ in the treatment of rheumatic disease are considered ambiguous[6-8]. In one double-blind, controlled study of 91 patients with rheumatoid arthritis[6], a statistically significant improvement in clinical disease among the recombinant IFNγ-treated patients was reported. In two other studies[7,9], which included 26 and 105 patients respectively, the IFNγ treated group showed some clinical improvement, but not statistically significant compared to the placebo group.

Although IFNγ has primarily immunomodulatory as well as antiviral and antiproliferative properties, a number of recent studies has shown that IFNγ is also able to modulate the metabolism of connective tissue cells. IFNγ induces the production of reactive oxygen species and PGE_2[10-12] and the expression of ICAM-1 and major histocompatibility complex class II antigens by human isolated chondrocytes and synovial fibroblasts[13-16]. IFNγ also inhibits the synthesis of cartilage-specific collagens, fibronectin and proteoglycans by chondrocytes[10, 11, 17, 18]. On the other hand, IFNγ inhibits TNFα and IL-1β-induced collagenase and stromelysin by chondrocytes, as well as IL-1 or TNF-stimulated proteoglycan degradation *in vitro*[11,19]. IFNγ has both proliferative and antiproliferative effects on synovial fibroblasts and stimulates chondrocyte proliferation. In addition, IFNγ is a potent inhibitor of bone resorption. In bone explants, it inhibits osteoclast differentiation[20], and reduces the activity of existing cells in a manner similar to calcitonin[21].

These observations suggest that IFNγ could be involved in cartilage degradation occurring in joint diseases, not only by acting on cartilage itself but also on neighboring tissues. Moreover, potent synergism and antagonism between IL-1β and IFNγ have been reported in reference to their actions on the metabolic function of chondrocytes. These findings led us to hypothesize that IFNγ could be a modulator

of the IL-1β bioactivity. IL-1β modifies regulation of the metabolic functions of chondrocyte, synovial and bone cells leading to joint destruction [22, 23]. It stimulates metalloproteases and reactive oxygen species production by chondrocytes and drastically inhibits cartilage matrix component synthesis [24, 25]. Furthermore, it stimulates the proliferation of fibroblasts and their production of type I collagen and fibronectin. Moreover, IL-1β also induced the production by fibroblasts and chondrocytes of soluble mediators (e.g, IL-6, IL-8, NO,...) involved in the pathogenesis of joint diseases [26, 27]. Some previous studies have reported that the regulation of IL-1 production and activity directly modulates cartilage resorption. IL-1β production is inhibited by IL-4, IL-10 and IL-13 but stimulated by TNFα [28-30]. IL-1β-induced cartilage resorption is down-regulated by two types of inhibitors: the IL-1 receptor antagonist (IL-1ra) which competitively binds to the receptor of IL-1 without inducing signal transduction and the soluble receptors (IL-1sR) which bind IL-1 and diminish the free concentration of soluble cytokines susceptible to bind the cell surface receptor. *In vitro*, IL-1β-stimulating effects on cartilage degradation are counterbalanced by TGFβ and osteogenic protein 1 (OP1) and partially reversed by NO synthase inhibitors [25, 31-33].

These findings prompted us to investigate the effect of IFNγ on NO, IL-6, IL-8, IL-10, IL-1ra, PGE_2, proteoglycans and stromelysin production by normal non-stimulated and IL-1β-stimulated human chondrocytes. At the present time, little is known about the regulatory functions of IFNγ on chondrocytes, or about its role in the pathophysiology of joint disease.

Materials and Methods

Human chondrocytes in culture

Chondrocytes were cultured at high density for 48 h in order to keep their phenotype. Cartilage was obtained from the knee of cadavers immediately after death via excision from the superficial and medium layer avoiding the calcified layer. Upon dissection, femoral and patellar articular surfaces were evaluated for gross pathological cartilage modifications. Cartilage fragments were sequentially treated by hyaluronidase (EC: 3.2.1.35; Sigma Chemie, Bornem, Belgium) dissolved in Dubbelcco's Modified Eagle's Medium (DMEM, Biowhittaker, Brussels, Belgium) (0.5 mg/ml-3 g of cartilage per 10 ml of enzyme solution) for 30 min at 37 °C and under constant agitation (200 rpm). Cartilage fragments were then put into pronase solution (1 mg per ml in DMEM-3 g of cartilage per 10 ml of enzymatic solution) (EC: 3.4.24.4; Merck-Belgolabo, Overijse, Belgium) and incubated with this enzyme for 1h at 37 °C, subsequently re-incubated under constant agitation (200 rpm) with collagenase (EC: 3.4.24.3; Sigma Chemie, Bornem, Belgium) (1 mg/ml - 3 g/10 ml of enzyme solution) dissolved in DMEM containing 1% of Ultroser (Gibco, Gent, Belgium) for 20 h at 37 °C.

The cells were then filtered through nylon mesh with pore of 70 μm, washed three times, counted and resuspended at 0.5 10^6 cells/ml of adequate culture medium. Nutrient medium was DMEM without red phenol containing 2 mM of gluta-

mine and 1 % of ITS+. ITS+ is a well defined culture supplement containing 0.625 mg/ml of insulin, 0.625 mg/ml of transferrin, 0.625 μg/ml of selenium acid, 125 mg/ml of bovine serum albumin (BSA) and 0.530 mg/ml of linoleic acid. Cells were kept in this culture medium for 48 h to eliminate *in vivo* contamination with drugs. After this washout period, cells were seeded into 24-well plates and maintained in culture for 48 h at 37 °C in a 95 %-5 % oxygen-CO_2 environment. Cells and supernatant were separated by centrifugation (160 g; 5 min).

The cells recovered after collagenase digestion of the tissue were 95 % viable as demonstrated by the vital stain with trypan blue. Cell pellets were washed twice with HBSS (Hank's Buffer Saline Solution, Gibco, Merelbeke, Belgium) and then homogenized in phosphate buffer saline (PBS) pH 7.5 by ultrasonic dissociation (10s pulses, power 50 W/cm^2). Culture media were directly assayed for NO, IL-6, IL-8, IL-10, prostaglandin E_2 (PGE_2), stromelysin, proteoglycan (PG) and IL-1ra. All the chemicals for which the source was not mentioned were of the purest grade available commercially.

Culture treatment

Human normal chondrocytes (± 0.5 10^6 cells/ml) were cultured for 48 h in the absence or in the presence of human recombinant IFNγ (rhIFNγ, Roche, Brussels, Belgium) (0-100 U corresponding to 0.05-5 ng/ml) added solely or in combination with interleukin-1β (IL-1β; 2.10^{-10} M, Roche, Brussels, Belgium) and/or N^G-monomethyl-L-arginine (L-NMMA; 0.5 mM). Four culture flasks were used for each concentration of cytokines as well as for the corresponding control. Each culture was run with chondrocytes from the knee joint of a single patient. Each culture condition was tested at least three times with cartilage samples coming from 3 different young (< 35 years) donors. Cell viability was superior to 95 % in all culture treatments.

Proteoglycan (PG) radioimmunoassay

PG released into culture medium was assayed as described previously[34]. Briefly, human cartilage PG was extracted according to the methods of Roughley *et al.*[35] and Bayliss and Venn[36]. Antiserum against human cartilage PG was raised in rabbits according to the method of Vaitukaitis *et al.*[37]. Antibodies were directed against the core protein of human cartilage PG. No cross-reaction was observed with glycosaminoglycans such as chondroitin sulfate or keratan sulfate nor with PG treated with various proteases. Only treatment with proteolytic enzymes (papain, trypsin, and chymotrypsin) abolished or diminished immunoreactivity as opposed to treatment with chondroitinase ABC and neuraminidase. Antiserum did not cross-react with other cartilage matrix constituents such as type II, type IX, and type XI collagens. The lack of cross-reaction with bone PG or skin, cornea, liver, heart or lung tissue extracts, showed that the antiserum used in the study contains only specific aggrecan antibodies.

The analytical sensitivity of the RIA was 0.6 ng per tube. The intra- and interassay coefficients of variation were < 10 and 20 %, respectively, for the linear part of the curve.

Immunoassay for stromelysin

Total stromelysin production was assayed using a EASIA (Emzyme Amplified Sensitivity Immunoassay) provided from Biosource Europe (Nivelles, Belgium). In this immunoassay, monoclonal antibodies are directed against pro-stromelysin, activated-stromelysin and stromelysin bound to TIMP-1 and TIMP-2. Antibodies do not cross-react with stromelysin bound with α2-macroglobulin, collagenase or gelatinase. The method was linear between 5 and 20 ng/ml with a limit of detection of 2.5 ng/ml.

NO assay

Nitrite is a stable end product of nitric oxide. Nitrite concentrations in conditioned media were determined by a spectrophotometric method based upon the Griess reaction [39]. Briefly, 200 µl of supernatant was added to 50 µl 6.5 M HCL and 50 µl 37.5 mM sulfanilic acid. After 10 min at room temperature, 50 µl of 12.5 mM N-(1-naphtyl)-ethylene diamine (Merck, Darmstadt, Germany) was added. The absorption was measured at 540 nm. Sodium nitrite ($NaNO_2$) was used for calibration.

PGE_2 radioimmunoassay

PGE_2 was assayed in conditioned culture medium without extraction according to a previously described radioimmunoassay (RIA) [39]. Polyclonal antiserum, obtained from rabbit, did not cross-react with other prostanoids (TxB2, 6-keto-PGF-1α, PGA_2) or with fatty acids (arachidonic, linoleic, oleic acids). Intra and interassay coefficients of variation are 6 and 10 %, respectively. The recovery of known amounts of PGE_2 added to the culture medium was satisfactory (90-97 % for the amounts of PGE_2 ranging from 2500 to 312 pg/ml). The limit of detection of the RIA was 20 pg/ml.

Immunoassays for cytokines and IL-1 receptor antagonist

IL-6, IL-8, IL-10 and IL1-ra released by chondrocytes in the culture media were assayed by four EASIA (Cytosets) designed by Biosource Europe (Nivelles, Belgium). The assays were based on the oligonal system in which several monoclonal antibodies were directed against distinct epitopes of the cytokines.

DNA assay

Chondrocyte DNA content was measured according to the fluorimetric method of Labarca and Paigen [40].

Statistical analysis

The results (mean +/- SD) were expressed per µg of DNA for proteoglycan, stromelysin, IL-1ra, PGE_2 and cytokines and NO measured in the culture medium. Comparison of mean values was performed for each culture individually using the unpaired t-test of Student with a limit of significance at $p < 0.05$. Multiple comparisons between treated groups were also achieved by analysis of variance test.

Correlations between the variables were investigated by linear regression analysis and one-way analysis of variance was performed (F-test).

Results

Effects of IFNγ on non stimulated normal chondrocytes

The effects of IFNγ were investigated on human chondrocytes isolated from young cartilage at concentrations ranged between 0.1 to 100 units per ml. These concentrations corresponded to the levels of IFNγ found in the synovial fluid of patients with OA or RA (values are ranged between 0.6 to 23.7 U/ml according the References 1 and 2). As shown in the Fig. 1, IFNγ at concentrations of 1, 10 and 100 U/ml significantly stimulated NO production ($0.05 < p < 0.001$). Furthermore, IFNγ dose-dependently increased IL-6, IL-1ra and PGE_2 production ($r = 0.85$ - 0.95; $p < 0.001$) but strongly inhibited IL-8. IL-8 production was depressed by 25 and 55 % at the IFNγ concentrations of 10 and 100 U/ml, respectively. On the other hand, PG production was dose-dependently inhibited by IFNγ ($r = 0.82$; $p < 0.01$) whereas stromelysin was slightly but significantly decreased by IFNγ only at the highest concentration of 100 U/ml ($p = 0.02$). IL-10 was undetectable in non stimulated human chondrocyte cultures.

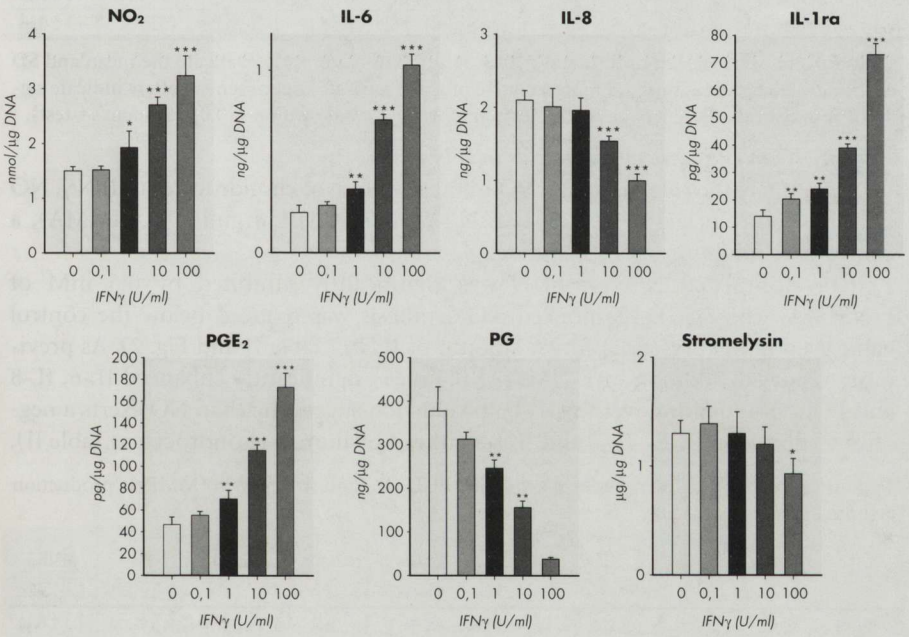


Fig. 1: *Effects of increasing doses of IFNγ on the production of NO, IL-6, IL-8, PGE_2, IL-1ra, PG and stromelysin in the culture medium of normal human chondrocytes (5.10^4 cells/ml). Data are the mean and S.D. of triplicate determinations and representative of three separate experiments (total N=9).*

Effects of IFNγ on IL-1ß -stimulated human chondrocytes

As previously reported [26, 27], IL-1β at the concentration of 2.10^{-10} M significantly stimulated NO, IL-6, IL-8, PGE_2, IL-10, IL-1ra and stromelysin production but drastically depressed PG synthesis ($p<0.05$, Table I). Addition of rhIFNγ at the concentrations of 10 and 100 U/ml significantly increased IL-1β-stimulated NO, IL-6 and IL-1ra production but strongly depressed the stimulatory effect of IL-1β on IL-8 and IL-10 productions, whereas IL-1β-induced stromelysin production was inhibited at the concentrations of 1, 10 and 100 U/ml of IFNγ. Finally, IFNγ acted synergically with IL-1β to depress PG production by human chondrocytes but did not significantly modify IL-1-stimulated PGE_2 production (Table I).

Table I: Effects of increased concentrations of IFNγ on cytokines, PG, PGE_2 and stromelysin production by human articular chondrocytes.

	NO_2	IL-6	IL-8	PGE_2	IL-10	IL-1ra	PG	MMP-3
	nmol/µg DNA	ng/µg DNA	ng/µg DNA	pg/µg DNA	pg/µg DNA	pg/µg DNA	ng/µg DNA	µg/µg DNA
Controls	1.5 ± 0.08a	0.22 ± 0.04a	2.01 ± 0.14a	47 ± 7a	0.13 ± 0.01a	14 ± 2.3a	378 ± 55a	1.3 ± 0.1a
IL-1β 2.10^{-10} M	2.9 ± 0.09b	19 ± 2b	97 ± 7b	1401 ± 244b	71 ± 3b	124 ± 5.2b	41 ± 4b	2.8 ± 0.1b
IL-1β + IFNγ 0.1U/ml	2.8 ± 0.1b	18 ± 1.5b	96± 9.8b	1668 ± 341b	62 ± 3c	131 ± 15b	34 ± 3.8c	2.8 ± 0.3b
IL-1β + IFNγ 1U/ml	3± 2b	20.1 ± 2.3b	97 ± 8b	1731 ± 160b	60 ± 5c	174 +/ 30c	33 ± 3.2c	1.8 ± 0.3c
IL-1β + IFNγ 10 U/ml	3.7 ± 0.3c	24 ± 1.4c	81 ± 11c	1454 ± 280b	39 ± 3c	254 ± 20d	35 ± 3c	1.3 ± 0.1d
IL-1β + IFNγ 100 U/ml	4.2 ± 0.1c	29 ± 5.3d	51 ± 8d	1534 ± 382b	23 ± 1c	429 ± 33e	32 ± 5c	1.1 ± 0.1d

Chondrocytes were treated or were not treated with IL-1ß (2.10^{-10} M). Data are the mean and SD of triplicate determinations and representative of three separate experiments. Letters indicate significant statistical differences between treatments. $a \neq b \neq c \neq d$, with $p < 0.05$ (Student's t-test).

Role of NO in chondrocyte responses to IFNγ

To evaluate the role played by NO in the response of chondrocytes to IFNγ, NO synthesis was blocked by 0.5 mM of N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NO synthase.

Non stimulated NO synthesis was significantly inhibited by 0.5 mM of L-NMMA, whereas IL-1β induced NO synthesis was reduced below the control value even in the presence of high amount of IFNγ (Table II and Fig. 2). As previously reported, addition of L-NMMA (0.5 mM) significantly enhanced IL-6, IL-8 and PGE_2 productions over the IL-1β production suggesting than NO exerts a negative feedback on PGE_2, IL-6 and IL-8 synthesis by human chondrocytes (Table II).

Table II: Effect of NO biosynthesis on cytokines, PGE_2, PG and stromelysin (MMP-3) production by human chondrocytes.

	NO_2	IL-6	IL-8	PGE_2	IL-10	IL-1ra	PG	MMP-3
	nmol/µg DNA	ng/µg DNA	ng/µg DNA	pg/µg DNA	pg/µg DNA	pg/µg DNA	ng/µg DNA	µg/µg DNA
Controls	1.5 ± 0.08a	0.22 ±0.04a	2.01 ± 0.14a	47 ± 7a	ND	14 ± 2.3a	378 ± 55a	1.3 ± 0.1a
L-NMMA	0.5 ±0.05b	0.18 ± 0.02a	1.94 ± 0.22a	54 ± 7a	ND	14.3 ± 2.3a	330 ± 23a	1.4 ± 0.11a
IL-1ß	2.9 ±0.06c	19 ±2c	97 ±7b	1401 ± 244c	71 ±3a	124 ± 5b	41 ± 4c	2.8 ± 0.08b
IL-1 + L-NMMA	0.7 ±0.05b	25 ± 2.1d	125 ± 8c	5732 ± 901d	72 ± 1a	97 ± 11c	44 ± 0.6c	2.6 ± 0.25b

Letters indicate significant statistical differences between treatments. $a \neq b \neq c \neq d$, with $p < 0.05$ (Student's t-test).

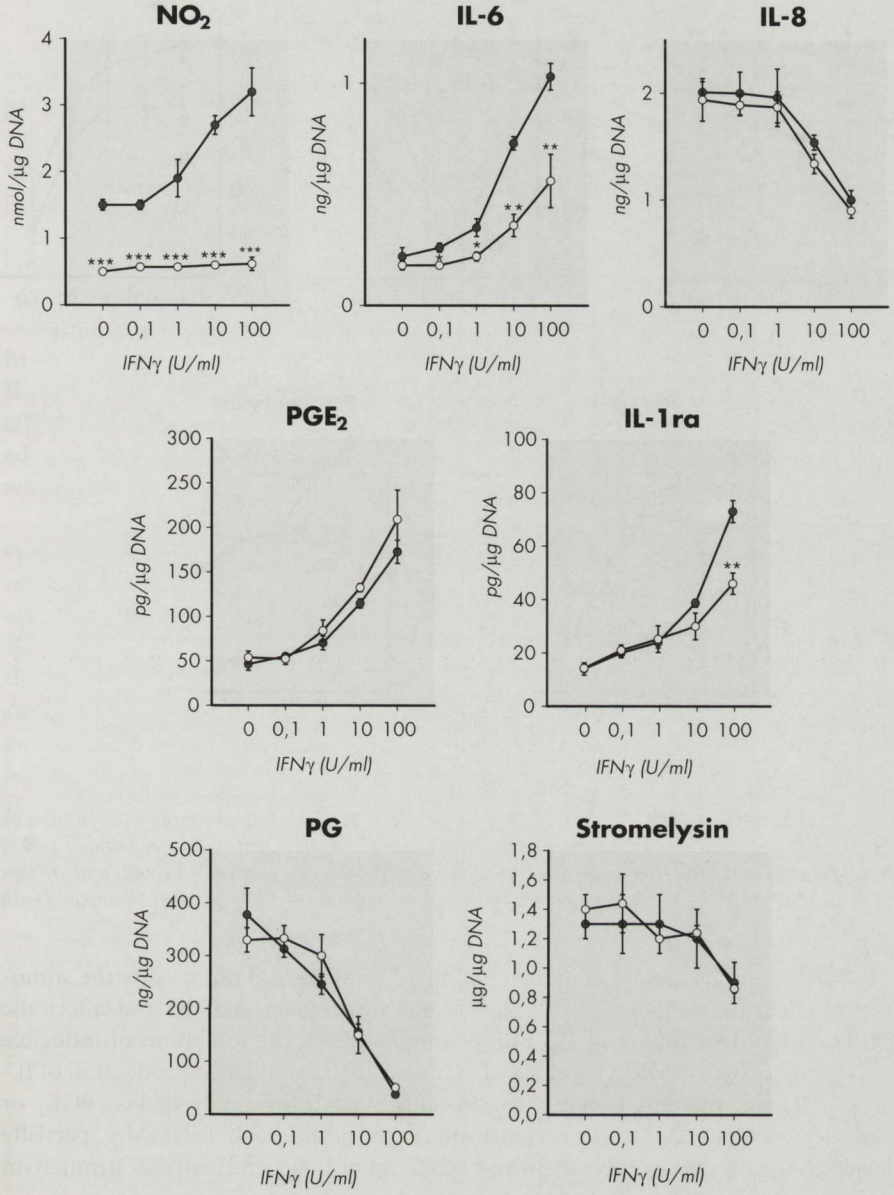


Fig. 2: *Production of NO, IL-6, IL-8, IL-10, IL-1ra, PG and stromelysin by human chondrocytes treated with rhIFNγ, in the presence (-○-) or absence (-●-) of L-NMMA 0.5 mM. Results are the mean ± SD values for four replicate wells and are representative of three similar experiments. * = p<0.05; ** = p < 0.01 and *** = p < 0.001 as compared with control.*

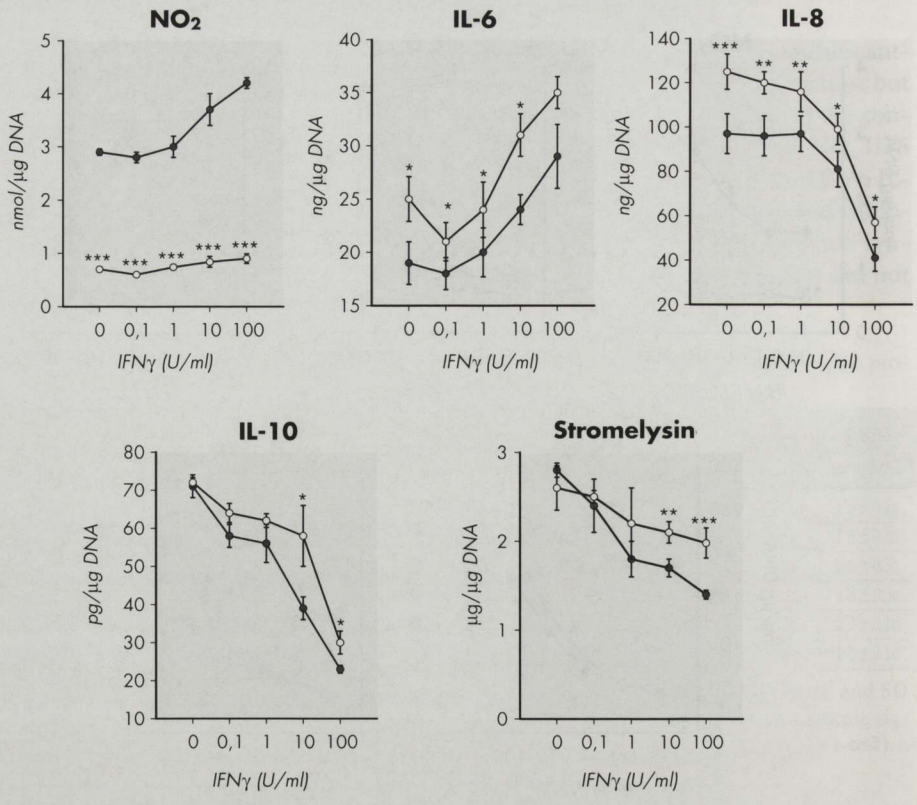


Fig. 3: *Effects of increased amounts of IFNγ on NO, IL-6, IL-8, IL-10 and stromelysin synthesis by IL-1ß-treated (2 x 10^{-10} M) human chondrocytes cultured for 48 h with (-○-) or without (-●-) L-NMMA 0.5 mM. Results are expressed as the mean ±SD values for four replicate wells and are representative of three similar experiments. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ compared with the control.*

Furthermore, here, we demonstrated that L-NMMA did not modify the stimulatory effect of IL-1β on IL-10, IL-1ra and stromelysin and did not affect the IL-1ß-induced inhibition of PG production. However, the inhibition of inducible NO synthase by L-NMMA significantly reduced IFNγ-stimulated production of IL-6 and IL-1ra, but did not modify the effects of IFNγ on IL-8, PG, PGE_2 or stromelysin (Fig. 2). In the presence of IL-1β, addition of L-NMMA, partially reversed the inhibitory effect of 10 and 100 U/ml of IFNγ on IL-10 and stromelysin (Fig. 3).

Discussion

Joint diseases are characterized by synovial membrane inflammation, cartilage degradation and subchondral bone remodeling. Cartilage plays a central role in the pathophysiology of joint diseases by producing a spectrum of soluble mediators able to regulate in an autocrine or paracrine manner its own metabolic functions as well as the metabolism of neighboring tissues such as bone or synovium. Inversely, chondrocyte functions are controlled by factors produced by synovium and bone cells. Among these factors, IFNγ is often described as a potent mediator of the cellular immune reaction with potential regulatory actions on connective tissues [41]. Although IFNγ was not expressed in cartilage [42], IFNγ is found in OA and RA synovial membrane suggesting that this cytokine could influence cartilage metabolism. In this work, we demonstrated that IFNγ synergized with IL-1β to stimulate NO, IL-6 and IL-1ra and to inhibit proteoglycans but antagonized the IL-1β stimulating effect on IL-8, IL-10 and stromelysin production. These observations were recorded at concentrations of IFNγ similar to those found in the synovial fluid of patients with OA or RA

In previous studies, similar opposite effects of IFNγ were reported on synoviocytes from RA and OA patients [43]. These observations assist in explaining that, relative to the circumstances, IFNγ may have inflammatory or anti-inflammatory activities. The antiinflammatory properties of IFNγ could be related to its effects on IL-8 and IL-1ra. The inhibition of IL-8 by IFNγ may lead not only to a reduction of the number of neutrophils and T-lymphocytes invading the joint cavity but also a decrease of neovascularization of the synovial membrane as suggested by a recent paper on the properties of IL-8 as an angiogenic factor [44]. On the other hand, we have shown that IFNγ was a potent stimulator of IL-1ra synthesis by chondrocytes. IL-1ra is a receptor-binding antagonist that block many of the effects observed during the pathological process of OA and RA, including prostaglandin synthesis by synovial cells, collagenase production by chondrocytes and cartilage matrix degradation [45-49]. Treatment of rats with IL-1ra can significantly suppress recurrence of swelling and severity of synoviotis and erosion induced by re-activation by i.v. injection of PG-APS (peptidoglycan-polysaccharide isolated from the cell wall of group A streptococci) [49]. Furthermore, intraarticular injection of IL-1ra also prevents cartilage lesions appearance in rabbit OA model [50]. Therefore, our study provides novel mechanistic data to explain the antiinflammatory properties of IFNγ.

On the other hand, IFNγ can also inhibit IL-10 production by IL-1-stimulated human chondrocytes. *In vitro*, IL-10 is a cytokine with the capacity to down-regulate the synthesis of proinflammatory cytokines (IL-1 and TNFα) by mononuclear cells [51] and completely reverse inhibition of cartilage PG synthesis induced by stimulated RA mononuclear cells [52]. However, the role of this anti-inflammatory cytokine in the pathogenesis of erosive joint diseases is not yet clearly demonstrated.

We also demonstrated that IFNγ may interfere with cartilage metabolism by inhibiting PG and stromelysin production. Our results are consistent with those found in the literature [10, 11, 17, 54, 55]. Destruction of cartilage in arthritis is partially attributable to metalloproteases released by the chondrocytes in response to IL-1.

Here, we demonstrated that IFNγ counteracts the stimulating effect of IL-1β on stromelysin synthesis suggesting that IFNγ could modulate the progression of tissue degradation and remodeling in inflammatory conditions as observed in RA. Furthermore, it appeared that IFNγ inhibited PG synthesis at a concentration 100 times lower than the concentration required to inhibit stromelysin. This latter observation suggests that IFNγ could have different effects on cartilage metabolism according to its relative concentration which may vary during the course of the disease and at different sites within the joint. In addition, Quintavalla *et al.* [53] have demonstrated that subpopulations of chondrocytes within the joint may differ in their responsiveness to cytokines, including IFNγ. Collectively, these observations could explain some events occurring in joint diseases such as OA. During OA development, phases of destruction and repair alternate. Therefore, we can hypothesize that in this disease, IFNγ contributes to repair reaction failure by inhibiting PG synthesis and has a protective role limiting cartilage degradation by metalloproteinases in inflammatory conditions. This hypothesis is supported by other work demonstrating that IFNγ partially counteracts the IL-1-induced cartilage degradation as measured by glycosaminoglycan released from cartilage fragments in culture, but acts synergically with TNFα to inhibit cartilage-specific type II, IX and XI collagens production by fetal and adult human chondrocytes [10, 11]. The effects of IFNγ do not appear to be due to cell death or proliferation. No decrease in cell viability, as determinated by trypan blue exclusion, was evident in culture of chondrocytes incubated with up to 100 U/ml of IFNγ (data not shown) and DNA levels remained stable regardless the concentrations of IFNγ employed.

Interestingly, we have observed that IFNγ stimulated NO production by chondrocytes suggesting that several effects of IFNγ could be mediated by NO. To verify this hypothesis, L-NMMA, a NO synthase inhibitor, was added to the chondrocyte cultures. As previously described, we have observed that the inhibition of endogenously NO production resulted in a significant enhancement of the IL-1β-stimulated IL-6, IL-8, and PGE_2 suggesting that NO down-regulates the synthesis of some inflammatory mediators [27]. Inversely, the effects of IFNγ on IL-8 and PGE_2 synthesis were not significantly affected by NO synthase inhibition whereas its stimulating effect on IL-6 and IL-1ra production was partially reversed. These data clearly show that IFNγ and IL-1β activate different signaling pathways in which NO plays different regulatory functions. On the other hand, neither IL-1β nor IFNγ effects on stromelysin and PG production were significantly modified by the addition of L-NMMA. These results differ from studies showing that L-NMMA partially reversed the effect of IL-1β on the synthesis of cartilage matrix molecules and on MMP activity [33, 54, 55]. This discrepancy can be explained by the difference in methodology. In our study, newly synthesized proteoglycans were quantified by RIA using an antiserum directed against the core protein of the aggrecans, whereas other studies revealed proteoglycans synthesis by the incorporation of $^{35}SO_4$ into glycosaminoglycans [55, 56]. In our work, we have quantified stromelysin by an immunoassay in which monoclonal antibodies were directed agains prostromelysin, activated-stromelysin

and stromelysin bound with TIMP-1 and TIMP-2. In other studies, metalloprotease activity was measured by enzymatic assay using labeled substrates. Metalloprotease activity is the result of the efficiency of both activation and inhibition systems and not only the result of the enzyme synthesis.

In conclusion, these results show that IFNγ has a number of effects on articular chondrocytes *in vitro* and suggest that IFNγ may modulate joint inflammation by modulating mediator production by chondrocytes. Moreover, we clearly show that the role played by IFNγ in joint disease pathophysiopathology may differ according to its own concentration but also to the concentration of other cytokines such as IL-1β.

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ANNEXE 5

Nitric oxide downregulates interleukin 1ß (IL-1ß) stimulated IL-6, IL-8 and prostaglandin E_2 production by human chondrocytes.

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Abstract

Objective: To investigate the effects of endogenously produced nitric oxide (NO) on interleukin-6 (IL-6), IL-8, prostaglandin E_2 (PGE_2) and proteoglycans production by human chondrocytes.

Methods: Human articular chondrocytes were isolated from their extracellular matrix by a triple successive enzymatic digestion of the cartilage and cultured for 48 h in a well defined culture medium. IL-6 and IL-8 were directly assayed into culture media by specific enzyme amplified sensitivity immunoassays (EASIAs). Proteoglycans and PGE_2 were quantified by specific radioimmunoassays. Cell culture media were assayed for NO_2- using a spectrophotometric assay based upon the Griess reaction.

Results: Unstimulated chondrocytes produced low levels of NO, IL-6, IL-8 and PGE_2. Production was significantly stimulated by IL-1ß and lipopolysaccharide (LPS). Furthermore, proteoglycans synthesis was profoundly inhibited by IL-1ß and LPS. Inhibition of NO synthesis with the competitive inhibitor N^G-monomethyl-L-arginine (L-NMMA) led to an enhancement of the IL-6, IL-8 and PGE_2 productions stimulated by either IL-1ß alone or in combination with LPS, whereas the inhibition of the proteoglycan production by IL-1ß was not modified by L-NMMA.

Conclusion: LPS and IL-1ß stimulated IL-6, IL-8 and PGE_2 production are downregulated by endogenously produced NO, which could limit the inflammatory reaction occurring in arthritis.

Key Indexing Terms: Nitrix oxyde, cytokines, proteoglycans, prosta-glandins, chondrocytes, osteoarthritis.

Introduction

Nitric oxide (NO) is a widespread intracellular and intercellular mediator involved in the pathophysiological mechanisms of some connective tissue diseases. In patients presenting either rheumatoid arthritis or osteoarthritis, increased levels of nitrite have been observed in the synovial fluid [1]. A significant source of NO production in these patients appeared to be articular chondrocytes. This finding is important since we known the brevity of NO half-life, defining its immediate sphere of influence to autocrine and paracrine effects. In cartilage, soluble mediators, including cytokines, prostanoids and NO, produced locally by chondrocytes play an important regulatory role in chondrocyte functions and contribute to the maintenance of tissue homeostasis. Nevertheless, under the influence of abnormal exogenous stimuli, these mediators are overproduced and participate in the development of synovial membrane inflammation, bone remodeling and cartilage lesions occurring in joint diseases.

Interleukin 1ß (IL-1ß) is one of the most important mediators involved in cartilage degradation processes. IL-1 is known to induce many catabolic responses in articular chondrocytes. Indeed, in response to IL-1ß, chondrocytes secrete neutral metalloproteinases [2], active oxygen species [3], pro-inflammatory cytokines (IL-6, IL-8) [4, 5], and nitric oxide (NO) [6, 7]. Moreover, IL-1ß is a potent inhibitor of proteoglycans and collagen synthesis [8, 9]. At the present, the intracellular events occurring after receptor occupancy by IL-1 are poorly understood. Studies show that endogenously produced NO can regulate some cellular responses to IL-1ß. Indeed, production of NO by NO synthases (NOS) leads to the inhibition of several important steps of cellular metabolism, specifically aerobic energy metabolism [10], DNA replication [11] and protein synthesis [12]. *In vitro*, the inhibition of endogenous NO production by N^G-monomethyl-L-arginine (L-NMMA), an established NO synthases inhibitor, leads to the suppression of gelatinase, collagenase and stromelysin production by IL-1ß stimulated isolated chondrocytes [7, 13]. Inhibition of the NO production also partially reduces the increase in the lactate production that occurs from exposure of chondrocytes to IL-1ß [14]. Treatment of cartilage fragments with L-NMMA partially reverses the IL-1ß inhibitory effect on glycosaminoglycans synthesis [15, 16], inhibits IL-1ß stimulated metalloprotease activities [13], and increases interleukin-1 receptor antagonist (IL-1ra) production [17]. These findings suggest that NO is a potent regulator of the IL-1ß effect and in this way may contribute to the pathophysiology of joint diseases. These results have encouraged the notion that specific inhibitors of NO synthases are potential novel drugs in the treatment of autoimmune, inflammatory or erosive joint disease. Nevertheless, investigators have yet to define the pathophysiological roles played by NO in the development and prolongation of joint inflammation. Our findings indicate that the inhibition of NOS by L-NMMA induced an overproduction of IL-6, IL-8 and PGE_2 suggesting that NO may downregulate the production of proinflammatory mediators, resulting in a control of the inflammatory reaction.

Materials and Methods

Short term suspension culture of human chondrocytes.

Cartilage obtained from the knee joints of normal adults shortly after death were used for this study. All donors were between 38 and 74 years old (mean 54). Avoiding the calcified layer, cartilage slices were excised from the superficial and medium layers of cartilage, cut into small fragments, and submitted to enzymatic digestion. Chondrocytes were released from cartilage by sequential digestion with hyaluronidase, pronase and collagenase. Cartilage pieces were incubated with hyaluronidase dissolved in Dubelcco's Modified Eagle's Medium (DMEM; Biowhittaker, Brussels, Belgium) (0.5 mg/ml: 3 g of cartilage per 10 ml of enzyme solution) for 30 min at 37 °C and continuously agitated (100 rpm). They were then placed into a pronase solution (1 mg/ml in DMEM - 3g per 10 ml of enzymatic digestion), incubated for 90 min at 37 °C and subjected to continuous agitation (100 rpm). They were reincubated with collagenase (0.5 mg/ml - 3g per 10 ml of enzymatic solution) dissolved in DMEM containing 1 % of Ultroser G (Gibco, Gent, Belgium) for 20 h at 37 °C. Cells were then filtered through 25 μm nylon mesh, washed three times, counted and seeded into 24 well plates at 0.05 to 1.6 x 10^6 cells/ml. Nutrient medium was DMEM containing 1 % of ITS+ and 1 % of glutamine (Flow Laboratories, Irvine, Scotland). ITS+ is a premixed cell growth system containing in 1 ml: 0.625 mg of insulin, 0.625 mg of transferrin, 0.625 μg of selenium acid, 0.125 g of bovine serum albumin and 0.535 mg of linoleic acid. Cells were maintained in culture for 48 h at 37 °C in an atmosphere of 95 % air 5 % CO_2. Cells and supernatants were separated by centrifugation (160 g, 5 min.). The cell pellets were washed two times with Hank's Buffer Saline Solution (Gibco) and then homogenized in PBS, pH 7.5 by ultrasonic dissociation at 4 °C for DNA assay. Cell viability was 95 % measured by trypan blue incorporation. Culture media were directly assayed for NO, IL-6, IL-8, prostaglandin E_2 (PGE_2), and proteoglycans.

Treatments.

Chondrocytes were cultured for 48 h with and without human recombinant IL-1ß (rhIL-1ß) and with and without lipopolysaccharide (LPS) and L-NMMA. Human recombinant IL-1ß was purchased from Boehringer (Mannheim, Germany) and tested at concentrations ranging from 10^{-12} to 10^{-9} M corresponding to 0.850 to 850 U/ml, respectively. LPS (Boehringer Mannheim) was tested at the final concentration of 10 μg/ml. L-NMMA was purchased from Calbiochem (Novabiochem, San Diego, USA) and tested at concentrations ranged from 0.5 to 2 mM.

Four culture flasks were used for each concentration of cytokine as well as for the cor-responding control. Each culture was run with chondrocytes from the knee joint of a single subject. Each culture condition was tested three times with cartilage samples coming from three different donors. Cell viability was superior to 90 % in all culture treatments.

NO assay.

Nitrite is a stable end product of nitric oxide. Nitrite concentrations in conditioned media were determined by a spectrophotometric method based upon the Griess reaction [18]. Briefly, 200 µl of supernatant was added to 50 µl 6.5 M HCL and 50 µl 37.5 mM sulfanilic acid. After 10 min at room temperature, 50 µl of 12.5 mM N-(1-naphtyl)-ethylene diamine (Merck, Darmstadt, Germany) were added. The absorption was measured at 540 nm. Sodium nitrite ($NaNO_2$) was used for calibration.

PGE_2 radioimmunoassay.

PGE_2 was assayed in conditioned culture medium according to a previously described radioimmunoassay (RIA) [19]. PGE_2 was directly measured in the culture medium without previous extraction. Polyclonal antiserum, obtained from rabbit, did not crossreact with other prostanoids (TxB_2, 6-keto-PGF-1α, PGA_2) or with fatty acids (arachidonic, linoleic and oleic acids). The ^3H labelled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule PGE_2 from Sigma Chemical Co (St Louis, MO, USA). PGE_2 was measured in duplicate on a 100 µl aliquot of each sample. After 48 h incubation at 4 °C, free antigen was separated from antibody-bound antigen by charcoal precipitation and centrifugation (20 min at 1000g; 4 °C). The supernatant was counted by liquid scintillation. Intra- and interassay coefficients of variation are 6 and 10 %, respectively. The recovery of known amounts of PGE_2 added to the culture medium was satisfactory (90 to 97 % for the amounts of PGE_2 ranging from 2,500 to 312 pg/ml). The limit of detection of the RIA was 20 pg/ml.

Proteoglycan radioimmunoassay.

Proteoglycan released in conditioned culture media were assayed by a previously described 1 RIA method [20]. The analytical sensitivity of the RIA was 0.6 ng/tube. Along the linear portion of the curve, the intra- and interassay coefficients of variation were less than 10 and 20 %, respectively. The antibodies were only directed against the antigenic determinant of the core protein, as shown by the lack of cross-reaction with chondroitin sulfate and keratan sulfate. Contrary to the treatment with chondroitinase ABC or neuraminidase, the treatment with proteolytic enzymes (papain, trypsin, chymotrypsin) diminished or abolished immunoreactivity. Antiserum did not cross react with other cartilage matrix constituents such as type II, IX and XI collagens, chondroitin and keratan sulfate, hyaluronic acid or fibronectin. On the other hand, as shown by the lack of crossreaction with bone proteoglycans or skin, cornea, liver, kidney, heart and lung tissue extracts, it appears that our antiserum contains only specific chondrocyte-aggrecan antibodies.

Cytokine assays.

IL-6 and IL-8 released by chondrocytes in the culture media were assayed by 2 enzyme amplified sensitivity immunoassays (EASIAs) designed by Biosource Europe (Fleurus, Belgium) [21]. Th assays were based on the oligonal system in which

several monoclonal antibodies were directed against distinct epitopes of the cytokines. The assays were directly performed in the culture media without previous extraction. The intra and interassay coefficients of variation were less than 5 %.

DNA assay.

Chondrocyte DNA content was measured according to the fluorimetric method of Labarca and Paigen [22].

Calculation and statistical analysis.

The results were expressed as amounts of NO, cytokines, proteoglycans and PGE_2 amounts measured in the culture medium per μg of DNA. The mean ± standard deviation (SD) of each variable was calculated. Comparison of mean values was by unpaired Student t-test with a limit of significance at $p < 0.05$. Multiple comparisons between treatment groups were also achieved by analyse of variance test. Correlations between the variables were investigated by linear regression analysis and one-way analysis of variance was performed (F test).

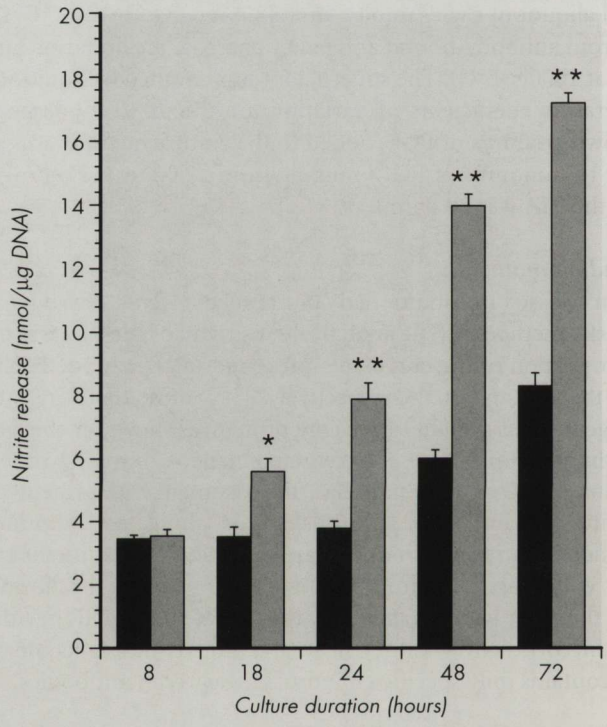


Figure 1: *NO production over time. Chondrocytes were unstimulated (black bars) or stimulated with 2.5 x 10-10 M of rhIL-1ß (grey bars). Results are expressed as mean ± SD of 4 experiments. IL-1ß treated groups are significantly different from the controls with a * $p < 0.05$, ** $p < 0.01$.*

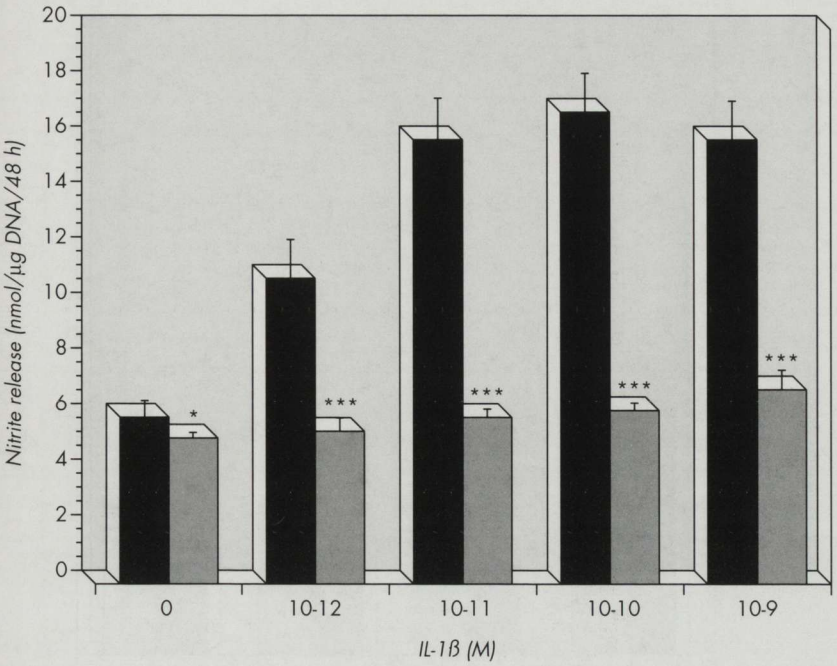


Figure 2: *Induction of NO generation in response to increasing amounts of rhIL-1ß. Freshly human chondrocytes were cultured for 48 h in the absence (black bars) or presence (grey bars) of L-NMMA at 0.5 mM concentration. Results are expressed as mean values of 4 cultures from the same donor. L-NMMA treated groups are significantly different from the untreated groups: * $p < 0.05$, *** $p < 0.001$.*

Table I: Effect of NO biosynthesis on cytokines, PGE_2 and proteoglycans production by human articular chondrocytes

	NO_2	IL-6	IL-8	PGE_2	PROTEOGLYCANS
	nmol/µg DNA/48 h	ng/µg DNA/48 h	ng/µg DNA/48 h	ng/µg DNA/48 h	µg/µg DNA/48 h
Controls	1.24 ± 0.17 a	0.08 ± 0.01 a	0.25 ± 0.04 a	0.60 ± 0.06 a	20.8 ± 2.4 a
L-NMMA 0.5 mM	1.11 ± 0.28 a	0.09 ± 0.01 a	0.22 ± 0.02 a	0.35 ± 0.04 b	17.1 ± 1.5 a
LPS 10 µg/ml	9.36 ± 0.17 b	35 ± 5.5 b	199 ± 13 b	28.2 ± 1.7 c	6 ± 0.5 b
LPS + L-NMMA	1.84 ± 0.15 c	65 ± 9 c	234 ± 25 b	38.6 ± 5 d	6.5 ± 0.4 b
IL-1 ß 2.5 10^{-10} M	12.8 ± 1.55 d	100 ± 21 d	249 ± 25 b	37.3 ± 2.9 d	5.3 ± 0.5 b,c
IL-1ß + L-NMMA	2.47 ± 0.28 e	201 ± 24 e	326 ± 38 c	70 ± 6 e	5 ± 0.1 b,c
IL-1ß + LPS	18.5 ± 1.28 f	139 ± 19 f	368 ± 81 c	68 ± 6 e	4.8 ± 0.6 c
IL-1ß + LPS + L-NMMA	3.14 ± 0.17 g	278 ± 38 g	539 ± 113 d	157 ± 22 f	4.7 ± 0.6 c

* Letters indicate significant statistical differences between groups with $p < 0.05$ (Anova test).

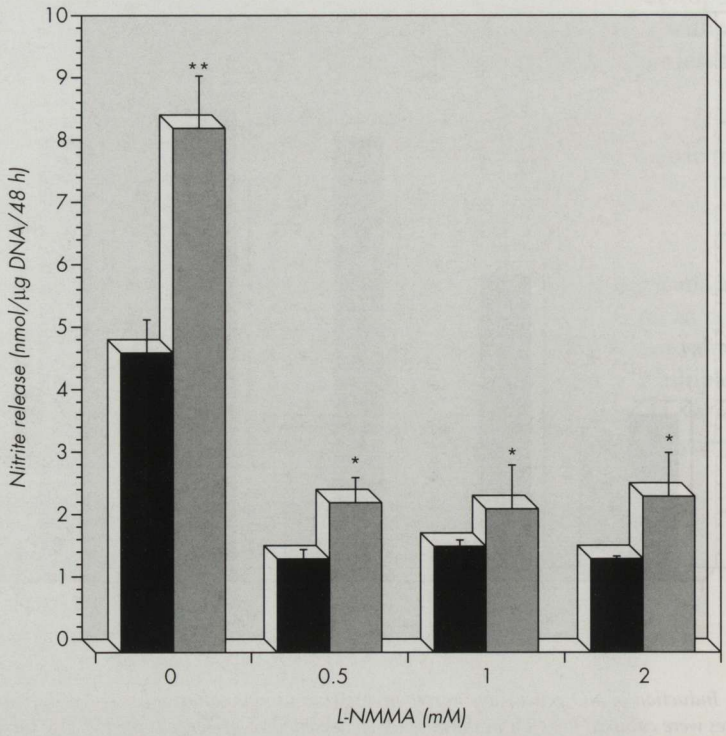


Figure 3: *Effect of increased amounts of L-NMMA on NO production by human chondrocytes cultured in the absence (black bars) or presence (grey bars) of 2.5 x 10^{-10} M of IL-1ß. IL-1ß treated groups are significantly different from the untreated groups: * $p < 0.05$ or ** $p < 0.01$.*

Results

Unstimulated chondrocyte culture spontaneously produced nitric oxide (1 - 10 nmol/µg DNA/48 h). In control conditions, NO production remained stable during the first 24 h of culture and slightly increased thereafter (Figure 1). As previously described, NO generation, measured as the accumulation of stable endproduct NO_2 was highly stimulated by rhIL-1ß (10^{-12} to 10^{-9} M) ($0.01 < p < 0.001$) (Figure 2). In the presence of rhIL-1ß, NO synthesis was significantly stimulated after 18 h of culture and reached a maximal rate of production after 48 h. Dose reponse experiments revealed a maximal production at 10^{-11} M of rhIL-1ß. No additional NO production was observed at the concentrations of 10^{-10} and 10^{-9} M of IL-1ß. NO production was also significantly stimulated by LPS (10 µg/ml) ($p < 0.001$). The combination of IL-1ß (2.5 10^{-10} M) and LPS (10 µg/ml) resulted in an increased NO synthesis (Table 1). Unstimulated NO synthesis was significantly inhibited by 0.5 mM of L-NMMA whereas IL-1ß induced NO synthesis was reduced at control level even in the presence of high doses of rhIL-1ß (10^{-9} M) (Figure 2).

Additional amounts of L-NMMA did not lead to a greater suppressive effect (Figure 3). On the other hand, NO synthesis was affected by the culture cell density. In the presence of IL-1ß or not, NO_2^- increased in the culture medium as a function of cell numbers and reached a maximum at 0.8 x 10^6 cells.

Nevertheless, when the NO production was expressed per µg of DNA, we observed that the rate of NO produced by unstimulated and IL-1ß stimulated chondrocytes decreased as a function of cell numbers, suggesting that NO production is inversely correlated to the cell density (r= - 0.90 to -0,94; $p < 0.001$) (Figure 4). The stimulating effect of IL-1ß was not greatly affected by cell culture density. The maximal IL-1ß stimulating effect was observed at the density of 0.1 x 10^6 cells per ml (240 % of the control value). On the other hand, no significant difference was recorded between the lowest (0.05 x 10^6 cells/ml; 170 % of the control value) and the highest cell density (1.6 x 10^6 cells/ml; 180 % of control value).

As described [5], IL-1ß stimulated PGE_2, IL-6 and IL-8 production (Figure 5). The maximal stimulating effect was obtained at the concentration of 10^{-11} M of IL-1ß. No additional IL-6, IL-8 or PGE_2 production was obtained with higher IL-1ß concentrations. Of interest, addition of L-NMMA (0.5 mM) resulted in a significant enhancement of IL-6, IL-8 and PGE_2 productions over the IL-1ß production ($0.01 < p < 0.001$) (Figure 5). As shown by figure 5, IL-6, IL-8 and PGE_2 production stimulated by 10^{-9} M of IL-1ß was more than doubled in the presence of L-NMMA. The same observations were made with LPS. Endotoxin (LPS 10 µg/ml) also significantly increased IL-6, IL-8 and PGE_2 production ($p < 0.001$) (Table I). LPS-stimulated production was markedly enhanced by L-NMMA. The combination of IL-1ß and LPS led to a synergic effect on cytokines and PGE_2 synthesis, and the effect of L-NMMA was even more profound when IL-1 and LPS were combined.

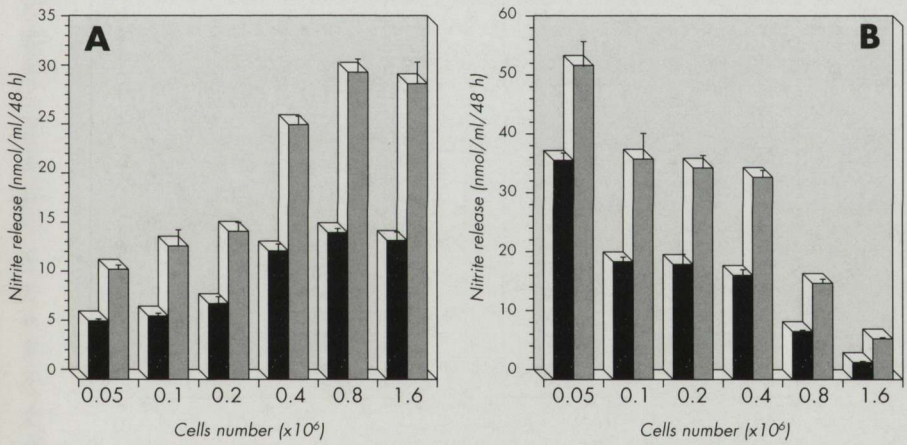


Figure 4: *Production of NO as a function of culture cell density. Chondrocyte cultures were untreated (black bars) or treated by 2.5 x 10^{-10} M (grey bars) of rhIL-1ß. (A) Results are expressed as the amounts of NO_2^- per ml of culture medium. (B) Results are expressed as the amounts of NO_2^- per µg of DNA. Results represent means ± SD of 4 experiments.*

Furthermore, treatment of unstimulated cells with L-NMMA led to a significant decrease of PGE_2 production but did not modify the basal IL-6 and IL-8 production (Table I).

IL-1ß provoked a marked inhibition of the proteoglycan production. This inhibitory effect was maximal at the concentration of 10^{-11} M of IL-1ß (70 % of inhibition at 10^{-11} M and 79 % at 10^{-9} M). The inhibitory effect of IL-1ß was not modified by addition of L-NMMA at the concentration of 0.5 mM.

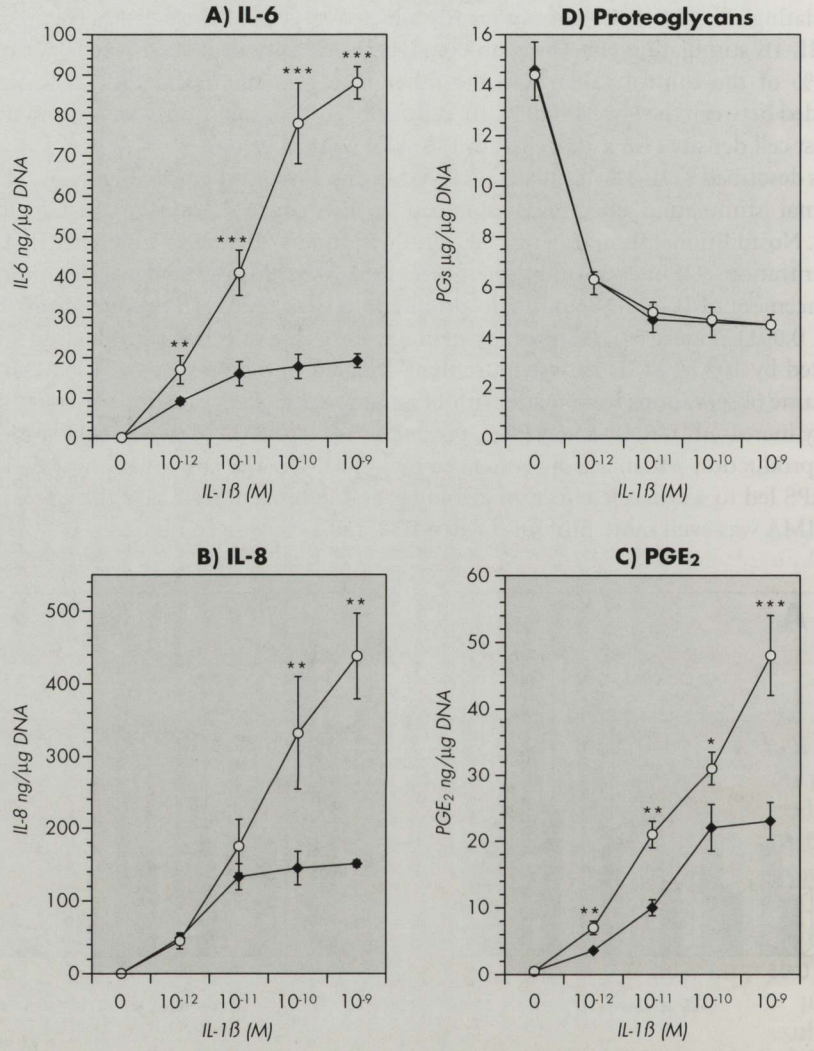


Figure 5: *Effects of rhIL-1ß on IL-6 (A), IL-8 (B), proteoglycan (C) and PGE_2 (D) production by human chondrocytes cultured for 48 h with (○) or without (●) L-NMMA at the concentration of 0.5 mM. L-NMMA treated groups are significantly different from the untreated groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

Discussion

We demonstrated that IL-1ß and LPS are potent stimulators of NO production by human articular chondrocytes. These data are in agreement with studies showing that LPS, tumor necrosis factor (TNF), and IL-1ß act synergistically to stimulate NO production by rabbit chondrocytes [7, 23]. This finding warrants attention since it is known that NO is involved in pathogenesis of joint diseases. Indeed, exposure of rabbit cartilage to exogenous NO suppresses glycosaminoglycans production at a level close to IL-1ß and increases metalloproteases production, suggesting that NO mediates cartilage breakdown [7, 13, 16]. We examined the role played by NO on the inhibitory effect of IL-1ß on proteoglycan production by freshly isolated human articular chondrocytes. In our culture conditions, the inhibition of endogenous NO production by human chondrocytes did not modify the spontaneous proteoglycan production or reverse the IL-1ß inhibitory effect. These results are in disagreement with the study of Taskiran *et al.* [16], showing that NO is a mediator of the inhibitory effect of IL-1 upon proteoglycan synthesis by rabbit chondrocytes. This discrepancy can be explained by the differences in methodology. Taskiran *et al.*, cultured rabbit cartilage fragments, whereas our study used freshly isolated human chondrocytes, and newly synthesized proteoglycans were quantified by RIA using an antiserum directed against the core protein of the aggrecans whereas in Taskiran *et al.*, proteoglycan synthesis was measured by the incorporation of $^{35}SO_4$ into glycosaminoglycans. The regulation of polysaccharide chains and protein synthesis occurs at different levels [24]. NO may modulate polysaccharide chain elongation or sulfatation without modifying protein synthesis. On the other hand, the inhibition of sulfated glycosaminoglycans production by NO observed by Taskiran *et al.* could be explained by NO's capacity to inhibit PGE_2 production. PGE_2 have been reported to enhance proteoglycans synthesis. Therefore, the increased PGE_2 production following L-NMMA treatment might result in an offsetting increase in proteoglycan synthesis.

Although there is general agreement that NO is involved in inflammation, the literature is confusing, as both pro-inflammatory and antiinflammatory properties have been ascribed to NO. NO was originally identified as the endothelium derived relaxing factor with possible proinflammatory effects including augmentation of vascular permeability in inflamed tissues [25], the generation of destructive free radicals such as peroxynitrite and hydroxyl radical [26] and the induction of inflammatory cytokines like IL-1 and TNF [27]. Further evidence that NO may be antiinflammatory under certain circumstances is provided by its ability to inhibit the synthesis of PGE_2 by chondrocytes [28], the production of thromboxane and IL-6 by kupffer cells [29] and to suppress the production of superoxide anions by neutrophils through a direct action upon NADPH oxidase [30]. We showed that the inhibition of NO production induced a significant overproduction of IL-6, IL-8 and PGE_2 by human chondrocytes suggesting that NO downregulates the synthesis of some inflammatory mediators. Studies have reported that NO inhibits the synthesis of IL-6 and cyclooxygenase products by rat Kupffer cells [29]. On the other hand, conflicting

results have been described on IL-8 expression in response to NO or NO synthase blockade. NO donors increase and NO synthase inhibitors decrease stimulated IL-8 production by human neutrophils [31], whole blood [32] or endothelial cells [33]. These factors are involved in the development and maintenance of inflammatory and immune reactions accompanying arthritis. Indeed, IL-6 is a potent B cell growth and differentiation factor [34]. Therefore, through augmentation of rheumatoid factor and other antibodies in the synovium, it could indirectly exacerbate inflammation. Moreover, it also induces acute phase protein synthesis such as C-reactive protein (CRP) and serum amyloid A (SAA) [35]. IL-8 is one of the most important chemotactic factors for neutrophils and T-lymphocytes. IL-8 also induces neutrophil degranulation and thus may participate in cartilage and bone destruction in rheumatoid arthritis [36, 37]. Finally, PGE_2 produced by chondrocytes may alter vascular permeability and exacerbate liquid extravasion in the joint cavity [38]. Our results also suggest that agents that inhibit NO synthesis may contribute to the development and persistence of synovial membrane inflammation by increasing the production of proinflammatory cytokines. Moreover, other studies reveal that L-NMMA is a poor therapeutic agent in rat adjuvant induced arthritis once the disease was fully established [39].

The mechanisms of cartilage growth, differentiation and repair involved both the synthesis and the degradation of extracellular matrix. These processes are modulated by growth factors, cytokines and cell-cell and cell-matrix interactions. Studies have reported that biosynthetic activities and the response of cells to growth factors are modulated by cell culture density [40]. These findings can be related to our observation that chondrocytes cultured in high density produce less NO than in low density. We hypothesized that NO synthase activity was regulated by cell-cell contacts and that the amounts of endogenously produced NO may modulate chondrocyte bioactivity.

In conclusion, our study confirms the negative feed-back of NO on PGE_2, IL-6 and IL-8 synthesis by human chondrocytes. This mechanism is important in the control of joint inflammation and suggests new therapeutic approaches.

Acknowledgments

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ANNEXE 6

Regulation by reactive oxygen species of interleukin-1ß, nitric oxide and prostaglandin E_2 production by human chondrocytes

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Summary

Objectives. To determine the effects of two drugs, N-monomethyl-L-arginine (L-NMMA) and N-acetylcysteine (NAC), on interleukin-1ß (IL-1ß), nitric oxide (NO) and prostaglandin E_2 (PGE_2) production by human chondrocytes. The effect of aceclofenac (ACECLO), a non steroidal anti-inflammatory drug (NSAID), was also examined.

Methods. Human chondrocytes were enzymatically isolated from osteoarthritic knee cartilage and then maintained in culture in suspension for 48h in the absence or in the presence of lipopolysaccharide (LPS) (10 µg/ml), L-NMMA (0.5 mM), NAC (1 mM) or ACECLO (6.10^{-6} M). IL-1ß and PGE_2 productions were quantified by specific immunoassays. Nitrite was measured in the culture supernatants by a spectrophotometric method based upon the Griess reaction. Cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS) and IL-1ß gene expressions were quantified by transcription of mRNA followed by real time and quantitative polymerase chain reaction. COX-2 protein expression was analysed by Western-blot.

Results. LPS markedly increased the expression of IL-1ß, iNOS and COX-2 genes. In parallel, NO_2 and PGE_2 amounts found in the culture supernatants were significantly enhanced whereas IL-1ß was immunologically undetectable. The addition of L-NMMA (0.5 mM) fully blocked LPS-induced NO production but greatly increased PGE_2 production suggesting a negative effect of NO on PGE_2 synthesis. Inversely, NO production was stimulated by NAC while PGE_2 production was not affected. Interestingly, NAC increased the IL-1ß and iNOS mRNA levels but did not significantly modify COX-2 mRNA expression. L-NMMA did not significantly affect the expression of IL-1ß, iNOS and COX-2. The amount of COX-2 protein did not change in the presence of the antioxidants. Finally, ACECLO fully blocked the production of PGE2 by chondrocytes without affecting the levels of COX-2 mRNA.

Conclusions. The stimulation of IL-1ß, NO and PGE_2 production by LPS is differentially controlled by reactive oxygen species (ROS). In fact, L-NMMA and NAC have different mechanisms of action on the regulation of NO and PGE_2 productions. L-NMMA fully inhibits NO but increases PGE_2 production whereas NAC upregulates NO but does not modify PGE_2 synthesis. The stimulating effect of L-NMMA on PGE_2 production is not controlled at the transcriptional level. These findings suggest that antioxidant therapy could have different effects according to the oxygen radical species targeted. ©2002 OsteoArthritis Research Society International

Key words: Reactive Oxygen Species, Chondrocyte, Nitric oxide, Cytokines, Arthritis

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Introduction

NO is a gaseous free radical, which is transformed to nitrite and nitrate in the presence of oxygen. NO is synthesized through L-arginine oxidation by a family of NO synthases. It rapidly reacts with superoxide anions ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^-$) which is a strong oxidant. It is well accepted that chondrocytes possess a NADPH oxidase and produced both ROS and NO [1, 2]. *In vitro*, ROS and NO productions are strongly stimulated by IL-1ß, which is a key cytokine in the pathogenesis of arthritis [3]. Nitric oxide is presented as a catabolic agent since it was demonstrated that intraarticular injection of L-NIL (n-iminoethyl-L-lysine), a selective inhibitor of inducible nitric oxide synthase, reduced the progression of cartilage erosion in an experimental osteoarthritic dog model [4]. *In vitro*, the inhibition of endogenous NO production by L-NMMA, another well established NO synthase inhibitor, led to the suppression of IL-1ß stimulating effects on metalloproteases [5] and partially reversed the IL-1ß inhibitory effect on glycosaminoglycan synthesis by chondrocytes [6]. Furthermore, NO inhibits IL-1 receptor antagonist (IL-1ra) production by chondrocytes, a mechanism possibly responsible for the enhancement of IL-1 biological activity [7].

Although there is a general agreement that NO is involved in cartilage destruction, the literature is unclear about the role played by NO in the initiation and progression of synovial membrane inflammation. NO was originally identified as the endothelium-derived relaxing factor with potential proinflammatory effects, including increase of vascular permeability, enhancement of PGE_2 synthesis [8-10] and the induction of catabolic cytokines like IL-1ß and TNFα. Further evidence that NO may be antiinflammatory under certain circumstances is provided by its ability to down-regulate PGE_2 [11-14], IL-6 and IL-8 production by chondrocytes and liver kupffer cells [13, 15] and to suppress the production of $O_2^{\cdot-}$ by neutrophils through a direct action upon NADPH-oxidase [16]. These conflicting results obtained with NO synthase inhibitor could be explained by the fact that intracellular signalling pathways are very sensitive to intracellular fluctuation of ROS levels. This hypothesis gains strong support from the finding that the two well studied nuclear transcription factors, NF-kB and AP-1, are activated when intracellular ROS are increased or decreased, respectively [17]. These factors are involved in rapid induction of gene transcription in response to a variety of pathogenic conditions, specifically chronic inflammation. Another explanation could be that NO reactivity could be strongly different according to its ability to react with other oxygen species to form new oxidative molecules. In fact, NO interacts easily with $O_2^{\cdot-}$ to form the most potent peroxynitrite but also derivative active free radicals such as peroxynitrous acid, hydroxyl radical and nitrogen dioxide [18].

This complex interdependent regulation of NO, IL-1ß and PGE_2 creates a dilemma for pharmacological intervention of NO. In pathological context, such as arthritis where both inducible NOS and COX-2 are activated, therapeutical strategies focused on regulating COX activity by NO may be of some benefit.

In view of these observations, this study was designed to investigate the effects of

antioxidant molecules on IL-1ß and PGE_2 productions by human chondrocytes and then to identify the regulatory role played by NO or derived reactive species.

Materials and Methods

Short term suspension culture of human chondrocytes

Cartilage specimens were obtained from the knee of 8 donors shortly after death (4 males and 4 females) with the mean age of 49 (47-52) years old, being excised from the superficial and medium layers and avoiding the calcified layer. Upon dissection, femoral and patellar articular surfaces were evaluated for the severity of the macroscopic cartilage lesions using a personal scale. Four different grades were considered: 0, normal white cartilage on all areas examined; I, presence of yellow-gray area with some superficial fibrillations on one or more articular surfaces; II, irregular surface with deep fibrillations on one or more articular surfaces; III, ulcers penetrating to subchondral bone on one or more articular surface. Four out of the eight cartilage samples collected showed lesion of grade III and four cartilage samples had lesion of grade I. Each culture was run with chondrocytes from a single patient. The cartilage slices were cut into small fragments and then subjected to sequential enzymatic digestions with hyaluronidase, pronase and collagenase as previously described [13]. The cells were then filtered through a nylon mesh (70 µm), washed three times, counted (1.10^6 cells/ml) and resuspended in 1 ml of adequate culture medium: DMEM without red phenol (Biowhittaker, Brussels, Belgium) supplemented with 10mM HEPES, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 1 % of ITS+ (ICN, Doornveld, Belgium). ITS+ is a culture supplement containing in one ml: 0.625 mg of insulin, 0.625 mg of transferrin, 0.625 µg of selenious acid, 125 mg of bovine serum albumin (BSA) and 0.535 mg of linoleic acid. The cells recovered after collagenase digestion of the tissue were 95 % viable (trypan blue test). Cells were then kept in culture medium for 48h in order to eliminate *in vivo* contamination with drugs that the donors may have taken before death.

After this washout period, cells (1.10^6 cells/ml in culture medium) were seeded in 15 ml polypropylene Falcon tubes and maintained under agitation on a gyratory shaker (100 rpm) in a 95 % air/5 % CO_2 environment. Chondrocytes were cultured for 48 h in the absence or in the presence of LPS (10 µg/ml, serotype 026: B6; Sigma-Aldrich, Bornem, Belgium) and with or without L-NMMA (0,5 mM; Calbiochem, San Diego, U.S.A.), NAC (1 mM); Sigma-Aldrich, Bornem, Belgium) or ACECLO (6.10^{-6} M; Prodesfarma, Spain). ACECLO was first dissolved in N,N-dimethylformamide (DMF, Sigma-Aldrich, Bornem, Belgium), and then diluted in culture medium to achieve the required concentration. The final concentration of DMF was 0.1 %. Similar amount of the vehicle was added to the controls. Cells and supernatant were then separated by centrifugation (1000 rpm; 5 min). Other chemicals were of the purest commercially grade available.

Nitric oxide assay

NO production was determined by quantifying its derived product nitrite in

conditioned medium using a spectrophotometric method based on the Griess reaction [19]. Briefly, 150 µl of conditioned culture medium or sodium nitrite ($NaNO_2$) standard dilutions were mixed with 100 µl of Griess reagent (0.5 % sulfanilanide, 0.05 % naphtyl ethylenediamine dihydrochloride, 2.5 % H_3PO_4) and incubated for 5 min at 37 °C. The limit of detection was 2 µM of nitrite. Before adding the Griess reaction, nitrate was converted to nitrite by treating sample with nitrate reductase (0.2 U/mL, Roche, Brussels, Belgium) [20]. The absorption was measured at 540 nm.

PGE_2 radioimmunoassay

PGE_2 level was determined in conditioned culture medium according to a previously described radioimmunoassay (RIA) [21] using a polyclonal antiserum, obtained from rabbit. The ^3H-labelled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule PGE_2 from Sigma-Aldrich (Bornem, Belgium). PGE_2 was measured in triplicate in a 100-µl aliquot of each sample. After 48 h of incubation at 4 °C, free antigen was separated from antibodies-antigen complex by charcoal precipitation and centrifugation (20 min at 1000g, 4 °C). The supernatant was counted by liquid scintillation. Intra- and interassay coefficients of variation were 6 and 10 %, respectively. The recovery of known amounts of PGE_2 added to the culture medium was satisfactory (90 to 97 % for the amounts of PGE_2 ranging from 2500 to 312 pg/ml). The limit of detection of the RIA was 20 pg/ml.

IL-1ß assays

IL-1ß production by chondrocytes was assayed in the culture media by two different enzyme amplified sensitivity immunoassays (EASIA) designed by R&D System (Abingdon, UK) and by Genzyme (Cambridge, U.S.A). The assays were directly performed in the culture media without previous extraction. The limits of detection of the two assays were 1 and 0.1 pg/ml, respectively.

DNA assay

Chondrocyte DNA content was directly correlated to the cell number of each culture. DNA content was measured in the cell pellet extracts using a fluorimetric method. This measurement ensures elimination of result variations due to the different number of chondrocytes [22].

Reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated by a single step guanidinium thiocyanate-phenol-chloroform method. Cells (3.10^6) were lysed using TRI REAGENT (Sigma-Aldrich, Bornem, Belgium) and the samples were processed following the manufacturer's protocol. RNA (2 µg) was reverse transcribed in a volume of 30 µl containing 1X RT buffer, 10 mM DTT, 1 mM dNTPs, 500 ng oligo(dT)15 primer, 140 U M-MLV Reverse Transcriptase and 40 U RNaseOUT (Life Technologies, Merelbeke, Belgium) for 60 min at 37 °C. The reaction was halted by an incubation at 95 °C for 5 min and by adding 100 µl of H_2O.

The polymerase chain reaction was carried out in an automatic DNA thermal cycler (Hybaid, Doornveld, Belgium). Primer sequences used to amplify the desired cDNA are as follows: hß-actin forward and reverse primers:5'-GTGGGGCGCC CCAGGCACCA-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3' ; hGAPDH forward and reverse primers: 5'-TTGGTATCGTGGAAGGACTCA-3' and 5'-TGTCATCATATTTGGCAGGTTT-3' ; hiNOS forward and reverse primers: 5'-CCATGGAACATCCCAAATAC-3' and 5'-TCTGCATGTACTTCATGAAGG-3' ; hCOX-2 forward and reverse primers: 5'-TTCAAATGAGATTGTGGGAAAA-3' and 5'-AGATCATCTCTGCCTGAGTATCTT -3' ; hIL-1ß forward and reverse primers: 5'-TCTCCGACCACCACTACAGCA A-3' and 5'-GGGGAACTGGGCAGACTCAA-3'.

The reaction mixture contained 2 µl cDNA (equivalent to 30 ng of RNA), 0.5 µM sense and antisense primer, 250 µM dNTPs, 1 x PCR buffer, 1.5 mM $MgCl_2$ and 1U Taq DNA polymerase (Life Technologies, Merelbeke, Belgium) in a final volume of 40 µl. The cycle program was set to denature at 95 °C for 45 s, to anneal at 55 °C for 45 s, and to extend at 72 °C for 90 s. The number of cycles was selected in the exponential phase of the amplification curve (20 cycles for ß-actin, 26 for iNOS, COX-2 and IL-1ß).

Electrophoresis of the PCR products was performed on 1.5 % agarose gels containing 0.1 µg/ml ethidium bromide.

Quantitative real-time PCR

PCR reaction was performed by using the LightCycler-FastStart DNA master Sybr Green I (Roche Diagnostics, Brussels, Belgium). This reaction mixture is provided as a 10-fold stock solution containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix, 10 mM $MgCl_2$ and SYBR Green I dye. For each LightCycler PCR, a mastermix of the following reaction components was prepared in a final volume of 18 µl: 2 µl LightCycler-FastStart DNA master Sybr Green I, 1.6 µl of 25 mM $MgCl_2$, 0.5 µM sense and antisense primer. The mastermix was loaded into glass capillary (Roche Diagnostics, Brussels, Belgium) and 2 µl of PCR template was added. The template source was either 3 ng first-strand cDNA or purified DNA standard.

Amplification was performed with a spectrofluorometric thermal cycler (LightCycler, Roche Diagnostics, Brussels, Belgium). After an initial denaturation step at 95 °C for 10 min, amplification was performed using 40 cycles of denaturation (95 °C for 15 s), annealing (temperature gradient: 68 °C to 58 °C with an increment of 0.5 °C per cycle during 20 cycles, continued by 20 cycles at 58 °C, for 5 s) and extension (72 °C for 15 s). For each run, a standard curve was generated from purified DNA ranging from 10^6 to 10 copies. To standardize mRNA levels, we amplified glyceraldehyde-3-phosphate deshydrogenase (GAPDH), a house keeping gene as internal control. Normalized gene expression was calculated as the ratio between sample and GAPDH cDNA copy number.

After amplification, a final melting curve was recorded by cooling the PCR mixture to 65 °C for 30 s and then slowly heating it to 95 °C at 0.1 °C/s. Fluorescence was measured continuously during the slow temperature rise to monitor the disso-

ciation of the dsDNA. Specificity of the expected IL-1ß, iNOS and COX-2 LightCycler products were demonstrated by melting curves analysis.

Amplification products performed in the LightCycler were checked by electrophoresis on 1.5 % ethidium bromide stained agarose gel. The estimated size of the amplified fragments matched the calculated size.

Western-blot analysis

After incubation with the different drugs, chondrocytes (5.10^6 cells) were washed three times with PBS and lysed by boiling for 5 min in 150 µl of lysis buffer (10 mM Tris-HCl, pH7.4, 1 % SDS, 0.1 mg/ml PMSF). Equal amounts of protein (20 µg) estimated by bicinchonimic acid reagent (Pierce, Rockford, USA) were loaded onto 10 % SDS-polyacrylamide gel and were transferred to nitrocellulose membrane (BioRad, Nazareth, Belgium). Sheep COX-2 purified enzyme (Cayman Chemical, Ann Arbor, U.S.A) was also loaded on the gel as positive control. The membrane was satured with 5% fat-free dry milk in phosphate-buffered saline (80 mM Na_2HPO_4, 25 mM NaH_2PO_4, 100 mM NaCl, pH7.5) with 1 % (v/v) Tween 20 (PBST) overnight at 4 °C. The membrane was incubated with purified polyclonal rabbit IgG antibody against a peptide corresponding to amino acids 567-599 of human COX-2 (Cayman Chemical, Ann Arbor, USA) at 1:1,000 dilution in PBST for 1h at room temperature. The blot was washed in PBST three times (10 min each). The blot was further incubated for 1 h at room temperature with goat anti-rabbit IgG antibody coupled to horse-radish peroxidase (Biosource, Nivelles, Belgium) at 1:20,000 dilution in PBST. After three washings (10 min) in PBST, the blot was incubated with chemiluminescence substrates (ECL Plus, Amersham, Rosendaal, The Netherlands) according to the manufacturer's instructions. The blot was exposed to a radiographic film for 15 s.

Calculation and statistical analysis

The results were expressed as amounts of NO_2 plus NO_3 and PGE_2 into supernatants per µg of DNA. The mean ± standard deviation (S.D.) of each variable was calculated. A multivariate repeated measures ANOVA model with calculation of contrast was performed on all of the experiments (MANOVA; SAS system; general linear models).

Results

Effects of L-NMMA, NAC and ACECLO on NO and PGE_2 productions by human chondrocytes

Chondrocytes were isolated from seven different donors. As previously described [13], NO and PGE_2 synthesis were significantly stimulated in the presence of LPS. In both basal and LPS-stimulated conditions the NO and PGE_2 productions varied markedly according to the donor considered. After 48h of incubation, the mean basal production of NO_2/NO_3 and PGE_2, calculated on seven different chondrocyte cultures, were 2.39 nmoles/µg DNA/48 h and 10.41 pg/µg DNA/48 h,

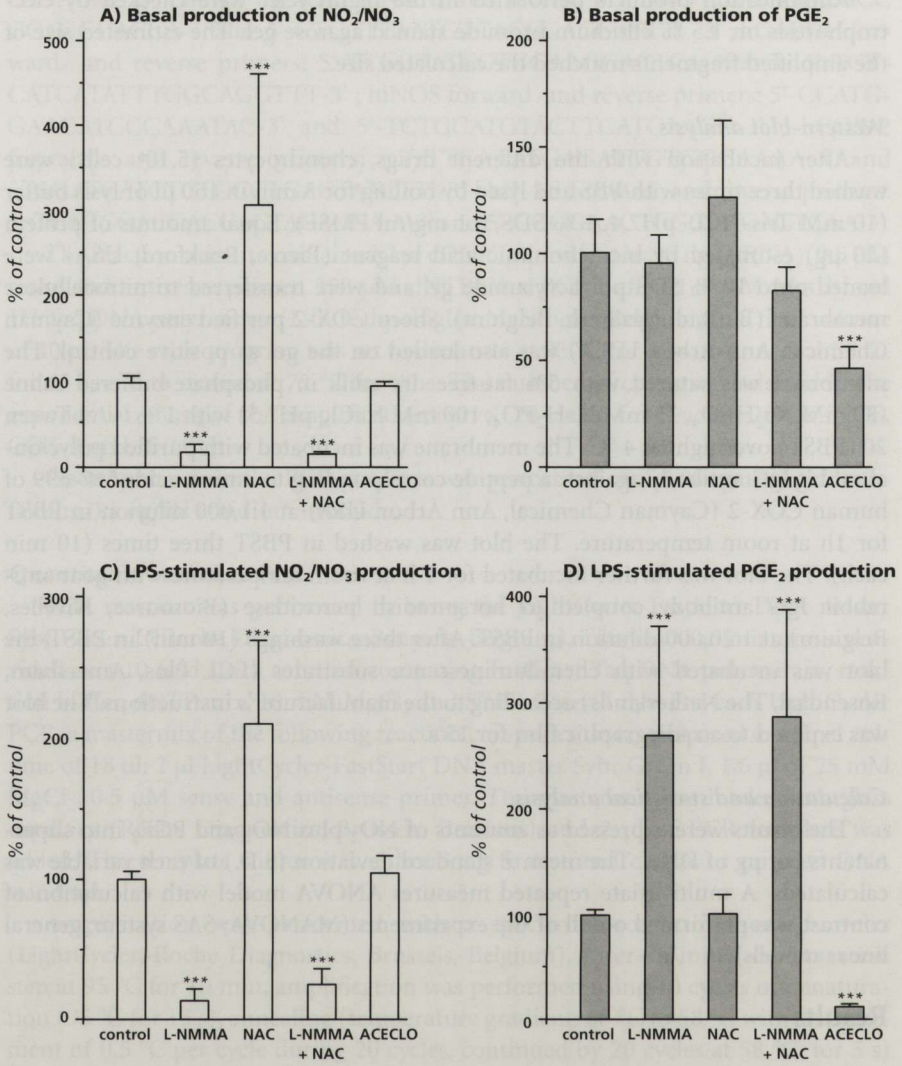


Fig. 1. *Effects of L-NMMA, NAC and ACECLO on the productions of NO_2/NO_3 and PGE_2 by human chondrocytes. Chondrocytes were cultured for 48 h in the absence (A, B) or the presence (C, D) of LPS (10 μg/ml) and with or without with L-NMMA (0.5 mM), NAC (1 mM), L-NMMA (0.5 mM) + NAC (1 mM) or ACECLO (6 .10^{-6} M). NO_2/NO_3 (white columns, A and C) and PGE_2 (hatched columns, B and D) concentrations were measured in conditioned culture supernatants and were normalized to the production obtained in the absence of the drug. Each condition was tested in triplicate. Results are the mean ± S.D. of seven experiments performed with cartilage specimens of different donors. Comparison of mean values was performed by ANOVA analysis. Drug treated groups are significantly different from the untreated groups: $^{***}p < 0.001$.*

respectively. LPS increased NO and PGE_2 productions by mean 2.8 and 7.2 times, respectively. Whichever donor was considered, IL-1ß was undectable.
As expected, L-NMMA reduced by 81 and 87 % the basal and LPS-stimulated NO production, respectively (Fig. 1A and 1C) ($p<0.001$). As previously described [13], the addition of L-NMMA resulted in a significant enhancement of LPS-stimulated PGE_2 production (Fig. 1D) ($p<0.001$) suggesting a negative effect of NO on PGE_2 synthesis.

Inversely, NAC increased both the basal and LPS-stimulated NO production (Fig. 1A and 1C) without significant effect on PGE_2 synthesis (Fig. 1B and 1D). When NAC and L-NMMA were added simultaneously, NO and PGE_2 productions varied in the same order of magnitude that when L-NMMA was added alone (Fig. 1). ACECLO, a non-steroidal antiinflammatory drug, strongly inhibited PGE_2 production by both untreated and LPS-treated chondrocytes but did not significantly modify NO production (Fig. 1).

Effects of L-NMMA, NAC and ACECLO on IL-1ß, iNOS and COX-2 mRNA expression

Real-time RT PCR was performed to quantify the drug effects on gene expression (Fig. 2). Under basal condition, both iNOS and COX-2 mRNA were expressed at low levels while IL-1ß mRNA was undetectable. As expected, LPS (10 µg/ml) induced a marked increase of IL-1ß, iNOS and COX-2 mRNA levels. After 48 h of incubation, LPS-stimulated chondrocytes expressed until 19,863 ± 642, 112,600 ± 2,326 and 11,283 ± 1,138 cDNA copies number per 3 ng of total RNA of IL-1ß, iNOS and COX-2 respectively.

L-NMMA (0.5 mM) did not significantly modify LPS-stimulated gene expressions (Fig. 2). NAC (1mM) enhanced IL-1ß ($p < 0.001$) and iNOS mRNA levels ($p < 0.01$) (Fig. 2A and B) without affecting the COX-2 gene expression (Fig. 2C). Finally, ACECLO (6.10^{-6} M) had a significant inhibitory effect on IL-1ß gene expression ($p < 0.01$) (Fig. 2A) but did not affect COX-2 and iNOS mRNA levels . These results were confirmed by classical RT PCR followed by agarose electrophoresis (Fig. 3).

Effects of L-NMMA and NAC on COX-2 protein

To determine the mechanism of action of L-NMMA on PGE_2 production, we studied the COX-2 protein expression by Western-blot analysis (Fig. 4). In the basal condition, COX-2 protein level was very low and was not modified by L-NMMA and NAC treatments. An LPS-inducible expression was observed. L-NMMA and NAC added separately or in combinaison had no significant effect on COX-2 protein level.

Discussion

We have demonstrated that the inhibition of NO_2 production by L-NMMA was accompanied by an increase of PGE_2 production suggesting a negative feedback of NO on PGE_2 synthesis. Our results are in accordance with previous studies showing

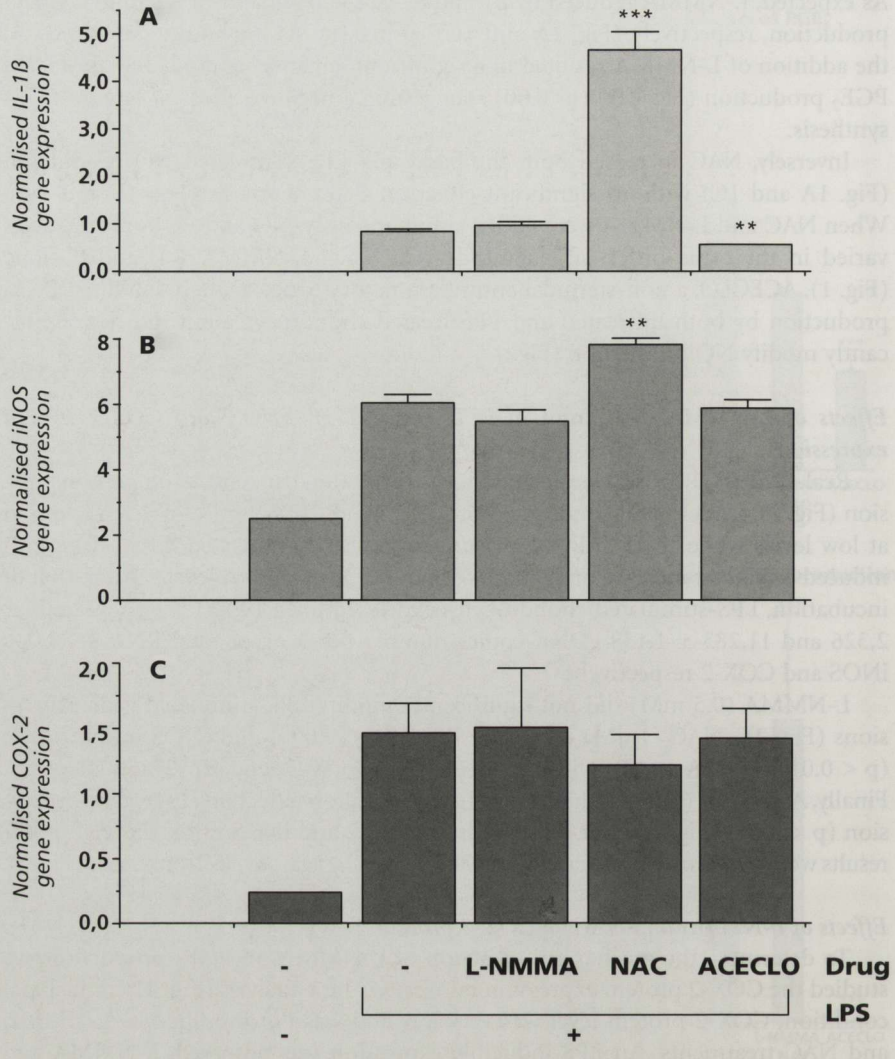


Fig. 2. *Effect of L-NMMA, NAC and ACECLO on IL-1ß, iNOS and COX-2 mRNA levels. Human chondrocytes were cultured for 48 h in the absence or the presence of LPS (10 µg/ml). Co-incubation with L-NMMA (0.5 mM), NAC (1 mM) or ACECLO (6 .10^{-6} M) were realized. Total RNA were isolated and mRNA of IL-1ß (A), iNOS (B), COX-2 (C) quantified by real-time RT PCR as described in Materials and Methods. The mRNA copy numbers were normalized against the corresponding copy number of GAPDH mRNA. Data represent mean ± SD of triplicate. Comparison of mean values was performed by ANOVA analysis (drug treated groups are significantly different from the untreated groups: ** $p < 0.01$,*** $p < 0.001$).*

by the inhibition of NO synthase or the incubation of cells with NO donors, that NO down-regulated PGE_2 production in cell line murine macrophages [23], in rat peritoneal macrophages [11], in human and bovine chondrocytes [13, 14] and in human osteoarthritic cartilage explants [12, 14]. In contrast, others have reported that NO enhanced COX-2 activity and PGE_2 production in different cell types [8-10]. Interestingly, Stadler [3] demonstrated a dual role of L-NMMA on PGE_2 production in rabbit chondrocytes culture. When low or high levels of NO were produced by cells activated by respectively IL-1ß alone or by a combination of IL-1ß plus LPS, an inhibition or an enhancement of the PGE_2 production was obtained. The Stadler's study provides a possible explanation for the discrepancy reported in the literature about the relation between NO and PGE_2 synthesis, suggesting a biphasic effect of NO according to its production level.

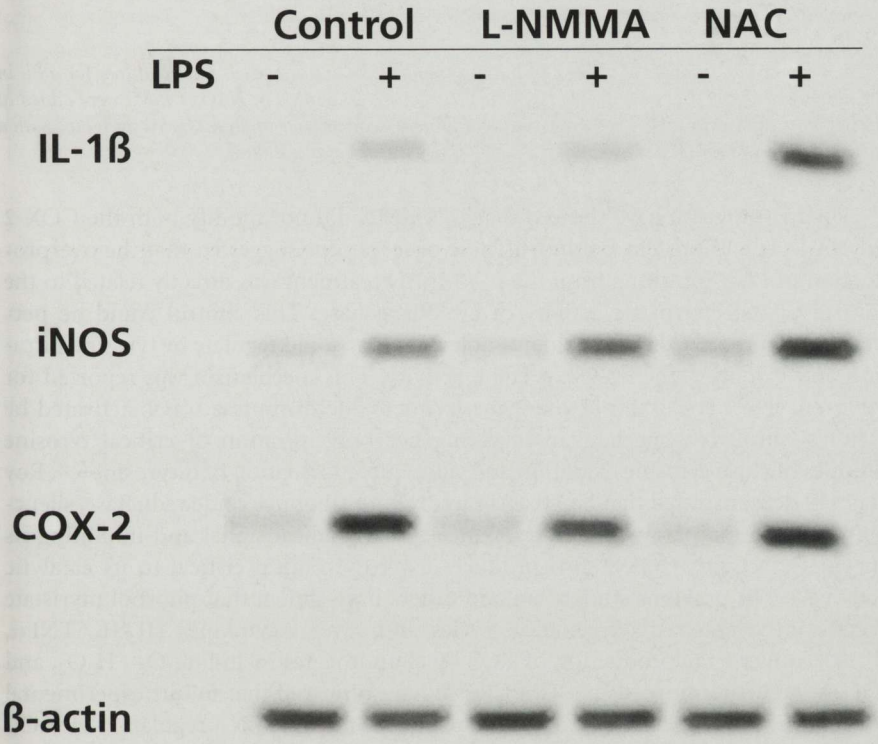


Fig. 3. *Effects of L-NMMA (0.5 mM), NAC (1 mM) and ACECLO (6.10^{-6} M) on gene expressions. RT-PCR products of IL-1ß, iNOS, COX-2 and ß-actin were fractionated by electrophoresis through a 1.5 % agarose gel and visualized by ethidium bromide staining.*

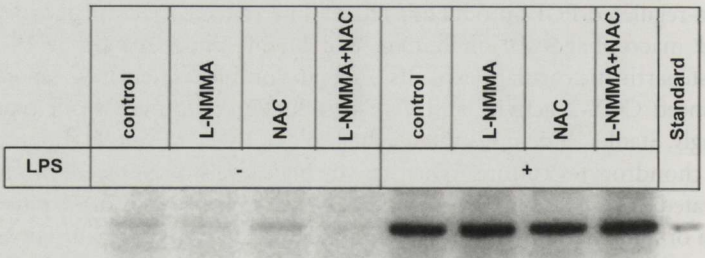


Fig 4. *Western blot analysis of COX-2 in human chondrocyte extracts. Cells were cultured for 48 h in the absence or in the presence of LPS(10 µg/ml). L-NMMA (0.5 mM) or NAC (1 mM) were added at the beginning of culture. The cells were lysed and protein extracts were analysed by Western blot with a specific antiserum for COX-2. Ovine COX-2 (400 ng) was used as standard.*

Furthermore, we have showed that L-NMMA did not modify both the COX-2 mRNA level and protein synthesis. These observations suggested that the overproduction of PGE_2 resulting from the L-NMMA treatment was directly related to the control of the enzymatic activity of cyclooxygenase. This control could be performed by nitrogen-derived oxidant molecules that could regulate by tyrosine nitration and oxidation the cyclooxygenase activity. This mechanism was reported for other enzymes. For example, manganese superoxide dismutase was inactivated by ONOO- through a mechanism involving not only nitration of critical tyrosine residues but also tyrosine oxidation and subsequent formation of dityrosine [24]. Roy *et al.* [25] demonstrated that NO reacts rapidly with ribonucleotide reductase, silencing its electronic paramagnetic resonance tyrosyl radical signal and inhibiting its enzymatic activity. COX-2 also produces a tyrosyl radical critical to its catalytic activity [26]. In previous studies, we and others have shown that phorbol myristate acetate, LPS, anoxia-reoxygenation cycles and several cytokines (IL-1ß, TNFα, IFNγ), induced the production of ROS by chondrocytes including $O_2^{\cdot-}$, H_2O_2, and $^{\bullet}OH$ [1, 2]. Furthermore, using RT-PCR, we have observed that in our experimental conditions, chondrocytes expressed the p22 sub-unit which is a regulatory element of the NADPH oxidase (data not shown). These data suggest that ROS, produced intracellularly by LPS-stimulated chondrocytes, could react with NO to form ONOO-, which is presented as a potential enzyme inhibitor. Finally, we can also suggest that NO or a nitric-oxide derived oxidant reacts with the phopholipase A2 and inhibits the release of arachidonic acid, the subtrate of cyclooxygenases. Nevertheless, Kosonen and colleagues [27] did not confirm this latter hypothesis showing that the addition of exogenous arachidonic acid to endothelial cell cultures

did not alter the inhibitory effect of NO donors on prostacyclin production, suggesting that phosholipases (PLA) are not the target of NO action. On the other hand, Martinez *et al.* [28] reported an increase of PLA2 activity in the membrane fraction of murine peritoneal macrophages submitted to SIN-1, a $NO/O_2^{\cdot-}$ donor. This stimulating effect was markedly inhibited in the presence of SOD while L-NMMA had no effect.

In our study, we have also demonstrated that NAC increased NO synthesis and iNOS gene expression but was without effect on PGE_2 production and COX-2 mRNA levels. These findings corroborate the results of Jiang and Brecher [29] demonstrating that N-acetyl-cysteine facilitated IL-1ß-induced nitric oxide synthase (iNOS) expression in rat vascular smooth muscle cells through a reduction/oxidation-related mechanism involving potentiation of cytokine activation of the p44/42 MAPK signalling pathway. Another explanation was given by Huang and colleagues [30], who reported that ascorbic acid enhanced endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin BH_4 content. Concerning the regulation of COX activity by antioxidants, our study was in disagreement with other studies which reported that known antioxidants, such as butylated hydroxyanisol or diphenylamine, were also potent inhibitors of arachidonic acid metabolism [31].

ACECLO is a NSAID of the phenylacetic acid class, commonly used in the treatment of arthritis. Interestingly, the mechanism of action of ACECLO is multifactorial. Indeed, this NSAID acts not only by inhibiting preferentially the COX-2 activity but also by decreasing proinflammatory cytokines synthesis and by scavenging reactive oxygen species [32]. At 1.10^{-5} M, ACECLO decreased luminol-enhanced chemiluminescence generated by PMA-activated neutrophils and accelerated the decomposition of ONOO-, suggesting a potential capacity of this drug to scavenge ONOO-. In contrast, and by comparison with other classical NSAID, ACECLO did not reduced lipid peroxydation induced by Fe^{2+}/ascorbate system and did not significantly reduced HOCl induced chemiluminescence. Furthermore, we have recently demonstrated that, at therapeutic concentration (ranged among 6 to 10 10^{-6} M) ACECLO did not react with •OH or $O_2^{\cdot-}$ (submitted data). Taken together, these findings demonstrated that ACECLO preferentially inhibited ONOO- but not $O_2^{\cdot-}$, •OH or HOCl. Considering these particular antioxidant properties, we have tested this drug on the expression of COX-2, IL-1ß and iNOS genes, and on the synthesis of PGE_2 and NO. Interestingly, ACECLO fully blocked PGE_2 synthesis without affecting COX-2 gene expression. Furthermore, we have previously reported that ACECLO strongly inhibited COX-2 activity ($IC_{50} = 8.10^{-7}$ M) in the whole blood test but showed an anecdoctal effect on purified cyclooxygenases [33]. These observations suggested that ACECLO probably exerts its inhibitory action on PGE_2 synthesis at the post-transcriptional level but not by a direct interaction with the catalytic site of COX.

Taken together these data clearly demonstrate that NO down-regulates PGE_2 synthesis at the post-transcriptional level and that antioxidant, such as N-acetyl-cysteine, increase NO synthase activity and expression. These findings suggest that

antioxidant therapy could have contradictory effects as a function of the molecule administered, especially the oxygen radical targeted. To block NO could result in an increase of PGE_2 production, which was, clearly identify as an inflammatory and catabolic factor. Increasing the antioxidant status of the cell could induce an over-production of NO.

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ANNEXE 7

Reactive oxygen species downregulate pro-inflammatory gene expressions by human chondrocytes

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ABSTRACT

Objectives: To determine the regulatory effects of reactive oxygen species (ROS) on the expression by human osteoarthritic chondrocytes of interleukin (IL)-1ß, -6 and -8, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene in response to interleukin (IL)-1ß or lipopolysaccharide (LPS).

Methods: Human chondrocytes in monolayer culture were incubated for 3 h with ROS generating molecules such as S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 100 µM), 3-morpholinosydnonimine (SIN-1, 100 µM), with chemically synthesised peroxynitrite (ONOO-, 10 µM) or hydrogen peroxide (H_2O_2, 100 µM). After treatment by ROS, chondrocytes were washed and then cultured for the next 24 h with or without lipopolysaccharide LPS (10 µg/ml) or IL-1ß (1.10^{-11}M). IL-1ß, IL-6, IL-8, iNOS and COX-2 gene expression was analysed by real time and quantitative RT PCR. IL-6, IL-8 and prostaglandin (PG) E_2 productions were assayed by specific immunoassays. Nitrite was measured in the culture supernatants by the Griess procedure.

Results: LPS and IL-1ß stimulated IL-1ß, IL-6, IL-8, iNOS and COX-2 gene expression. SNAP significantly downregulated LPS induced overall gene expressions, whereas SIN-1 had no effect. ONOO- inhibited iNOS and COX-2 gene expression but not that of the cytokine genes. When chondrocytes were incubated with IL-1ß, SIN-1 and ONOO- dramatically decreased all gene expressions while SNAP was inefficient. H_2O_2 treatment inhibited both LPS and IL-1ß induced gene expressions.

Conclusions: These data give an evidence that ROS may have anti-inflammatory properties by depressing inflammatory gene expressions. Further, we demonstrate that ROS effects are dependent on the nature of radical species and the signalling pathway, which is activated. These findings should be taken into consideration for the management of antioxidant therapy in treatment of inflammatory joint diseases.

Key words: Reactive Oxygen Species, chondrocyte, nitric oxide, cytokines, arthritis
Running title: ROS on human chondrocytes

INTRODUCTION

The progressive deterioration and loss of cartilage leading to joint impairment are the common final pathogenic events in arthritis. Cartilage degradation is induced by soluble factors secreted by chondrocytes but also by cells of the inflamed synovium. It is clearly established that cytokines, mainly IL-1ß and TNF-α, can stimulate chondrocytes and synoviocytes to produce reactive oxygen species (ROS), factors responsible for cartilage degradation[1, 2]. Indirect evidence for ROS implication in cartilage degradation come from the presence of lipid peroxidation products[3], nitrite[4, 5, 6], nitrotyrosine[7], modified low-density lipoprotein and oxidised IgG[8, 9] in the biological fluids of patients with arthritis.

Recent studies have shown that chondrocytes possess NADPH-oxidase and •NO synthase[10, 11], and are capable to produce •NO and superoxide anions ($O_2^{\cdot}$) in response to cytokines (i.e. IL-1ß, TNFα or IFNγ) or endotoxin (i.e. LPS)[12, 13, 14]. The intracellular presence of ONOO- has been demonstrated by immunohistochemistry[15]. ROS contribute to cartilage degradation by acting directly on cartilage matrix components[16], by activating zymogen[1] but also by dysregulating chondrocyte metabolic functions. Furthermore, ROS serve as regulatory agents for intracellular signal transduction of catabolic cytokines. The binding activity of transcription factors such as nuclear factor kappa B (NF-κB) and activated protein-1 (AP-1), is increased by IL-1ß through a mechanism involving ROS[17]. Recently, ROS have been suggested to act as second messengers molecules transducing some of the deleterious effects of IL-1ß in chondrocytes [18].

Although, there is a general agreement that ROS are involved in cartilage breakdown[19, 20], the literature is unclear about the role played by ROS in the initiation and progression of synovium inflammation in arthritis. Further evidence that •NO may be anti-inflammatory under certain circumstances is provided by its ability to downregulate PGE_2[21, 22, 23, 24], IL-6 and IL-8 production by chondrocytes and liver kupffer cells [23, 25] and to suppress the production of $O_2^{\cdot}$ by neutrophils through a direct action upon NADPH-oxidase[26]. Furthermore, some investigators have found that deletion of the iNOS gene in mutant mice renders these animals more susceptible to adjuvant[27] and septic arthritis[28, 29]. *In vivo*, the treatment of streptococcal cell wall-induced arthritis in rat with N-iminoethyl-L-lysine (L-NIL), a specific competitive inhibitor of the inducible isoform of •NO synthase, failed to reduce the acute response and exacerbated the chronic inflammatory response, as reflected by profound tissue destruction and loss of bone and cartilage[30].

Here, we postulated that •NO, but also other ROS, might modulate synovium inflammation by regulating the synthesis of cytokines by chondrocytes and that this effect is dependent on the ROS nature and the signalling pathway activated. To test this hypothesis, human osteoarthritic articular chondrocytes were exposed to different ROS for a short-term period, prior incubation with IL-1ß or LPS. For the first time, we showed that IL-1ß stimulating effect on pro-inflammatory cytokine synthesis is preferentially regulated by peroxynitrite.

MATERIALS AND METHODS

Human osteoarthritic chondrocyte culture

Cartilage was obtained from the knees immediately after death, being excised from the superficial and medium layers and avoiding the calcified layer. Upon dissection, femoral and patellar articular surfaces were evaluated for gross pathological cartilage modifications. The severity of pitting was recorded for each donor using the scale of Moskowvitz *et al.*[31]. Four different grades were considered: 0, normal white cartilage in all areas examined; I, the presence of a yellow-grey area with some superficial fibrillations on one or more articular surfaces; II, irregular surface with deep fibrillations on one or more articular surfaces and III, ulcers penetrating to the subchondral bone on one or more articular surfaces. Experiments were performed with OA cartilage from 5 donors (1 female and 4 males) with the mean age of 57 (46 - 74) years. All donors showed OA cartilage lesions of grade II (n = 2) or III (n = 3). The cartilage was cut into small fragments and then subjected to enzymatic digestions sequentially with hyaluronidase, pronase and collagenase as previously described[23]. After collagenase digestion, cell viability was estimated by trypan blue exclusion and in all cases was superior to 95 %. The cells were then filtered through a nylon mesh with a pore diameter of 70 mM, and then washed three times, counted and suspended in Dubbelcco's Modified Eagle medium (DMEM; Biowhittaker, Brussels, Belgium) supplemented with 10 % fetal calf serum (FCS) (Biowhittaker, Brussels, Belgium), 10mM HEPES, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 2mM glutamine. Cells were then seeded in a 6-well plate at 1.3 10^6 cells per well and grew until confluence. The confluent cells were washed with phosphate buffer saline (PBS) and the FCS-containing medium was replaced by a nutrient medium composed by DMEM without red phenol (Biowhittaker, Brussels, Belgium), supplemented with 2mM glutamine, 10mM HEPES, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 1% of ITS+ (ICN, Doornveld, Belgium). ITS+ is a culture supplement containing in one ml: 0.625 mg of insulin, 0.625 mg of transferrin, 0.625 µg of selenious acid, 125 mg of bovine serum albumin (BSA) and 0.535 mg of linoleic acid. Cells were then maintained in this culture medium for 24 h in order to eliminate the serum components. Human chondrocytes were washed and then incubated for 3 h in Hank's Balanced Saline Solution (HBSS) with S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 100 µM, ICN, Doornveld, Belgium), a •NO donor; 3-morpholinosydnonimine (SIN-1, 100 µM, ICN, Doornveld, Belgium), a compound generating both •NO and $O_2^{\cdot}$; peroxynitrite (ONOO-, 10 µM) chemically synthesised as described by Kohnen *et al.* [32] or hydrogen peroxide (H_2O_2, 100 µM, Sigma-Aldrich, Bornem, Belgium). After washings, the cells were cultured for the next 24 h in the ITS+ supplemented culture medium with or without lipopolysaccharide (LPS, 10 µg/ml, serotype 026:B6, Sigma-Aldrich, Bornem, Belgium) or recombinant human interleukin-1ß (IL-1ß, 1.10^{-11}M, Roche Diagnostics, Brussels, Belgium). In our experimental conditions, 100 µM SIN-1, 100 µM SNAP and 10 µM ONOO– generated ± 80 µM of NO_2-/NO_3-. This production was in the same order of magnitude than the level of NO_2-/NO_3- found in the supernatants of primary

human chondrocyte cultures stimulated by IL-1ß or LPS. H_2O_2 was added at the concentration of 100 µM, which is the concentration currently used in the literature [33, 34, 35].

DNA assay

Chondrocyte DNA content was directly correlated to the cell number of each culture. DNA content was measured in the cell pellet extracts using a fluorimetric method [36]. This measurement ensures elimination of result variations due to the different number of chondrocytes.

LDH release assay

Cell viability was estimated by the release of lactate deshydrogenase (LDH) in the culture supernatant. 100 µl of the supernatant or dilutions of standard solution of LDH from rabbit muscle were mixed with 50 µl of Tris buffer (10 mM Tris-HCl (pH 8.5), 0.1% BSA) containing 800 mM lactate. 100 µl of colorimetric reagent (1.6 mg/ml iodonitrotetrazolium chloride (Sigma-Aldrich, Bornem, Belgium), 4 mg/ml nicotinamide adenine dinucleotide (Roche Diagnostics, Brussels, Belgium), 0.4 mg/ml phenazine methosulfate (Sigma-Aldrich, Bornem, Belgium) was added and the absorbance at 492 nm was read after 10 min of incubation at room temperature.

Analysis of the DNA fragmentation

Fragmentation of internucleosomal DNA was used as a marker of apoptosis. Cellular DNA was purified by the Apoptotic DNA ladder kit (Roche Diagnostics, Brussels, Belgium) and then, subjected to electrophoresis on 1.5 % agarose gel containing 0.1 mg/ml of ethidium bromide.

Quantitative Real-time RT PCR

RNA from 3.10^6 cells was isolated by SV total RNA isolation system (Promega, Leiden, The Netherlands). RNA (0.5 µg) was reverse transcribed in 30 µl of RT buffer containing 10 mM DTT, 1 mM dNTPs, 500 ng oligo(dT)15 primer, 140 U M-MLV Reverse Transcriptase and 40 U RNaseOUT (Life Technologies, Merelbeke, Belgium) for 60 min at 37 °C. The reaction was stopped by incubating the samples at 95 °C for 5 min and by adding 100 µl of H_2O.

Polymerase chain reaction (PCR) was performed by using the Light Cycler-FastStart DNA Master Sybr Green I (Roche Diagnostics, Brussels, Belgium). The PCR template source was either 3 ng first-strand cDNA or purified DNA standard. Primer sequences used to amplify the desired cDNA were as follows: hGAPDH forward and reverse primers: 5'-TTGGTATCGTGGAAGGACTCA-3' and 5'-TGTCATCATATTTGGCAGGTTT-3'; hiNOS forward and reverse primers: 5'-CCATGGAACATCCCAAATAC-3' and 5'-TCTGCATGTACTTCATGAAGG-3'; hCOX-2 forward and reverse primers: 5'-TTCAAATGAGATTGTGGGAAAA-3' and 5'-AGATCATCTCTGCCTGAGTATCTT-3'; hIL-1ß forward and reverse primers: 5'-TCTCCGACCACCACTACAGCAA-3' and 5'-GGGGAACTGGGCAGACTCAA-3'; hIL-6

forward and reverse primers: 5'-CAATGAGGAGACTTGCCTGG-3' and 5'-GCACAGCTCTGGCTTGTTCC-3'; hIL-8 forward and reverse primers: 5'-TGACTTCCAAGCTGGCCGTG-3' and 5'-CGCAGTGTGGTCCACTCTCA-3'. Amplification was performed with a spectrofluorometric thermal cycler (LightCycler, Roche Diagnostics, Brussels, Belgium). After an initial denaturation step at 95 °C for 10 min, amplification was performed using 40 cycles of denaturation (95 °C for 15 s), annealing (temperature gradient: 68 °C to 58 °C with an increment of 0.5 °C per cycle during 20 cycles, continued by 20 cycles at 58 °C, for 5 s) and extension (72 °C for 15 s). For each run, a standard curve was generated from purified DNA ranging from 10^6 to 10 copies. To standardize mRNA levels, we amplified glyceraldehyde-3-phosphate deshydrogenase (GAPDH), a house keeping gene, as internal control. Gene expression was normalized by calculating the ratio between the number of cDNA copies of IL-1ß, IL-6 and IL-8, iNOS, COX-2 and the number of cDNA copies of GAPDH.

After amplification, a final melting curve was recorded by cooling the PCR mixture to 65 °C for 30 s and then slowly heating it to 95 °C at 0.1 °C/s. Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of the dsDNA. Specificity of the expected LightCycler products was demonstrated by melting curves analysis.

Amplification products performed in the LightCycler were checked by electrophoresis on 1.5 % ethidium bromide stained agarose gel. The estimated size of the amplified fragments matched the calculated size.

•NO assay

•NO production was determined by measuring nitrite (NO_2^-) in conditioned culture medium. Nitrite is the stable end product of •NO. It was measured by a spectrophotometric method based on the Griess reaction[37]. Briefly, 100 µl of the culture supernatant or sodium nitrite ($NaNO_2$) standard dilutions were mixed with 100 µl of the Griess reagent (0.5 % sulfanilanide, 0.05 % naphtyl ethylenediamine dihydrochloride, 2.5 % H_3PO_4) and incubated for 5 min at 37°C. Before adding the Griess reaction, nitrate was converted to nitrite by adding nitrate reductase (0.2 U/ml, Roche Diagnostics, Brussels, Belgium) to the culture supernatants. The absorption was measured at 540 nm. The limit of detection was 2 µM of nitrite.

Immunoassays for IL-6 and IL-8

IL-6 and IL-8 were assayed in the culture supernatants using two Enzyme Amplified Sensitivity Immunoassays (Cytosets, EASIA) designed by Biosource Europe (Nivelles, Belgium). The assays were directly performed in the culture supernatants without previous extraction. The intra and interassay coefficients of variation were less than 5 %. Recovery of cytokines in a chondrocytes conditioned culture medium was comprised of between 87 to 111 % for amounts of cytokines ranged in the linear part of the curve. The sensitivity of the assay was 8 pg/ml for IL-6 and 2 pg/ml for IL-8.

Prostaglandin (PG) E_2 radioimmunoassay

PGE_2 was assayed in conditioned culture medium according to a described radioimmunoassay (RIA)[38]. PGE_2 was directly measured in the culture medium without previous extraction. The ^3H-labeled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule PGE_2 from Sigma Chemical Co. (St Louis, MO, USA). After 48 h of incubation at 4 °C, free antigen was separated from antibodies bound-antigen by charcoal precipitation and centrifugation (20 min at 1000g, 4 °C). The supernatant was counted by liquid scintillation. Intra and interassay coefficient of variations were 6 and 10 %, respectively. The recovery of known amounts of PGE_2 added to the culture medium was satisfactory (90 to 97 % for the amounts of PGE_2 ranging from 2500 to 312 pg/ml). The limit of detection of the RIA was 20 pg/ml.

Calculation and statistical analysis

Results were expressed as the mean ± SD of three experiments. Statistical significance was assessed using the ANOVA test, and significant differences were confirmed only when the probability was ≤ 5 %.

RESULTS

Effect of ROS on cells viability, DNA content and DNA fragmentation.

Pre-treatment of chondrocytes with ROS donors followed by a 24 h incubation with IL-1ß (1.10^{-11} M) or LPS (10 µg/ml) did not induced the release of LDH activity in culture supernatants (data not shown). Further, this sequence of treatments did not significantly modify the content of DNA in culture (Table 1) and did not induced DNA fragmentation.

Table 1. Effect of ROS pre-treatment on DNA content (mg)

	Controls	LPS (10 µg/ml)	IL-1ß (1.10^{-11} M)
HBSS	6.16 ± 0.16	5.53 ± 0.41	5.53 ± 0.25
SNAP	6.11 ± 0.14	5.34 ± 0.44	5.69 ± 0.59
SIN-1	5.70 ± 0.50	5.91 ± 0.44	6.69 ± 0.16
ONOO-	6.99 ± 0.73	6.28 ± 0.46	6.27 ± 0.46
H_2O_2	5.99 ± 0.31	6.23 ± 0.68	6.19 ± 0.17

Chondrocytes were incubated for 3 h in HBSS with the different reactive oxygen species and then cultured for 24 h in ITS+ supplemented DMEM culture medium with or without LPS or IL-1ß. The content of DNA was measured at the end of the culture. Each experimental condition was tested in triplicate. The results are expressed as the mean ± SD.

Effects of ROS on LPS induced gene expression

In the basal conditions, OA chondrocytes spontaneously expressed per ng of total RNA, 235 ± 16 cDNA copies of IL-6, 6332 ± 92 cDNA copies of IL-8, 114 ± 13 cDNA copies of iNOS and 70 ± 2 cDNA copies of COX-2 whereas IL-1ß gene was

not expressed. At 10 µg/ml, LPS induced IL-1ß gene expression and increased IL-6, IL-8, iNOS, COX-2 mRNA levels by 23, 25, 7.5 and 11.2 times, respectively (figure 1). The pre-incubation with 100 µM SNAP significantly down-regulated LPS induced IL-1ß ($p < 0.05$), IL-6 ($p < 0.01$), IL-8 ($p < 0.01$), iNOS ($p < 0.001$), and COX-2 gene expression ($p < 0.001$), whereas 100 µM SIN-1 was inefficient. At 10 µM, ONOO– significantly decreased LPS stimulated iNOS and COX-2 mRNA levels ($p<0.001$ and $p<0.05$) but had no effect on IL-1ß, IL-6 and IL-8 gene expression. Finally, 100 µM H_2O_2 down-regulated the LPS stimulated IL-6, IL-8, iNOS ($p< 0.001$) and COX-2 ($p<0.01$) gene expression but had no effect on IL-1ß gene.

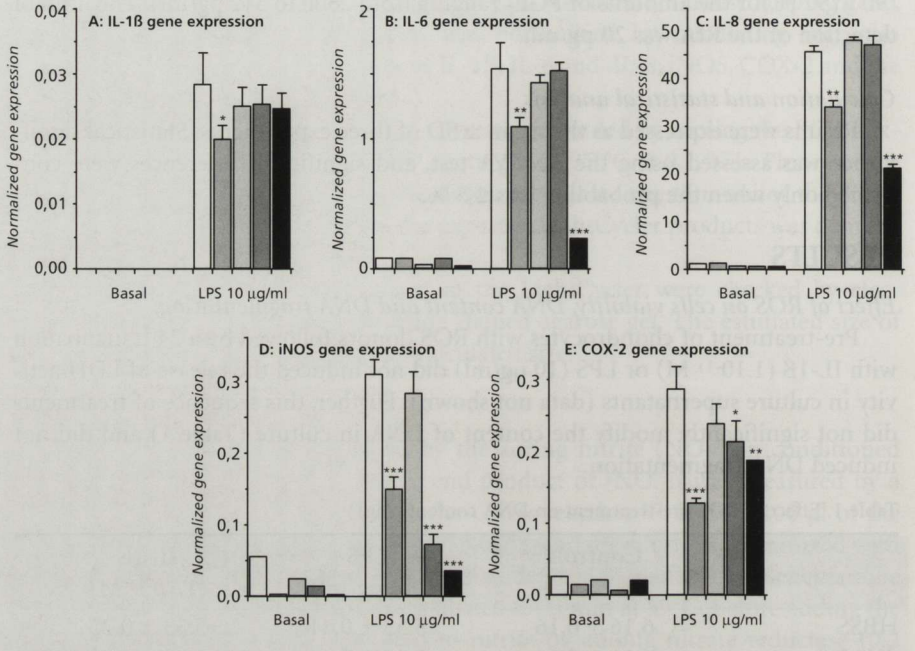


Fig. 1. *Effect of exogenous ROS treatment on LPS chondrocyte gene activation. Chondrocytes were incubated for 3h in HBSS (□)(control) or with SNAP (100 µM) (■), SIN-1 (100 µM) (□), ONOO- (10 µM) (■), or H_2O_2 (100 µM) (■) and then, cultured for 24 h in the absence (basal) or in the presence of 10 µg/ml of LPS. Total RNA were isolated and mRNA of IL-1ß (a), IL-6 (b), IL-8 (c), iNOS (d) and COX-2 (e) were quantified by real-time RT-PCR. The mRNA copy numbers were normalized against the corresponding copy number of GADPH mRNA. Comparison of mean values was performed by ANOVA test (drug treated groups are significantly different from the control group with * $p < 0.05$; ** $p < 0.01$ or *** $p < 0.001$).*

Effects of ROS on IL-1ß induced gene expression

At 1.10^{-11} M, IL-1ß increased IL-6, IL-8, iNOS and COX-2 mRNA levels by 42, 20, 5.3 and 8.9 times, respectively (figure 2). When chondrocytes were pre-incubated for 3 h with 100 µM SIN-1, 10 µM $ONOO^-$ or 100 µM H_2O_2 before IL-1ß stimulation, the effects of IL-1ß on IL-6, IL-8, IL-1ß, COX-2 and iNOS gene expression are significantly decreased (figure 2). In contrast, IL-1ß induced gene expression was not modified by 100 µM SNAP.

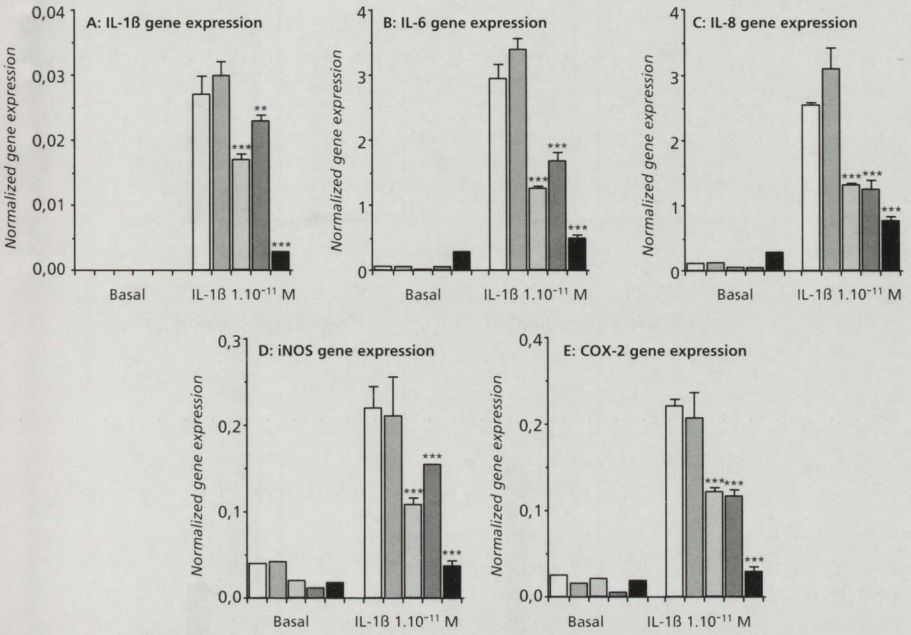


Fig. 2. *Effect of exogenous ROS treatment on IL-1ß chondrocyte gene activation. Chondrocytes were incubated for 3h in HBSS (□)(control) or with SNAP (100 µM) (▨), SIN-1 (100 µM) (□), $ONOO^-$ (10 µM) (▨), or H_2O_2 (100 µM) (■) and then, cultured for 24 h in the absence (basal) or in the presence of 1.10^{-11} M IL-1ß. Total RNA were isolated and mRNA of IL-1ß (a), IL-6 (b), IL-8 (c), iNOS (d) and COX-2 (e) were quantified by real-time RT-PCR. The mRNA copy numbers were normalized against the corresponding copy number of GADPH mRNA. Comparison of mean values was performed by ANOVA test (drug treated groups are significantly different from the control group with ** $p < 0.01$ or *** $p < 0.001$).*

Effects of ROS on the IL-1ß or LPS-induced IL-6, IL-8, •NO and PGE_2 synthesis

To determine whether inflammatory genes downregulation by ROS pre-incubation was associated with a decrease of IL-1ß, IL-6, IL-8, •NO and PGE_2 secretion, these factors were measured in the culture supernatants. Both LPS and IL-1ß significantly increased IL-6, IL-8, •NO and PGE_2 chondrocyte productions whereas IL-1ß was undetectable. Pre-treatment of chondrocytes with 100 µM of SNAP significantly inhibited LPS stimulated IL-6 and PGE_2 without affecting IL-8 and •NO

productions (figure 3). On the other hand, $ONOO^-$ only decreased PGE_2 production. Overall mediators were decreased by H_2O_2 pre-incubation while SIN-1 had no effect.

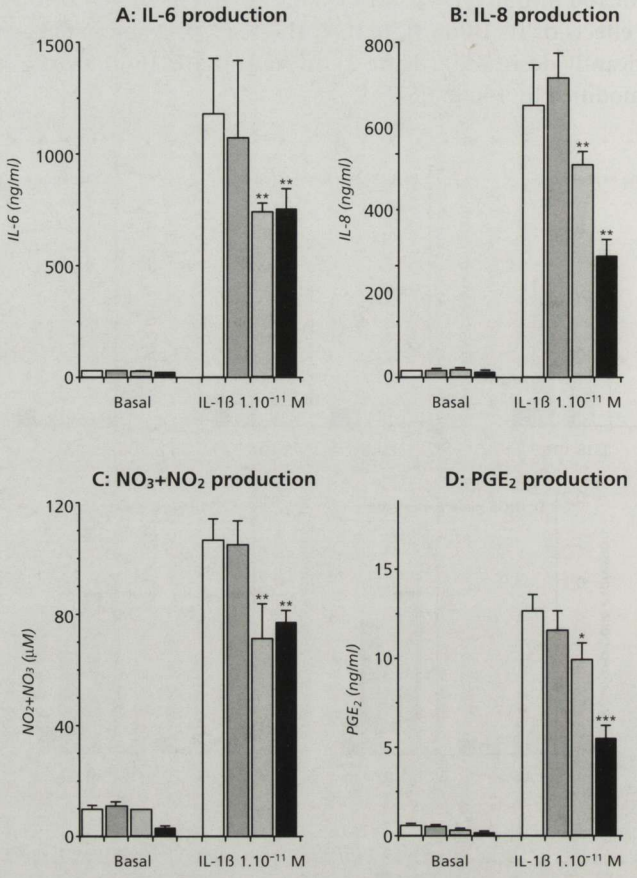


Fig. 3. *Effect of exogenous ROS treatment on LPS-induced inflammatory mediators production. Chondrocytes were incubated for 3h in HBSS (□)(control) or with SNAP (100 µM) (▨), SIN-1 (100 µM) (▨), ONOO⁻ (10 µM) (▨), or H_2O_2 (100 µM) (■) and then, cultured for 24 h in the absence (basal) or in the presence of 10 mg/ml of LPS. Total RNA were isolated and mRNA of IL-1ß (a), IL-6 (b), IL-8 (c), iNOS (d) and COX-2 (e) were quantified by real-time RT-PCR. The mRNA copy numbers were normalized against the corresponding copy number of GADPH mRNA. Comparison of mean values was performed by ANOVA test (drug treated groups are significantly different from the control group with ** $p < 0.01$ or *** $p < 0.001$).*

A good correlation between ROS effects on IL-1ß-induced gene expression and synthesis was observed (figure 5). SIN-1 and H_2O_2 pre-incubation resulted in an inhibition of IL-1ß induced IL-6, IL-8, •NO and PGE_2 production while SNAP remained inefficient.

Discussion

In previous studies, we have demonstrated that chondrocytes produced •NO in response to IL-1ß and LPS and that the inhibition of •NO production by L-N-monomethyl-L-arginine (L-NMMA) resulted in an increase of IL-6, IL-8 and PGE_2 synthesis[23]. These observations indicated that •NO might have anti-inflammatory properties by inhibiting inflammatory cytokines and prostanoids synthesis. We have also reported that the antioxidant N-acetylcysteine (NAC) molecule amplified LPS-induced IL-1ß and iNOS gene expression in human chondrocyte cultures[39] suggesting that ROS, other than •NO, were also involved in the regulation of inflammatory gene expressions. To check this hypothesis, we have submitted primary

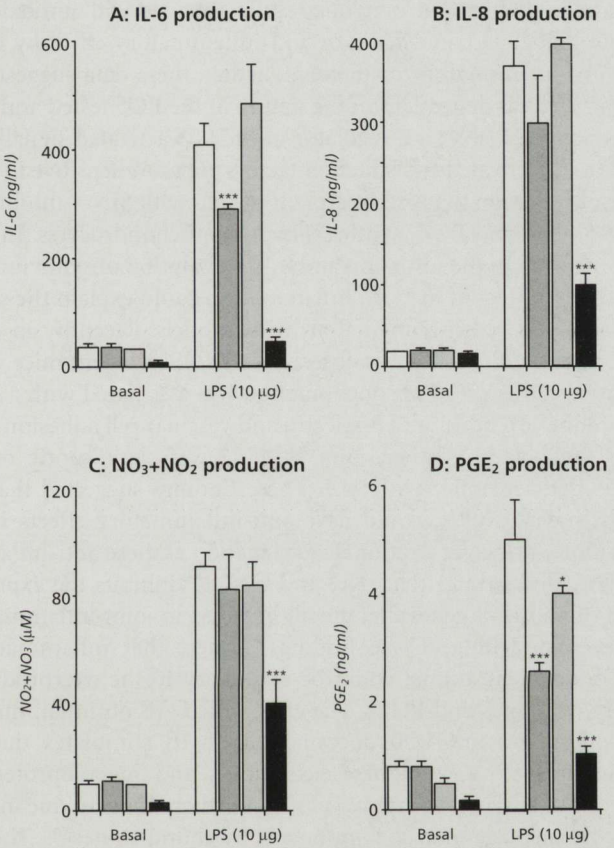


Fig. 4. *Effect of exogenous ROS treatment on IL-1ß chondrocyte gene activation. Chondrocytes were incubated for 3h in HBSS (□)(control) or with SNAP (100 µM) (▨), SIN-1 (100 µM) (□), $ONOO^-$ (10 µM) (▨), or H_2O_2 (100 µM) (■) and then, cultured for 24 h in the absence (basal) or in the presence of 1.10^{-11} M IL-1ß. Total RNA were isolated and mRNA of IL-1ß (a), IL-6 (b), IL-8 (c), iNOS (d) and COX-2 (e) were quantified by real-time RT-PCR. The mRNA copy numbers were normalized against the corresponding copy number of GADPH mRNA. Comparison of mean values was performed by ANOVA test (drug treated groups are significantly different from the control group with $p < 0.05$; ** $p < 0.01$ or *** $p < 0.001$).*

chondrocyte cultures for a short time to sublethal levels of different ROS, such as •NO, $ONOO^-$ and H_2O_2. After washings, chondrocytes were then treated by IL-1ß or LPS, two stimulators of the expression of IL-6, IL-8, iNOS and COX-2 genes. In these experimental conditions, none of the ROS tested significantly modified DNA content, induced LDH release from cells or DNA fragmentation. These data demonstrate that the effects of ROS reported in this study were not the consequence of cell death processes. SNAP pre-treatment downregulated LPS stimulated IL-1ß, IL-6, IL-8, iNOS and COX-2 gene expressions but had no effect on the IL-1ß-induced overexpression of these genes. In contrast, SIN-1 and $ONOO^-$ solution inhibited the IL-1ß stimulated expression of cytokine genes but did not modify LPS stimulating effect. H_2O_2 diminished both LPS and IL-1ß stimulated gene expressions. These findings support our previous results obtained with antioxidant molecules, indicating that ROS could have an anti-inflammatory effect by inhibiting the synthesis of pro-inflammatory mediators. Further, these data suggest that this anti-inflammatory effect is dependent of the nature of the ROS tested and of the signalling pathway activated. •NO is a regulator of the LPS activated signalling pathway whereas the IL-1ß activated transduction factors are more sensitive to $ONOO^-$. Interesting is the observation that pre-treatment of cells with SIN-1 inhibited IL-1ß induced IL-6, IL-8, •NO and PGE_2 synthesis by human chondrocytes. In arthritis, these factors are involved in the initiation and in the evolution of synovium inflammation. A net negative effect of ROS on inflammation could explain the somewhat baffling results observed in experimental arthritis models. Recently, an enhanced leukocyte adhesion and infiltration was observed in iNOS-deficient mice with antigen-induced arthritis (AIA)[29]. This phenomenon was associated with a increased expression of the adhesion molecules P-selectin and vascular cell adhesion molecule 1 (VCAM-1) by leukocytes. Further, joint swelling was significantly more pronounced in iNOS deficient mice with AIA. These findings suggested that •NO or derived products, such $ONOO^-$, could have anti-inflammatory effects in experimental arthritis. Our work gives a rationale explanation at these anti-inflammatory actions of ROS. It demonstrates that •NO and $ONOO^-$ inhibits the expression of COX-2, IL-1ß, IL-6 and IL-8 genes that are all involved in joint inflammation and associated tissues remodelling. These findings suggest that inflammatory flare observed in iNOS deficient animal could be secondary to the overproduction of pro-inflammatory cytokines and PGE_2. The effects of IL-1ß on inflammation and cartilage degradation are now well documented. IL-1ß stimulates the massive release of pro-inflammatory cytokines, eicosanoids and metalloproteinases by inflammatory cells but also by connective cells[26]. Further, this cytokine inhibits the synthesis of specific cartilage matrix components by chondrocytes[40]. IL-8 is considered as one of the most potent chemoattractant factor of neutrophils and T lymphocytes. It also activates neutrophils to release degradative enzymes and ROS[41]. *In vitro*, IL-8 induced cartilage explant degradation by stimulating the synthesis of matrix-degrading enzymes[42]. IL-6 is involved in propagation and chronicity of joint inflammation[43]. In the presence of its soluble receptor, IL-6 can also promote cartilage matrix degradation. The release of high level of PGE_2 at the inflammatory

site contributes to oedema and inflammatory pain.

Our study provides strong *in vitro* evidence that sublethal ROS production may have anti-inflammatory effect by down-regulating the synthesis of pro-inflammatory mediator at a transcriptional level. The regulatory actions of ROS seem to be species specific and, for a same ROS, dependent of the signalling pathway activated.

These findings should be considered when evaluating antioxidants as potential therapeutic agents for the treatment of arthritis.

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ANNEXE 8

In vitro differences among Nonsteroidal Antiinflammatory Drugs in their activities related to Osteoarthritis pathophysiology

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Osteoarthritis and Cartilage 1999; 7: 355-7

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used for the treatment of osteoarthritis (OA). These drugs are recognized as often relieving the symptoms of OA. Nevertheless, several types of NSAIDs, (e.g. as sodium salicylate), inhibit the synthesis of proteoglycans (PGs) and collagens by chondrocytes *in vitro*. Hence, they may diminish the potential of cartilage repair in OA.

These *in vitro* observations are compatible with *in vivo* studies suggesting that some NSAIDs (e.g. aspirin), accelerate the progression of OA. Therefore, it is important to determine the effects of a new NSAID on the anabolic functions of the chondrocytes. Since there are no validated non-invasive methods for quantifying the anabolic and catabolic activities of cartilage *in vivo*, much information on the adverse effects of NSAIDs on chondrocyte functions can be obtained by *in vitro* methods using cartilage tissue or differentiated chondrocytes. The aims of *in vitro* studies should be the screening of the effects of NSAIDs on chondroformation and chondroresorption processes but also on cytokines activity and synthesis and by this way allow the classification of these drugs according their pharmacological *in vitro* effects.

Dingle and collaborators [1] have divided NSAIDs in three categories, with respect to their *in vitro* action upon anabolic functions of OA cartilage: (1) those such as aceclofenac, which can stimulate matrix component synthesis; (2) those such as piroxicam, which express a neutral effect on cartilage anabolism; (3) those like ibuprofen, naproxen and indomethacin, which significantly inhibit anabolic functions of chondrocytes. However, caution must be taken with regard of these conclusions because they come from short-term organ culture with plasmatic concentration of NSAIDs. Moreover, they results vary on isolated chondrocyte culture systems. Some NSAIDs which show a anabolic effect after short-term treatment, may have adverse effects on cartilage integrity when administrated during long term treatment or at higher doses.

Some NSAIDs, such as indomethacin, may exert their deleterious effects on cartilage matrix synthesis by inhibiting prostaglandin E_2 (PGE_2) production [2]. PGE_2 have been demonstrated to enhance collagen and proteoglycan synthesis by human chondrocytes secondary to the production of IGF-I [3]. Moreover, PGE_2 inhibits the synthesis of IL-1. IL-1 is the most important catabolic cytokine in OA, capable to overcome the stimulating effect of IGF-I on matrix components synthesis [4]. Therefore, the inhibition of PGE_2 synthesis by NSAIDs may induce the release of IL-1 and then inhibit cartilage matrix protein synthesis (Fig. 1).

Although, all NSAIDs inhibit PGE_2 but only some show an inhibitory effect on matrix component synthesis. Other mechanisms deleterious include the inhibition of glycosyltransferase activity [5], the uncoupling of mitochondrial oxidative phosphorylation [6], the activation of cAMP-dependent kinase A [7] or the disruption of protein-protein interactions at the cellular membrane level [8].

On the other hand, the stimulatory effect of some NSAIDs may be related to their inhibitory effect on IL-1 production or IL-1 receptor expression [9] and thus could promote growth factor activity, including IGF-I and TGF-β. Moreover, a number of NSAIDs as tiaprofenic acid, naproxen and indomethacin inhibit

aggrecans degradation in cartilage tissue and suppress cartilage breakdown in animal models of OA. These beneficial actions of NSAIDs seem to be related to their inhibiting effect on the metalloproteinases synthesis and activity.

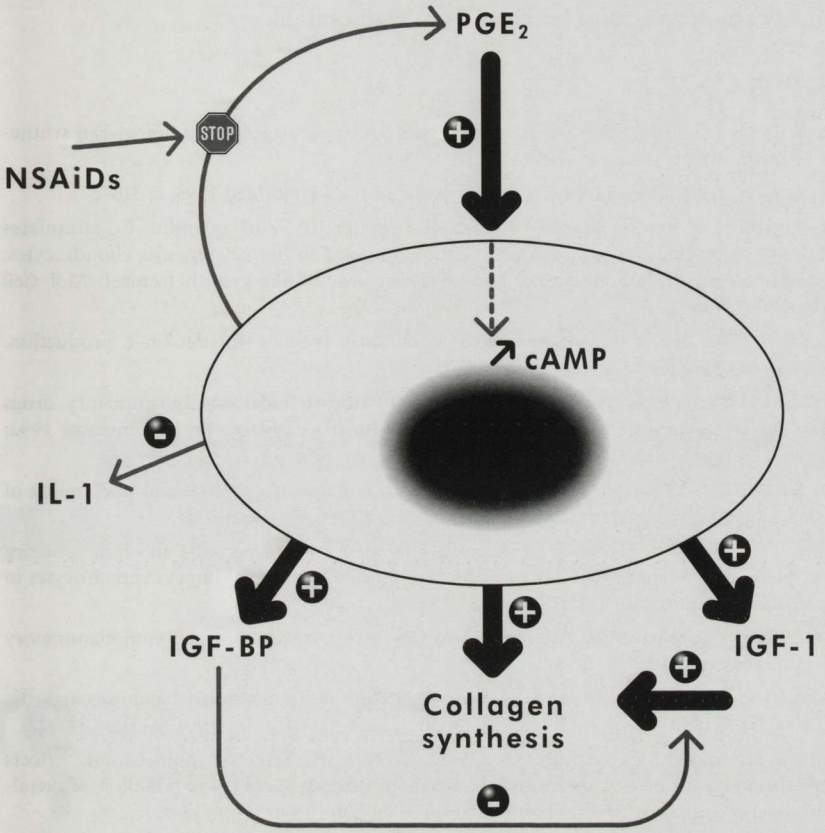


Figure 1: *Mechanisms of action of NSAIDs in cartilage anabolism: interaction between catabolic cytokines, growth factors and prostaglandins. IL-1, Interleukin-1; IGF-1, Insulin-like growth factor-1; IGF-BP, Insulin-like growth factor binding protein; cAMP = Cyclic adenosine monophosphate.*

The metalloproteinase activity may be modulated by NSAIDs stimulating action on the production by chondrocytes of tissue inhibitor of metalloproteinases-1 (TIMP-1) or by their inhibiting action on plasminogen activator synthesis [10].

Cartilage injury can also be mediated by active oxygen species ($O_2^{\cdot}$, H_2O_2, $^{\cdot}OH$) produced by chondrocytes and inflammatory cells present in the synovial membrane. Several NSAIDs, such as nimesulide, have the faculty to scavenge oxygen radicals and/or inhibit their production by NADPH oxidase [11].

Finally, it is interesting to note that NSAIDs effects on chondrocyte metabolic activities widely vary as a function of the concentration tested, the age of the donor, the metabolic activity of the chondrocytes and the severity of cartilage lesions.

It has been reported that osteoarthritic cartilage is more vulnerable to the influence of NSAIDs than intact cartilage.

In conclusion, *in vitro* research yields valuable informations on the bioactivity profile of NSAID drugs on chondrocyte metabolism. Nevertheless, extrapolation from *in vitro* to *in vivo* must be carried out with caution.

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ANNEXE 9

The inhibition of metalloproteinases to treat osteoarthritis: reality and new perspectives

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Abstract

The objective of this paper is to analyse the potential therapeutic action of MMP inhibitors in the management of osteoarthritis (OA), based on a critical review of the literature. The role played by metalloproteinases (MPs) in the pathophysiology of osteoarthritis is discussed as well as their regulation by tissular inhibitors, activators and cytokines. The evidences that commonly used drugs for treating osteoarthritis are also active on MPs synthesis is also reported. Finally, this paper provides an analysis of the recent patents published with promising therapeutic potential and gives new perspectives for anti-MPs therapy.

Key words : cartilage, inhibitors, metalloproteinases, osteoarthritis.

1. Introduction

Articular cartilage is essentially composed of an extensive hydrated matrix, synthesized and turned over by chondrocytes, the unique cell found in the cartilage. Cartilage matrix is mainly composed of aggrecan and type II collagen, whose synthesis is controlled by hormones, cytokines and mechanical stimuli. In normal conditions, cartilage matrix remodelling is a slow process consisting of a phase of chondrocyte resorption, directly compensated by a phase of chondrocyte repair [1]. In pathological conditions, as osteoarthritis (OA), cartilage turnover may accelerate, leading to an early repair reaction followed by cell decompensation and cartilage degradation. The appearance of fibrillation, matrix depletion, cell clusters and changes in matrix composition reflect the aberrant behavior of resident chondrocytes [2, 3].

Current knowlegde indicates an important involvement of metalloproteinases (MPs) in OA, mainly matrix metalloproteinases (MMPs) including collagenases (MMP-1, -8, -13), aggrecanases (ADAMTS-4, -5), membrane-type MPs (MT-MMPs), stromelysin-1 (MMP-3) and gelatinases (MMP-2, -9) which are all found to be elevated in human OA cartilage (Table 1) [4-8]. In OA, MMPs are secreted by chondrocytes, but also synovium cells including fibroblasts and macrophages. MMPs are synthesized as inactive proenzymes which are activated *in situ* by other MMPs, serine and cysteine proteinases [9-13]. Under physiologic conditions, the activity of MMPs is balanced by natural inhibitors that act at different levels of the proteolytic cascade of MMPs activation [14-16]. In contrast, in OA, a shift of this equilibrium leads to excessive matrix degradation [17]. This paper focuses on MPs activity regulation by natural activators and inhibitors but also by drugs used in the treatment of OA in human. Finally, this paper provides an analysis of the recent patents and debates on the relevance of anti-MPs therapy.

2. Metalloproteinases and cartilage homeostasis

Before discussing the role of MPs in the degradation of cartilage during OA progression, it is important to note that there is some evidence for a role of these

enzymes in the homeostasis of the normal cartilage [18-23]. In support of this hypothesis, *in situ* hybridization experiments indicate that mRNAs for MMP-1, -3 and -13 are constitutively expressed in human knee cartilage regardless of age [18]. Moreover, the aggrecan neoepitopes generated by MMPs (VDIPEN) and aggrecanase (NITEGE) as well as the neoepitopes formed by collagenases in type II collagen have been found in normal cartilage [19-21]. Finally, MMPs and products of aggrecan degradation are quantifiable in synovial fluid and serum of healthy adults [22, 23], suggesting the role of MPs in the turnover of normal cartilage. In rabbit, MMP-1, -3 , -13 and aggrecanases-1 and -2 (ADAM-TS4 and -TS5) genes are expressed in normal cartilage and meniscus [6, 18]. These data suggest that therapy by MP inhibitors could also alter the normal turnover of connective tissue, causing systemic connective tissue toxicity.

3. Metalloproteinases in osteoarthritis

In OA, MMPs activity is found to be increased in cartilage, suggesting the role of these enzymes in the processing of aggrecan and type II collagen [24-28].

3.1. Collagenases in matrix degradation

The enzymatic type II collagen cleavage is enhanced in OA cartilage and is correlated with MMP activity in cartilage explant culture [24]. Type II collagen breakdown is primarily mediated by collagenases that cleave the triple-helical collagens between residues Gly^{775} and Leu^{776}. The three collagenases involved in OA are the interstitial or fibroblast collagenase (collagenase-1 ; MMP-1), the neutrophil collagenase (collagenase-2; MMP-8) and collagenase-3 (MMP-13). All these enzymes are produced by human chondrocytes but also by synovial cells (macrophages and fibroblasts). A collagenase that may be of particular importance in the degradation of cartilage in pathologic conditions is MMP-13, because this MMP is highly expressed at inflammatory site and strongly up-regulated by pro-inflammatory mediators such as IL-1ß and TNFα [29, 30]. Interestingly, MMP-13 can be distinguished from the other MMPs in term of topographic distribution, regulation of synthesis and functional behavior :

- A study using inhibitors for different MMPs showed that MMP-13 is the predominant enzyme involved in the degradation of resident type II collagen molecules in OA cartilage explants [28].
- MMP-13 has the broadest substrate specificity, cleaving type I, II, III, IV, X and XIV collagens, gelatin, tenascin, fibronectin and aggrecan core protein. Furthermore, it has been shown to be 5 to 10-fold more active on type II collagen than MMP-1 and MMP-8 [29, 31].
- MMP-13 is expressed almost exclusively by chondrocytes [31], whereas MMP-1 is found in most adult normal tissues.
- MMP-13 shows a differentiated topographic distribution within OA cartilage. In OA dogs, the number of chondrocytes synthesising either MMP-1 or MMP-3 is enhanced in the early stage of OA throughout the cartilage layers [32].

Nevertheless, the percentage of chondrocytes staining positive for MMP-1 or MMP-13 antibody (cell score) is different according the cartilage layer considered. The proportion of cells staining for MMP-13 is higher in lower intermediate and deep layers of OA fibrillated cartilage than in the superficial and the upper intermediate layers. In contrast, MMP-1 is mainly expressed by the cells of the superficial layers [32]. A correlation of the second order is found between the cell score for MMP-1 for each layer and the full thickness histological grading of OA lesions, with the strongest coefficient found for the superficial layers. Inversely, the most significant degree of correlation between MMP-13 and the histological grading is found in the deep layers of cartilage. These findings suggest the selective involvement of each collagenase at preferential sites during the disease process.

- MMP-13 may be differentially regulated relative to other MPs. For example, TGF-ß1 inhibits the expression of all three collagenases in culture of chondrocytes isolated from areas proximal to the macroscopically identified OA lesion, but upregulates MMP-13 and down-regulates MMP-1 and -8 in human chondrocytes from uninvolved area of the OA joint [33]. Tardif and collaborators [34] classified human OA chondrocytes according their basal MMP-13 production and their responsiveness to cytokines. Based on these criteria, they distinguished two categories of chondrocytes: the L chondrocytes, showing low MMP-13 basal synthesis and high sensitivity to IL-1ß stimulation; and the H chondrocytes with high MMP-13 basal synthesis level and low IL-1ß inducibility. In L chondrocytes, PTH, IGF-1, PDGF, and TGF-ß1 stimulate MMP-13 production. In H chondrocytes, PTH, IGF-1 and TGF-ß1 have little or no impact, whereas bFGF and PDGF BB stimulate it.
- IL-1 induction of MMP-13 requires p38 activity, c-Jun N terminal kinase (JNK) activity and NF-kB translocation, suggesting that both NF-kB and AP-1 are necessary for IL-1 induction of MMP-13. In contrast, IL-1 induction of MMP-1 requires the involvement of p38 MAPK and MEK but not JNK or NF-kB [35]. Recently, Catterall and collaborators, have suggested that IL-1α/oncostatin M combination is required for MMP-1 gene induction and involved STAT, c-fos and AP-1 transcription factors [30].

3.2. The A disintegrin and metalloproteinase with thombospontin motifs family

One of the earliest changes observed in arthritis is the depletion of cartilage aggrecan due to increased proteolytic cleavage of its core protein, mainly by aggrecanases. The initial investigation of naturally occurring degradation showed that the core protein of aggrecan is predominantly cleaved at one site within the interglobular domain indentified between Glu^{373}-Ala^{374}. This cleavage site is different from the MMP-3 site (Asn^{341}-Phe^{342}), which is also located within the interglobular domain (IGD) [36]. Aggrecanases are glutamyl endopeptidases that cleave the aggrecan core protein at the Glu^{373}-Ala^{374} site. The aggrecanases are members of the ADAM-TS family. ADAM-TS are MP disintegrin/cysteine-rich proteins (ADAMs) containing a varying number of carboxy thrombospondin (TS) motifs. So far, 12 mammalian ADAM-TS proteinases have been identified [37, 38]. The aggrecanases show the following characteristics:

Table 1. Main MPs involved in cartilage degradation.

Name	MMP number	Substrates
Interstitial collagenases		
Colllagenase-1	MMP-1	Collagen types I, II, III, VII, VIII, X, aggrecan, gelatin, pro-MMP-2,-9
Collagenase-2	MMP-8	Collagen types I, II, III, VII, VIII, X, aggrecan, gelatin
Collagenase-3	MMP-13	Collagen types I, II, III, gelatin, aggrecan
Stromelysins		
Stromelysin-1	MMP-3	Collagen types II, III, IV, IX, X, XI, elastin, pro-MMP-1, -α7,-8,-9,-13, aggrecan, fibronectin, laminin
Membrane-type-MMPs		
MT1-MMP	MMP-14	Pro-MMP-2,-13, collagen types I, II, III, gelatin, aggrecan, laminin, fibronectin
MT2-MMP	MMP-15	Pro-MMP-2, gelatin, laminin, fibronectin
MT3-MMP	MMP-16	Pro-MMP-2
Gelatinases		
Gelatinase A (72 kDa)	MMP-2	Collagen types I, II, III, IV, V, VII, X, XI, XIV, gelatin, elastin, fibronectin, aggrecan, pro-MMP-9, -13
Gelatinase B (92 kDA)	MMP-9	Collagen types IV, V, VII, X, XIV, gelatin, elastin, aggrecan
ADAM-TS		
Aggrecanase-1	ADAM-TS4	Aggrecan, brevican, versican
Aggrecanase-2	ADAM-TS5	Aggrecan

- Like many ADAM-TS proteinases, aggrecanases have high affinity for sulfated glycosaminoglycans through interactions of with the TS motifs [39]. Heparin, but also chondroitin sulphate, dose-dependently (10-100 μg/ml) inhibit aggrecanase activity in synovium-conditioned medium, suggesting that aggrecanases could be stocked in an inactive form in the extracellular matrix and then activated *in situ* [40, 41]. These enzymes cleave the aggrecan core protein in the interglobular domain at the Glu373-Ala374 site, but not at the Asn^{341}- Phe^{342} site that is cleaved by most MMPs. ADAM-TS4 and -TS5 also cleave at Glu^{1480}-Gly^{1481}, Gly^{1667}-Gly^{1668} and Glu^{1871}-Leu^{1872}, which are located in the chondroitin sulphate attachment region [42].
- Neither TIMP-1 nor TIMP-2 inhibit aggrecanases activity at concentrations ≤ 1 μM, which would be sufficient to completely inhibit the activity of the other MMPs, whereas TIMP-3 inhibits aggrecanase-1 (ADAM-TS4) and -2 (ADAM-TS5), with Ki values in the subnanomolar range [43].

- Recently, mRNAs for ADAM-TS1, -TS2, -TS3, -TS4, -TS5, -TS6 and -TS11 have been detected in bovine cartilage explants, but only three have been shown to cleave cartilage aggrecan including ADAM-TS1 [44], ADAM-TS4 (aggrecanase-1) and -5 (aggrecanase-2) [45, 46]. ADAM-TS4 and ADAM-TS5 are expressed in OA cartilage and synovium and seem to be involved in aggrecan breakdown [21, 41, 45]. In cartilage, ADAM-TS5 is constitutively expressed whereas ADAM-TS4 is induced following IL-1 and TNF treatment [42].

3.3. The membrane type metalloproteinases

Another group of MMPs, localised at the cell membrane surface, has recently been discovered, and named membrane type MMPs (MT-MMPs). Four members have been identified, and named MT1-MMP to MT4-MMP. MT1-MMP (MMP-14) production has been found upregulated in OA cartilage [47]. MT1-MMP possesses collagenase activity [48] and both MT1-MMP and MT2-MMP are able to activate MMP-2 and MMP-13 [49].

3.4. The A disintegrin and metalloproteinase family

Another pathophysiological pathway of cartilage degradation, is the activation of pro-TNF-α by TNF-α convertase enzymes (TACE or ADAM-17). TACE is a member of the ADAM family. ADAM-9, -10, -12, -15, and -17 are all constitutively expressed at low levels in the normal cartilage but are overexpressed in OA cartilage [50-54]. ADAM-15 (also called metargidin) mRNA expression does not show preference for certain zones of OA cartilage, although the extended calcified zone remains mostly negative, and is not correlated with lesion severity [53]. However, evidence of strong mRNA signals in the mildly damaged cartilage samples and even in the unloading area distant from the lesion suggest that the upregulation of ADAM-15 is rather an early event than a phenomenon restricted to late-stage disease. ADAM-10 protein is present in human articular cartilage and is upregulated in OA in response to IL-1α in bovine nasal cartilage [54]. The strongest immunostaining is found in cytoplasm and/or cell membranes of the superficial and upper middle layer of normal adult human cartilage, cell clusters and fibrillated areas of OA cartilage [34].

4. Metalloproteinases activation and inhibition.

MMPs are produced as inactive proenzymes that can be stocked in the extracellular matrix [9] until their activation. There is evidence for at least three activation pathways of latent MMPs *in vivo*.

4.1. Metalloproteinase pathway.

Active MMPs can activate pro-MMPs. For example, MMP-3 (stromelysin-1) can activate pro-MMP-1, pro-MMP-8, and pro-MMP-13 [10, 11, 55]. MT1-MMP, together with MMP-2, can activate proMMP-13 on the cell surface [10]. MT-1, -3, -5 and -6 MMPs are activators of pro-MMP-2 [56-60]. MT-MMPs and MMP-11 are activated intracellularly by furin [56, 61].

4.2. Serine proteinase pathway.

Urokinase and plasmin are found to be elevated in human OA cartilage [15, 16]. Plasmin can activate several members of the MMPs including pro-MMP-1, pro-MMP-3, pro-MMP-9, and pro-MMP-13 [12, 13, 55, 62]. Both urokinase-type (uPA) and tissular-type plasminogen activators (tPA) process plasminogen into plasmin. It is thought that the primary role of tPA is to generate plasmin for thrombolysis, while it is mainly uPA that generates plasmin in events involving extracellular matrix degradation. Urokinase plasminogen activator is secreted as a zymogen (pro-u-PA) that binds to cell surface receptor (u-PAR), which concentrates u-PA activity at the plasma membrane after activation by plasmin [63-67]. Plasminogen activators activity is regulated by two specific inhibitors (PAI-1 and PAI-2) [14-16, 68]. Plasminogen, plasminogen activators and plasminogen activator inhibitors have been detected in the synovial fluid of patients with various arthritis [15]. Increases in plasmin activity and PAs have been detected in OA cartilage along with a decrease in PAI [16]. In addition IL-1 has been shown to upregulate PAs and downregulate PAI in chondrocytes [68].

4.3. Cysteine proteinase pathway.

Some studies, using histochemistry and activity measurements, have confirmed cathepsin B as the major, active cysteine peptidase in OA cartilage, particularly at sites where matrix neosynthesis takes place [69]. However, cathepsin L and cathepsin K activity, of either osteoclastic or macrophages, is also found subchondrally in association with cathepsin B in osteophytes, in zone undergoing bone remodeling, and at site of inflammation. Cathepsin B, L and K are found to be elevated in synovial fluid of patients with OA and strongly expressed in OA synovial membrane [70, 71]. Cathepsin B and L are both capable of degrading aggrecans [70, 71], and bone matrix component such as osteocalcin [72] but also to activate pro-MMP-1 and MMP-3 [69]. Recently, cathepsin B was demonstrated to degrade TIMP-1 and TIMP-2 [73]. Cystatin C is one of the most powerful natural inhibitor of cysteine peptidases. This inhibitor is widely distributed in human tissues and extracellular fluids [56], including synovial membrane and fluid [74, 75]. In synovium, cystatin C is produced by both macrophage-like and fibroblast-like synoviocytes and is found to be overexpressed in arthritis [74, 75].

4.4. Tissue inhibitors of metalloproteinases

The biologic activity of MMPs is controlled by tissue inbibitors of matrix metalloproteinases (TIMPs). There are currently four TIMPs that have been identified in human OA joint named TIMP-1, -2, -3 and -4. In general, most TIMPs appear to be able to inhibit most active MPs, including MMPs and ADAM-TS [43, 76]. In OA tissue there is an imbalance between the amount of TIMPs and MMPs synthesis, that could lead to an increase of MMP activity [17]. In Ad-TIMP-1 virus-injected mice with collagen-induced arthritis, TIMP-1 lacks to prevent inflammation

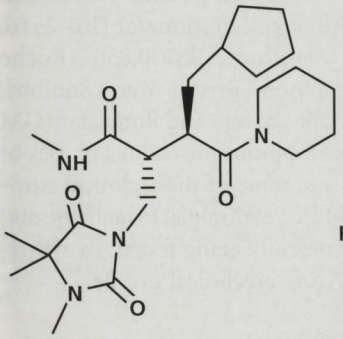
or joint cartilage degradation and even significantly aggravated bone erosion, suggesting that TIMP-1 is probably not the main inhibitor target. [77]

5. Management of osteoarthritis by inhibiting matrix metalloproteinases

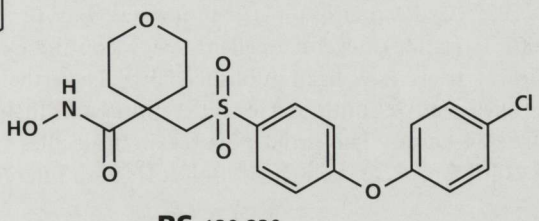
The goal of pharmacological treatment is usually to relief symptoms of the disease, pain and limitation of function, which is traditionally achieved with analgesic agents or non-steroidal anti-inflammatory drugs (NSAIDs). Drugs for the treatment of OA are classified as symptom-modifying drugs and also as structure-modifying drugs if they are able to favourably interfere with the structural changes of joint tissues [78, 79]. We have recently demonstrated in a randomised, double-blinded and placebo-controlled clinical study that oral glucosamine sulfate (GS, 1,500 mg on daily for 3 years ; Dona, Viartril-S, or Xicils, Rotta Research Group, Monza, Italy) improves OA symptoms (WOMAC algofunctional index) and prevents joint space narrowing of the medial compartment (measured on weight-bearing knee x-ray by computerised method) in patients with OA of the knee [80]. After three years the placebo had a progressive joint-space narrowing, with a mean joint space loss of 0.31 mm, whereas no significant joint space loss was observed in patients receiving GS. These results have been confirmed in a independent study including 202 patients with knee OA randomised and receiving either placebo or GS 1,500 mg once a day for three years. The 3-year JSN observed with placebo was - 0.2 mm and significantly higher that with GS, for which no JSN occurred in average [81]. Several other agents, including avocado/soybean unsaponifiables (PiasclédineTM, Laboratoire Pharmasciences, Courbevoie, France) and diacerein (ArthrylTM, Negma, France) have also been reported to be efficient at relieving joint pain in patients with knee and hip OA [82, 83]. These agents are under investigation for their actions on the disease progression in long-term clinical trials.

Interestingly, all these agents have been demonstrated to decrease MMP synthesis by chondrocytes or synovium cells and to prevent or reduce cartilage degradation *in vitro* and *in vivo* in animal models [84-93]. In human chondrocytes, GS causes a consistent decrease in the levels of MMP-1 and -3 mRNA [84]. GS also reduces the protein levels of MMP-1 and -3 found in culture supernatants of OA chondrocytes [85]. D(+)-Glucosamine significantly inhibits the IL-1ß-mediated aggrecan degradation in bovine cartilage explants estimated by the formation of the aggrecanase generated G1-NITEGE doublet in culture supernatants and explants [85]. Avocado/Soybean unsaponifiables have been demonstrated to stimulate plasminogen activator inhibitor-1 (PAI-1) production by bovine articular chondrocytes in monolayer [86] and to decrease crude stromelysin-like and collagenase-like activity in human primary chondrocytes cultures in suspension [87]. More recently, it has been demonstrated, using specific immunoassays, that avocado/soybean unsaponifiables decreases MMP-3 synthesis but increases TIMP-1 production by human chondrocytes cultured for 12 days in alginate beads [88]. Finally, rhein, an active metabolite of diacerein, downregulates the IL-1α-induced production of pro-

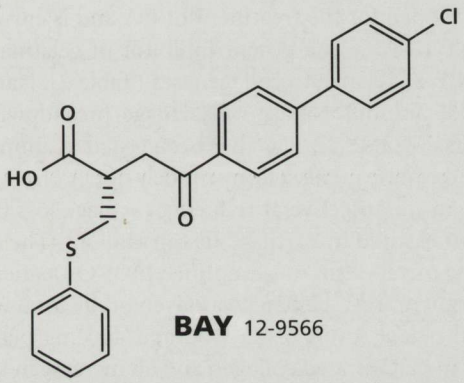
MPs-1, -3, -9 and -13 and upregulates the production of TIMP-1 by rabbit chondrocytes in monolayer. The suppressed production of pro-MMPs is accompanied by a decrease in the crude total activity of MMPs in the culture medium [89].



RO 32-3555



RS 130,830



BAY 12-9566

Table 2. MMPs inhibitors in clinical arthritis trials

Compounnd	K_i (nM)				
	MMP-1	MMP-2	MMP-3	MMP-9	MMP-13
Ro32-3555	3	154	527	59	3.4
RS-130, 83	590	0.22	9.3	0.58	0.52
BAY 12-9566	>5,000	11	143	301	1,470

6. Experimental matrix metalloproteinases inhibitors

One approach for controlling abnormal MMP activity is through the use of small molecular weight synthetic inhibitors, which bind directly to the catalytic site of the appropriate enzyme. For this purpose, several peptidic and non-peptidic MMP inhibitors have been developed on the basis of two principles; to mimic part of the peptide sequence surrounding the cleavage site of the MMPs and to chelate zinc atom in the active site of the enzyme with a metal binding group such as

hydroxamate, reverse hydroxamate, carboxylate, thiol, or phosphonic acid.

Some synthetic inhibitors have been shown to prevent or reduce cartilage degradation in *in vitro* and in animal models without inducing cytotoxicity or systemic adverse effects, and have been tested in clinical trials. The compounds included in clinical trials are batimastat (BB-94, British Biotechnology), marimastat (BB-2516, British Biotechnology), prinomastat (AG 3340, Agouron), RS130,830 (Roche Biosciences), BMS-275, 291 (D-2163, Celtech Group plc and Bristol-Myers Squibb), D-1927 (Celltech Group plc), tanomastat (Bay 12-9566, Bayer) and ilomastat (GM 6001, Glyomed). Several excellent reviews on these compounds in various phases of clinical trials have been published [94-97]. Nevertheless, some of these clinical studies have been discontinued for different reasons including arthralgia, myalgia or disease worsening. This article focuses on molecules currently being tested in arthritis clinical trials and patented molecules with interesting preclinical profile.

6.1. Matrix metalloproteinase inhibitors in OA clinical trials

BAY 12-9566 (Tanomastat, Bayer Corporation), containing a carboxylic zinc-binding group, was developed for the treatment of OA and is currently included in clinical trials [98, 99]. BAY 12-9566 is a potent inhibitor of gelatinases (MMP-2, -9) and stromelysin-1 (MMP-3) relative to collagenases (Table 2). Nanomolar concentrations of BAY 12-9566 inhibits release of cartilage breakdown products from equine cartilage explants [100]. BAY 12-9566 has been tested in animal models of OA, including guinea pig and canine menisectomy models of OA (101). At daily doses of 1-10 mg/Kg BAY 12-9566 was effective at reducing cartilage loss by 50-60 % compared with controls, as measured by cartilage histopathology. The effect of BAY 12-9566 on human cartilage metabolism was examined in 35 OA patients scheduled for elective total hip replacement. BAY 12-9566 was given orally for 3 weeks to placebo-controlled cohorts at the dose levels of 25, 100 and 400 mg per day. At dose of 100 mg /day, a decrease in denaturated collagen and an increase in total collagen was observed in cartilage biopsies [102]. It is interesting to note that BAY 12-9566, which is a poor inhibitor of collagenase-1 and -3, prevents cartilage loss suggesting a less important role for these MMPs in OA cartilage degradation. To date, BAY 12-9566 has been tested in studies in 300 osteoarthritic patients for up to 3 months [98]. No musculoskeletal adverse effects have been reported but a mild, reversible lowering of platelets counts has been observed [102]. Curiously, at this time, no data on the symptomatic effects of BAY 12-9566 have yet been published. Recently, long-term trials with BAY 12-9566 have been initiated in OA to evaluate its effect on structural changes, but in our knowledge, no results are yet available.

The compound Roche selected for OA, RS-130,830, preferentially inhibits collagenase-3 (MMP-13) and gelatinases (MMP-2 and MMP-9) (Table 2) [103]. This compound has no effect on TACE activity and does not inhibit the IL-1α-induced release of aggrecan from bovine cartilage explants, however it does inhibit collagen loss as demonstrated in several animal models. In a rabbit medial menisectomy model of OA, a dose of 3 or 10 mg/kg/day prevents cartilage degradation, whereas a dose of 1 mg/kg/day does not produced significant effect (104). Also, it is interest-

ing to note that despite not inhibiting aggrecan breakdown, inhibition of collagen breakdown in itself seems enough to prevent cartilage degradation. This observation reinforces the concept that collagen network breakdown represents the most important step of cartilage degradation and that inhibition of enzymes involved in the processing of collagen fibers are key targets for MMP inhibitors. The compound is currently in phase II trials for the prevention and retardation of joint damage in OA. The results are not yet available.

Table 3: *In vitro* activity (IC_{50}, nM) of new hydroxamate MMP inhibitors derived from functionalized 4-aminoproline.

	Structure	IC_{50} (nM) MMP-1	MMP-2	MMP-3	MMP-13
A	1N-(4-N-Propoxybenzenesulfonyl)-2(R)-N-hydroxycarboxyamido-4(S)-(morpholin-1N-yl) pyrrolidine.	490	nd	7	1
B	1N-(4-N-Butoxybenzensulfonyl)-2(R)-N-hydroxycarboxamido-4(S)-(morpholin-1N-yl)pyrrolidine.	920	4	5	0.6
C	1N-4-N-Butoxybenzensulfonyl)-2(R)-N-hydroxycarboxamido-4(S)-1N-(3N-methylhydantoin-1N-yl)pyrrolidine.	920	nd	2	0.3
D	1N-(4-(2-Methoxyethoxy)benzensulfonyl)-2(R)-N-hydroxycarbo-xamido-4(S)-1N-(3N-methylhydantoin-1N-yl)pyrrolidine.	1,800	1	26	4.4
E	1N-(4-N-Butoxybenzensulfonyl)-2(R)-N-hydroxycarboxamido-4(S)-1N-(3N-allylhydantoin-1N-yl)pyrrolidine.	480	nd	7	0.4

The drugs A to E have been developped by Procter and Gambles Pharmaceuticals (Ohio, USA) and the IC_{50} values reported by Natchus et al [109]. nd = not determined.

Roche has also developed a succinyl hydroxamic acid derivative for treating arthritis, RO 32-3555 (Trocade™, Roche), which was in phase III clinical trials for rheumatoid arthritis until recently. RO 32-3555 is a orally administrated MMPs inhibitor which competitively inhibits human collagenase -1, -2, -3 (MMP-1, -8, 13) and is selective for these MPs over stromelysin-1 (MMP-3) and gelatinases (MMP-2, -9). RO 32-3555 is presented as a selective inhibitor of collagenases (Table 2). *In vitro*, Ro 32-3555 inhibits IL-1α-induced cartilage collagens degradation in bovine nasal cartilage explants with a IC_{50} = 60 nM [105]. In an animal model in which bovine nasal cartilage is implanted subcutaneously in male rats, RO 32-3555 reduces granuloma-induced collagen loss from the implant with an IC_{50} = 10 mg/kg, when given p.o., b.i.d, for 14 days. Interestingly, RO 32-3555 inhibits collagen degradation by 79 % but reduces proteoglycans loss by only 7 % [105].

In an *in vivo* arthritis model in which monoarthritis is induced by injection of propionibacterium acnes in female rat, RO 32-3555 (50 mg/kg p.o., b.i.d., for 14 days) significantly inhibits degradation of articular cartilage [105]. In a similar model in rabbit, a clear protective effect of RO 32-3555 is observed by MRI technique [95]. Ro 32-3555 treatment (10-50 mg/Kg daily for 12 weeks) leads to significant protection of cartilage, inhibition of osteophyte formation, reduction of JSN and a decrease of the severity of histological score of OA lesions in the STR/ORT mice model of OA [106]. On the basis of these encouraging preclinical data, Ro 32-3555 has been included in human trials. Pharmacokinetic studies in healthy male volunteers revealed good oral bioviability and no serious adverse effects after administration of single oral dose ranging from 10 to 150 mg [107]. Small phase II studies in patients with rheumatoid arthritis have also suggested that the drug is well tolerated with no reports of muskuloskeletal symptoms seen with the use of broad spectrum MMP inhibitors [108].

6.2. Newly patented matrix metalloproteinase inhibitors

6.2.1. Compounds with MMP-13 selectivity

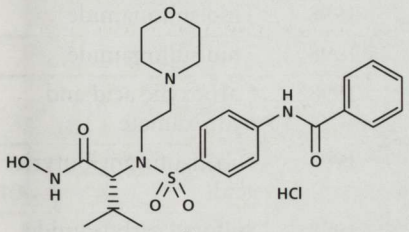
The recent findings on cartilage degradation by MMPs, give evidence for developing new inhibitors that could powerfully inhibit MMP-13 while sparing MMP-1. Collagenase -1 (MMP-1) inhibition is negatively selected against because of its widespread expression, its role in physiology and potential for adverse effects. On the other hand, MMP-13 is secreted in inflammatory conditions and almost exclusively expressed in cartilage, suggesting its role in cartilage degradation. Nevertheless, at this time, no *in vitro* or *in vivo* proofs have been published demonstrating that specific MMP-13 inhibition blocks cartilage degradation. Procter and Gamble has developed new aminopyrridinoline-based hydroxamate containing heterocyclic scaffolds or hydantoin moieties (Table 3). Two compounds of the heterocyclic group (compounds A and B) and three of the hydantoin group (compounds C, D, E) showed good selectivity for MMP-3 and MMP-13 and were tested for their capacity to protect cartilage in a iodoacetate-induced arthritis rat model [109]. Compounds were administrated p.o. b.i.d at a dose of 35 mg/kg for the first 7 days after intra-articular injection of iodoacetate. Animals were sacrified 21 days after iodoacetate administration and joint damage was assessed. The compounds containing a heterocyclic scaffold (A and B) had a weak inhibition (21-26 %) whereas those with hydantoin moities had little or no effect. This lack of efficacy was related to their poor bioavaibility and short half-lives.

Monsanto (now Pharmacia) has developed a series of sulfonamide hydroxamates showing a good selectivity for MMP-2 and MMP-13 (Table 4). Compound 1 [201] shows the best selectivity with MMP-1/MMP-2 and MMP-1/MMP-13 ratios equal to 20,000 and 13,333, respectively (Figure 1).

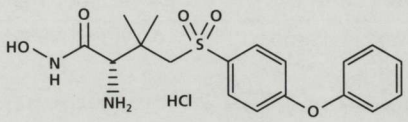
Warner Lambert (now Pfizer Global R&D) has reported a novel dibenzofuranylbutyric acid derivative 5 [208] with a carboxylate zinc-binding functionality that is a

selective inhibitor of the MMP-2 (IC_{50} = 17 nM) , but does not inhibit MMP-1 (IC_{50} = 100,000 nM). This compound shows a weak activity against MMP-13 (IC_{50} = 5,600 nM) (Table 4 and Figure 1).

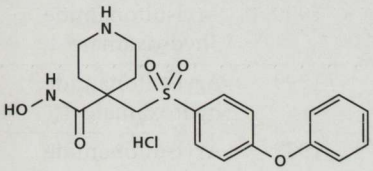
A series of sulfone hydroxamate has been patented by GD Searle in 1999 and 2000. These compounds are highly specific for MMP-2 and MMP-13. For example, coumpound 6 [215] inhibits MMP-2, MMP-9 and MMP-13 with a IC_{50} = 0.2, 1.5 and 0.5 nM, respectively, and MMP-1 with a IC_{50} of 9,000 nM. This compound is 45,000 times more active against MMP-2 than MMP-1, and 18,000 times more active against MMP-13 than MMP-1.



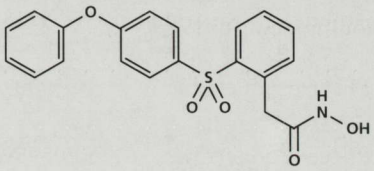
1 [201]



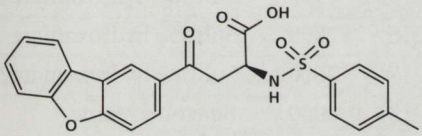
2 [202]



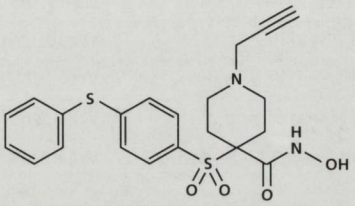
3 [203]



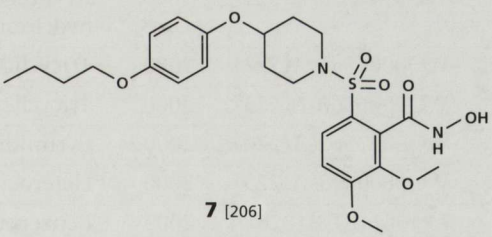
4 [204]



5 [208]



6 [205]



7 [206]

Figure 1. Drugs with selectivity for MMP-2, MMP-9 and MMP-13

Table 4. *In vitro* activity (IC_{50}/Ki nM) of newly patented MMP inhibitors.

Company	Patent number and ref.	Year	Class
Monsanto Co	WO9839329(201)	1998	Sulfonamide hydroxamate
Monsanto Co	WO9839316(202)	1998	Phenylsulfonylbutanamide
Monsanto Co	WO9839315(203)	1998	Aromatic sulfonyl hydroxamate
Monsanto Co	WO9838859(204)	1998	Sulfonyl aryl hydroxamate
Monsanto Co	WO9803166(205)	1998	Thiolsulfonamide
Monsanto Co	WO9803164(206)	1998	Thiolsulfonamide
Warner-Lambert Co	WO9809957(207)	1998	Carboxylic acid and hydroxamate
Warner-Lambert Co	WO9806711(208)	1998	Dibenzofuranylbutyric acid
American Cyanamid Co	WO9838163(209)	1998	Sulfonyl carboxamide
American Cyanamid Co	WO9816520(210)	1998	Arylsulfonamide hydroxamate
American Cyanamid Co	WO9918076-A(211)	1999	Arylsulfonamide 3hydroxamate
Pfizer Products Inc	WO9907675-A(212)	1999	Arylsulfonamide hydroxamate
Pfizer Products Inc	EP0895988-A(213)	1999	Arylsulfonamide hydroxamate
Pfizer Products Inc	EP0935963-A(214)	1999	Phosphonic acid
GD Searle & Co	WO9925687-A(215)	1999	Sulfone hydroxamate
GD Searle & Co	WO0069819-A(216)	2000	Sulfone hydroxamate
GD Searle & Co	WO0069821-A(217)	2000	Sulfone hydroxamate
American Cyanamid Co	WO0044730-A(218)	2000	Benzodiazepine hydroxamate
American Cyanamid Co	WO0044740-A(219)	2000	Sulfone hydroxamate
American Cyanamid Co	WO0044713-A(220)	2000	Acetylenic hydroxamate
Bayer AG	WO0040539-A(221)	2000	Aryl carboxylic acid hydroxamate
Warner-Lambert Co	WO0006561-A(222)	2000	Tricyclic sulfonamide
Warner Lambert Co	WO0006560-A(223)	2000	Tricyclic hydroxamate
Hoffmann -La Roche	WO0047565-A(224)	2000	Pyrimidine-2,4,6-triones
Pfizer Products Inc	WO0009492-A(225)	2000	Heterocyclic hydroxamate
Pfizer Products Inc	EP1081137-A(226)	2001	Benzensulfonamide
Novartis AG	WO0110827-A(227)	2001	Arylsufonamide hy droxamate

Nd = Not determined.

MMP-1	MMP-2	MMP-3	MMP-9	MMP-13	TACE
4,000	0.2	90	4.5	0.3	nd
3,300	0.3	nd	nd	1.6	nd
2,400	2.8	158	30	8	nd
900	0.3	nd	nd	2	nd
30	nd	nd	nd	0.2	nd
370	nd	nd	nd	0.2	nd
3,500	3.8	12	nd	32	nd
100,000	17	760	nd	5,600	nd
598	nd	nd	10.3	2.2	71.9
1,227	nd	nd	15	47	294
96	nd	nd	3	2.4	138
200	nd	nd	nd	0.6	nd
195	nd	nd	nd	1.7	nd
40,000	nd	nd	nd	40	nd
9000	0.2	nd	1.5	0.5	nd
> 10,000	0.8	nd	nd	1.6	nd
> 10,000	100	nd	nd	2.1	nd
125	nd	nd	2	7	33
> 10,000	nd	nd	607	478	11
1,400	nd	nd	142	69	15.7
639§	0.5§	3.7§	0.7§	5.6§	nd§
25,000	18	21	28,000	1,345	nd
100,000	59	114	32,000	1,206	nd
nd	26	930	23	47	nd
1,400	nd	nd	nd	nd	4.5
1,000	nd	nd	nd	<10	< 10,000
622	0.7	nd	1.2	nd	nd

§value given is Ki rather than IC50

6.2.2. MT-MMP inhibitors

A series of tricyclic derivatives with selective action for MT1-MMP (MMP-14) have been proposed by Warner Lambert Co. in 2000 (Figure 2). The specified compounds 8 [222] and 9 [223] inhibit MMP-14 with IC_{50} values of 240 and 64 nM, respectively. The ratios MMP-1/MMP-14 and MMP-13/MMP-14 are equal to 417 and 5 for compound 8 [222] and to 390 and 21 for compound 9 [223]. These compounds also show a good selectivity for MMP-2 with a ratio of MMP-1/MMP-2 equal to 1,388 and 1,694, respectively, but spare MMP-9 (IC_{50} = 28,000 nM for 8 and IC_{50} 32,000nM for 9) (Table 4 and Figure 2).

A novel benzenesulfonamide is presented by Pfizer Products as a inhibitor of the ^{35}S-glycosaminoglycans release from primary porcine chondrocytes in monolayer. This test was presented as an aggrecanases assay and used to determine the IC_{50} values. The specified compound 10 [226] is also active against MMP-13 with IC_{50} values of < 10 nM for aggrecanases and MMP-13, of < 10 μM for TACE and 200-1000 nM for MMP-1 (Figure 2).

6.2.3. The inhibitors of TACE

TACE is a adamalysin that contains both disintegrin and MMP domain, involved in the processing of membrane-associated pro-TNFα to the mature monomer. This enzyme is also required for shedding the TNF receptors. An upregulation of TACE mRNA has been reported in human OA cartilage [110]. Furthermore, TNF-α has been found in elevated amounts in OA synovial membrane [111], synovial fluid [112] and cartilage [113], suggesting that TNF-α could play an important role in OA pathogenesis. Nevertheless, its exact role remains obscure and controversial. It is still unclear whether TNF-α acts independently, or in concert with other cytokines, such as IL-1ß, to induce OA pathogenesis, or if a functional hierarchy exists between these pro-inflammatory cytokines. In animal models, it has been shown that blocking IL-1 synthesis or its activity is very effective in preventing cartilage destruction [114, 115], whereas blocking TNF-α results in decreased inflammation [116, 117]. In OA horse, the activity of TNF-α found in the synovial fluid is not correlated to the degree of cartilage lesions [112]. On the other hand, in chondrocyte cultures, TNF-α has the potential not only to increase the synthesis of MMPs and PA, but also to decrease the synthesis of collagens and proteoglycans [118]. Taken together, these findings support the concept of TACE inhibition for the treatment of OA. Even now, anti-TNF therapies are limited to rheumatoid arthritis (RA), ankylosing spondylitis and psoriatic arthritis treatment. In the last two years, the U.S. Food and Drug Administration and the EC have approved two biological agents (etanercept and infliximab) with anti-TNF activities. Etanercept is a fusion protein, composed of Fc portion of immunoglobulin and the extracellular domain of a TNF receptor (p75). Infliximab is a neutralizing chimaeric (mouse-human) TNF-specific mAb. In placebo-controlled trials, both agents have proven to be efficient at relieving symptoms and to slow-down the radiological progression of RA [119, 120], suggesting the interest of blockage TNF-α activity. Nevertheless, the use of TACE inhibitors is questionable since specific drugs that block TNF-α are already

used in practice.

Recently, several inhibitors for TACE have been developed by American Cyanamid (Table 4). The compound 11 [219], is of particular interest because it powerfully inhibits TACE (IC_{50} = 11 nM) and shows a good selectivity for this enzyme relative to MMP-1 and MMP-13 (Figure 3). Pfizer Products Inc proposed a novel heterocyclic derivative that is [146] strongly active against TACE (IC_{50} = 4.5 nM), but weakly against MMP-1 (IC_{50} = 1,400 nM).

7. Expert opinion and new perspectives

MPs are responsible for cartilage matrix degradation occurring in joint diseases and constitute an important target for drug design. Several MMP inhibitors with a broad spectrum of activity have been developed and included in clinical trials. In OA cartilage cultures and animal models of cartilage degradation, these agents have shown chondroprotective effects. However, the results from clinical trials in cancer have been un-equivocal, with some studies being discontinued because of lack of efficacy or safety concerns. The failure of these clinical trials was attributed to the lack of enzyme specificity of the inhibitors and particularly to the suppression of normal connective tissue collagen turnover by collagenase-1. Collagenase inhibitors without collagenase-1 activity should therefore have no or reduce connective tissue toxicity.

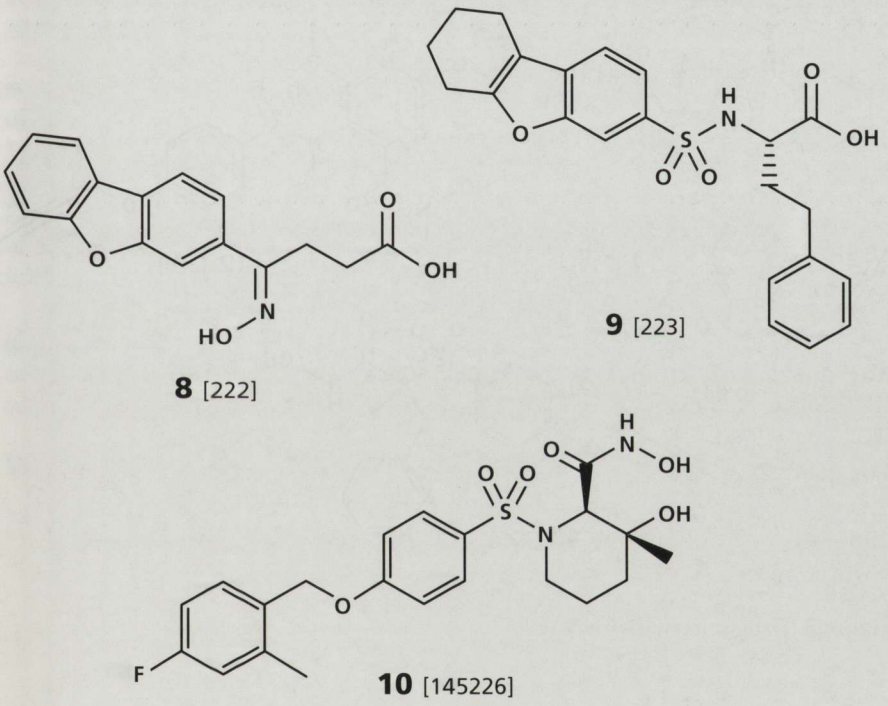


Figure 2. Structure of aggrecanases and MMP-14 inhibitors.

BAY 12-9566, which is a powerful inhibitor of gelatinases and stromelysins relative to collagenases, shows good safety in human and is effective at preventing cartilage loss *ex-vivo*. Inversely, RO 32-3555, which competitively inhibits collagenase -1, -2, -3 and is selective for these enzymes over stromelysin-1 and gelatinases, is also effective at preventing cartilage degradation in *in vitro* and animal models. Taken together, these findings suggest that there are more than one important enzyme family involved in cartilage degradation in arthritis. Cartilage degradation is a complex mechanism, involving a vast number of MPs which interact to degrade cartilage. Therefore, the specific inhibition of one particular MP is questionable. What is the best target? MMP-13, ADAM-TS or TACE are potential candidates.

MMP-13 has been targeted because this enzyme seems to be expressed mainly in cartilage under pathological conditions, shows a higher affinity for type II collagen than MMP-1 and is almost exclusively expressed in cartilage. Some molecules, inhibiting MMP-13 but sparingly MMP-1, have been designed, but none is specific for MMP-13. Furthermore, the efficacy of specific MMP-13 inhibition in the management OA has not been clearly evidenced until now.

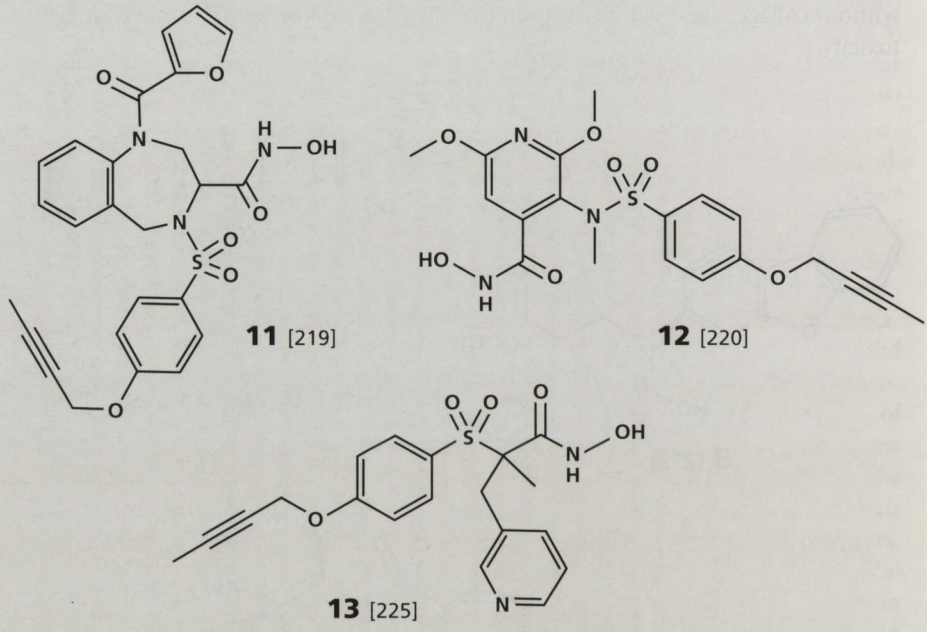


Figure 3. Drug selectivity for TACE

The increase catabolism of aggrecan is a principal process which leads to the degeneration of articular cartilage in OA. The consequential loss of aggrecan compromises both the functional and the structural integrity of the cartilage matrix and ultimately renders the tissue incapable of resisting the compressive loads applied to joint during locomotion. It is now accepted that aggrecanases are primary responsible for the catabolism and loss of aggrecan in the early stage of the OA that precede collagen catabolism and disruption of tissue integrity. Therefore, the inhibition of aggrecanases could be of interest in the management of OA. Until now, no drug has been made available that specifically blocks the activity of aggrecanases. Furthermore, we have no information about the adverse effects linked to their systemic administration and no proof that aggrecanases inhibition prevent cartilage degradation, mainly during the late stage of the disease. The early phase of the disease is asymptomatic, preclinical and currently untreated. At later stages of the disease, it is recognised that the degradation of aggrecan remaining in the tissue occurs independently of continued aggrecanase activity [38]. This later finding sustains that MMPs, other than aggrecanases, are involved in aggrecan degradation when the disease reaches a symptomatic, morbid phase.

Another strategy that could be used for blocking the proteolytic cascade of MMPs activation by inhibition is MT-MMPs or urokinase. Nevertheless, MMP activation is a complex process that involves a network of MMPs, serine and cysteine proteinases that act at different levels of the activation process. Therefore, the blockage of one particular pathway could be compensated by another.

Finally, some studies have been devoted on the inhibition of TACE. This enzyme is required for the activation of TNF-α, a cytokine involved in the pathophysiology of OA. Recently, GW3333 [(2R,3S)-3-(formyl-hydroxyamino)-2-(2-methyl-1propyl)-4-methylpentanoic acid [(1S,2S)-2-methyl-1-(2-pyridylcarbamoyl)-1-butyl] amide], a dual inhibitor of TACE and MMPs, completely inhibited the IL-1α-induced hydroxyproline release from bovine nasal plugs and the appearance of cartilage lesions in adjuvant arthritis [121]. This data suggest that drugs that can inhibit both MMP-13 and TACE could be advantageous.

The inhibition of MPs to treat OA remains speculative and questionable. The global inhibition by systemic administration of inhibitors is accompanied by severe adverse effects. On the other hand, the efficacy of specific inhibitor, directed against one particular metalloproteinase, has not yet been demonstrated.

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ANNEXE 10

Evaluation of classical NSAIDs and COX-2 selective inhibitors on purified ovine enzymes and human whole blood

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Summary

Cyclooxygenase is the key enzyme in the biosynthesis of prostanoids, biologically active substances involved in several physiological processes and also in pathological conditions such as inflammation. It has been well known for 10 years that this enzyme exists under two forms: a constitutive (COX-1) and an inducible form (COX-2). Both enzymes are sensitive to inhibition by conventional non-steroidal anti-inflammatory drugs (NSAIDs). Observations were made that COX-1 was mainly involved in homeostatic processes, while the COX-2 expression was associated with pathological conditions leading to the development of COX-2 selective inhibitors. Several methods have been reported for the evaluation of the COX-1 and COX-2 inhibitory potency and selectivity of conventional or COX-2 selective NSAIDs. In this study, we evaluated the COXs inhibitory profile of both conventional NSAIDs and COX-2 selective inhibitors using two different *in vitro* methods: the first test was performed using purified enzymes while the second method consisted of a whole blood assay. The results obtained with reference drugs in these two assays will be discussed and compared in this article. © 2001 Harcourt Publishers Ltd

INTRODUCTION

Cyclooxygenase (prostaglandin endoperoxide synthase or COX) catalyses the transformation of arachidonic acid into prostaglandin H_2 (PGH_2) as the first step in the biosynthesis of prostanoids (prostaglandins, prostacyclin and thromboxane). Until recently, one COX was thought to be responsible for both the physiological production of prostaglandins (PGs) and their increased production when inflammation occurs. In 1990 however, an inducible COX was identified as a different isoform from the constitutive enzyme and called COX-2 in opposition to the constitutive form: COX-1.[1] Although these two enzymes are involved in the biosynthesis of the same molecules, the roles played by the prostanoids deriving from COX-1 and COX-2 are different. High levels of COX-1 are expressed in platelets,[2] vascular endothelial cells,[3] stomach [4, 5] and kidney collecting tubules.[4, 6, 7] Prostanoids deriving from COX-1 play a major role in thrombogenesis [8] and in the homeostasis of the gastro intestinal tract and kidney,[9-11] In contrast, COX-2 is almost undetectable in physiological conditions but its expression is induced by a wide range of stimuli such as growth factors,[12, 13] phorbol esters,[14] interleukin-1 (IL-1)[15, 16] or lipopolysaccharide (LPS).[17, 18] High levels of COX-2 are detected in exudates and in the spinal cord in different models of inflammation.[19] Moreover, specific COX-2 inhibitors exhibit anti-inflammatory, analgesic and antipyretic properties in animal and human models. On the other hand, several types of cancers are characterzed by the presence of elevated levels of COX-2,[20-23] suggesting the involvement of PGs deriving from COX-2 in cancerogenesis and, subsequently, the interest of selective COX-2 inhibitors in cancer prevention. This approach is supported by several epidemiological studies of regular use of NSAIDs indicating their effectiveness in chemoprevention of colorectal cancer.[24,25] All those points clearly demonstrate that COX-2 is implicated in different pathologies such as inflammation or cancer proliferation whereas COX-1 is mainly implicated in homeostatic processes.

Acting as non selective COX inhibitors, classical NSAIDs have been widely used in the treatment of inflammation states for a long time. However, all those agents cause untoward side-effects related to COX-1 inhibition of which gastrointestinal irritation [26] is the most common. So, the evidence of therapeutical benefit deriving from selective COX-2 inhibition led to the development of COX-2 selective inhibitors.

In order to evaluate the potency and COX-2 selectivity of these new drugs, several *in vitro* and *in vivo* methods have been reported. *In vitro* assays can be classified in two distinct categories: assays using enzymes or cells. Enzymatic methods use purified or recombinant enzymes [27] while cellular models use various mammalian cell types.[28] *In vivo* assays such as carageenan-induced paw oedema or carrageenan-induced algesia in rats [29] are currently used in order to evaluate the anti-inflammatory and analgesic properties of drugs, respectively.

In the present study, we have evaluated the COXs inhibitory potency of reference drugs in an enzymatic assay using purified ovine enzymes and in a cellular assay using human whole blood. As reference drugs, we used both classical NSAIDs

(Diclofenac, Indomethacin and Tenoxicam) and selective COX-2 inhibitors such as Celecoxib, Rofecoxib, NS-398 and Nimesulide. The results obtained with these reference drugs on both purified enzymes and human whole blood assays and the differences observed between these two methods of evaluation of the selectivity and potency of COX inhibitors will be discussed in this paper.

MATERIALS AND METHODS

COX-1 and COX-2 enzyme inhibition test

One unit of cyclooxygenase enzyme (COX-1 from ram seminal vesicles and COX-2 from sheep placenta, Cayman Chemical Company, Ann Arbor, MI, USA) was suspended in TRIS buffer (1 mL, 100 nmol/L, pH 8) containing phenol (2 nmol/L) and hematin (1 μmol/L, Sigma-Aldrich, Bornem, Belgium) as cofactors. They were preincubated with substract alone (controls) or with the investigated drugs (0.01 to 100 μmol/L) for 60 minutes at 37°C. Arachidonic acid sodium salt (10 μmol/L, Calbiochem, Belgium) was added and further incubated for 2 minutes at 37%. Reaction was halted by placing the tubes on ice and by adding diclofenac sodium salt (Federa, Belgium) at a final concentration of 1 mmo1/L. Prostaglandin E_2 (PGE_2) production was quantified by a specific radioimmunoassay (RIA).[30]

Human whole blood assay

In vitro COX-1 assay

Fresh blood was collected into tubes containing no anticoagulants by venipuncture from normal volunteers who had not ingested any drugs within the previous two weeks. One millilitre blood aliquots were immediately transferred into siliconized microcentrifuge tubes preloaded with 4 μL vehicle (DMSO) or test compounds at final concentrations ranging from 0.01 to 100 μmol/L. The tubes were vortexed and incubated at 37 °C under constant agitation for 1 h to allow the blood to clot. At the end of incubation, the serum was obtained by centrifugation (10.000 g for 10 min) and was assayed for thromboxane B_2 (TXB_2) production using an enzyme immuno -assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions.

In vitro COX-2 assay

Fresh blood was collected i n heparinized tubes by venipuncture from normal volunteers who had not ingested any drugs within the two previous weeks One mL aliquots of blood were transferred in heparinized tubes preloaded with either 4 μL vehicle (DMSO) or 4 μL of test compounds at final concentrations ranging from 0.01 to 100 μmol/L. The tubes were vortexed and incubated under constant agitation for 15 min at 37%. This first treatment was followed by incubation of the blood with 20 μL lipopolysaccharide (LPS) at fina1 concentration of 100 μmol/L (Sigma-Aldrich, Bornem, Belgium, LPS from E. Coli serotype B4, diluted in phosphate buffer saline (PBS)) under constant agitation for 24 h at 37°C in order to induce COX-2 expression. Appropriate controls (LPS replaced by 20 μL PBS) were used as blanks. After incubation, the tubes were centrifugated at 2,500 g for 20 min in order

to obtain plasma. Three hundred µL aliquots of plasma were mixed with 300 µL methanol for protein precipitation. The supernatants were obtained and assayed for PGE_2 production by a specfic radioimmunoassay [30].

RESULTS

In this study, we evaluated the COXs inhibitory profile of seven reference drugs including both conventional NSAIDs represented by indomethacin, diclofenac and tenoxicam (Fig. 1) and COX-2 selective inhibitors such as nimesulide, NS-398, celecoxib and rofecoxib (Fig. 2). The COX inhibitory selectivity and potency of these drugs were measured using two different methods.

First, we evaluated the profile of these reference drugs on purified ovine enzymes (Table 1). In this model, only nimesulide and rofecoxib did not decrease the PGE_2 production by COX-1 while these two drugs dose-dependently inhibited the PGE_2 production by COX-2. NS-398, celecoxib and diclofenac inhibited both COX-1 and COX-2 but showed an enhanced inhibitor potency against COX-2. Furthermore, for concentrations below 10^{-6} M, celecoxib and NS-398 selectively inhibited COX-2. Finally, tenoxicam appeared as a weak COX-1 and COX-2 inhibitor while indomethacin showed a greater inhibitor potency against COX-1 than COX-2.

Secondly, we evaluated the inhibitor potency of these reference drugs at a cellular level using human whole blood (Table 2). Results obtained in this test can be summarized as folows: inhibitor potency measured was greater compared to the results obtained cm purified enzymes for each drug investigated. This enhanced potency permitted the estimation of drug concentration corresponding to an inhibition of 50% of both TXB_2 and PGE_2 production (IC_{50}) for all the drugs considered (Table 3). However, none of these drugs selectively inhibited COX-2. Nevertheless, for drug concentration lower than 10^{-6} M, nimesulide, rofecoxib, celecoxib and NS-398 selectively inhibited COX-2 activity. Diclofenac also preferentially inhibited COX-2 but not selectively, whatever the drug concentration considered. Finally, tenoxicam showed a similar inhibitor potency against COX-1 and COX-2 while indomethacin preferentially inhibited COX-1.

DISCUSSION AND CONCLUSIONS

In this study, we have tested seven reference drugs in two different models in order to evaluate both their potency and selectivity against COX isozymes. Results obtained on purified ovine enzymes demonstrated that nimesulide and rofecoxib selectively inhibited the PGE_2 production by COX-2. On the other hand, celecoxib and NS-398 selectively inhibited COX-2 activity for drug concentrations lower than 10^{-6} M. Finally, diclofenac, indomethacin and tenoxicam did not selectively inhibit COX-2 whatever the drug concentration investigated, although diclofenac appeared more potent against COX-2 than COX-1. These results correlated the classification of the reference drugs investigated in conventional NSAIDs and selective COX-2 inhibitors.

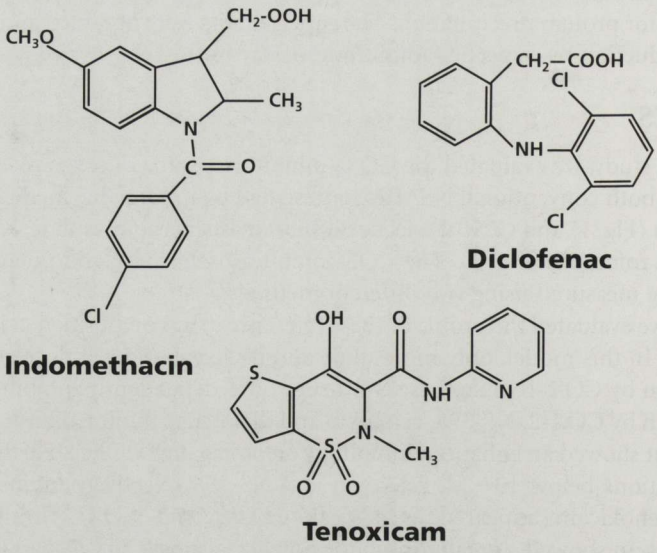


Fig. 1: *Structure of indomethacin, diclofenac and tenoxicam, three conventional NSAIDs.*

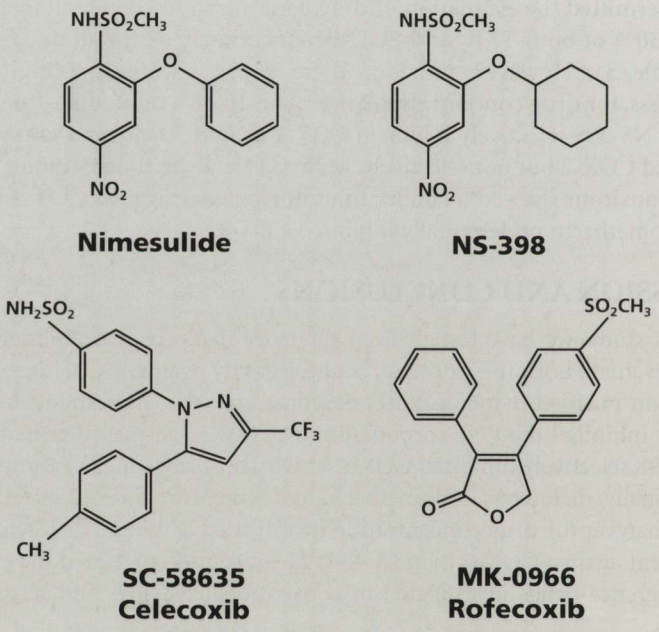


Fig. 2: *Structure of nimesulide, NS-398, rofecoxib and celecoxib, four reference drugs presented as COX-2 selective inhibitors.*

Table 1. Effects of the investigated reference drugs on the PGE_2 production by ovine COX-1 and COX-2

		COX-1 (%)	COX-2 (%)
Nimesulide	10^{-4} M	101.62±4.10	65.21±3.54
	10^{-5} M	99.30±1.92	75.44±2.11
	10^{-6} M	100.08±5.04	79.93±3.24
	10^{-7} M	97.52±3.01	90.02±3.87
	10^{-8} M	95.14±2.69	98.96±3.62
NS-398	10^{-4} M	79.94±5.35	47.35±2.63
	10^{-5} M	92.20±0.67	60.99±0.16
	10^{-6} M	97.41±6.29	66.06±0.70
	10^{-7} M	98.55±0.41	76.36±0.56
	10^{-8} M	94.25±2.77	93.09±0.96
Celecoxib	10^{-4} M	72.10±4.89	56.95±4.55
	10^{-5} M	87.65±10.04	59.63±3.79
	10^{-6} M	107.85±2.03	82.80±3.10
	10^{-7} M	111.69±1.98	91.18±2.19
	10^{-8} M	103.45±9.70	97.19±2.88
Rofecoxib	10^{-4} M	107.62 ± 6.49	64.31 ± 2.57
	10^{-5} M	107.06 ± 5.97	75.49 ±2.19
	10^{-6} M	107.07±12.28	80.32 ± 3.01
	10^{-7} M	97.94 ±1.62	91.72 ± 1.73
	10^{-8} M	96.96 ± 7.78	102.44 ± 0.56
Indomethacin	10^{-4} M	7.32 ± 1.67	18.09 ±2.08
	10^{-5} M	17.09 ± 1.20	31.20 ±3.31
	10^{-6} M	27.41 ± 0.92	42.01 ±3.40
	10^{-7} M	58.70 ± 2.80	96.57 ±2.54
	10^{-8} M	85.94 ± 4.22	100.01 ±3.32
Diclofenac	10^{-4} M	60.00±12.34	38.23 ±2.26
	10^{-5} M	62.36±5.84	42.27 ±1.69
	10^{-6} M	69.73±5.74	48.62 ± 3.91
	10^{-7} M	81,45±2.51	56.07±1.84
	10^{-8} M	97.69 ±1.74	89.21 ±2.53
Tenoxicam	10^{-4} M	73.11±1.16	87.68±1.16
	10^{-5} M	97.15±1.59	84.46 ± 2.85
	10^{-6} M	97.08 ± 3.28	95.93 ± 0.70
	10^{-7} M	98.15 ± 7.66	97.59 ±1.43
	10^{-8} M	99.31 ±0.03	100.04 ± 4.31

Results are expressed as percent of control values. Values are means ± S.D. of triplicates.

The second step of our study consisted of the evaluation of the same reference drugs at a cellular level using human whole blood. Results obtained revealed two major differences with those obtained on ovine purified enzymes. First, all the investigated drugs in this model showed an enhanced inhibitor potency against COX isozyme. This greater inhibition of PGE_2 production by both COX-I and COX-2 permitted the estimation of IC_{50} for all the tested compounds. This enhanced potency can be explained by the different nature of the COX enzymes used in the

Table 2. Effects of the investigated reference drugs on human whole blood

		COX-1 (%)	COX-2 (%)
Nimesulide	10^{-4} M	6.53±2.95	0.31±0.56
	10^{-5} M	58.10±16.09	2.00±1.02
	10^{-6} M	93.57±11.92	52.34±6.97
	10^{-7} M	103.20±7.94	99.77±7.02
	10^{-8} M	99.80±4.11	102.34±4.49
NS-398	10^{-4} M	4.24±1.96	0.54±0.02
	10^{-5} M	44.06±6.27	0.93±0.09
	10^{-6} M	68.66±9.84	7.73±4.29
	10^{-7} M	78.08±12.25	72.76±15.25
	10^{-8} M	92.88±12.31	84.51 ±8.76
Celecoxib	10^{-4} M	4.31±0.47	9.36±1.57
	10^{-5} M	50.73±20.38	13.18 ±3.54
	10^{-6} M	99.68±17.27	42.30±2.94
	10^{-7} M	104.07±6.41	67.06±10.73
	10^{-8} M	98.24 ±12.31	82.05±13.03
Rofecoxib	10^{-4} M	18.35±l.26	0.89±0.72
	10^{-5} M	31.97±0.53	1.81±0.31
	10^{-6} M	95.40±19.28	16.89±0.40
	10^{-7} M	97.95±4.48	77.19±8.12
	10^{-8} M	95.45±7.78	104.96±8.86
Indomethacin	10^{-4} M	2.22±0.67	1.07±2.79
	10^{-5} M	3.21±0.15	1.10±1.82
	10^{-6} M	23.14±1.92	7.51±4.91
	10^{-7} M	36.21±9.61	58.87±16.41
	10^{-8} M	76.63±2.98	88.16±9.49
Diclofenac	10^{-4} M	0.87±0.10	0.13 ±0.07
	10^{-5} M	1.55±0.38	0.31 ±0.20
	10^{-6} M	17.62±3.79	0.88±0.26
	10^{-7} M	84.27±7.34	3.08±3.06
	10^{-8} M	86.77±22.66	97.99±9.18
Tenoxicam	10^{-4} M	6.27±6.01	0.30±0.24
	10^{-5} M	29.77±8.82	18.05±2.95
	10^{-6} M	91.76 ±13.02	93.12±13.32
	10^{-7} M	98.15 ±7.66	105.98±8.56
	10^{-8} M	106.24±9.08	103.27±4.79

Results are expressed as percent of control values. Values are means ± S.D. of triplicates.

two tests performed. Indeed, ovine and human COX enzymes show about 90% sequence homology[31] and minor differences in the sequences between species can modify the drug-enzyme interaction. Furthermore, tests performed on purified enzymes only show a purely drug-enzyme interaction whereas other interactions can exist and modify the measured potency at a cellular level. For example, binding of the investigated drug to plasmatic proteins, enzymatic degradation of the drug into active metabolites [32] or interaction between the drug and mediators of inflammation that can induce COX-2 [33, 34] can drastically modify the inhibitor potency

measured at a cellular level. Secondly, none of the investigated drugs appeared as a purely selective COX-2 inhibitor. However, at therapeutic concentration, nimesulide, rofecoxib and celecoxib can almost be considered as COX-2 selective inhibitors. Indeed, C_{max} at steady state after multiple doses of 25 mg rofecoxib is 321 ng/mL (1.02×10^{-6} M). In regard to the results obtained on human whole blood, at this concentration, rofecoxib inhibits 19.67 % of the COX-1 activity while it decreases of 83.98% the PGE_2 production by COX-2. The same observations can be realized concerning celecoxib. The plasmatic C_{max} of celecoxib is measured 3 h after ingestion of 100 mg and reaches 705ng/mL (2×10^{-6} M). At this concentration and in regard to the results obtained on human whole blood, celecoxib inhibits 13.46 % of the COX-1 activity while it decreases of 65.94 % the PGE_2 production by COX-2. The same reasoning can be led with nimesulide, demonstrating that this drug preferentially inhibits COX-2 On the other hand, indomethacin, for which plasmatic levels measured currently reach 1×10^{-6} M, inhibits, in regard to the results obtained in the whole blood model, 72.59 % of the COX-1 activity while this drug only inhibits 57.99 % of the COX-2 activity. This estimation demonstrates that indomethacin preferentially depresses COX-1 activity.

Table 3. IC_{50} estimated for each reference drug in the human whole blood assay

	IC_{50}	
	COX-1 (%)	COX-2 (%)
Nimesulide	2.2×10^{-5} M	1.3×10^{-6} M
NS-398	8.3×10^{-6} M	3.4×10^{-7} M
Celecoxib	1.2×10^{-5} M	7.7×10^{-7} M
Rofecoxib	7.8×10^{-6} M	4.3×10^{-7} M
Diclofenac	6.0×10^{-7} M	4.0×10^{-8} M
Tenoxicam	7.2×10^{-6} M	5.6×10^{-6} M
Indomethacin	6.6×10^{-8} M	1.7×10^{-7} M

Several conclusions can be realized on the results obtained on both ovine enzyme test and human whole blood assay. First, results obtained with the ovine purified enzymes did not permit the determination of IC_{50} for the investigated drugs. However, this test permitted the comparison of the COXs inhibitory potency of the investigated drugs at a defined concentration. On the other hand, this test has been led using ovine enzymes. Although there is about 90% homology in the amino acid sequences between ovine and human COX enzymes, slight differences in the protein sequence can lead to variations of the inhibitory potency measured. Considering the nature of the enzymes, human whole blood appears more interesting. Moreover, results obtained using this test gave us the possibility to estimate IC_{50} for all of the investigated drugs and, consequently, permitted the estimation of COX-1/COX-2 ratios by division of the COX-1 IC_{50} by the COX-2 IC_{50} measured in the human whole blood assay (Table 4). These ratios classify the COX-2 selectivity of the investigated drugs. Using this classification, NS-398, rofecoxib, celecoxib,

nimesulide and diclofenac obtained high scores, clearly demonstrating that these compounds preferentially inhibit COX-2. This method also allows the comparison of the inhibitory potency and selectivity of drugs at doses corresponding to the plasmatic levels measured *in vivo*. In regard to these considerations, human whole blood assay seems to be an interesting method for the evaluation of the inhibitory selectivity and potency of drugs against COX isozymes although optimal investigation method should be a human whole blood assay performed *ex vivo* after intake of drugs during several days. However, such investigations can not be performed with drugs in pre-clinical trials.

Table 4. COX-1/COX-2 ratios for each reference drug determined from the results obtained in the human whole blood assay

	COX-1/COX-2 ratios
Nimesulide	16.92
NS-398	24.41
Celecoxib	15.59
Rofecoxib	18.14
Diclofenac	15
Tenoxicam	1.29
lndomethacin	0.39

Finally, literature concerning the COXs inhibitory potency of NSAIDs clearly demonstrates that minor differences in the evaluation method used leads to differences in the inhibitory potency and selectivity measured.[35] This observation suggests that reference drugs should always be included to the trials led on original molecules.

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ANNEXE 11

Comparison of the effects of nimesulide and nimesulide-L-lysine on PGE_2 production by COX-1 and COX-2 and on chondrocyte metabolism *in vitro*

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Abstract

Nimesulide, a non-steroidal anti-inflammatory drug and one of a promising class of the selective COX-2 inhibitors, has a very interesting therapeutic profile. Unfortunately, it is poorly soluble in water, which leads to important difficulties in the formulation of injectable solutions. This problem can also affect the bioavailability of nimesulide. To increase the aqueous solubility of the drug, a nimesulide-L-lysine salt was synthesized in our laboratory ; its aqueous solubility was greater than that of nimesulide (solubility in purified water 7.5 mg mL^{-1} and 0.01 mg. mL^{-1} , respectively).

The aim of this study was to compare the anti-inflammatory profiles of nimesulide and nimesulide-L-lysine salt in a two-step *in vitro* investigation. First, we evaluated the COX-2 selectivity of the drugs by a method using purified COX-1 and COX-2 enzymes. In a second step we evaluated the effects of the drugs on the production of prostaglandin (PGE_2) and proteoglycans by human chondrocytes. The results obtained confirmed the COX-2 selectivity of the two compounds. Nimesulide-L-lysine had the same anti-inflammatory profile as nimesulide on chondrocyte cultures and better water solubility.

Nimesulide-L-lysine should, therefore, be used to prepare injectable preparations and should ameliorate bioavailability of oral treatments.

Introduction

Cyclooxygenase (prostaglandin synthase) catalyzes the oxygenation of arachidonic acid to prostaglandin H_2 (PGH_2) as a first step in the biosynthesis of prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$), prostacyclin (PGI_2) and thromboxane (TXA_2). The enzyme is expressed in mammalian cells as two distinct isozymes (COX-1 and COX-2) that have approximately 60% amino acid sequence similarity (Herschman, 1996; Smith *et al.*, 1996). COX-1 is constitutively expressed in healthy tissue and is important in thrombogenesis (COX-1 is the only isoform expressed in platelets) and in the homeostasis of the gastrointestinal tract and kidneys (Funk *et al.*, 1991; O'Neill & Ford-Hutchinson 1993). In contrast, COX-2 is almost undetectable under physiological conditions but its expression is induced by a wide range of stimuli such as cytokines, growth factors and hormones (Fu *et al.*, 1990; Kujubu *et al.*, 1991; Jones *et al.*, 1993; Rimarachin *et al.*, 1994).

Most conventional non-steroidal anti-inflammatory drugs (NSAIDs) inhibit both enzymes. The observation that COX-2 is associated with inflammatory conditions and that COX-1 is mainly expressed as a 'housekeeping' enzyme led to the development of selective COX-2 inhibitors in order to reduce the risk of gastric irritation, ulceration and renal damages associated with the chronic use of non selective NSAIDs (Griswold & Adams 1996, Richardson & Emery 1996).

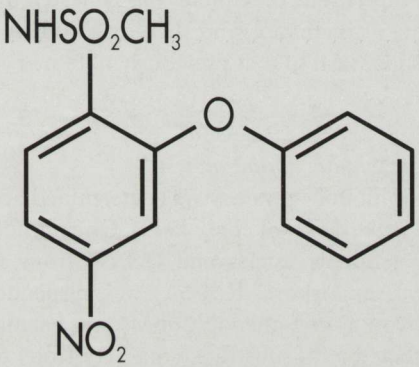


Figure 1: *The chemical structure of nimesulide.*

Nimesulide (4'-nitro-2-phenoxymethanesulphonanilide) (Figure 1) belongs to this promising class of NSAIDs . It has been shown to inhibit COX-2 selectivity both *in vitro* (Barnett *et al.*, 1994; Famaey 1997; de Leval *et al.*, 1999) and in man (Famaey 1997; Cullen *et al.*, 1998) and to have antinflammatory, antipyretic and analgesic properties (Ward & Brogden 1988; Davis & Brogden 1994) without inducing marked gastric side effects (Cipollini *et al.*, 1989; Marini & Spotti 1993; Porto *et al.*, 1994). Nimesulide differs chemically from most carboxylic NSAIDs by its weakly acidic sulphonanilide group (pka 6.8). Unfortunately, the very poor water solubility (0.01 mg.mL-1) and wettability of the drug cause difficulties in the formulation of injectable solutions and can lead to variable bioavailability after oral

and rectal treatment (Piel *et al.*, 1997). A nimesulide L-lysine salt has been synthesized (Pirotte *et al.*, 1995) and shown to increase the aqueous solubility of nimesulide to 7.5 mg mL^{-1} (Piel *et al.*, 1997). The aim of this study was to compare the anti-inflammatory profiles of nimesulide and nimesulide-L-lysine *in vitro* by using an enzymatic method based on evaluation of their inhibitory potency on prostaglandin (PG)E_2 production induced by COX-1 and COX-2. We also studied the effects of both drugs on PGE_2 and proteoglycans production by human articular chondrocytes.

Materials and Methods

Drugs and Chemistry

Nimesulide (free acid form) was purchased from Stragen (Genève, Switzerland) and L-lysine was obtained from Janssen Chimica (Geel, Belgium).

Nimesulide-L-lysine salt was prepared as described by Pirotte *et al.*, (1995). Briefly, a methanolic solution (50 mL) of L-lysine monohydrate (1.64 g; 10^{-2} mol) was added to a hot (60 °C) methanolic solution (100 mL) of nimesulide (3.08 g; 10^{-2} mol). The orange-colored solution was filtered if necessary and concentrated under reduced pressure to a small volume (80 mL). The resulting suspension was cooled at 4 °C for 2 hours. The precipitate was collected by filtration, washed twice with a small vo-lume of methanol, and then dried (yield, 82 %; mp, 200-204 °C). The compound was identical to that previously obtained (Pirotte *et al.*, 1995).

Pharmacology

COX-1 and COX-2 enzymatic inhibition test

Cyclooxygenase inhibitory potency was determined according to an enzymatic test described elsewhere (de Leval *et al.*, 1999). One unit of cyclooxygenase enzyme (COX-1 from ram seminal vesicles and COX-2 from sheep placenta; Cayman Chemical Company, Ann Arbor, MI, USA) was suspended in Tris buffer (1 mL, 100 mmol/L, pH 8) containing phenol (2 mM) and haematin (1 μM) as cofactors. They were pre-incubated with substrate alone (controls) or with different concentrations (0.01, 0.1, 1, 10 and 100 μM) for 5 min at 37°C. Arachidonic acid sodium salt (10 μM; Calbiochem) was added and further incubated for 2 min at 37 °C. The reaction was halted by placing the tubes on ice and by adding diclofenac sodium salt (final concentration 1 mM). PGE_2 production was assayed by a specific radioimmunoassay (RIA) and expressed as mean +/- s.d. The intra-assay precision of the method expressed as the coefficient of variation,was 3.8 % (n=3) and the reproducibility expressed as the inter-assay coefficient of variation was 2 % (n=3).

Human cartilage

Short term suspension culture of human chondrocytes. Cartilage was obtained from the knee joints of one adult shortly after death. Slices were excised from the superficial and medium layers of knee cartilage, avoiding the calcified layer, cut into small fragments, submitted to three successive enzymatic digestions and cultured as

according to a method described elsewhere (Henrotin *et al.*, 1998). The culture medium obtained was assayed directly for PGE_2 and proteoglycans.

Treatment. Chondrocytes were cultured at high density for a short time to maintain their phenotype. In brief, chondrocytes (1-2 x 10^6 cells mL^{-1}) were cultured for 72 h in the presence or absence of interleukin-1ß (IL-1β) (concentration 10^{-12} M; cytokine from Boerhinger Mannheim, Germany) and with or without nimesulide or nimesulide-L-lysine (at concentrations of 1, 10 and 100 μM). Both drugs were dissolved in dimethylsulfoxide (DMSO), and then further diluted with culture medium to the required final concentration. The final concentration of DMSO in each culture flask did not exceed 0.1 %.

DNA assay. The chondrocyte DNA content was measured according to the fluorimetric method of Labarca and Paigen (1980). Each flask was assayed twice.

PGE2 radioimmunoassay. PGE_2 was assayed directly in conditioned culture medium, i.e. without previous extraction, by a radioimmunoassay (RIA) method described elsewere (Serteyn *et al.*, 1988). Polyclonal antiserum, obtained from rabbit, did not cross-react with other prostanoids (TXB_2, 6-keto-PGF-1α) or with fatty acids (arachidonic, linoleic and oleic acid). ^3H labelled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and standard PGE_2 from Sigma. (St. Louis, MO). PGE_2 was measured in duplicate on 100 μL samples. After 48 h incubation at 4 °C, free antigen was separated from antibody-bound antigen by charcoal precipitation and centrifugation (20 min at 1000 g and 4 °C). The supernatant was counted by liquid scintillation. The limit of detection of the RIA was 19 pg mL^{-1}. The intra- and inter-assay coefficients of variation were 6 % and 10 %, respectively.

Proteoglycan radioimmunoassay. Proteoglycans released into the culture medium and present within the cellular phase were assayed by a method described elsewere (Gysen & Franchimont 1984). Briefly, anti-serum against human proteoglycan was raised in rabbits by a method of Vaitukaitis (1971). The radioimmunoassay (RIA) was performed in a 0.4 mL incubation volume consisting of 0.1 mL tracer solution (15,000-20,000 counts min^{-1}) containing proteoglycans labelled with ^{125}I by the chloramine-T method (Greenwood *et al.*, 1963); 0.1 mL anti-proteoglycans antiserum diluted 1:5000 in phosphate buffered saline (0.15 μM NaCl) and containing 5 g L^{-1} BSA (bovine serum albumine); and 0.2 mL of several dilutions of conditioned culture medium, chondrocyte cluster extract or the unlabelled antigen ranging from 0.1 to 500 ng/tube (reference curve). After 4 days at 4 °C, the labelled proteoglycan anti-body complexes were separated by double precipitation. The analytical sensitivity of the RIA was 0.6 ng/tube. The intra- and inter-assay coefficients of variation were less than 10 % and 20 %, respectively, along the linear part of the curve.

Results and Discussion

In a first set of experiments we compared the selective inhibition of COX-2 by nimesulide and nimesulide-L-lysine using an enzymatic method based on evaluation of PGE_2 production by arachidonic acid incubated with purified enzymes COX-1 and COX-2 in the presence or absence of the drug at different concentrations (0.01, 0.1, 1, 10 and 100 μM). In this test, neither nimesulide nor nimesulide-L-lysine, irrespective of concentration, inhibited PGE_2 production induced by COX-1 whereas at concentrations above 10 μM drugs inhibited COX-2 (Table 1). In contrast, indomethacin, a non-specific NSAID used as reference, caused dose-dependent inhibition of PGE_2 production by COX-1 (indomethacin compared with COX-1: $r = 0.97$; $y = -198x + 39.4$; $p = 0.0001$; 92,7 % inhibition of PGE_2 production at a concentration of 100 μM: IC_{50}: 0.1 μM) and COX-2 (indomethacin compared with COX-2: $r = 0.95$; $y = -22.67x + 57.4$; $p = 0.0001$; 81,9 % inhibition of PGE_2 production at a concentration of 100 μM: IC_{50}: 0.5 μM) (Table 1).

Table 1: *Effect of indomethacin, nimesulide, and nimesulide-L-lysine on the production of prostaglandin E_2 by COX-1 and COX-2.*

Drugs	Concn (μM)	COX-1	COX-2
INDOMETHACIN	0.01	85.9±4.2	100.0±3.3
	0.1	58.7±2.8	96.6±2.5
	1.0	27.4±0.9	42.0±3.4
	10.0	17.1±1.2	31.2±3.3
	100.0	7.3±1.7	18.1±2.1
NIMESULIDE	0.01	95.1±2.7	99.0±3.6
	0.1	97.5±3.0	90.0±3.9
	1.0	100.1±5.0	79.9±3.2
	10.0	99.3±1.9	75.5±2.1
	100.0	101.6±4.1	65.2±3.5
NIMESULIDE-L-LYSINE	0.01	103.4±4.1	104.1±4.6
	0.1	102.6±3.9	99.1±2.4
	1.0	97.9±1.8	99.2±3.2
	10.0	98.5±3.8	87.4±3.7
	100.0	98.9±3.1	79.3±2.1

Results (%) are means ± s.d. from triplicate measurements.

This enzymatic *in vitro* test showed that nimesulide-L-lysine and nimesulide were both selective COX-2 inhibitors. To determine their anti-inflammatory potency at the cellular level we evaluated their effects on PGE_2 production by human chondrocytes. Compared with the controls, nimesulide and nimesulide-L-lysine strongly reduced PGE_2 production by unstimulated (controls 61.7 ± 13.3 pg mL^{-1}) or IL-1ß-stimulated (controls 240.3 ± 34.5 pg mL^{-1}) chondrocytes to undetectable values (< 19 pg mL^{-1}) whatever the concentrations used (Table 2). The results of this test confirm the cyclooxygenase inhibitory potency of both drugs on chondrocytes,

even at low concentrations in contrast with the test realized on purified COX enzymes. This contrasting results might arise because of sligth differences between the structures of COX enzymes from man and sheep, or because nimesulide might have other activity, for example inhibition of COX-2 gene expression. Compared with reference values the two compounds did not significantly modify the DNA content of the cellular phase of unstimulated or IL-1β-treated chondrocyte cultures (differences between DNA content of control cultures and cultures treated with the drugs at 10 μM and 1 μM < 13 %) indicating the absence of cellular toxicity (Table 2).

Table 2. *PGE_2 production by unstimulated or IL-1ß-stimulated chondrocytes and the DNA content of the human chondrocytes.*

Drugs	Concn (μM)	PGE_2 (pg mL^{-1})	PGE_2 +IL-1ß (pg mL^{-1})	DNA (μg mL^{-1})	DNA +IL-1ß (μg mL^{-1})
Control values		61.7±13.3	240.3±34.5	8.8±0.8	8.7±0.9
Nimesulide	100	<19	<19	7.8±0.7	6.6±0.3
Nimesulide	10	<19	<19	8.4±0.4	7.6±0.3
Nimesulide	1	<19	<19	8.4±0.4	9.3±0.3
Nimesulide L-lysine	100	<19	<19	7.5±0.4	6.4±0.9
Nimesulide L-lysine	10	<19	<19	9.0±0.5	8.4±1.0
Nimesulide L-lysine	1	<19	<19	8.3±0.4	7.6±0.9

Results are means ± s.d. from triplicate measurements.

Table 3. *Extracellular and intracellular proteoglycan production by human unstimulated and IL-1ß-stimulated chondrocytes from man.*

Drugs	Concn (μM)	Extracellular proteoglycans		Intracellular proteoglycans	
		Unstimulated (μg mL^{-1})	+IL-1β (μg mL^{-1})	Unstimulated (μg mL^{-1})	+IL-1β (μg mL^{-1})
Control values		2579±40.7	1410±70.3	1370±59.59	65±20.8
Nimesulide	100	1883±75.5	1008±65.1	1080±47.4	802±70.9
Nimesulide	10	2650±62.4	1201±56.7	1334±17.1	948±21.8
Nimesulide	1	2793±123.2	1410±51.9	1348±49.1	981±40.5
Nimesulide L-lysine	100	1722±103.8	883±53.9	949±62.8	699±91.2
Nimesulide L-lysine	10	2777±106.2	1371±18.4	1408±72.1	891±13.7
Nimesulide L-lysine	1	2695±92.3	1298±23.1	1395±51.7	905±68.1

Results are means ± s.d. from triplicate measurements.

At therapeutic concentrations, nimesulide has been shown to inhibit PGE_2 production without affecting proteoglycan production by human articular chondrocytes, whereas several types of NSAID for example salicylates accelerated cartilage cata-bolism by suppressing proteoglycan synthesis (Pelletier *et al.*, 1993, Henrotin *et al.*, 1999). We compared the effects of nimesulide-L-lysine and nimesulide on

proteoglycan synthesis by chondrocytes from man; as indicated in Table 3, at 100 μM both drugs weakly inhibit release of proteoglycan into the culture medium and reduce the levels present in the cellular phase of unstimulated or IL-1β-treated chondrocyte cultures whereas at therapeutic doses (10 and 1 μM) they did not significantly affect proteoglycan production. These findings suggest that nimesulide-L-lysine which, is more water soluble, has a similar anti-inflammatory profile to nimesulide with regard to COX-2 specificity and cartilage safety. Nimesulide-L-lysine could, therefor, be used to replace nimesulide in the pharmaceutical formulation of injectable solutions, oral treatment with nimesulide-L-lysine should ameliorate the bioavailability without affecting the clinical efficiency of the drug.

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ANNEXE 12

Effects of nimesulide and sodium diclofenac on interleukin-6, interleukin-8, prostaglandin E_2 and proteoglycan production by human articular chondrocytes *in vitro*.

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Abstract

Objectives The aim of this study was to investigate the effects of two nonsteroidal anti-inflammatory drugs (NSAIDs), nimesulide and sodium diclofenac, on the production of proteoglycans (PG), prostaglandin E_2 (PGE_2) and cytokines (IL-6 and IL-8) by human articular chondrocytes *in vitro*.

Methods Enzymatically isolated chondrocytes were cultured under constant agitation in a well defined culture medium. Specific radioimmunoassays were used to quantify PG and PGE_2 production. Cytokine production (IL-6 and IL-8) was assayed by enzyme amplified sensitivity immunoassays (EASIAs).

Results At a concentration of 3 μg/ml, nimesulide did not affect the PG production by chondrocytes. This concentration was superior to the highest level of nimesulide found in the synovial fluid of patients with rheumatoid arthritis 3 hours after the last oral administration of nimesulide (100 mg twice daily for 7 days). At 6 μg/ml a significant reduction in the PG content was obtained in the cellular phase in 5 out of the 8 cultures investigated. No similar effect was observed in the culture supernatants. Above this concentration nimesulide inhibited PG production in a dose-dependent manner

At concentrations ranging from 0.005 to 1 μg/ml diclofenac did not significantly alter PG production. At therapeutic concentrations PGE_2 production was totally inhibited by nimesulide, thus suggesting that PG inhibition is not linked to PGE_2 production. Nimesulide inhibited PGE_2 production by unstimulated (IC_{50}= 6 ng/ml) and IL-1ß-stimulated (IC_{50} = 6.9 ng/ml) chondrocytes. At these concentrations, PGE_2 production was fully inhibited by diclofenac.

Furthermore, both nimesulide and diclofenac at therapeutic concentrations significantly decreased spontaneous and IL-lß stimulated IL-6 production by human chondrocytes, but did not modify IL-8 production.

Conclusion From the results of this study we conclude that nimesulide and diclofenac at therapeutic concentrations are potent inhibitors of PGE_2 and IL-6 production, while they do not modify proteoglycan or IL-8 production.

Key words Osteoarthritis, chondrocytes, cytokines, nimesulide, diclofenac.

Introduction

Osteoarthritis (OA) is the result of an imbalance between cartilage resorption and cartilage repair processes. This imbalance may be reproduced *in vitro* by interleukin-1 (IL-1ß) which is capable, at sub-nanomolar concentrations, of inhibiting the synthesis of cartilage matrix components and of stimulating the production of enzymes and free radicals [1, 2, 3]. Moreover, IL-1ß increases the synthesis of many soluble mediators including prostanoids and cytokines which are capable of regulating chondrocyte functions but also neighbouring tissue metabolism [4]. Some therapeutic drugs have recently been developed for the treatment of OA, their aim being to restore cartilage homeostasis and hence to prevent, retard, reverse, or stabilize the progression of OA.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used for the treatment of OA. These drugs are now recognized to be efficacious in relieving the symptoms of OA. Nevertheless, several types of NSAIDs inhibit the synthesis of proteoglycans (PGs) and collagen by chondrocytes *in vitro* [5, 6] and hence may diminish the potential for cartilage repair in OA. These *in vitro* observations are compatible with *in vivo* studies suggesting that some NSAIDs such as aspirin accelerate the progression of OA [7, 8]. NSAIDs may exert their deleterious effects on cartilage ma-trix synthesis by inducing IL-1 synthesis [9], inhibiting prostaglandin E_2 (PGE_2) production [10], inhibiting glycosyltransferase activity (11) or reducing mitochondrial oxidative phosphorylation, as recently suggested by Mahmud *et al.* [12]. However, it has also been reported that a number of NSAIDs inhibit PG degradation in cartilage tissue and suppress cartilage breakdown in animal models of OA [13]. These beneficial actions of NSAIDs seem to be linked to their inhibiting effect on metalloprotease synthesis and their stimulating action on the production of the tissue inhibitor of metalloproteases-1 (TIMP-1) by chondrocytes [14].

Nimesulide is an innovative NSAID which is chemically different from the other drugs in this class because of the functional non-acidic group in its sulfonanilide group. This drug exerts its anti-inflammatory action through the inhibition of prostanoid synthesis more potent on cyclooxygenase-2 (COX-2) than COX-l [15]. Other possible mechanisms of action include inhibition of the release of active oxygen species [16], histamine [17] or tumor necrosis factor [18], and platelet activating factor synthesis [19]. Some of these effects are related to the drug's ability to inhibit phosphodiesterase type IV [20]. Nimesulide also has a direct scavenging effect on hypochlorous acid and hydroxyl radicals [21, 22]. In OA nimesulide is effective in reducing the clinical symptoms associated with the disease [23]. Nevertheless, little is known about the effects of this drug on cartilage metabolism. When added to cartilage explant *in vitro*, nimesulide at concentrations within the therapeutic range reduced the breakdown of OA cartilage by the inhibition of metalloproteinase synthesis [24].

Although many studies have been published on the NSAIDs, no information is yet available concerning the safety of nimesulide in terms of anabolic chondrocyte functions. Moreover, to the best of our knowledge no study has provided data on the

action of NSAIDs on IL-6 and IL-8 production by human chondrocytes. The purpose of this study was to assess the effect of nimesulide and sodium diclofenac on the production of cytokines (IL-6, IL-8), PGE_2 and PGs by unstimulated and IL-1ß-stimulated normal and OA human chondrocytes.

Materials and methods

Preparing the chondrocyte suspension culture

Cartilage was obtained from the knees of cadavers immediately after death, being excised from the superficial and medium layers of cartilage and avoiding the calcified layer. Upon dissection the femoral, patellar and tibial articular surfaces were evaluated for gross pathological cartilage modifications. Using the scale of Moskowitz *et al.* [25], the severity of pitting was recorded for each patient.

Four different grades were considered: 0, normal white cartilage in all areas examined; I, the presence of a yellow-grey area with some superficial fibrillations on one or more articular surfaces: II, irregular surface with deep fibrillations on one or more articular surfaces; and III: ulcers penetrating to the subchondral bone on one or more articular surfaces.

Chondrocytes were cultured at high density for a short period in order to maintain their phenotype. The procedure was as follows. Cartilage was cut into small fragments and then subjected to enzymatic digestion sequentially with hyaluronidase, pronase, and collagenase. First, the pieces were incubated for 30 min. at 37 °C under constant agitation (200 rpm) with hyaluronidase (Sigma Chemie, Bornem, Belgium), previously dissolved (0.5 mg/ml) in Dulbecco's Modified Eagle's Medium (DMEM, Biowhittaker, Bruxelles, Belgium) (10 ml enzyme solution per 3 g cartilage). The fragments were then incubated for one hour at 37 °C in pronase solution (1 mg/ml of DMEM; enzyme obtained from Merck-Belgolabo, Overijse, Belgium) (3 g cartilage per 10 ml enzyme solution). Finally the cartilage fragments were incubated for 20 hours at 37 °C under constant agitation (200 rpm) with collagenase (Sigma Chemie) dissolved in DMEM (1 mg/ml) containing 1% Ultroser G (Gibco, Gent, Belgium) (3 g cartilage per 10 ml enzyme solution).Ultroser G is a serum substitute designed to replace foetal calf serum in nutrient culture medium. The cells were then filtered through nylon mesh with a pore diameter of 25 pm, washed three times, counted (range: 1 to 2 10^6 cells/ml) and resuspended in 1ml of the appropriate culture medium (DMEM supplemented with 1 % of ITS+ and ascorbate 50 µg/ml). ITS+ is a well defined culture supplement containing 0.625 mg/ml insulin, 0.625 mg/ml transferrin, 0.625 µg selenium acid, 125 mg/ml bovine serum albumin (BSA), and 0.530 mg/ml linoleic acid.

Cells were seeded in 10 ml polypropylene Falcon flasks and maintained in suspension by constant agitation on a gyratory shaker (100 rpm) in a 95% air + 5% CO_2 atmosphere. The cells and supernatant were separated by centrifugation at 1000 rpm for 5 min. Cell viability, estimated by trypan blue incorporation, was 95 % in both the treated and untreated groups. PGs, PGE_2 and cytokines (IL-6 and IL-8) were directly assayed in culture media previously supplemented with protease

inhibitors. The protease inhibitor mixture used for this study was composed of 6-amino-hexanoic acid 0.1 mole/L, benzamidine chlorhydrate 0.05 mole/L, trypsin inhibitor 5 x 10^{-8} mole/L, EDTA 0.01 mole/L, and sodium azide 6.7 x 10^{-3} mole/L. Cell pellets were washed twice with HBSS (Hank's buffer saline solution, Gibco, Merelbeke, Bel-gium) and then homogenized in phosphate buffer saline (PBS) pH 7.5 by ultrasonic dissociation (10 s pulses, power 50 W/cm^2). All chemicals for which the source was not mentioned were of the purest grade commercially available.

Treatments

Chondrocytes were cultured at high density for a short period in order to maintain their phenotype. In brief, chondrocytes (1 to 2 10^6 cells/ml) were cultured for 24 to 72 hours in the presence or absence of IL-1ß (at concentrations ranging from 10^{-12} to 10^{-9} M; cytokine from Boehringer Mannheim, Germany) and with or without nimesulide (at concentrations ranging from 0.005 to 120 µg/ml) or diclofenac (0.005 to 6 µg/ml). Nimesulide was kindly provided by Therabel Research (Bruxelles, Belgium) while the sodium diclofenac was a gift of Novartis (Basel, Switzerland).

Both NSAIDs were first dissolved in dimethylformamide (DMF), and then further diluted in culture medium to achieve the required final concentration. The final concentration of DMF in each culture flask was 0.1%. These cultures were grown using chondrocytes isolated from cartilage samples excised from 15 different donors. The cultures from 13 donors were treated with various concentrations of nimesulide, while the cultures from 3 donors were treated with various concentrations of diclofenac. Three culture flasks were used for each concentration of NSAID as well as for the corresponding controls. Each culture was run with chondrocytes from the knee joint of a single patient.

The peak concentrations of nimesulide reported in the plasma of RA patients who were taking 100 mg orally twice a day ranged from 3 to 6 mg/L (after one or repeated 100 mg doses b.i.d; 3 mg/L is above the highest level noticed 3 hrs after the last dose in the synovial fluid of RA patients receiving 100 mg b.i.d orally for 7 days) [26]. The maximum observed plasma concentrations after the administration of oral diclofenac 50 mg twice daily ranged from 0.5 to 3 mg/L [27, 28].

Radioimmunoassay for proteoglycans

PGs released into the culture medium (CM) and PGs present within the cellular phase (CP) were assayed according to a method previously described [29]. Briefly, anti-serum against human cartilage was raised in rabbits according to the method of Vaitukaitis *et al.* [30]. The radioimmunoassay (RIA) was performed in a 0.4 ml incubation volume consisting of 0.1 ml tracer solution (15,000 - 20,000 cpm) containing PGs labelled with ^{125}I by the chloramine-T method [31]; 0.1 ml anti-PGs antiserum diluted 1: 5000 in PBS (0.15 mol/1 NaCl) and containing 5 g/l BSA (incubation buffer); and 0.2 ml of several dilutions of conditioned culture medium, chondrocyte cluster extract or the unlabelled antigen ranging from 0.1 to

500 ng/tube (reference curve).

After 4 days at 4 °C, the labelled PG-antibody complexes were separated by double precipitation. The analytical sensitivity of the RIA was 0.6 ng/tube. The intra- and inter-assay coefficients of variation were less than 10 % and 20 %, respectively, along the linear part of the curve. The antibodies were only directed against the antigenic determinant of the core protein, as shown by the lack of cross reaction by chondroitin sulfate, keratan sulfate and treatment with various enzymes [29]. Only treatment with proteolytic enzymes (papain, trypsin, and chymotrypsin) abolished or diminished immunoreactivity as opposed to treatment with chondroitinase ABC and neuraminidase [29]. The anti-serum did not cross react with other cartilage matrix constituents such as type II, type IX, and type XI collagens. On the other hand, as suggested by the lack of cross reaction with bone PGs or skin, cornea, liver, heart and lung tissue extracts, it appears that our anti-serum was tissue-specific [29].

The sum of PGs in the CM and CP corresponded to the total production (TP) of the molecule.

The culture media were directly assayed for PGs. Chondrocyte pellets were washed three times in PBS containing enzymatic inhibitors and were then homogenized by ultrasonic dissociation (three pulses of 10 s, power: 50 watts/cm^2). This extraction method allowed us to recover 80 % of the total HCl-guanidine extractable PGs. All of the chemicals used were of the purest grade commercially available.

Physico-chemical forms of the PGs produced by chondrocytes

$^{35}Na_2SO_4$ (5 µci/ml; Amersham, Gent, Belgium) was added to the culture medium during the last 24 hours of culture.

The culture media were dialysed 3 times against Tris-HCL 0.05 M, pH 9 containing protease inhibitors (listed above) and were then directly eluted onto a Sepharose CL2B column (Pharmacia, Sweden). PGs contained in the cellular phase were extracted by treatment with 4 M guanidinium hydrochloride and 0.5 M sodium acetate, pH 5.8 containing protease inhibitors (listed above) and kept for 24 hrs at 4 °C under constant agitation. The extracts of 3 cell pellets treated under the same conditions were pooled and extensively dialysed against distilled water and Tris-HCL 0.05, pH 9. For analytical purposes, a column (0.8 x 60 cm) was packed with Sepharose CL2B. The column was equilibrated and eluted with Tris-HCL 0.05 M, pH 9 containing enzymatic inhibitors (listed above). The flow rate was 6 ml/h and a 1 ml fraction was collected. The column was calibrated under the same conditions with a 1 ml sample of $^{35}Na_2SO_4$ adjusted to 20,000 cpm. The peak fraction containing aggregated ^{35}S-PG and the $^{35}Na_2SO_4$ counts were used to determine the void volume of the column (Vo) and the saline volume of the column (Vs), respectively. The chromatogram was established by determining the radioactivity in 0.3 ml of each fraction. All of the chemicals used were of the purest grade commercially available.

Prostaglandin E_2 radioimmunoassay

PGE_2 was assayed in conditioned culture media according to a previously described RIA [32]. PGE_2 was directly measured into the culture media without previous extraction. In this assay, polyclonal anti-serum obtained from rabbit cross reacts neither with other prostanoids (TxB_2, 6-keto-PGF1α, PGA_2) nor with any fatty acids (arachidonic, linoleic or oleic acids) [32]. ^{3}H-labelled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule (PGE_2 from Sigma Chemical Company (St. Louis, USA). PGE_2 was measured in duplicate on a 100 µl aliquot of each sample. After 48 hrs of incubation at 4 °C, the free antigen was separated from the antibody-bound antigen by charcoal precipitation after centrifugation (20 min. at 1,000 g, 4 °C). The supematant was counted by liquid scintillation.

The intra- and inter-assay coefficients of variation were 6 % and 10 %, respectively. The recovery of the known amounts of PGE_2 added to the culture medium was satisfactory (97 % - 90 % for amounts of PGE_2 ranging from 2,500 to 312 pg/ml). The sensitivity was 20 pg/ml.

Immunoassays for cytokines

IL-6 and IL-8 were assayed in the culture media by specific EASIAs (Enzyme Amplified Sensitivity Immunoassays) provided by Biosource Europe (Fleurus, Belgium). The methods and type of tracer used, and the sensitivity, precision, reproducibility and accuracy of the technique have been previously described [33].

DNA assay

The chondrocyte DNA content was measured according to the fluorimetric method of Labarca and Paigen [34]. Each flask was assayed twice.

Statistical analysis

The results were expressed as the rates of PGs, PGE_2 and cytokines released in the CM per µg of DNA and as the CP extract content per µg of DNA. The mean ± SD for each variable was calculated. The total PG production corresponded to the sum of the PG amounts found in the culture supernatant and in the corresponding cellular phase. Comparison of the treated groups with the controls was performed for each culture individually using the unpaired Student's t-test, with a significance limit set at $p < 0.05$. A generalised statistical analysis was also carried out on all of the experiments by the ANOVA test. The correlation between the variables was investigated by linear regression analysis, and a one-way analysis of variance was performed (F-test).

Results

Effect of nimesulide and diclofenac on proteoglycan production

The quantitative and kinetic studies of PG synthesis showed that the amounts of PG released in the supernatants and contained in the cellular phase of the chon-

drocyte culture increased as a function of the duration of the culture period (Fig. 1). A large part of the newly synthesized PGs (10% to 20%) was immobilized in the cellular phase of the culture, suggesting the formation of a new matrix surrounding the chondrocytes.

Table I shows the effect of nimesulide on PG production by cultured unstimulated chondrocytes isolated from 13 different cartilage samples. The doses of nimesulide (3 and 6 μg/ml) tested were within the range of concentrations found in the plasma after oral administration of either one or repeated b.i.d 100 mg doses of nimesulide. When a global statistical analysis was performed on all the chondrocytes cultures ($n = 13$; ANOVA test), nimesulide at these concentrations did not significantly modify proteoglycan synthesis. On the other hand, individual analysis of each experiment revealed that nimesulide at 3 μg/ml and 6 μg/ml resulted in a significant decrease in the amount of PGs released into the culture medium in only 1 out of the 13 chondrocyte cultures investigated (donor 12; $p < 0.05$). In the cellular phase, the PG content was significantly diminished by nimesulide at 3 μg/ml in one culture (donor 1) and at 6 μg/ml in 5 out of the 8 chondrocyte cultures studied (donors 1, 2, 3, 12, and 13; $0.05 < p < 0.01$).

Total PG production, which corresponds to the sum of the PG contents in the supernatant and in the corresponding cell pellet, was also significantly diminished by nimesulide (6 μg/ml) in 4 out of 8 cultures (donors 1, 2, 3, and 12; $p < 0.05$) (data not shown). Furthermore, we separately analysed the effects of nimesulide on PG

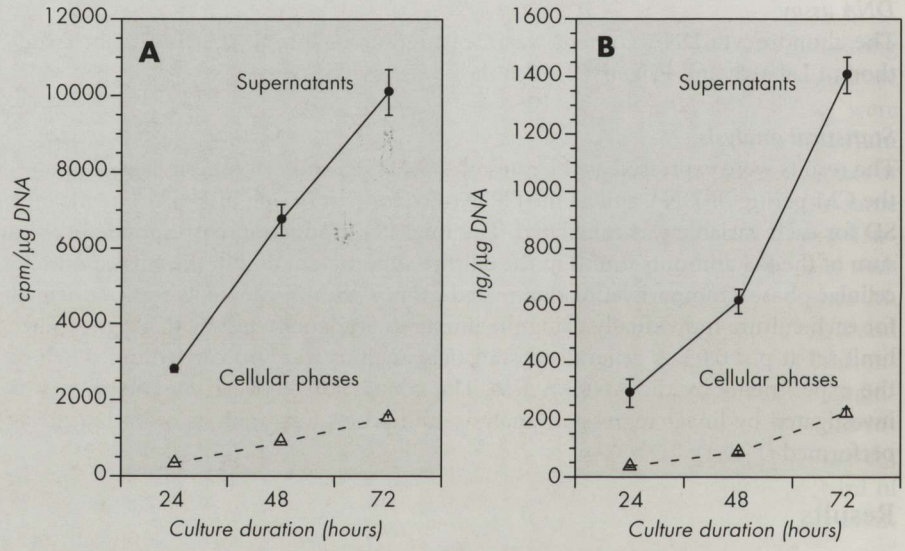


Fig. 1: *The time course of proteoglycans (PG) production by human normal chondrocytes in a 35 years old donor. PG synthesis was measured by ^{35}S incorporation into the newly synthesized glycosaminoglycans (A) and by immunological detection (radioimmunoassay) (B). Results are expressed as means ± SD of triplicate cultures.* ● *= PG released into the culture supernatants;* △ *= PG retained in the cellular phase.*

production in normal donors (donors 1, 2, 3). Interestingly, a significant decrease in PG was found in the culture supernatants in the presence of 6 µg/ml of nimesulide (control vs 6 µg/ml: 997 ± 92 vs 893 ± 72; p = 0.0174; ANOVA test) and in the cellular phase of cultures with 3 and 6 µg/ml of nimesulide (control vs 3 µg/ml: 82.8 ± 8.4 vs 71 ± 12; p = 0.038; control vs 6 µg/ml: 82.8 ± 8.4 vs 59 ± 14; p = 0.0005; ANOVA test). The inhibition was more marked on chondrocytes isolated from the cartilage of a 16 year old donor (donor 1; less 44 % at the 6 µg/ml concentration). At the highest concentrations, ranging 12 to 120 µg/ml, nimesulide decreased in a dose-dependent manner the amount of PG released into the culture medium (r = 1; p = 0.005) and the content in the cellular phase (r = 1; p = 0.011) of one representative donor (donor 4; Fig. 2). Whatever the concentration of nimesulide used, the DNA content in the cellular phase of the culture and trypan blue incorporation were not significantly modified as compared with the controls, suggesting that the inhibition of PG synthesis was not due to a loss of cell viability (data not shown).

We next investigated the effect of nimesulide on ten chondrocyte cultures treated with IL-1ß. IL-1ß had a marked suppressive effect on PG production. At the concentration of 85 pg/ml, IL-1ß decreased by 40% the amount of PG released in the culture supernatants and by 50 % the PG content in the cellular phase (Fig. 3). At 3 µg/ml in two (donors 2 and 12; $p < 0.05$) and at 6 µg/ml in 3/10 chondrocytes cultures (donors 2, 12, and 13; $p < 0.05$), nimesulide partially reversed the IL-1ß effect on the PG content in the cellular phase. No such effect was observed on the PG amounts released into the culture supernatants (Table II).

Table I: Effect of nimesulide on proteoglycans production by human chondrocytes.

			ng/pg DNA/72 hr.					
Pt./ N°	Age/Sex	Lesion gravity	Culture medium - Controls	3µg/ml	6 µg/ml	Culture phase Controls	3 µg/ml	6 µg/ml
1	16/F	0	908±47	1027±107	815±29	82±2	64±2*	45±7**
2	45/M	0	1041±86	1202±92	946±75	93±3	87±9	76±3*
3	46/F	0	1054±61	959±31	919±21*	73±4	64±4	56±4**
4	48/M	I	538±16	-	510±34	148±23	-	130±14
5	51/M	I	380±15	330±58	-	49±4	45±3	-
6	54/M	I	1072±136	1115±112	1051±89	107±6	124±13	121±14
7	54/M	I	966±159	1090±176	-	87±9	90±1	-
8	59/F	I	436±13	429±25	443±28	29±2	28±3	27±5
9	62/M	II	375±22	407±64	-	48±2	43±6	-
10	64/F	II	245±17	234±16	-	24±3	24±5	-
11	70/M	I	442±42	444±52	-	94±11	96±8	-
12	74/M	I	780±87	637±64*	635±66*	87±11	76±13	72±3*
13	75/M	III	841±66	867±138	828±70	108±6	95±4	90±7*

** $p < 0.05$, ** $p < 0.01$ compared with the controls. The unpaired Student's t-test was used for the statistical analysis. Proteoglycan production was measured by a radioimmunoassay on the culture supernatants and cellular extracts. The results are expressed as the means ± SD of triplicate cultures.*

At the highest concentrations (above 6 µg/ml) tested in one donor nimesulide accentuated the IL-1ß inhibitory effect (Fig. 2).

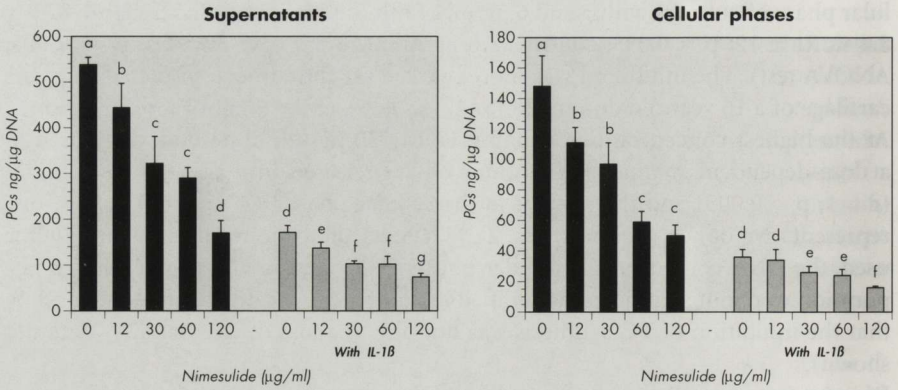


Fig. 2: *In vitro effects of increase concentrations of nimesulide on the proteoglycan (PG) production by untreated and IL-1ß treated (10^{-9}M) human articular chondrocytes in donor 4. PGs were assayed in the supernatants and in the cellular phases by a specific radioimmunoassay. The unpaired Student's t-test was used for the statistical analysis. Results are expressed as means ± SD (n=3). Letters refer to a statistically significant difference between treatments ($a \neq b \neq c \neq d \neq e \neq f \neq g$ with $p < 0.05$).*

Qualitative analysis of the newly synthesized ^{35}S-GAGs was performed in one donor (donor 3) by elution of the dialysed culture media and guanidinium cel-lular extracts through a Sepharose CL2B chromatography column. Radioactivity profiles were divided into three groups: PG-hyaluronic acid complexes (Kd ≤ 0.13), PG monomers and small complexes (0.13 < Kd < 0.7), and small PGs and PG fragments (Kd 0.7). As shown in Fig. 4, newly synthesized PGs were eluted in two major peaks, one excluded from the column and the other with a Kd ≥ 0.7. The majority of ^{35}S-PGs contained in the culture supernatants (56 %) and extracted from the cellular phase (57 %) were eluted with a Kd ≤ 0.13 and corresponded to aggregated proteoglycans. Nimesulide at concentrations ranging from 0.3 to 6 µg/ml did not significantly modify the physico-chemical form of the newly synthesized proteoglycans (data not shown).

Table III shows that in two different donors diclofenac up to the therapeutical concentration of 1 µg/ml (concentrations ranging from 0.005 to 0.03 µg/ml were tested in donor 13 and concentrations ranging from 0.125 to 1 µg/ml in donor 14) did not significantly modify the PG production by human chondrocytes and did not reverse the IL-1ß inhibitory effect. On the other hand, comparison of the effects of diclofenac and nimesulide on one culture (from donor 8) which was not affected by nimesulide, showed that diclofenac at the concentrations of 3 and 6 µg/ml induced a profound inhibition of PG production (Fig. 5).

Table II: Effect of therapeutical concentrations of nimesulide on proteoglycans production by IL-1ß-treated human chondrocyte cultures.

			ng/µg DNA/72H (IL-1ß alone)					
Pt./		Lesion	Culture medium			Culture phase		
N°	Age/Sex	gravity	Controls	3µg/ml	6 µg/ml	Controls	3 µg/ml	6 µg/ml
1	16/F(a)	0	453±16	428±15	459±19	23±2.5	21±2	23±1
2	45/M	0	509±17	508±47	529±11	45±5	60±2*	55±3*
3	46/F (a)	0	533±21	494±17	514±35	20±2	21±1	19.5±2
6	54/M	I	507±38	527±29	477±46	58±6	60±7	53±4.5
7	54/M	I	556±38	505±69	-	48±2	46±5	-
8	59/F	I	267±21	299±37	253±24	18±1	20±3	16±1
10	64/F	II	183±22	187±22	-	15±2	14±2	-
11	70/M	I	132±16	123±9	-	18±1	19±1	-
12	74/M	I	308±28	283±28	283±35	25±3	30±2*	32±2.5*
13	75/M	III	349±27	389±54	378±16	25±1	24±3	31±1.5*

*(a) Chondrocytes cultures treated by 10^{-11} M IL-1ß. * $p<0.05$ in comparison with the controls. The unpaired Student's t-test was used for statistical analysis.*

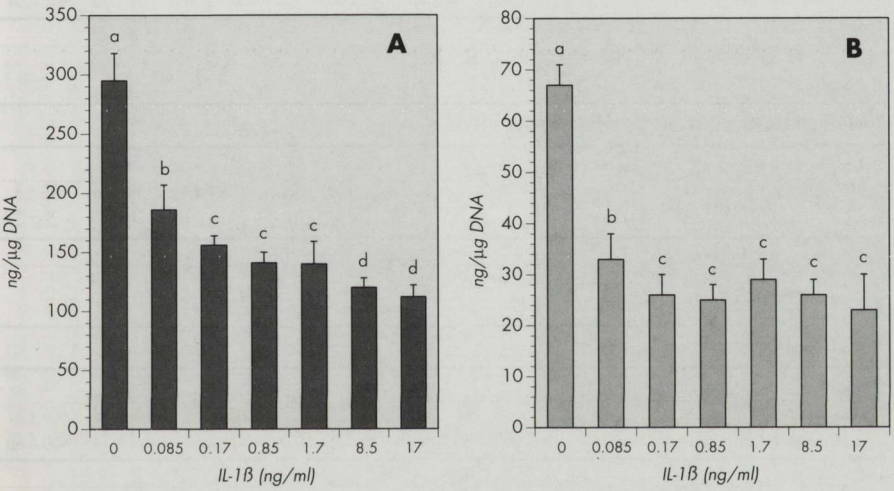


Fig. 3: *Effects of increased amounts of rIL-1ß on normal human chondrocytes (35 years old donor). Chondrocytes were incubated for 72 hours in Dulbecco's Modified Eagle Medium (DMEM) + 1 % ITS+ containing various amounts of human rIL-1ß (0.085 to 17 ng/ml). Proteoglycans were assayed in the supernatants (A) and in the cellular phases (B) by a radioimmunoassay. Results are the means ± SD (n=3). The unpaired Student's t-test was used for the statistical analysis. Letters indicate the statistical differences between groups ($a \neq b \neq c \neq d$ with $p < 0.05$).*

Effect of nimesulide and diclofenac on PGE_2 production

Under unstimulated conditions, PGE_2 was produced in trace amounts by chondrocytes (9.7 ± 0.7 pg/µg DNA; n = 5). Upon IL-1ß stimulation (10^{-9} M), chondrocytes produced about 20 times more PGE_2 than unstimulated chondrocytes (202 ± 12.3 pg/µg DNA). Nimesulide, at concentrations ranging from 2.5 to 10 ng/ml, reduced in a dose-dependent manner the basic (IC_{50} = 6 ng/ml) and IL-1ß-stimulated (IC_{50} = 6.9 ng/ml) PGE_2 production by chondrocytes (Fig. 6). At concentrations superior to 20 ng/ml, nimesulide totally inhibited PGE_2 production. The addition of diclofenac totally blocked the PGE_2 production already at the lowest tested concentration of 5 ng/ml (data not shown).

Table III: Effects of increased concentrations of diclofenac on proteoglycans production by human articular chondrocytes.

		Nimesulide				Diclofenac			
	Controls	0.005 µg/ml	0.01 µg/ml	0.02 µg/ml	0.03 µg/ml	0.125 µg/ml	0.250 µg/ml	0.5 µg/ml	1 µg/ml
Proteoglycans released in the culture supernatants (ng/µg DNA)									
Donor 13									
Basal	841 ± 66					819 ± 144	778 ± 51	836 ± 96	812 ± 90
With IL-1ß	349 ± 27					363 ± 7	401 ± 30	398 ± 75	416 ± 51
Donor 14									
Basal	1072 ± 154	1186 ± 49	1024 ± 17	1048 ± 81	1021 ± 79				
With IL-1ß	480 ± 42	491 ± 38	483 ± 22	413 ± 18	481 ± 27				
Proteoglycans contained in the cellular phases (ng/µg DNA)									
Donor 13									
Basal	108 ± 6					102 ± 5	106 ± 2	85 ± 16	95 ± 3
With IL-1ß	25 ± 0.6					25 ± 1	30 ± 2.6	25 ± 1.5	29 ± 2.6
Donor 14									
Basal	108 ± 5	110 ± 9.3	114 ± 8	113 ± 11	118 ± 11				
With IL-1ß	20 ± 0.6	19 ± 1.5	20 ± 1.5	19.4 ± 0.4	19 ± 0.4				
Total proteoglycans production (ng/µg DNA)									
Donor 13									
Basal	948 ± 72					882 ± 172	883 ± 51	940 ± 89	906 ± 87
With IL-1ß	374 ± 27					388 ± 7	431 ± 31	424 ± 74	450 ± 46
Donor 14									
Basal	1181 ± 159	1296 ± 158	1138 ± 13.6	1161 ± 92	1139 ± 69				
With IL-1ß	500 ± 43	511 ± 40	503 ± 23	432 ± 18	500 ± 27				

Diclofenac was tested on chondrocytes isolated from two different donors. Donor 13 was 75 years old and showed grade III cartilage lesions. Donor 14 was 62 years old and had only stage I catilage lesions. Chondrocyte cultures were or were not treated by IL-1ß 10^{-9} M. Values represent the means ± SD (n=3).

Effects of nimesulide and diclofenac on IL-6 and IL-8 production

Under our culture conditions, human articular chondrocytes constitutively produced detectable amounts of IL-6 and IL-8. Addition of IL-1ß to the chondrocyte

cultures stimulated IL-6 release 4-fold and IL-8 release 2-fold. Basic IL-8 production was not affected by nimesulide, while diclofenac slightly decreased thisproduction at the concentration of 6 µg/ml ($p < 0.01$) (Fig. 7). On the other hand,both NSAIDs markedly reduced IL-6 production. The lowest concentration inducing a significant inhibition was 6 µg/ml for nimesulide, whereas diclofenac was already efficient at the concentration of 5 ng/ml. Interestingly, both NSAIDs reduced IL-lß-stimulated IL-6 production in a dose-dependent manner ($r = 1$, $y = -0.3 x + 4949$, $p = 0.0001$

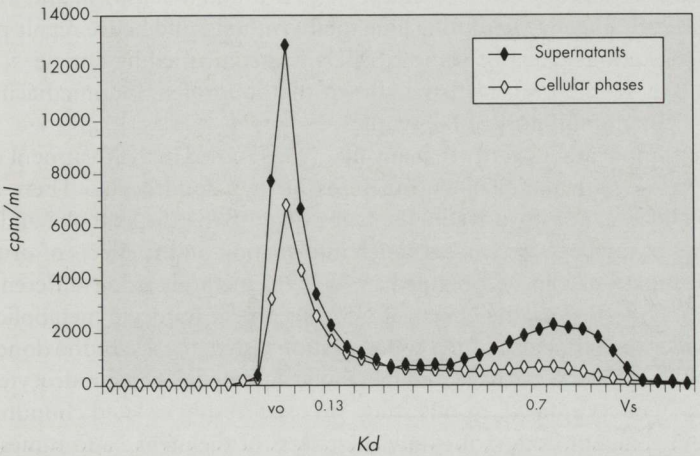


Fig. 4: *Associative size exclusion chromatograms of ^{35}S-Proteoglycans (PG) produced by normal chondrocytes (isolated from the knee cartilage of donor 3) during the last 24 hours of the culture. (♦) ^{35}S-PG released into the culture supernatants and (◊) ^{35}S-PGs retained in the cellular phases.*

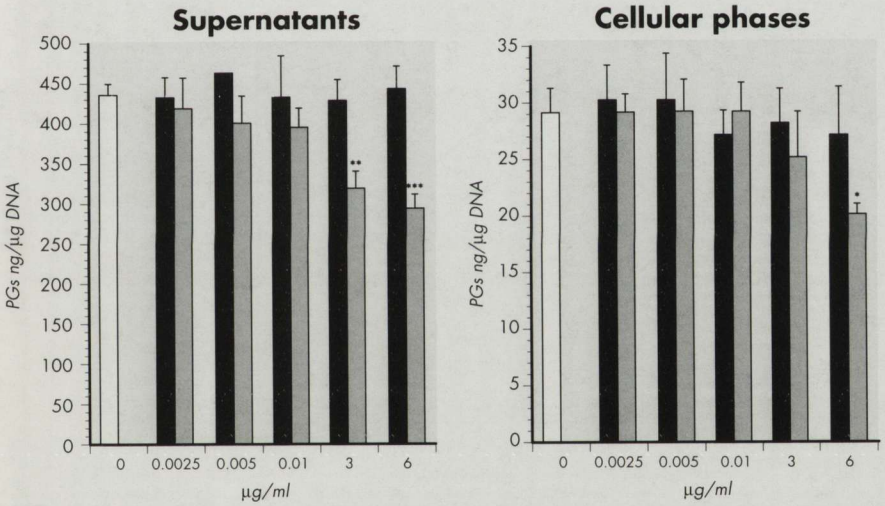


Fig. 5: *Comparative effects of nimesulide (black bars) and sodium diclofenac (grey bars) on one single chondrocyte culture (donor 8, 58 years old) on proteoglycans production. Results are expressed as the mean values of three cultures from the same donor. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ in comparison with the controls. The unpaired Student's t-test was used for the statistical analysis.*

for diclofenac). At concentrations between 5 ng/ml and 6 µg/ml, diclofenac was significantly more effective than nimesulide on this para-meter. Neither nimesulide nor diclofenac modified IL-8 production by IL-1ß treated chondrocytes.

Discussion

Brandt in 1987 [5], using organ cultures of canine cartilage, was the first to demonstrate that a number of NSAIDs are capable at therapeutic concentrations of inhibiting the synthesis of PGs by chondrocytes. He found that aspirin and ibuprofen are particularly effective in diminishing their synthesis, and hence repair potentiality. This deleterious effect of some NSAIDs was confirmed by Dingle [6] using human cartilage. In these works it was shown that ibuprofen, indomethacin, and naproxen are potent inhibitors of PG synthesis.

Therefore, it is important to verify that any new NSAID used in the treatment of OA does not interfere with the anabolic functions of the chondrocytes. There are at present no validated, non-invasive methods for quantifying the anabolic and catabolic activities of cartilage *in vivo*, but much information on the effects of drugs on chondrocyte functions can be obtained by *in vitro* methods using differentiated chondrocytes. Nevertheless, the effects of NSAIDs on chondrocyte metabolic activities vary widely as a function of the concentration tested, the age of the donor, the pathological status of the cartilage, the metabolic activity of the chondrocytes and the culture system considered. In this study we used freshly isolated chondrocytes obtained from different donors to study the effects of diclofenac and nimesulide. Under our culture conditions, chondrocytes produced spontaneously aggregating PGs and type II collagen, thus suggesting that these chondrocytes are well differentiated.

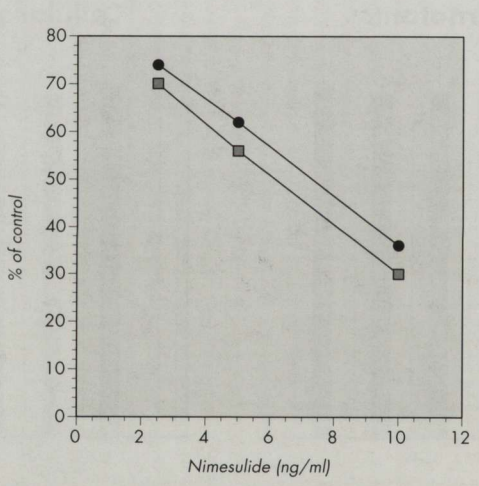


Fig. 6: *Effect of increased concentrations of nimesulide on PGE_2 production by unstimulated (-■-) and IL-1ß stimulated (-●-) human chondrocytes. Results are expressed as percent of the control value.*

We have demonstrated that nimesulide at 3 µg/ml did not affect the PG productionby human OA chondrocytes, but decreased the PG accumulation in the newly synthesised matrix of normal chondrocytes in culture. This nimesulide concentration is above the maximum found in the synovial fluid of patients with rheumatoid arthritis 3 hours after the last oral administration of nimesulide 100 mg twice daily

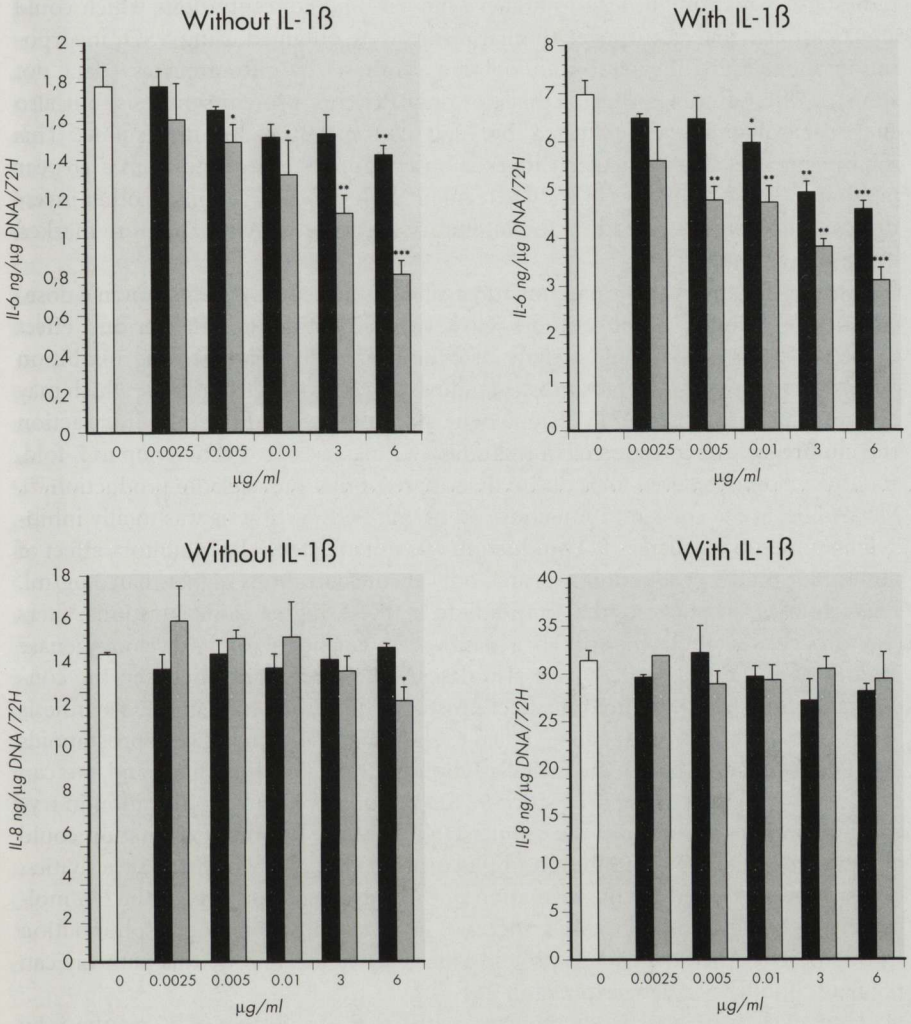


Fig. 7: *Effects of nimesulide (black bars) and sodium diclofenac (grey bars) on interleukin-6 (IL-6) and interleukin-8 (IL-8) production by human chondrocytes isolated from the knee cartilage of a 59 years old woman. Chondrocytes were cultured for 72 hours in the absence or presence of 10^{-9} M of IL-1ß. The data are expressed as the amounts of cytokines found into culture supernatants per µg of DNA and are presented as the mean ± SD of triplicate cultures. NSAID-treated groups are significantly different from the control group with a * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The unpaired Student's t-test was used for the statistical analysis.*

for 7 days, which is the recommended daily therapeutic dose [26]. These findings suggest that nimesulide used at therapeutic concentrations does not modify anabolic chondrocyte functions.

On the other hand, at 6 µg/ml, corresponding to the maximal plasma concentration,nimesulide may induce a significant decrease in PG production, suggesting that overdoses or the prolonged administration of non-recommended high doses of nimesulide which might lead to higher synovial fluid concentrations which could affect cartilage matrix turnover. Similar results were obtained using $^{35}SO_4$ incorporation to measure the glycosaminoglycan synthesis by chondrocytes (data not shown). These findings suggest that not only PG core protein synthesis, but also sugar or sulfatation are affected by high concentrations of nimesulide. This inhibitory effect was particularly marked on chondrocytes isolated from a 16 year old donor. These results are in good agreement with the observations of other investigators who demonstrated that the inhibitory effects of NSAIDs are more marked in young cartilage [5].

At concentrations above 6 µg/ml, nimesulide inhibited PG production in a dose-dependent manner. Some authors have suggested that the deleterious effect of NSAIDs on the synthetic activity of chondrocytes is related to the inhibition of PGE_2 synthesis [9, 10]. In fact, prostaglandins can control IL-1 synthesis, which may explain why misoprostol, a PGE1 analogue, partially reversed the inhibitory action of indomethacin. It is suggested that this NSAID increases IL-1 activity up to 7-fold, and this action is believed to be due to the suppression of prostagladin production [9]. Meanwhile, at the concentration above 30 ng/ml, PGE_2 synthesis was totally inhibited by nimesulide whereas PG production was not affected. The inhibitory effect of nimesulide on PG production appeared only at concentrations higher than 3 µg/ml. This observation suggests that nimesulide at these higher concentrations alters chondrocyte metabolism through a pathway independent of its cyclooxygenase inhibitory properties. Others have also described a discordance between the concentration of NSAID required to affect prostaglandin production and PG synthesis [35, 36]. Moreover, Mitrovic *et al.* [37] failed to show a correlation between prostanoids and PG production by bovine articular chondrocytes. On the other hand, we can exclude a toxic effect of the drug since we did not observed any modification in trypan blue incorporation or in the cellular DNA content. Another explanation could be advanced, such as an inhibition of glucuronyl- and xylosyltransferase activities, two enzymes required for the formation of the polysaccharide part of the PG molecule [17]. Finally, some NSAIDs increase intracellular protein phosphorylation through cyclic AMP-dependent protein kinase activation and by this pathway can regulate chondrocyte gene expression [38].

Human chondrocytes are extremely sensitive to the inhibition of PG synthesis by subnanogram concentrations of IL-1ß. In our culture model, 85 pg/ml of IL-1ß decreased by 40 % the amount of PGs released into the culture medium and by 50 % the PG content in the cellular phase. At therapeutic concentrations, nimesulide did not significantly modify the IL-1ß inhibitory effect. On the other hand, high concentrations of nimesulide significantly increased the suppression of PG

synthesis induced by IL-1ß. These results suggest that high NSAID doses may increase the inhibitory effect of IL-1 ß on PG production.

In our model, nimesulide showed a weak inhibitory activity on cyclooxygenase in comparison with diclofenac. In fact, at 5 µg/ml diclofenac fully inhibited, while nimesulide only decreased by 46 %, IL-1ß-induced PGE_2 production. These results are in accordance with previous studies demonstrating that nimesulide has less potency than other NSAIDs at similar concentrations on prostaglandin production tested *in vitro*. Our results are well in line with two other studies which showed that nimesulide is considerably less active than indomethacin (50 % inhibitory concentration (IC_{50}) 2.3 mmol/L vs 2 µmol/L) against microsomial cyclooxygenase [39] and on PGE_2 production by murine macrophage (40). In our culture model, nimesulide inhibited by the same order of magnitude basic PGE_2 production and IL-1ß-induced PGE_2. Several other studies have reported that nimesulide has a much higher selectivity for COX-2 than COX-1 in intact cell systems as well as on purified enzymes [15]. We could explain this discrepancy by our experimental conditions. In fact, we cannot exclude the possibility that COX-2 was previously activated under our control conditions.

NSAIDs are used in the treatment of pain and inflammation in OA. Therefore it would be interesting to know the effects of NSAIDs on the synthesis of immunomodulatory mediators, such as IL-6 and IL-8, by human chondrocytes. Our data reveal that both nimesulide and diclofenac inhibit baseline and IL-1ß-stimulated IL-6 production. These results are not fully in agreement with the study by Smith *et al.* showing that diclofenac did not modify IL-6 production [41]. On the other hand, Pelletier *et al.* [42] demonstrated that nimesulide reduced IL-6 production by human OA synovial fibroblasts. Depending on the exact role of IL-6, the clinical relevance of this observation remains unclear. Nevertheless, IL-6 was recently demonstrated to potentiate IL-1ß and TNFα effects on chondrocyte metabolism, and then decreased IL-6 levels may be of benefit [43]. On the other hand, it has been reported that IL-6 stimulates TIMP-1 production, which is beneficial in that it inhibits the metalloproteases [44]. Surprisingly, these NSAIDs at therapeutic concentrations did not affect IL-8 production, suggesting that IL-6 production and IL-8 production are regulated by two different pathways.

In conclusion, this study demonstrates that nimesulide at therapeutical concentrations (< 3 µg/ml) does not affect PG synthesis by normal and OA adult chondrocytes, but significantly inhibits the production of inflammatory mediators. Nevertheless, it appears that the principal mechanism of nimesulide and diclofenac is the inhibition of PGE_2 synthesis, since the effect on IL-6 is only marginal at the therapeutic concentrations used. Previously, Pelletier *et al.* [24] have demonstrated that nimesulide is a potent inhibitor of metalloprotease production by chondrocytes. The combination of these data suggests that, *in vitro*, nimesulide might reduce cartilage catabolism by reducing metalloprotease synthesis without affecting the repair capacity of the chondrocytes. Although it is hazardous to extrapolate such results to physiopathological conditions involved in OA, these results allow to consider nimesulide as an interesting therapeutical tool for this disease.

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ANNEXE 13

In vitro Effects of Aceclofenac and its Metabolites on the Production by Chondrocytes of Inflammatory Mediators

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Abstract

Objectives. To investigate the mechanisms of action underlying the anti-inflammatory action of aceclofenac *in vivo*, we studied *in vitro* the effect of aceclofenac and its main metabolite, 4'-hydroxyaceclofenac, in comparison with diclofenac, another metabolite, on cyclooxygenases activity as well as interleukin-1ß, -6 and -8, nitric oxide, and prostaglandin E_2 production by human osteoarthritic and normal articular chondrocytes.

Methods. Enzymatically isolated human chondrocytes were cultured for 72 h in the absence or presence of interleukin-1ß (IL-1ß) or lipopolysacharride (LPS) and with or without increased amounts (1 to 30 µM) of aceclofenac or metabolites. The production of different cytokines was measured by Enzyme Amplified Sensitivity Immunoassays (EASIA). Prostaglandin E_2 was quantified by a specific radioimmunoassay. Nitrite and nitrate concentrations in the culture supernatants were determined by spectrophotometric method based upon the Griess reaction. Cyclooxygenase-2, inducible NO synthase and IL-1ß gene expression were quantified by reverse transcription of mRNA followed by real time and quantitative polymerase chain reaction. Finally, cyclooxygenase inhibitory potency of the drugs was also tested in both a cell-free system using purified ovine cyclooxygenase-1 and -2 (COX-1 and COX-2) and at a cellular level using human whole blood assay.

Results. We have demonstrated that aceclofenac, 4'-hydroxyaceclofenac and diclofenac significantly decreased interleukin-6 production at concentrations ranged among 1 to 30 µM and fully blocked prostaglandin E_2 synthesis by IL-1ß- or LPS-stimulated human chondrocytes. Aceclofenac and diclofenac had no effect on interleukin-8 production while 4'-hydroxyaceclofenac slightly decreased this parameter at the highest dose (30 µM). Aceclofenac was without effect on IL-1ß- or LPS-stimulated nitric oxide production. At 30 µM, 4'-hydroxyaceclofenac inhibited both IL-1ß or LPS-stimulated nitric oxide production while diclofenac inhibited only the LPS-stimulated production. Finally, at 30 µM, the three drugs significantly decreased IL-1ß mRNA. In the whole blood test, aceclofenac and 4'-hydroxyaceclofenac weakly inhibited COX-1 with IC_{50} values superior to 100 µM, but decreased by 50% COX-2 activity at the concentration of 0.77 and 36 µM, respectively. Diclofenac strongly inhibited both COX-1 and COX-2 with IC_{50} values of 0.6 and 0.04 µM, respectively. On the other hand, aceclofenac and diclofenac weakly inhibited purified ovine cyclooxygenases with IC_{50} values superior to 100 µM, whereas 4'-hydroxyaceclofenac was without effect.

Conclusions. These results suggest that aceclofenac actions are multifactorial and that metabolites could contribute to its anti-inflammatory actions.

Keywords. Inflammation - Arthritis - Chondrocytes - Cytokines - Nitric oxide - Prostaglandins

Introduction

Aceclofenac (ACE) is a nonsteroidal anti-inflammatory drug (NSAID) of the phenylacetic acid class (2-{(2,6-dichlorophenyl)amino}phenylacetoxyacetic acid) which has been shown to exhibit a good clinical efficacy in osteoarthritis, ankylosing spondylitis, and rheumatoid arthritis in several clinical trials [1-4]. Furthermore, it showed a similar efficacy profile to that of diclofenac, naproxen, indomethacin, piroxicam or tenoxicam and an improved safety profile in all these studies in terms of gastrointestinal tolerance [5-9]. In a multi-centre, double blind, parallel group, placebo controlled study over a period of 4 weeks, ACE was effective in improving clinical outcomes in patients with rheumatoid arthritis to a greater extent than in the placebo group and with an side effect profile similar to that of placebo [10].

In vitro, ACE inhibited the production of prostaglandin E_2 (PGE_2) by human synovial cells [11] and reduced lipopolysacharride (LPS)-stimulated interleukin-1 beta (IL-1ß) and tumor necrosis factor alpha (TNFα) production whereas indomethacin was ineffective. The action of ACE in suppressing IL-1 activity has also been demonstrated in an *ex vivo* study by Gonzalez *et al.* [12]. These authors demonstrated that long term administration (6 months) of therapeutic doses of ACE reduced IL-1ß and TNFα activity by blood cells from patients with knee OA in whom basic cytokine levels were high. ACE has also been reported to interfere with cell adhesion molecules implicated in neutrophils adhesion and motility [13]. In the presence of serum or insulin-like growth factors (IGF-1), ACE increased glycosaminoglycan synthesis (+ 22 %) by osteoarthritic human cartilage, partially reversed IL-1ß inhibitory effect and facilitated the recovery of GAG synthesis from a episode of IL-1 [14, 15]. Furthermore, ACE reduced luminol-enhanced chemiluminescence generated by phorbol myristate acetate-activated neutrophils, suggesting that it could limit the oxidative stress generated by neutrophils [16]. On the other hand, ACE did not affect metalloproteases (MMP-1 and MMP-3) synthesis by rheumatoid synovial cells [17].

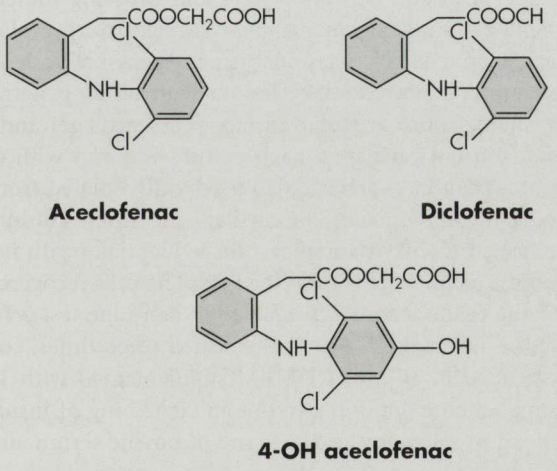


Fig. 1: *Structural formulae of aceclofenac, 4-OH aceclofenac and diclofenac.*

In healthy human, ACE is rapidly metabolized in human hepatocytes and human microsome to form 4'-hydroxyaceclofenac (4'-HOACE) as the major metabolite [18] (Fig. 1).

Minor metabolites were diclofenac (DICLO), 5-hydroxydiclofenac (5-HODICLO), 4'-hydroxydiclofenac (4'-HODICLO) and 5-hydroxyaceclofenac (5-HOACE). The average percentage of these compounds in the plasma measured at t_{max} after an oral intake of 100 mg ^{14}C-ACE, was 66.1 ± 7.2 % for ACE, 23.2 ± 5.6 % for 4'-HOACE and only 5.8 ± 1.3 % for DICLO [18]. The cytochrome involved in the oxidation of ACE at 4'-position was clearly identified as the isoform CYP2C9 whereas the minor metabolite DICLO results from the breakage of ACE ester bond. Furthermore, it was reported that ACE and 4'-HOACE could be transformed at the site of the inflammation into DICLO and 4'-HODICLO by polymorphonuclear leukocytes [19], monocytes and rheumatoid synovial cells [11]. Therefore, it was suggested that ACE metabolites could contribute to its activity. At that point, the effects of ACE metabolites had been poorly studied. Recently, it was reported that 4'-HOACE down-regulated both basal and IL-1ß-induced production of procollagenase (pro-MMP-1) and prostromelysin (proMMP-3) by rheumatoid synovial cells whereas DICLO and 4'-HODICLO had no effect [20]. Our study was designed to investigate the effects of ACE metabolites, 4'-HOACE and DICLO, on PGE_2, interleukin-6 (IL-6), interleukin-8 (IL-8), IL-1ß and nitric oxide (NO) synthesis by normal and osteoarthri-tic human articular chondrocytes. All these mediators contribute to the development of chronic synovium inflammation, and then, their inhibition constitutes an interesting therapeutic focal point.

Materials and methods

Short-term suspension culture of human chondrocytes

Cartilage specimens were obtained from the knee of 10 donors shortly after death (8 males and 2 females) with the mean age of 62 (23-87) years old, being excised from the superficial and medium layers and avoiding the calcified layer. Upon dissection, femoral and patellar articular surfaces were evaluated for the severity of the macroscopic cartilage lesions using a personal scale [21]. Four out of the ten cartilage samples showed severe lesions with ulcers penetrating to subchondral bone on one or more articular surface (OA cartilage) and six had no macroscopic lesions (normal cartilage). Each culture was run with chondrocytes from a single patient. When OA cartilage was used, cells isolated from non-fibrillated and fibrillated areas were mixed. The cartilage slices were cut into small fragments and then subjected to enzymatic digestion sequentially with hyaluronidase, pronase and collagenase as previously described [21]. The cells recovered after collagenase digestion of the tissue were 95 % viable (trypan blue test). The cells were then filtered through a nylon mesh (70 µm), washed three times, counted (1.10^6 cells/ml) and resuspended in 10 ml of DMEM supplemented with 1 % of ITS+. ITS+ is a culture supplement containing in one ml: 0.625 mg of insulin, 0.625 mg of transferrin, 0.625 µg of selenious acid, 125 mg of bovine serum albumin (BSA)

and 0.535 mg of linoleic acid. Cells remained in this culture medium for 48 h as a precaution, in case of *in vivo* contamination occurred with drugs that the donors may have taken before death.

After this washout period, cells (1 10^6 cells/ml) were seeded in 10 ml polypropylene Falcon tubes and maintained under agitation on a gyratory shaker (100 rpm) in a 95 % air/5 % CO_2 atmosphere. Cells and supernatant were then separated by centrifugation (1000 rpm; 5 min). Cell pellets were washed twice with HBSS (Hank's Buffer Saline Solution, Gibco, Merelbeke, Belgium) and then homogenized in phosphate buffer saline (PBS) pH 7.5 by ultrasonic dissociation (10s, 50 W/cm^2). All the chemicals for which the source was not mentioned were of the purest grade commercially available.

Culture treatment

Human chondrocytes were cultured for 72 h in the absence or in the presence of human recombinant IL-1ß (5 U/ml corresponding to 100 pg/ml, Roche, Brussels, Belgium) or lipopolysaccharide (LPS from E. Coli serotype, Sigma-Aldrich, Bornem, Belgium; 10 µg/ml final concentration) and with or without ACE, 4'-HOACE or DICLO (1 to 30 µM corresponding to 0.3 to 10 µg/ml). After oral administration of a single 100 mg dose of ACE to healthy volunteers, estimated C_{max} values were 8.1 and 0.1 mg/l (corresponding to 24.3 and 2.1 µM) for ACE and DICLO respectively [22]. Three flasks were used for each concentration of the drug and for the corresponding controls.

PGE_2 radioimmunoassay

PGE_2 was assayed in conditioned culture medium according to a described radioimmunoassay (RIA) [23]. PGE_2 was directly measured in the culture medium without previous extraction. The ^{3}H-labeled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule PGE_2 from Sigma Chemical Co. (St. Louis, MO, USA). PGE_2 was measured in duplicate on a 100 µl aliquot of each sample. After a 48 h incubation at 4 °C, free antigen was separated from antibodies bound-antigen by charcoal precipitation and centrifugation (20 min at 1000g, 4 °C). The supernatant was counted by liquid scintillation. Intra and interassay coefficient of variations were 6 and 10 %, respectively. The recovery of known amounts of PGE_2 added to the culture medium was satisfactory (90 to 97 % for the amounts of PGE_2 ranging from 2500 to 312 pg/ml). The limit of detection of the RIA was 20 pg/ml.

Real-time Quantitative PCR

Total RNA was isolated by a single step guanidine thiocyanate-phenolchloroform method. Cells (3.10^6) were lysed using TRI REAGENT (Sigma, Bornem, Belgium) and the samples were processed following the manufacturer's protocol. RNA (2 µg) was reverse transcripted in a volume of 35 µl containing 1X RT buffer (Gibco, Merelbeke, Belgium), 10 mM DTT, 1 mM dNTPs, 500 ng oligo(dT) 15 primer, 140 U M-MLV Reverse Transcriptase and 40 U RNAseOUT (Gibco,

Merelbeke, Belgium) for 60 min at 37 °C. The reaction was stopped by incubation at 95 °C for 5 min and the volume was adjusted to 100 µl.

Real-time quantitative PCR was performed using LightCycler FastStart DNA Master SYBR Green I technology (Roche Molecular Biochemicals, Bruxelles, Belgium). The reaction mix is provided as a 10-fold stock solution containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix, 10 mM $MgCl_2$ and SYBR Green I dye. For each LightCycler PCR a mastermix of the following reaction components was prepared in a final volume of 18 µl: 2 µl LightCycler-FastStart DNA master Sybr Green I, 1.6 µl of 25 mM $MgCl_2$, 0.2 µM sense and antisense primer. The mastermix was loaded into glass capillary (Roche Molecular Biochemicals, Bruxelles, Belgium) and 2 µl of cDNA (corresponding to 4 ng of the initial RNA) was added as PCR template. In this work, the following primers were used:

hGAPDH sense 5'-TTG GTAT CGT GGA AGG ACT ACA-3'
hGAPDH antisense 5'-TGT CAT CAT ATT TGG CAG GTTT-3'
h iNOS sense 5'-CCA TGG AAC ATC CCA AAT AC-3'
h iNOS antisense 5'-TCT GCA TGT ACT TCA TGA AGG-3'
h COX-2 sense 5'-TTC AAA TGA GAT TGT GGG AAA A-3'
h COX-2 antisense 5'-AGA TCA TCT CTG CCT GAG TAT CTT-3'
h IL-1ß sense 5'-TCT CCG ACC ACC ACT ACA GCA A-3'
h IL-1ß antisense 5'-GGG GAA CTG GGC AGA CTC AA-3'

Amplification was performed with a spectrofluorometric thermal cycler (LightCycler, Roche Molecular Biochemicals, Bruxelles, Belgium). After an initial denaturation step at 95 °C for 10 min, amplification was performed using 40 cycles of denaturation (95 °C for 15 s), annealing (temperature gradient: 68 °C to 58 °C with an increment of 0.5 °C per cycle during 20 cycles, continued by 20 cycles at 58 °C, for 5 s) and extension (72 °C for 15 s). For each run, a standard curve was generated from purified DNA ranging from 10^6 to 10 copies. The fluorescent signal was measured at the end of elongation and was automatically plotted for each sample. These curves were used to determine the threshold value corresponding at the fluorescence level at which the PCR was in the log phase. For each reaction, the software determined the cycle number (crossing point: CP) corresponding to the point of intersection with the threshold value. A logarithmic-linear correlation was then plotted between the logarithmic concentration of the standards and the CP.

After amplification, a final melting curve was recorded by cooling the PCR mixture to 65 °C for 30 s and then slowly heated to 95 °C at 0.1 °C/s. Fluorescence was measured continously during the slow temperature rise to monitor the dissociation of the dsDNA. Melting curve was converted to melting peak by plotting the negative derivative of the fluorescence with respect to temperature against temperature: (-dF/dt) vs T.

DNA assay

Chondrocyte DNA content was directly correlated to the cell number of each culture. DNA content was measured in the cell pellet extracts using a fluorimetric

method. This measurement ensures elimination of result variations due to the different number of chondrocytes [24].

Nitric oxide measurement

Nitrite is the stable endproduct of nitric oxide. Nitrite concentrations in conditioned media were determined by a spectrophotometric method based upon the Griess reaction [25]. Briefly, 200 µl of supernatant was added to 50 µl 6.5 M HCl and 50 µl 37.5 mM sulfanilic acid. After 10 min at room temperature, 50 µl of 12.5 mM N-(1-naphtyl)-ethylene diamine (Merck, Darmstadt, Germany) was added. Nitrate reductase (0.2 U/ml; Roche, Brussels, Belgium) was added to the culture supernatants to convert nitrate to nitrite. The absorption was measured at 540 nm. Sodium nitrite ($NaNO_2$) was used for calibration.

Immunoassays for Cytokines

IL-6 and IL-8 released by chondrocytes in the culture supernatants were assayed by two Enzyme Amplified Sensitivity immunoassays (Cytosets, EASIA) designed by Biosource Europe (Nivelles, Belgium). The assays were directly performed in the culture supernatants without previous extraction. The intra and interassay coefficients of variation were less than 5%. Recovery of cytokines in a chondrocytes conditioned culture medium was comprised of between 87 to 111 % for amounts of cytokines ranged in the linear part of the curve. The sensitivity of the assay was 8 pg/ml for IL-6 and 2 pg/ml for IL-8.

COX-1 and COX-2 enzyme inhibition test

Cyclooxygenase inhibitory potency was determined according to a reported enzymatic test described elsewhere [26]. Briefly, one unit of cyclooxygenase enzymes (COX-1 from ram seminal vesicle and COX-2 from sheep placenta) (Cayman Chemical Company, Ann Arbor, MI, USA) was suspended in Tris buffer (0.8 ml, 100 mM, pH 8) containing phenol (2 mM) and hematin (1 µM) as cofactors. They were pre-incubated with substrate alone (controls) or with increased concentrations (0.01, 0.1, 1, 10 and 100 µM) of drugs for 1 h at 37 °C. Arachidonic acid sodium salt (10 µM; Euro Biochem, Bierges, Belgium) was added and incubated for 2 min at 37 °C. Reaction was halted by placing the tubes on ice and by adding diclofenac sodium salt (Federa, Bruxelles, Belgium) at the final concentration of 1 mM. PGE_2 production was assayed by the previously specific radioimmunoassay [23] and results expressed as the percentage of the control values.

Whole Blood assay

In vitro COX-1 assay. Fresh human blood was collected into tubes containing no anticoagulants by venipuncture from three healthy volunteers who had not taken any medications for at least 14 days prior to blood collection. Aliquots of 1 ml were immediately transferred into siliconized microcentrifuge tubes preloaded with 4 µl vehicle (DMSO) or the investigated compound at final concentration ranging between 0.01 to 100 µM. The tubes were vortexed and incubated at 37 °C under con-

stant agitation for 1 h to allow blood clotting. At the end of the incubation, serum was obtained by centrifugation (15,000 rpm for 10 min). The serum was assayed for TXB_2 production using an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions.

In vitro COX-2 assay. Fresh blood was collected in heparinized tubes by venipuncture from three male healthy volunteers who had no apparent inflammatory conditions and had not taken any medications for at least 14 days prior blood collection. One ml aliquots of blood were transferred into heparinized tubes preloaded with either 4 µl vehicle (DMSO) or 4 µl of the tested drugs at the final concentration ranged among 0.01 to 100 µM. The tubes were then mixed and incubated under constant agitation for 15 min at 37 °C. This step was followed by incubation of the sample with 20 µl of lipopolysaccharide (LPS from E. Coli serotype B4, Sigma-Aldrich, Bornem, Belgium; 10 µg/ml final concentration) for 24 h at 37 °C under constant agitation in order to induce COX-2 expression. Thereafter, the tubes were centrifuged at 3,300 rpm for 20 min and 300 µl of plasma were mixed with 300 µl methanol in order to precipitate proteins. The supernatants were then collected and then assayed for PGE_2 using a specific radioimmunoassay [23].

Statistical analysis

The results were expressed as the concentration of PGE_2, cytokines and NO_2 plus NO_3 into supernatants per µg of DNA. The mean ± SEM of each variable was calculated. A multivariate repeated measures ANOVA model with calculation of contrasts was performed on all of the experiments (MANOVA; SAS system; General Linear Models).

Results

Effects of ACE and metabolites on cytokines production by human articular chondrocytes

We have investigated the effects of increase doses of ACE on eight chondrocyte cultures. Chondrocytes were isolated from cartilage specimens of eight different donors. Four specimens showed OA lesions (OA) whereas the others were considered as normal (N). In the basal conditions, OA chondrocytes significantly produced more IL-8 than N chondrocytes (OA 1.48 ± 1 ng/µg DNA / 72 h vs N 0.53 ± 0.16 ng/µg DNA/72 h ; $p = 0.01$), whereas IL-6 production was not significantly different (0.28 ± 0.23 ng/µg DNA / 72 h vs N 0.18 ± 0.13 ng/µg DNA/72 h) (Fig. 2). IL-1ß (5 U/ml) significantly ($p = 0.0001$) enhanced IL-6 and IL-8 synthesis in both N and OA chondrocyte cultures. The simulating effect of IL-1ß on IL-6 production was significantly more marked in OA than in N chondrocyte cultures (N vs OA; $p = 0.02$). No significant difference was observed between N and OA IL-1ß-stimulated IL-8 production.

In the basal conditions, ACE, at the concentration of 30 μM, significantly decreased IL-6 production in both N and OA chondrocyte cultures by 30 and 22 %, respectively ($p < 0.05$). No significant effect was observed at the lowest concentrations (data not shown). In the presence of IL-1ß, ACE at the concentrations of 1 and 6 μM significantly inhibited IL-6 production in OA but not in N chondrocyte cultures (Fig. 3). At 30 μM, it reduced IL-1ß-stimulated IL-6 in both N and OA chondrocyte cultures by 21 and 43 %, respectively (N vs OA; $p = 0.01$). IL-8 production was not significantly modified by ACE whatever the concentration tested and the origin of the cartilage.

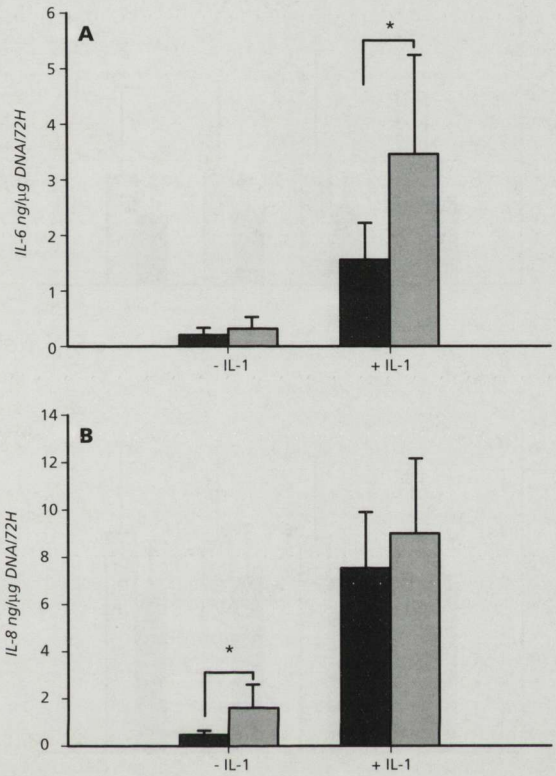


Fig. 2: *IL-6 (A) and IL-8 (B) production by human N (black column) or OA (grey column) chondrocyte cultures for 72 h with or without 5 U/ml of IL-1ß. The data are expressed as the amounts of cytokines found in the culture supernatants per μg of DNA and are presented as the mean ± SEM of four different chondrocyte cultures. Each culture was performed with chondrocytes isolated from a single cartilage sample. OA productions are significantly different from the corresponding controls with * $p < 0.05$.*

We have also compared the effect of ACE on IL-6 and IL-8 production with those of its metabolites on two OA chondrocyte cultures. Interestingly, both

4'-HOACE and DICLO were potent inhibitors of both basal and IL-1ß-stimulated IL-6 production. At the concentrations of 30 µM, DICLO and 4'-HOACE were significantly more effective than the parent molecule ($p < 0.05$) (Fig. 4). ACE and DICLO were uneffective on IL-8 production while 4'-HOACE depressed by 45 % both basal and IL-1ß-stimulated productions at the concentration of 30 µM ($p < 0.05$). No significant effect on IL-8 production were recorded at the lowest concentrations (data not shown).

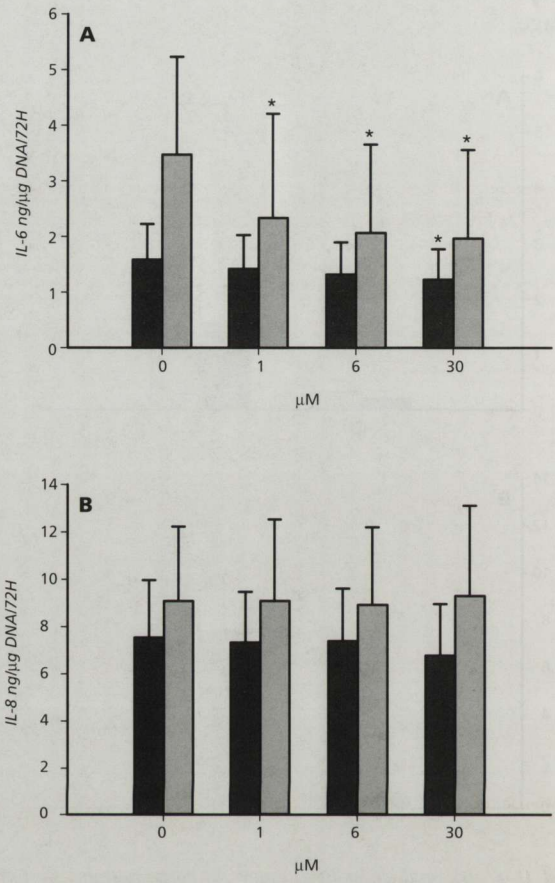


Fig. 3: *Effects of increased doses of ACE on IL-6 (A) and IL-8 (B) production by human N (black column) or OA (grey column) chondrocytes. Chondrocytes were cultured for 72 h in the presence of IL-1ß (5 U/ml). The data are expressed as the amounts of cytokines found in the culture supernatants per µg of DNA and are presented as the mean ± SEM of four different chondrocyte cultures. Each culture was performed with chondrocytes isolated from a single cartilage sample. NSAIDS-treated groups were significantly different from the controls (IL-1ß treated culture) with* $*p < 0.05$.

Effects of ACE and metabolites on PGE_2 and NO_2 production by human chondrocytes.

Under basal conditions, PGE_2 production was significantly higher in OA than in N chondrocyte cultures (N 7.6 ± 5.1 pg/µg DNA/72 h vs OA 31 ± 14 pg/µg DNA/72 h; $p < 0.05$) *(Table 1)*. Upon IL-1ß (5U/ml) stimulation, PGE_2 amounts produced by N and OA chondrocytes were enhanced by 2 and 6 times, respectively. IL-1ß stimulating effect was significantly more marked in OA than in N chondrocyte cultures ($p < 0.01$). ACE decreased in a dose-dependent manner both basal and IL-1ß-stimulated PGE_2 production. DICLO and 4'-HOACE fully inhibited PGE_2 at all concentrations tested (data not shown).

Nitric oxide production was determined by quantifying NO_2^- and NO_3^- into the culture supernatants. ACE did not significantly modify NO production whatever the dose tested. On the other hand, at 30 µM, 4'-HOACE decreased by 26% the IL-1ß stimulation and by 17 % the LPS-stimulation. DICLO inhibited by 34 % the LPS-stimulated production but was without significant effect on IL-1ß-stimulation (Fig. 5).

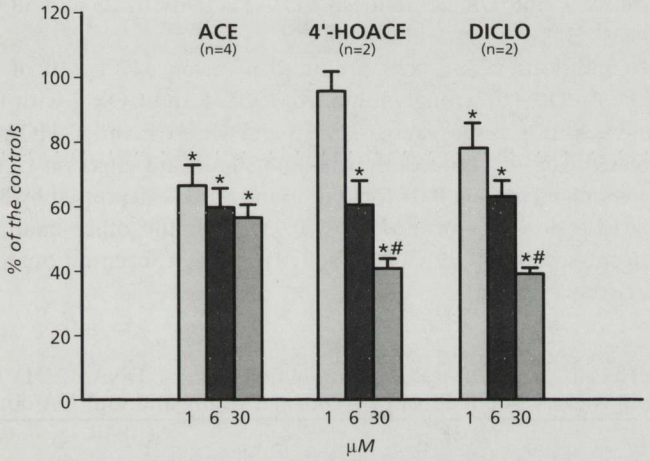


Fig. 4: *Effect of increased doses of ACE, DICLO and 4'-HOACE on IL-6 production by human OA chondrocytes cultured for 72 h in the presence of IL-1ß (5 U/ml). The data are expressed as the % of the control values and are presented as the mean ± SEM of four different cultures for ACE and of two cultures for 4'-HOACE and DICLO. NSAIDs-treated groups were significantly different from the controls with *p < 0.05. 4'-HOACE and DICLO inhibitory effects were significantly more important than ACE effect with a # p < 0.05.*

Effects of ACE and metabolites on iNOS, IL-1ß, COX-2 gene expression.

We have also determined the effect of ACE and its metabolites on iNOS , IL-1ß and COX-2 gene expressions by LPS-stimulated normal young chondrocytes (Fig. 6). In the basal conditions, chondrocytes expressed 4193 and 1526 copies of mRNA coding for iNOS and COX-2, respectively, whereas IL-1ß gene was not expressed. LPS (10 µg) greatly increased iNOS and COX-2 gene expressions by 11

and 19 times, respectively. Furthermore, LPS induced IL-1ß expression. Interestingly, at the concentration of 30 µM, 4'-HOACE and DICLO significantly decreased iNOS mRNA level by 25 and 40 %, respectively, whereas ACE was without effect. At the concentration of 3 µM, all drugs tested were ineffective on iNOS mRNA expression (Fig. 6). At the concentration of 30 µM, IL-1ß mRNA was significantly decreased by all drugs tested. The inhibition was equal to 25 %, 30 % and 70 % for ACE, 4'-HOACE and DICLO, respectively. At 3 µM, only DICLO was efficient. Finally, ACE and DICLO at the concentration of 30 µM significantly depressed COX-2 mRNA level whereas 4'-HOACE had no effect.

Effects of ACE and metabolites on cyclooxygenase activities

The effects of ACE and metabolites on cyclooxygenase activities were also tested in both cell free system and in whole blood. In our control conditions, one unit of purified ovine COX-1 and COX-2 produced on average 11 and 14 ng of PGE_2, respectively. ACE and DICLO weakly inhibited COX-1 activity with IC_{50} values higher than 100 µM whereas 4'-HOACE was ineffective (Table 2). At the concentration of 10 µM, ACE and DICLO reduced COX-2 activity by 25 and 58 %, respectively, whereas 4'-HOACE was without effect.

In the basal conditions, blood cells produced in mean 347 ng/ml of TXB_2 and 46 ng/ml of PGE_2. DICLO strongly inhibited COX-1 and COX-2 with IC_{50} values of 0.6 µM and 0.04 µM, respectively (Table 3 and 4). ACE and 4'-HOACE preferentially decreased COX-2. Indeed, they had no significant effect on COX-1 at the concentrations ranged among 0.01 to 10 µM and weakly depressed by 30 % TXB_2 production at the dose of 100 µM ($p < 0.05$). On the other hand, ACE and 4'-HOACE decreased by 50 % COX-2 activity at the concentrations of 0.8 and 36 µM, respectively (Table 4).

Table 1 : Effects of ACE on PGE_2 production (pg/µg DNA/ 72H) by human articular chondrocytes isolated from two normal young and four OA donors.

	- IL-1ß Aceclofenac (µM)				+ IL-1ß Aceclofenac (µM)			
	0	1	6	30	0	1	6	30
N (n=2)	7.6 ±5.1	1.6 ± 0.05a	ND	ND	13 ± 3.7	1.8 ±0.5	ND	ND
OA (n=4)	31 ± 14b	8 ± 6.5a	5.9 ± 5a	5.4 ± 4.2a	193 ± 67b	21 ± 4a	10 ± 5a	8 ± 4.3a

Chondrocytes were cultured in the absence or in the presence of 5 U/ml of rhIL-1ß and with or without increased amounts of drugs. Values are means ± SEM. Means comparison was performed by the MANOVA test. Treated groups and controls were statistically different with a $p < 0.01$. OA production was significantly higher than N production with b $p < 0.01$.

Discussion

Our work demonstrated that the antiinflammatory action of ACE is multifactorial. Indeed, ACE not only depressed PGE_2 production, but also reduced IL-6 synthesis by human chondrocytes. IL-6 is involved in the development and maintenance of inflammatory and immune reactions accompanying arthritis. IL-6 is a potent B cell growth and differentiation factor [27] and an inducer of acute phase protein synthesis by hepatocytes [28]. Furthermore, IL-6 has been proposed as a contributor to the OA pathological process [29] by increasing the amount of inflammatory cells in synovial tissue [30], by stimulating the proliferation of chondrocytes and

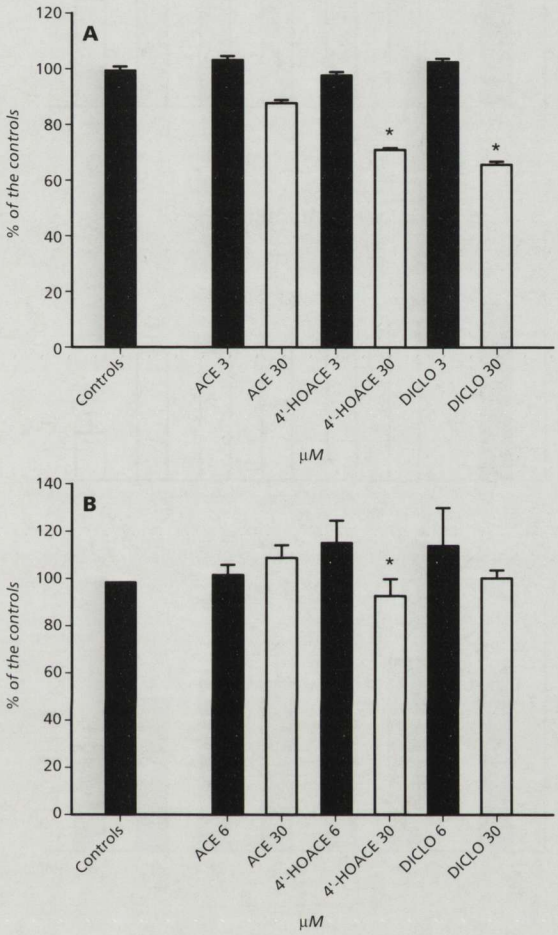


Fig. 5: *Effect of NSAIDs on NO_2^-/NO_3^- production by human chondrocytes. Chondrocytes were untreated (controls) or incubated with (A) LPS (10 µg/ml) or (B) IL-1ß (5 U/ml) alone or in combinaison with ACE, DICLO and 4'-HOACE. Results are expressed as the percentage of the LPS- or IL-1ß- stimulated values (controls) and as the mean ± SEM of two different cultures. NSAIDS-treated groups were significantly different from the controls with $*p < 0.05$.*

by inducing an amplification of the IL-1 effects on the increased MMP synthesis and inhibition of proteoglycan production [31]. Therefore, the inhibition of IL-6 synthesis could be an important mechanism for reducing the inflammatory reaction and associated connective tissue damages.

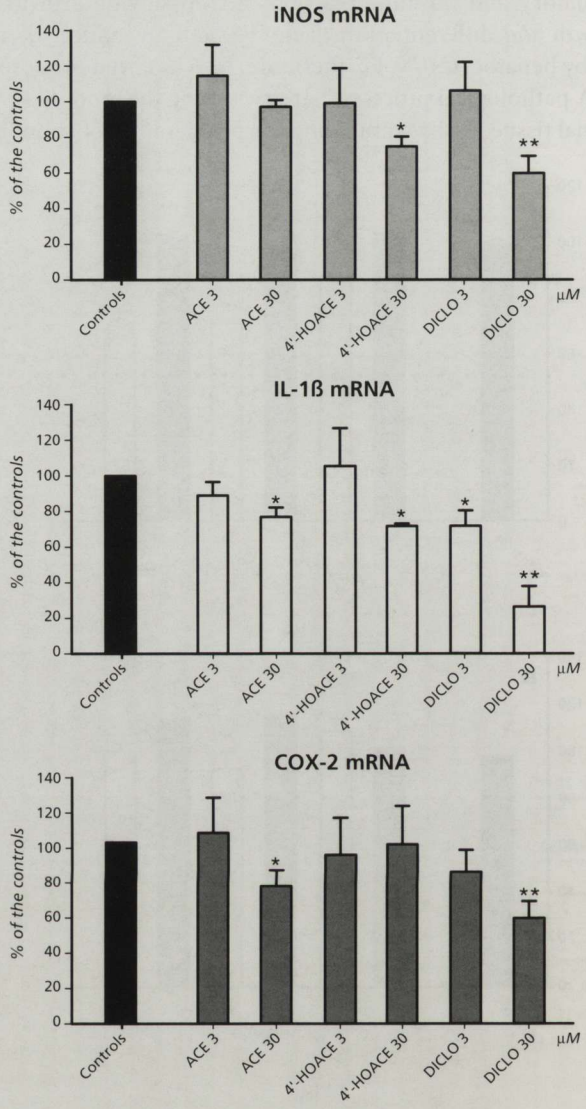


Fig. 6: *iNOS, IL-1ß and COX-2 mRNA levels were assayed by a real time and quantitative PCR. Results are expressed as the percentage of the LPS-stimulated values (controls) and as the mean of 6 cultures from two young, normal donors. NSAIDS-treated groups were significantly different from the controls with $p < 0.05$.*

4'-HOACE, the major metabolite of ACE in blood, together with DICLO, another metabolite, decreased iNOS and IL-1ß mRNA and inhibited IL-6 production. Furthermore, our results showed that DICLO and 4'-HOACE were potent inhibitors of PGE_2 synthesis by blood cells and chondrocytes. These findings suggest that these metabolites could play an active part in the anti-inflammatory properties of ACE. This observation is important since it was demonstrated that ACE can be converted into active metabolites by some inflammatory-related cells such as polymorphonuclears, monocytes, and rheumatoid synovial cells [19]. Taken together, these findings suggest that in arthritis, ACE, in addition to its well recognised anti-inflammatory properties, could be locally converted by joint cells into active metabolites. By this way, ACE could potentiate its effect at the inflammatory site.

Table 2 : Effects of ACE, 4'-HOACE and DICLO on the activity of ovine purified cyclooxygenases (COX-1 and COX-2).

Drug concentrations µM	COX-1 % of the control values	COX-2 % of the control values
ACE		
0.01	96.52 ± 1.78	101.44 ± 0.62
0.1	89.98 ± 11.01	97.32 ± 0.61
1	81.74 ± 2.89[a]	90.81 ± 1.75
10	68.30 ± 2.92[a]	75.00 ± 2.50[a]
100	68.77 ± 8.31[a]	73.25 ± 0.17[a]
4'-HOACE		
0.01	98.76 ± 4.08	99.94 ± 4.21
0.1	98.83 ± 3.32	96.81 ± 5.61
1	97.19 ± 4.42	98.26 ± 5.68
10	101.14 ± 6.03	105.42 ± 3.80
100	109.67 ± 4.62	116.62 ± 10.53
DICLO		
0.01	97.69 ± 1.74	89.21 ± 2.53
0.1	81.45 ± 2.51[a]	56.07 ± 1.84[a, b]
1	69.73 ± 5.74[a]	48.62 ± 3.91[a, b]
10	62.36 ± 5.48[a]	42.27 ± 1.69[a, b]
100	60.00 ± 12.34[a]	38.23 ± 2.26[a, b]

Results are expressed as the percentage of PGE_2 production of the control values. Values are means ± SEM of three independent experiments. a $p<0.05$ in comparison with the controls. b ACE and DICLO values were significantly different with $p < 0.05$.

In whole blood test, ACE preferentially inhibited COX-2 activity suggesting a relative selectivity of ACE for this COX isoform. Our results are consistent with those obtained in similar *in vitro* study by Lidbury *et al.* [32], showing that ACE has an apparent selectivity towards the inhibition of COX-2 slightly superior to that of celecoxib. This finding contributes to explain the better gastrointestinal tolerance of ACE when it is compared with other conventional NSAIDS.

Table 3 : Effects of ACE, 4'-HOACE and DICLO on the TBX_2 and PGE_2 production by human whole blood.

Drug concentrations μM	TBX_2 % of the control values	PGE_2 % of the control values
ACE		
0.01	104 ± 9.9	87.5 ± 23
0.1	97.5 ± 12.9	72.9 ± 31.8[a]
1	100.7 ± 11.1	46.2 ± 13[a]
10	101.9 ± 9.2	3.04 ± 2[a]
100	67.4 ± 17[a]	0.68 ± 0.1[a]
4'-HOACE		
0.01	103.4 ± 5.2	96 ± 10
0.1	102.8 ± 9.3	103.2 ±8.7
1	106.0 ± 11	108 ± 19.4
10	96.9 ± 3.7	59.5 ± 12.2[a, c]
100	71.2 ± 2.6[a]	18.2 ± 5.9[a, c]
DICLO		
0.01	86.8 ± 22.7	97.8 ± 9.2
0.1	84.3 ± 7.3	3.08 ± 3.1[a, b]
1	17.6 ± 3.8[a, b]	0.88 ± 0.3[a, b]
10	1.6 ± 0.4[a, b]	0.31 ± 0.20[a, b]
100	0.9 ± 0.1[a, b]	0.13 ± 0.07[a, b]

Results are expressed as the percentage of TXB_2 or PGE_2 production of the control values. Values are means ± SEM of three tests performed with three independent blood samples. a $p<0.05$ in comparison with the controls. b ACE and DICLO values were significantly different with $p < 0.05$. c ACE and 4'-HOACE values were significantly different with $p < 0.05$.

Inversely, ACE weakly depressed the activity of purified COX-2 and showed an anecdotical effect on COX-2 mRNA level. Our results are in agreement with the study of Yamazaki *et al.* [11] demonstrating that ACE had no inhibitory effects on COX-2 gene expression and protein synthesis in the rheumatoid synovial cells whereas ACE significantly reduced PGE_2 production. Taken together, these findings suggest that ACE probably exerts its main inhibitory action on PGE_2 synthesis at the post-transcriptional level but not by a direct interaction with the catalytic site of

COX. Indeed, ACE was inefficient on tests performed on purified enzymes which investigate drug-enzyme interaction excluding other interactions that the drug could have at the cellular level. For example, the intracellular enzymatic transformation of the drug into active metabolites, the inhibition of COX-2 inducers or a direct action on phospholipase A2 could explain the higher inhibitor potency of ACE in cell models.

In summary, the present results demonstrated that (i) ACE has a relative selectivity for COX-2 (ii) that its anti-inflammatory activity is multifactorial and (iii) that some of its effects could be mediated by its metabolites.

Table 4 : Effects of ACE, 4'-HOACE and DICLO on COX-1 and COX-2 activity in the whole blood test.

	IC_{50} (μM)	
	COX-1	COX-2
ACE	> 100	0.8
DICLO	0.6	0.04
4'-HOACE	> 100	36

COX-1 activity was determined by the measurement of TXB_2 in the sera of three normal volunteers and COX-2 activity by assaying the daily production of PGE_2 by LPS-stimulated whole blood cells.

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ANNEXE 14

In vitro Study of the Antioxidant Properties of Nimesulide and 4-OH Nimesulide: Effects on HRP-and Luminol-dependent Chemiluminescence Produced By Human Chondrocytes

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Osteoarthritis and Cartilage 2000; 8: 419-425

Abstract

Objectives. Reactive Oxygen Species (ROS) are now recognised to play an important role in the pathogenesis of rheumatic diseases and constitute an interesting therapeutic target for drugs. This *in vitro* study was designed to evaluate the antioxidant properties of nimesulide (NIM), a nonsteroidal antiinflammatory drug of the sulfonanilide class, and its main metabolite 4-OH nimesulide (4-OHNIM).

Methods. The scavenging effects of NIM and 4-OHNIM on hydroxyl radical (°OH) and superoxide anions ($O_2^{\cdot-}$) were investigated by Electron Spin Resonance (ESR), using 5,5-dimethylpyrroline-N-oxide (DMPO) as spin trap agent. The quenching properties of these drugs on hypochlorite anion was studied by luminol enhanced chemiluminescence. Finally, the effects of NIM and 4-OHNIM on the reactive oxygen species production by human articular chondrocytes were recorded by HRP and luminol enhanced chemiluminescence.

Results. By this method, it has been demonstrated that NIM and 4-OHNIM, at concentrations ranging from 10 to 100 μM, are potent scavengers of •OH whereas only 4-OHNIM was capable to scavenge $O_2^{\cdot-}$. Chemiluminescence generated by HOCl was also significantly and dose-dependently inhibited by both NIM and 4-OHNIM. Nevertheless, at each concentration tested, the inhibitory effect of 4-OHNIM was significantly more marked, even at the highest concentration (100 μM). Furthermore, when chondrocytes were pre-incubated for 48 - 96 h with NIM or 4-OHNIM, the luminol- and HRP-dependent CL produced by the cells was significantly inhibited in a dose-dependent manner.

Conclusions. NIM and 4-OHNIM may protect cartilage against oxidative stress, by scavenging ROS but also by inhibiting their production by chondrocytes.

Key words: Antioxidant, Chondrocytes, Nimesulide, Chemiluminescence

Introduction

Nimesulide (NIM) is a nonsteroidal antiinflammatory drug (NSAID) commonly used in the treatment of osteoarthritis (OA) [1,2]. The compound is weakly acidic (pka=6.5) and differs from other NSAIDs in that its chemical structure contains a sulfonanilide moiety as the acidic group [3]. In human, the drug is mostly biotransformed into 4-hydroxynimesulide (4-OHNIM), a metabolite which appears to contribute to the antiinflammatory activity of the compound [4]. NIM appears to exert its antiinflammatory activity through a variety of mechanisms including a relatively selective inhibition of prostaglandin synthesis by COX-2 [5-11], the inhibition of histamine release from human basophils and tissue mast cells [12], the reduction of neutrophil aggregation [13] and adherence to endothelial cells as well as the inhibition of PAF (Platelet Activating Factor) production by stimulated human neutrophils [14]. Moreover, NIM reduced the generation of superoxide anion by stimulated polymorphonuclear leukocytes through the inhibition of protein kinase C translocation and phosphodiesterase type IV activity , the principal enzyme responsible for the degradation of leucocyte cAMP [15]. In a study assessing the antioxidant activity of NIM and its main metabolites, it was reported that 4-OHNIM possessed antilipoperoxidant activity at a much lower concentration than the parent compound and another metabolite recently identified in human urine, 2-(4'-hydroxyphenoxy)-4-N-acetylaminomethansulfonanilide [16]. Using an *in vitro* model of erythrocyte membrane lipoperoxidation induced by cumene hydroperoxide (CuOOH), 4-OHNIM protects erythrocyte membrane by directly quenching peroxyl and alkoxyl radical species [17]. NIM is also a potent scavenger of hypochlorous acid which is responsible for large part of tissue injury and inflammation mediated by PMNs [18]. Nimesulide also exhibited favourable hydroxyl radical scavenging activity in a membrane phosphatidylcholine liposome [19]. Recently, NIM was reported to induce the intracellular phosphorylation and activation of glucocorticoid receptors and to activate their binding to target genes in human synovial fibroblasts [20]. This mechanism may contribute largely to its antiinflammatory activity.

In addition to its antiinflammatory action, nimesulide may also exert beneficial effects on osteoarthritic (OA) cartilage metabolism. When added to human articular cartilage explant *in vitro*, NIM at a therapeutic concentration (10 μM), reduced the degradation of the matrix by inhibiting the synthesis of metalloproteinases such as collagenase and stromelysin [21]. Using primary human chondrocytes cultured in suspension, we showed that therapeutic concentrations of NIM could inhibit the synthesis of IL-6, while it did not modify proteoglycan or IL-8 production [22]. With human osteoarthritic synovial fibroblasts in culture, NIM also reduced the synthesis of urokinase and increased plaminogen activator inhibitor-1 (PAI-1), suggesting that this drug decreases the degradation of cartilage matrix not only by reducing metalloproteases synthesis but also by inhibiting one of the mechanism involved in metalloprotease activation [23].

The antioxidant properties of NIM suggest that this drug could also protect cartilage by inhibiting reactive oxygen species (ROS) production by chondrocytes.

Recently, it was reported that chondrocytes possess an NADPH oxidase complex [24, 25] and produced ROS in response to phorbol 12-myristate 13-acetate (PMA), calcium ionophore ionomycin, anoxia-reoxygenation cycles and cytokines (TNFα, IL-1β, IFNγ) [26-29]. ROS are able to degrade matrix components (e.g. type II collagen, proteoglycans, hyaluronic acid) and DNA by direct action [30-32] and by indirect activation of latent collagenase [33]. Furthermore, α1-proteinase inhibitor , the major inhibitor of elastase, is inactivated by exposure to the PMNs-derived oxygen radicals species [34], lessening defences against the degradative actions of proteases on connective tissues. Exposure of the chondrocytes to oxygen radicals inhibits protein and DNA synthesis in these cells [35], and depletes intracellular ATP as a result of a simultaneous inactivation of glyceraldehyde-3-phosphate dehydrogenase [36]. These findings suggest that ROS produced by chondrocytes could play a key role in the cartilage degradation and, therefore, constitute a potential target for drugs, such as NSAIDs, that are used in the treatment of joint diseases and possess antioxidant side-effects.

In this work, we have investigated the capacities of NIM and 4-OHNIM to scavenge hypochlorous anion, hydroxyl radicals and superoxide anion and to modulate the production of ROS by human articular chondrocytes.

Materials and Methods

Human chondrocytes in Culture

Cartilage were obtained from the knees of eight adult donors shortly after death (three males and five females with the mean age of 58 years), being excised from the superficial and medium layers and avoiding the calcified layer. Chondrocytes were cultured for a short period in order to maintain their phenotype. Cartilage was cut into small fragments and then subjected to enzymatic digestion sequentially with hyaluronidase, pronase and collagenase. First, the fragments were incubated for 30 min at 37 °C under constant agitation (200 rpm) with hyaluronidase (Sigma Chemie, Bornem, Belgium) dissolved (0.5 mg/ml) in Dubbelcco's Modified Eagle's Medium (DMEM, Biowhittaker, Bruxelles, Belgium) (10 ml enzyme solution per 3 g of cartilage). The fragments were then incubated for one hour at 37 °C in pronase solution (1 mg/ml of DMEM; enzyme obtained from Merck-Belgolabo, Overijse, Belgium) (3 g cartilage per 10 ml enzyme solution). The cartilage fragments were incubated for 20 h at 37 °C under constant agitation (200 rpm) with collagenase (Sigma chemie) dissolved in DMEM (1 mg/ml) containing 1 % of Ultroser G (Gibco, Gent, Belgium) (3g cartilage per 10 ml enzyme solution). Ultroser G is a serum substitute designed to replace fetal calf serum in nutrient culture medium. The cells were then filtered through nylon mesh with a pore diameter of 70 μm, washed three times, counted (5 x 10^6 cells/ml) and resuspended in 10 ml of DMEM supplemented with 1 % of ITS+. ITS+ is a well defined culture supplement containing 0.625 mg/ml insulin, 0.625 mg/ml transferrin, 0.625 μg/ml selenium acid, 125 mg/ml bovine serum albumin (BSA) and 0.535 mg/ml linoleic acid. Cells were then kept at 37 °C for 48 h under constant agitation.

After this washout period, cells (0.5 x 10^6 cells/ml) were then seeded in 24-well plates treated for low cell attachment and cultured for 24, 48, 72 or 96 h in DMEM without red phenol containing 1 % ITS+, 1 mM glutamine and the drugs tested. NIM and 4-OHNIM (Therabel Research, Bruxelles, Belgium) were added to the culture medium at the concentrations ranging from 0.3 to 30 µg/ml. The peak concentrations of nimesulide reported in the plasma of RA patients who were taking 100 mg orally twice a day ranged from 10 to 20 µM. After a single or repeated 100 mg doses b.i.d, 10 µM was above the highest level noted in the synovial fluid of patients receiving 100 mg b.i.d orally for 7 days, 3 h after the last dose [37]. On the other hand, the mean plasma concentration of 4-OHNIM after single-dose administration of NIM 100 mg tablets to 12 healthy male volunteers was 1.4 mg/L [38]. NIM and 4-OHNIM were dissolved in 0.1 M NaOH (10 mg/ml) and diluted in DMEM (without phenol red) at the concentration of 10 µg/ml. pH was adjusted slowly back to 7.4 with 0.1 M HCl. After incubation, the chondrocytes suspension was collected by centrifugation and washed three times with HBSS (pH 7.4). The chondrocyte pellets were resuspended in 4.0 ml of HBSS (pH 7.4) and mixed gently (cells density +/-5.10^4/500 µl). A 500 µl chondrocytes suspension was added to a polypropylene cuvette for CL measurement.

Electron Spin Resonnance (ESR) experiments

The potential scavenging effect of NIM and 4-OHNIM on $O_2^{\cdot-}$ and •OH was investigated by direct addition of NIM and 4 OHNIM to the $O_2^{\cdot-}$ or •OH generating systems. Hydroxyl radicals (•OH) was generated by the Fenton reagents resulting from the mixture of the following agents: 0.1 mM H_2O_2, 0.01 mM $FeSO_4.7H_2O$ and 10 µM EDTA (Merck-Belgolabo, Overijse, Belgium). On the other hand , superoxide anion was produced from xanthine (1 mM)-xanthine-oxidase (0.08 U/ml) reaction in 50 mM phosphate buffer (pH 7.8). The scavenging effect of NIM and 4 OH-NIM on •OH and $O_2^{\cdot-}$ was monitored by EPR trapping technique, using 5,5-dimethylpyrroline-N-oxide (DMPO; 100 mM; Sigma-Aldrich, Bornem, Belgium) as spin trap agent. After preparation, the mixture was immediately transferred to a flat cell and placed in the TM 110 cavity of the ESR spectrometer (final volume 1 ml). The spectra were recorded at room temperature using a Bruker ESP 300 E spectrometer (Karlsruhe, Germany) operating at X-band with 100 kHz modulation frequency. These measurements were performed with non saturating 20 mW microwave power and microwave frequency 9.75 GHz. The instrumental settings were: center field 3480 G, time constant 40.96 ms, time conversion 40.96 ms, receiver gain 2 x 10^4 and sweep width 100 G.

Chemiluminescence (CL) measurement

The *in vitro* effects of NIM and 4-OHNIM on the light emission induced by sodium hypochlorite solution (NaOCl; 10^{-4}M; BDH Laboratory, London, U.K) were investigated. The assays were performed at 25 °C in phosphate buffer (pH 7.4). Light emission was produced by the direct reaction of NaOCl (10^{-4} M) with luminol (2.5 x 10^{-5} M; 5-amino-2, 3-dihydro-1, 4-phthalazinedione). Chemiluminescence generated by chondrocytes was also recorded according the following protocole:

luminol (5 μM), chondrocyte suspension (5.10^4 cells/0.5 ml) and horse radish peroxidase (HRP, 0.05 U/0.5 ml) were sequentially added to a circular polystyrene reaction vessel. To verify that light emission was the consequence of reactive oxygen species production, superoxide dismutase (SOD; 0.01-100 U/0.5 ml) and catalase (0 - 500 U/0.5ml) were added to the reaction mixture. CL was followed at 37°C for 10 min. CL was measured with a BioOrbit 1251 Luminometer (Turku-Finland) and the data were expressed in arbitrary units (1U = 1 mV).

Statistical analysis

The mean +/- SD for each variable was calculated. Comparison of the treated groups with the controls was performed using the unpaired Student's *t-test*, with a significant limit set at $p<0.05$.

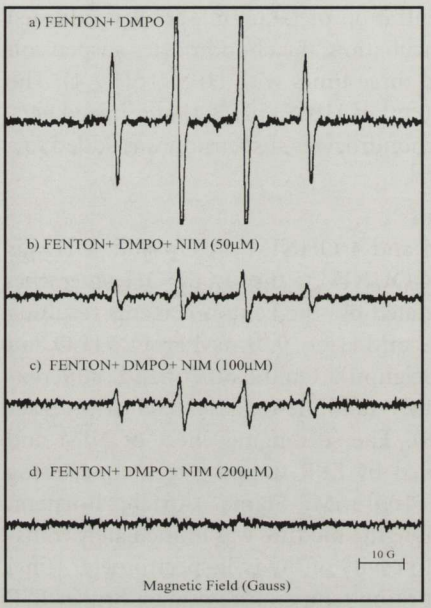


Fig 1: *ESR spectra (DMPO 100 mM) of the spin adduct of hydroxyl radical generated by the Fenton reaction (H_2O_2 0.1 mM, $FeSO_4.7H_2O$ 0.01 mM, EDTA 0.01 mM). (a) Normal spectrum (hyperfine splittings for DMPO-°OH adduct: aH=aN=14.87 G); (b) DMPO-°OH spectrum in the presence of NIM 50 μM; (c) DMPO-°OH spectrum in the presence of 100 μM; (d) DMPO-°OH signals was fully inhibited by NIM 200 μM.*

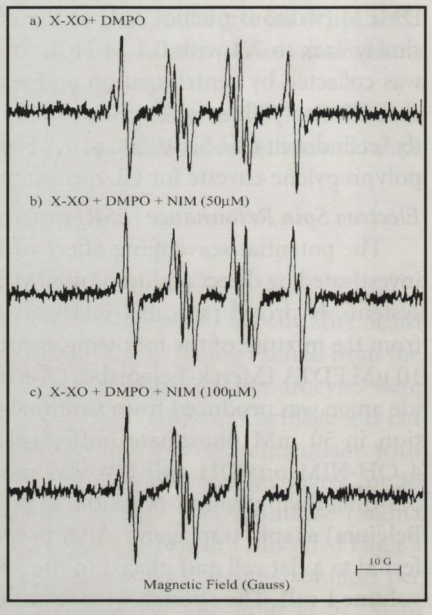


Fig 2: *ESR spectra (DMPO 100 mM) of superoxide adduct (DMPO-OOH) generated by xanthine (1mM)/xanthine oxidase (0.08 mU/ml) system in 50 mM phosphate buffer pH 7.4. (b,c) Complete system xanthine/xanthine-oxidase generating superoxide radical in the presence of NIM 50 μM (b) or 100 μM (c). No significant variation of the ESR signals was observed in the presence of NIM.*

Results

Scavenging effects of NIM and 4-OHNIM on HO° and $O_2^{\cdot-}$ radicals

The reaction of DMPO with •OH generated by the Fenton reaction ($FeSO_4$ and

H_2O_2) resulted in a four-line ESR spectrum (DMPO-•OH adduct). This ESR signal decreased in the presence of increasing concentrations (10 to 200 µM) of NIM (IC_{50} = 5.2 µM) and 4-OHNIM (IC_{50} = 4.9 µM) (Fig 1). In contrast, no inhibiting effect was observed on the $O_2^{\cdot-}$ produced by the xanthine/xanthine oxidase system in the presence of NIM (Fig 2) while, at similar doses, its metabolite (4-OHNIM) caused a decrease of the ESR spectrum intensity (IC_{50} = 17 µM) (Fig 3a and 3b). At 10 µM, which corresponds at the peak plasmatic concentration after oral administration of a therapeutic dose of NIM, ESR signal height generated by •OH was reduced by 70% whereas $O_2^{\cdot-}$ ESR signal was unaffected. Inversely, at 10 µM, 4-OHNIM decreased $O_2^{\cdot-}$ ESR signal height by 38 %.

Scavenging effects of NIM and 4-OHNIM on chemically-induced chemiluminescence

The chemiluminescence generated by OHCl was dose-dependently inhibited by NIM (IC_{50} > 100 µM) and 4-OHNIM (IC_{50} = 17 µM). Nevertheless, at each concentration tested, the inhibition was significantly higher in the presence of 4-OHNIM than NIM ($0.01 < p < 0.001$) (Fig 4). At therapeutic concentration (25 µM), NIM slighly quenched (-16 %) OHCl whereas 4-OHNIM decreased by 70 % light emission. At 100 µM, the inhibition of OHCl-induced CL is almost complete for 4-OHNIM (- 95 %) but not for NIM (only 38 % of inhibition).

Effects of NIM and 4-OHNIM on luminol and HRP-dependent chemiluminescence

To verify that the chemiluminescence resulted from the production of ROS by chondrocytes, we have introduced two antioxidant enzymes, superoxide dismutase (SOD) and catalase, in the reaction mixture (Fig 5). Interestingly, CL generated by chondrocytes was dose-dependently depressed by both, catalase ($r = 0.96$; $p = 0.01$; $y = -23.02x + 51,8$) and superoxide dismutase ($r = 0.91$; $p = 0.0125$; $y = -12.8\ x + 22.6$).

With the aim of evaluating the effects of NIM and its metabolite, 4-OHNIM, on the ROS generating systems of living cells, chondrocytes were pre-incubated for 24-96 h with increased amounts of NIM and 4-OHNIM. At the concentrations ranging from 0.4 to 10 µg/ml, NIM inhibited in a time and dose-dependent manner CL generated by chondrocytes (Fig 6). In the therapeutic range of concentrations (6 - 12 µM), the inhibition was already superior at 50%. After 48 h of incubation, 4-OHNIM also decreased chemiluminescence but in a lesser extent than NIM (Fig. 7). At plasmatic concentrations of 4-OHNIM, the inhibition of the HRP- and luminol-enhanced chemiluminescence produced by chondrocytes was less than 50 % even after 96 h of incubation.

Discussion

Reactive oxygen species have been shown to play a significant role in the degradation of cartilage in arthritis. Recent studies suggested that rabbit, bovine, or porcine chondrocytes are potent sources of oxygen free radicals [25, 27, 29]. Previous work from our laboratory showed that isolated human chondrocytes were also capable of producing reactive oxygen species such as hydrogen peroxide and

hydroxyl radicals when they were submitted to anoxia-reoxygenation cycle or PMA stimulation [28].

As demonstrated by ESR method, NIM is a potent scavenger of hydroxyl radical, but is ineffective on superoxide anions. In contrast, 4-OHNIM quenched both •OH and $O_2^{\cdot-}$. This finding can be explained by the presence of a phenolic structure in 4-OHNIM which is particularly susceptible to further $O_2^{\cdot-}$ attack. In a previous study, Maffei-Facino *et al.* demonstrated that NIM and its metabolites could prevent •OH-induced depolymerisation of hyaluronic acid and that the phenolic metabolite displayed maximun scavenging activity in the peroxidation model,

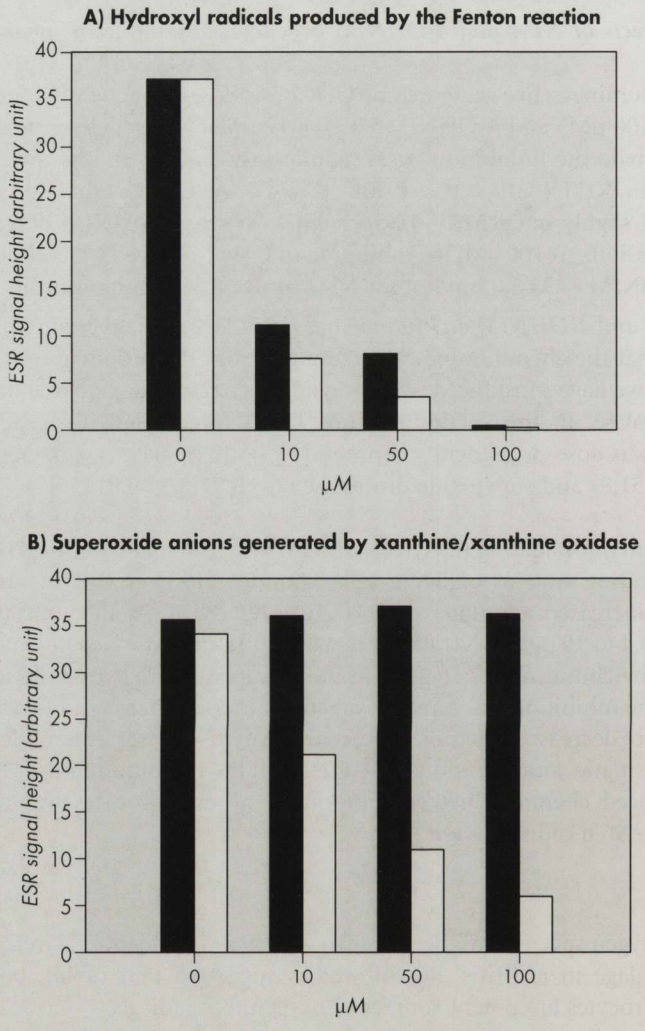


Fig. 3: *Effects of NIM (black columns) and 4-OHNIM (white column) on ESR signal height (DMPO 100 mM) generated by hydroxyl radicals resulting from the Fenton reaction (a) and by superoxide radical produced by xanthine/xanthine-oxidase system (b).*

where the pro-ximate reactive damaging species were oxygen- (RO• and ROO•) and carbon-centered (R•) lipid radicals [16]. Furthermore, since we know that NIM and 4-OHNIM can bind Fe^{2+}, the mechanism of action of NIM on the Fenton reaction could be the chelation of Fe^{2+} [16, 17].This chelating property of NIM and 4-OHNIM may also contribute to its therapeutic potency by reducing "free" iron at the inflammatory site when inflammatory stimuli induce respiratory bursts of invading leukocytes [39, 40, 41].

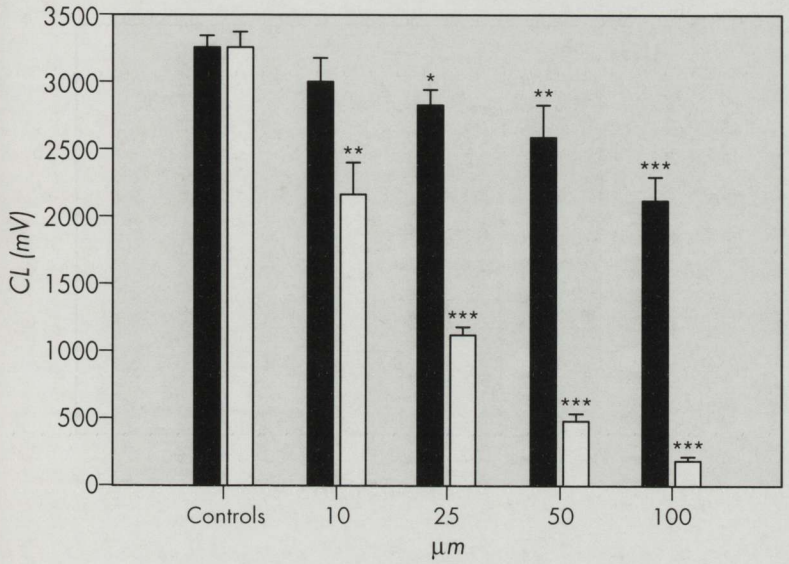


Fig. 4: *Effects of increase concentrations of NIM (black columns) and 4-OHNIM (white columns) on the peak value of luminol (10^{-4} M)-enhanced chemiluminescence curve generated by OHCl (NaOCl). Results are expressed as the mean values of triplicate. Statistically significant difference compared with the controls: *p<0.05; **p<0.01; ***p<0.001.*

Neutrophils, recruited to tissue sites of inflammation, release a variety of oxidants and enzymes, that are responsible for tissue damage. Among the oxidants released are potent chlorinated compounds such as hypochlorous acid, which induce tissue cell damage and inactivate protease inhibitors, particularly α1-antiprotease, the specific inhibitor of neutrophil elastase. In our study, NIM and 4-OHNIM quenched in a dose-dependent manner hypochlorous acid as demonstrated by luminolenhanced chemiluminescence method. Nevertheless, at each concentration tested, the inhibition was significantly more marked in the presence of 4-OHNIM than in that of NIM. Other studies have also shown that NIM, at therapeutic concentrations (10-100 µmol/l), prevented the inactivation of α1-antiprotease by neutrophils by effectively scavenging the hypochlorous acid released by neutrophils [42]. In contrast, aspirin and naproxen were completely ineffective, suggesting that the presence of an NH group, particularly exposed at the surface of the

NIM and therefore prone to chlorination by OHCl, probably accounts for the observed effect of NIM [42].

On the other hand, our results also demonstrate that 4-OHNIM is a stronger OHCl scavenger than NIM, suggesting that the OH group could also react with OHCl. At 10 μmol/l (equal to 3.08 mg/l) which correspond at the level of NIM found in plasma after a single 100 mg oral dose, NIM and 4-OHNIM inhibited OHCl-induced chemiluminescence by 11 and 38% respectively, suggesting that *in vivo*, 4-OHNIM could be the active compound on this process.

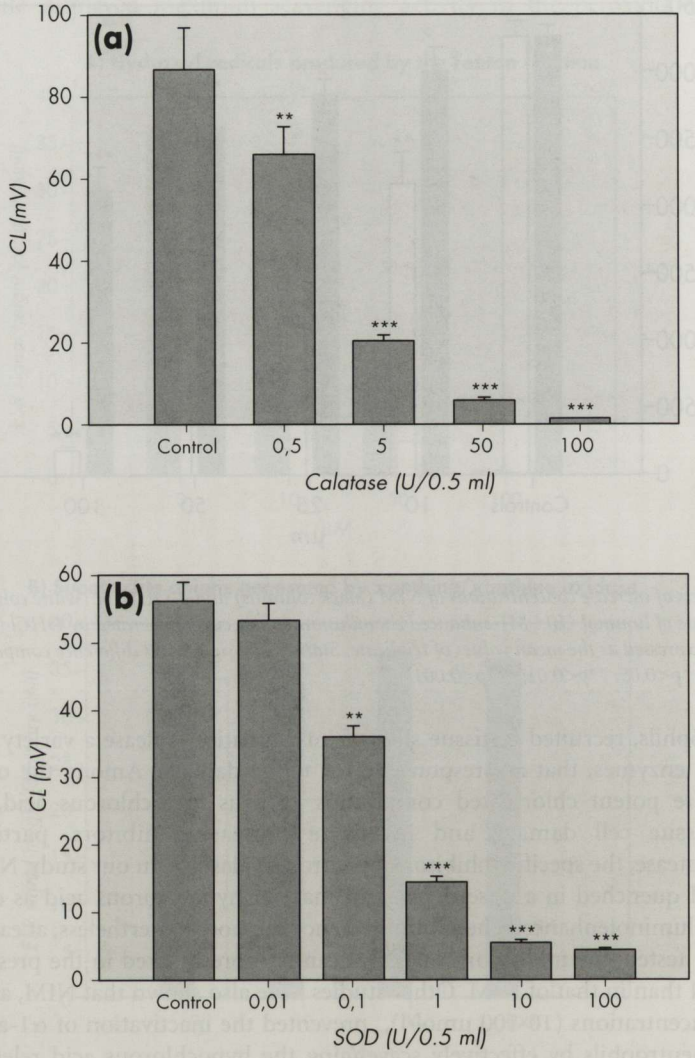


Fig. 5: *Effects of increase amounts of catalase (A) and superoxide dismutase (B) on HRP- and luminol-enhanced chemiluminescence. Luminol was used at the concentration of 5 μM and the reaction was started by the injection of 0.05 U of HRP.*

The production of ROS by isolated chondrocytes can be estimated by measurement of the chemiluminescence [27, 29]. Due to the low quantum yield of ROS, it is difficult to measure the intrinsic CL produced by chondrocytes. Luminescence enhancers such as luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) and lucigenin are susceptible to oxygenations and of high CL yield. Luminol-dependent or lucigenin-dependent CL in articular chondrocytes have been established by Rathakrishnan *et al.* [27,29]. Luminol-dependent CL is considered to reflect the production of hydrogen peroxide, peroxynitrite, superoxide and singlet oxygen [43-47]. In this study, we have developed an HRP- and luminol- dependent CL assay to measure the production of ROS by cultured human chondrocytes. Both luminol and HRP are necessary to the CL measurement. In the absence of these chemiluminescence enhancers, chondrocytes failed to emit chemiluminescence. Both catalase

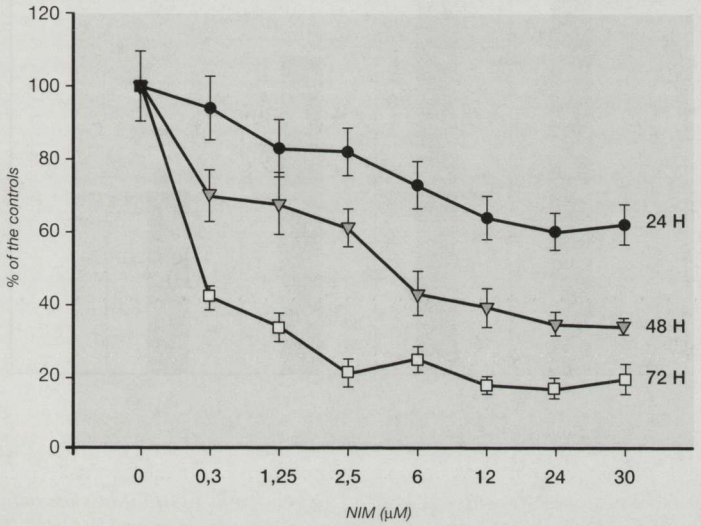


Fig. 6: *Effects of increasing concentrations of NIM on HRP- and luminol-enhanced chemiluminescence generated by human chondrocytes. Chondrocytes were pre-incubated for 24 (- ● -), 48 (- ▽ -) or 72 (-□ -) hours with nimesulide. Results are means of five experiments conducted with chondrocytes isolated from cartilage of five different donors expressed as the percentage of the control values. *Statistically significant difference compared with the controls * $p<0.05$; ** $p < 0.01$; *** $p<0.001$.*

and superoxide dismutase fully suppressed chemiluminescence generated by chondrocytes, indicating that this assay measures the production of hydrogen peroxide and superoxide anions by chondrocytes. When chondrocytes were pre-incubated with NIM and 4-OHNIM, HRP- and luminol-dependent chemiluminescence was dose- and time-dependently decreased. These findings suggest that NIM not only acts as a scavenger of ROS, but also inhibits the intracellular generating system of ROS. At the present time, two mechanisms have been advanced to explain the inhibitory effect of NIM on ROS production by leukocytes. By analogy, we can speculate that the effect of NIM on oxygen free radicals (superoxide anion, $O_2^{\cdot-}$) may be related to its inhibitory activity on cytosolic phosphodiesterase type IV (cAMP-specific), thus increasing cytosolic cAMP and, as a consequence, protein kinase A

activity, which promotes the phosphorylation of a number of intracellular factors and inhibits the assembly of NADPH-oxidase in the plasma membrane [15]. Another mechanism of action of NIM, could be a direct interference with the adenosine receptor system on the cell membrane. Recently, it was demonstrated that the pre-incubation of cells with the specific adenosine receptor antagonist theophylline was able to significantly reverse the inhibitory activity of NIM on free radicals production sustained by FMLP and the ionophores [45], suggesting the possibility that NIM interferes directly with the adenosine receptor system on the cell membrane.

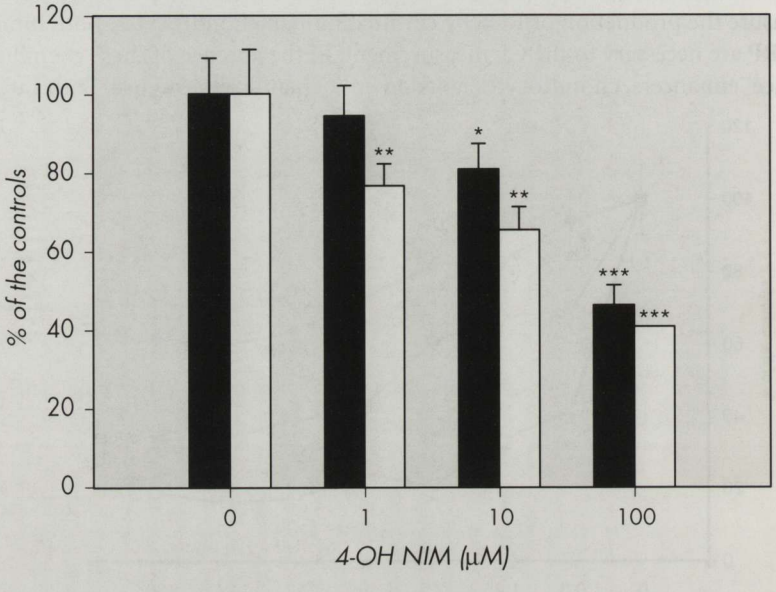


Fig. 7: *Effects of increase concentrations of NIM (black columns) and 4-OHNIM (white columns) on the peak value of luminol (10^{-4} M)-enhanced chemiluminescence curve generated by OHCl (NaOCl). Results are expressed as the mean values of triplicate. Statistically significant difference compared with the controls: *p<0.05; **p<0.01; ***p<0.001.*

In conclusion, our study demonstrates for the first time that an NSAID may inhibit ROS production by human chondrocytes. By this way, nimesulide could locally protect cartilage against the deleterious effects of ROS on the cartilage matrix integrity. This study also shows that the parent drug nimesulide, and its metabolite may have different scavenging effects suggesting that drug metabolization could also influence the drug efficacy. Finally, we must keep in mind that the reaction of NSAIDs with ROS could also conduct to the formation of transient radical species which could act as electron donors and initiate oxidative chain reaction [48]. The presence of excited state of the NSAID should be researched before definitively conclude on their antioxidant properties.

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ANNEXE 15

In vitro study of the antioxydant properties of non steroidal anti-inflammatory drugs by chemiluminescence and electron spin resonance (ESR).

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Free Radical Research 2000; 33: 607-21.

Objectives. To determine the antioxidant activities of nonsteroidal anti-inflammatory drugs (NSAIDS), we examined by chemiluminescence (CL) and electron spin resonance (ESR) their scavenging properties towards lipid peroxides, hypochlorous acid and peroxynitrite.

Methods. The antioxidant properties of nimesulide (NIM), 4-hydroxynimesulide (4-HONIM), aceclofenac (ACLO), 4-hydroxyaceclofenac (4-HOACLO), diclofenac (DICLO) and indomethacin (INDO) were tested on four different reactive oxygen species (ROS) generating systems: (I) phorbol-myristate acetate (PMA)-activated neutrophils, (II) Fe^{2+}/ascorbate-induced lipid peroxidation, (III) HOCl-induced light emission, (IV) the kinetics of $ONOO^-$ decomposition followed by spectrophotometry. ROS production was monitored by luminol-enhanced CL or by ESR using two different spin traps.

Results. At 10 μM, ACLO, NIM, 4-HONIM, 4-HOACLO, and DICLO decreased luminol-enhanced CL generated by PMA-activated neutrophils. Inversely, INDO increased the luminol enhanced CL. Interestingly, hydroxylated metabolites were more potent antioxidants than the parent drugs. Furthermore, all drugs tested, excepted ACLO, lowered lipid peroxidation induced by Fe^{2+}/ascorbate system. ACLO and DICLO, even at the highest concentration tested (100 μM), did not significantly lower the HOCl induced CL whereas the other drugs were potent scavengers. Finally, all the NSAIDS accelerated the decomposition of $ONOO^-$, suggesting a potential capacity of the molecules to scavenge peroxynitrite.

Conclusion. The NSAIDs possess variable degrees of antioxidant activities, linked to their ability to react with HOCl, lipid peroxides or $ONOO^-$. These antioxidant activities could offer interesting targeted side-effects in the treatment of joint inflammatory diseases.

Key Words: Antioxidants, NSAIDs, neutrophils, reactive oxygen species, lipoperoxidation, peroxynitrite.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used in the treatment of arthritis. They act by inhibiting prostaglandins, plasminogen activator, leukotrienes and pro-inflammatory cytokines (IL-1, IL-6, IL-8) synthesis by infiltrated and resident cells [1-6]. Furthermore, some NSAIDs have been demonstrated to be potent scavengers of reactive oxygen species (ROS) and inhibitors of the intracellular oxidase activity [7-16]. Since we know that ROS produced in excess at the inflammatory site play key roles in the progression of the rheumatoid inflammation, they constitute potential targets for drugs used in rheumatoid arthritis (RA) treatment. Some studies have evidenced the therapeutic effects of antioxidant agents in the treatment of RA. Therapeutic remission of RA was obtained following intraarticular administration of the copper-zinc-containing enzyme superoxide dismutase (SOD) [17]. A significant alleviation of articular pain and morning stiffness was obtained following selenium and vitamin E supplementation in a double-blind study on RA patients [18].

More recently, researchers attention was focused on peroxynitrite anion, a newly identified strong oxidant resulting from the reaction of nitric oxide (•NO) with superoxide anion ($O_2^{\bullet}$) [19]. It was demonstrated to be formed in rheumatoid joint where it can oxidize essential biomolecules implicated in the homeostasis of cells and tissues [20, 21]. Peroxynitrite can also inactivate α1-antiproteinase, degrade glycosaminoglycans and induce nitration of tyrosine [22-24]. At this time, the reactivity of NSAIDs with $ONOO^-$ has not yet been investigated.

For instance, few informations are available on the antioxidant properties of NSAIDs. Nimesulide (NIM), indomethacin (INDO) and diclofenac (DICLO) were reported to exhibit an antioxidant profile on the oxidation of liposomes taken as membrane model [7]. In addition, Capecchi *et al.* showed that NIM was able to reduce free cytosolic calcium intake induced by N-formyl-methionyl-leucyl-phenylalanine and ionophore ionomycin [8]. It also exhibited similar inhibiting effects on the lysis of red blood cells photosensitized by tiaprofenic acid [9]. Moreover, NIM reduced the generation of $O_2^{\bullet}$ by stimulated polymorphonuclear leukocytes through the inhibition of protein kinase translocation and phosphodiesterase type IV activity, the principal enzyme responsible for the degradation of leukocyte cAMP [10]. NIM is also a potent scavenger of hypochlorous acid, which is responsible for a large part of tissue injury and inflammation [11]. Using an *in vitro* model of erythrocyte membrane lipoperoxidation induced by cumene hydroperoxide (CuOOH), 4-HONIM, the main metabolite of NIM, was demonstrated to protect erythrocyte membrane by directly quenching peroxyl and alkoxyl radical species [12]. On the other hand, 4-HONIM was identified as a chelating and antiradical agent on the model of peroxidation of phosphatidylcholin-liposomes induced by Fe^{2+} via the Fenton reaction [13]. Iron excesses are found *in vivo* in the synovial fluid of rheumatoid arthritis patients and are capable of exacerbating the arthritic inflammation, probably throught their pro-oxidant potentials [14-16]. Therefore, iron chelation could be a limiting factor of the inflammation reaction.

Nevertheless, the link between antioxidant profile of NSAIDs and a potential chelating effect is not yet clearly established.

The work presented herein was designed to investigate the mechanisms of action of some NSAIDs and their metabolites towards diverse oxidant species that are susceptible to be produced at the level of joint inflammation. The effects of NSAIDs were tested in 4 types of experiments: (i) production of ROS by phorbol-myristate acetate-activated neutrophils, monitored by chemiluminescence (CL) and electron spin resonance (ESR) techniques, (ii) Fe^{2+}/ascorbate-induced lipid peroxidation of linoleic acid monitored by ESR, (iii) HOCl-induced light emission monitored by CL, (iiii) the kinetics of $ONOO^-$ decomposition followed by spectrophotometry. NIM and its metabolite 4-HONIM were compared to INDO, DICLO, aceclofenac (ACLO) and its metabolite 4-hydroxyaceclofenac (4-HOACLO).

MATERIAL AND METHODS

Reagents.

All experiments were carried out in phosphate buffer (0.066 M Na_2HPO_4; 0.066 M KH_2PO_4; pH 7.4) or in Hanks' Balanced Salt Solution (HBSS, pH 7.4). Ethylenediamine tetraacetic acid (EDTA), $FeSO_4.7H_2O$, hydrogen peroxide (H_2O_2), sodium nitrite ($NaNO_2$), sodium chloride (NaCl), sodium hydrogenophosphate (Na_2HPO_4), sodium hydrogenocarbonate ($NaHCO_3$) and potassium dihydrogenophosphate (KH_2PO_4), ammonium chloride (NH_4Cl) and manganese dioxide (MnO_2) were analytical grade and purchased from Merck (Bornem, Belgium). 5,5-Dimethyl-1-pyrroline-N-Oxide (DMPO), α-(4-pyridyl-N-oxide)-tert-butylnitrone (4-POBN), ascorbic acid (vitamin C) and 3-[(Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and INDO were from Aldrich (Bornem, Belgium). DMPO was purified with activated charcoal as previously described [25]. NIM and 4-HONIM were gifts from Helsinn Healthcare (Lugano, Switzerland). Sodium hypochlorite (NaOCl) was obtained from BDH Laboratory (Poole, England). Linoleic acid, phorbol 12-myristate 13-acetate (PMA), superoxide dismutase (SOD), mannitol, Ficoll solution and luminol were purchased from Sigma (Bornem, Belgium).

Neutrophils isolation and activation.

Buffy coats were obtained from healthy volunteers (Transfusion Center-CHU, Liège) and diluted (2:1) with Ficoll solution [26]. After 20 min of centrifugation at 450 g (20 °C), the supernatant was diluted (2:1) with 0.9 % NaCl and further centrifuged for 20 min at 1250 g (20 °C). The pellet was treated with a hypotonic solution (NH_4Cl 0.15 mM; EDTA 0.1 mM; $NaHCO_3$ 10 mM) to eliminate the remaining erythrocytes. Neutrophils were then suspended in HBSS, counted and kept at room temperature. NIM and 4-HONIM were dissolved in alkaline solution (0.1 M NaOH), further diluted with phosphate buffer at pH 7.4, and tested at concentrations ranging from 10 to 200 μM. Drugs were preincubated with neutrophils for 10 min at 37 °C before cell activation. The ROS and free radical production by activated neutrophils was monitored by CL and ESR techniques, respectively.

Chemiluminescence assay.

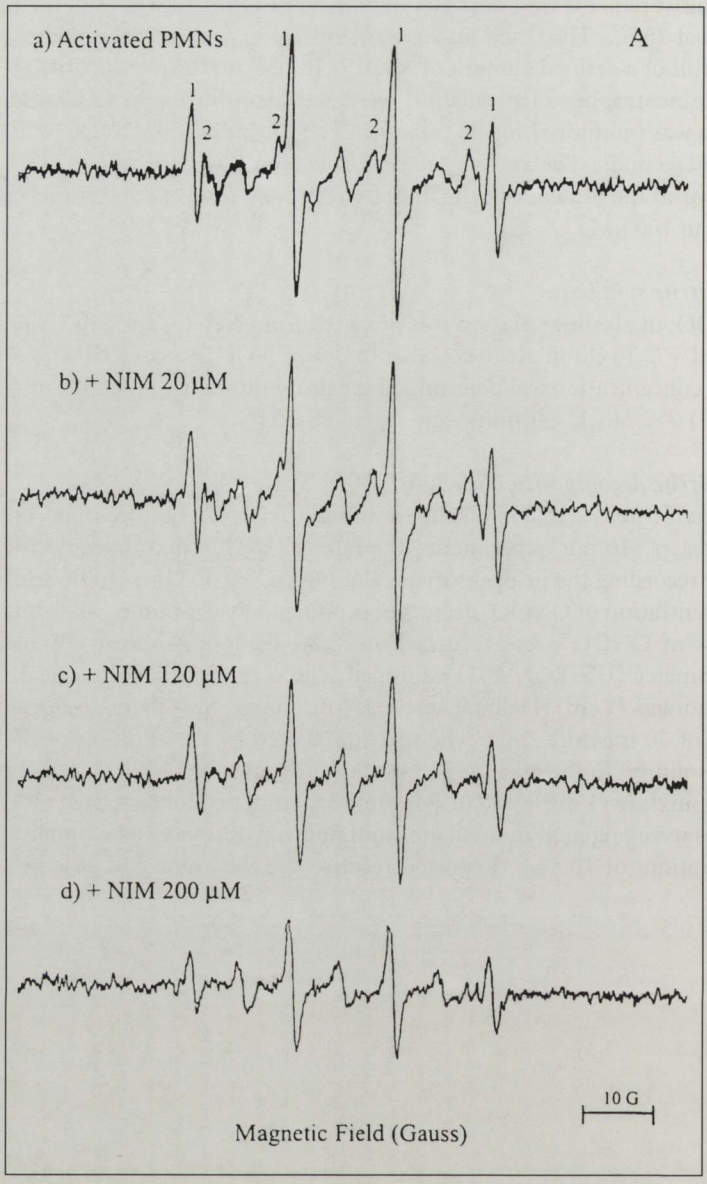
CL measurements were carried out with a Bio-Orbit 1251 luminometer (Turku-Finland) at 25 °C. The reactions were performed in circular polystyrene tubes with neutrophils or a defined amount of NaOCl (10^{-5} M), in the presence of 10^{-3} M luminol. The neutrophils (10^6 cells/ml) were activated by 10^{-6} M PMA and the light emission was monitored for 20 minutes. The CL induced by NaOCl was recorded during 60 seconds. The assays were performed in phosphate buffer (pH 7.4), in the absence or in the presence of the drugs, which were used at concentrations ranging from 10 to 100 μM.

Peroxynitrite synthesis.

$ONOO^-$ in alkaline solution was prepared from $NaNO_2$ and H_2O_2 as previously described [27]. To eliminate the excess of H_2O_2, $ONOO^-$ was treated with MnO_2, and $ONOO^-$ concentration was determined spectrophotometrically at 302 nm ($E = 1700\ M^{-1}\ cm^{-1}$) [28]. Stock solutions were stored at -80 °C.

Peroxynitrite decomposition analysis.

Peroxynitrite is stable in alkaline solution (pH > 12) but decomposes when the pH decreases. In our experiments, the rate of $ONOO^-$ decomposition was monitored by recording the peak absorbance of $ONOO^-$ at 302 nm. In these conditions, the concentration of $ONOO^-$ decreased exponentially with time. The kinetics of the reactions of $ONOO^-$ were recorded at 25 °C using a Kontron UV-visible spectrophotometer (UVIKON 941) equipped with a thermostated (circulating water) cuvette holders (1 cm) (Hellma, Aartselaar-Belgium). Absorbance was monitored at the rate of 50 measures/min. The reaction started by the addition of the buffered NSAID solution to the alkaline solution of 0.28 mM $ONOO^-$. The final pH of the reaction mixture (1 ml) was 8.0. For these experiments, concentrated (10^{-2} M) drug solutions were prepared in methanol, and diluted with phosphate buffer to the final concentration of 10^{-5} M. Reported results were the average of at least 4 separate assays.


a) Activated PMNs
A
1
2
2
1
2
1
2
1
b) + NIM 20 µM
c) + NIM 120 µM
d) + NIM 200 µM
10 G
Magnetic Field (Gauss)

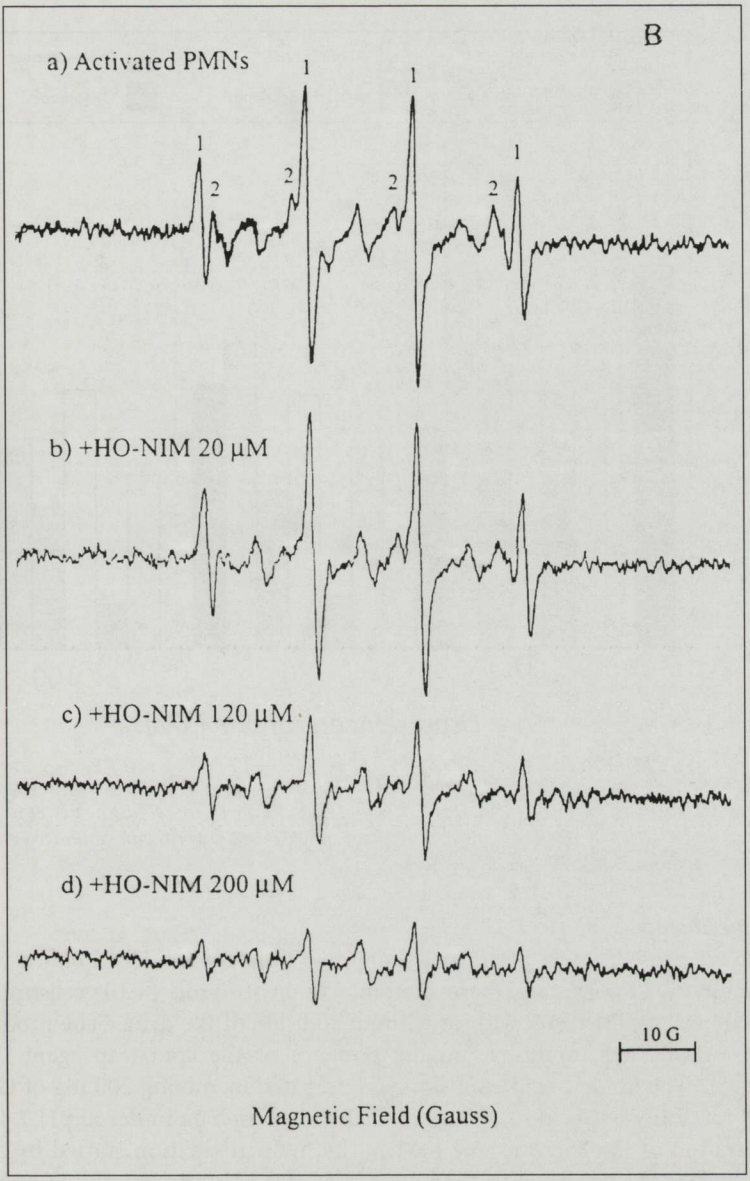


Figure 1. *ESR spectra resulting from PMA-activated human neutrophils (PMNs) incubated in the presence of DMPO (100 mM), with or without increasing amounts of NIM (A) or 4-HONIM (B). The experimental settings were: receiver gain 2.10⁴, time constant 81.56 ms, conversion time 164 ms. The number of scans was 4. 1. DMPO-OH adduct ($a_N = a_N = 14.89$ G) ; 2. DMPO-OOH adduct ($a_N = 14.3$ and $a_H = 1.3$ G).*

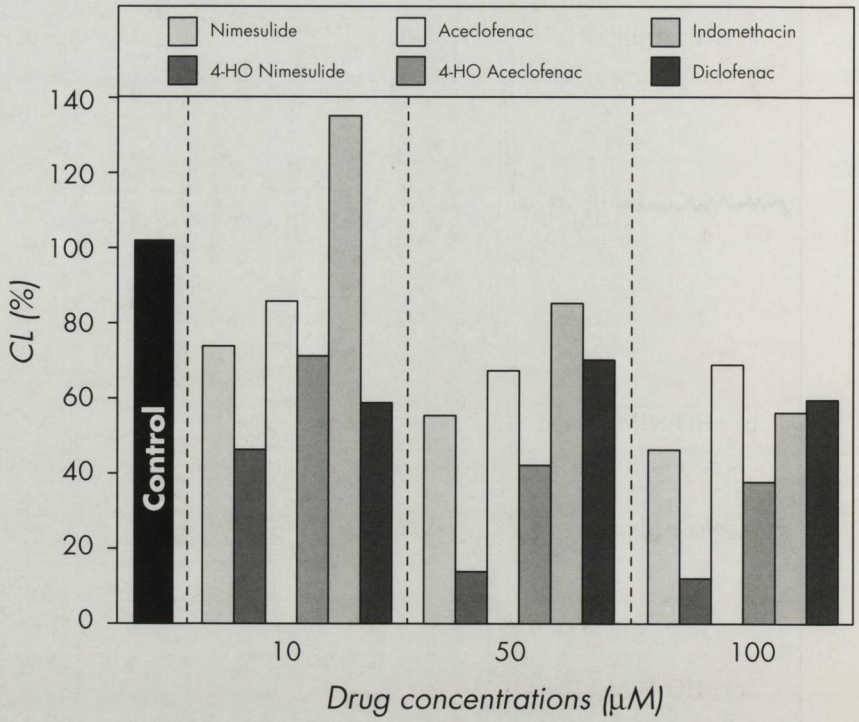


Figure 2. *Effects of increasing concentrations of NSAIDs on luminol-enhanced chemiluminescence generated by PMA-activated neutrophils (10^6 cells/ml). Results are expressed as the percentage of the control value (100%). The values are the means of at 3 independent experiments conducted with neutrophils coming from 3 different healthy donors.*

ESR experiments.

ESR experiments were performed using two different models: isolated human neutrophils and linoleic acid peroxidation. The neutrophils (4.10^6 cells/ml) were activated by 5.10^{-7} M PMA, with or without addition of the drug. The production of free radicals was monitored in the presence of the spin trap agent DMPO (100 mM). The linoleic acid emulsion was prepared by mixing 200 mg of CHAPS and 0.2 ml of linoleic acid in 100 ml of chelexed phosphate buffer at pH 7.4 (final concentration of linoleic acid: 6.4 mM). The lipoperoxidation started by adding 10^{-5} M Fe^{2+} to the emulsion in the presence of 10^{-3} M ascorbate and 50 mM spin trap 4-POBN (final volume 1 ml). The reaction mixture was immediately transfered into the flat cell in the TM110 cavity of the spectrometer. ESR spectra were monitored with a Bruker spectrometer, operating at non-saturating microwave power (20 mW). The experimental settings were as follows: 9.75 GHz microwave frequency, 3480 G centre of field, 100 G sweep width. The other parameters are listed in the legends of the figures.

Statistical analysis.

The results were expressed as the mean values with the standard deviation. Statistical analysis was performed by the unpaired Student's t test with a limit of significance at $p < 0.05$. Dose-reponse curves were fitted by regression models (Pearson's test) using drug concentrations in logarithmic scale.

RESULTS

Effects of NSAIDs on ROS and free radical production by PMA-activated neutrophils.

Figure 1 shows the high intensity ESR spectrum resulting from the activation of neutrophils by PMA. This spectrum was characteristic for DMPO-OOH radical adducts (a_N = 14.3 G, a_H = 1.3 G) and/or their decomposition adducts DMPO-OH ($a_H = a_N$ = 14.89 G) with a major part of the spectrum (75 %) formed by the DMPO-OH adducts. The intensity of the ESR spectrum was strongly reduced in the presence of 30 U/ml SOD (90 % inhibition) and to a lesser degree by •OH scavengers (30 % inhibition with 10^{-4} M mannitol). The addition of NIM (Figure 1A) or its metabolite 4-HONIM (Figure 1B), at the concentrations ranging from 10 to 200 μM, resulted in a dose-dependent inhibition of the ESR signal intensity. At 200 μM, NIM and 4-HONIM decreased the signal height by 52 and 73 % respectively. By comparison, ACLO exhibited a weak effect (7 % inhibition) and 4-HOACLO reduced by 35 % the signal intensity (Table 1).

The chemiluminescence results are presented on figure 2: the control value (without drug addition) is the mean of 3 assays performed with 3 different cell batches, with the individual absolute values obtained for each cell batch ranging from 2100 to 1950 mV. This mean value was taken as 100 % and the effects of drugs were expressed in % of this control value. NIM and 4-HONIM exhibited a significant dose-dependent inhibiting effect on the CL. The inhibiting effect was more important compared to ESR studies. At 10 μM, corresponding to the plasmatic NIM peak value after therapeutic dose administration [29], NIM inhibited by 25 ± 5 % and 4-HONIM by 55 ± 9 % the CL intensity. By comparison, ACLO or 4-HOACLO (10 μM) reduced the CL emission by 16 ± 4 % and 30± 7 %, respectively and DICLO by 40 ± 9 %. Surprisingly, INDO showed a biphasic effect on this parameter. At low concentration (10 μM), INDO increased by +30 % the CL resulting from activated PMN but induced a progressive inhibition at high concentration (100 μM).

Compound	Inhibition of the ESR signal (%)
None	0
Nimesulide	52
4-HO-nimesulide	73
Aceclofenac	7
4-HO-aceclofenac	35

Table 1. *Comparative effects of 200 µM of NSAIDs on the ROS production by PMA-activated neutrophils (4.10^6 cells/mL). The production of free radicals was monitored by ESR experiment using DMPO as spin trap agent. The results are expressed as the % of inhibition of the ESR signal height. The experimental settings were: receiver gain 2.10^4, time constant 81.92 ms, conversion time 164 ms. The number of scans was 6.*

Compound	Inhibition of the ESR signal (%)
None	0
Nimesulide	50
4-HO-nimesulide	50
Aceclofenac	0
4-HO-aceclofenac	45
Indomethacin	34
Diclofenac	38

Table 2. *Comparative effects of 10 µM NSAIDs on the inhibition of ESR signal resulting from linoleic acid peroxidation induced by Fe^{2+}/ascorbate system. In this model, 4-POBN was used as spin trap agent. The experimental settings were: receiver gain 2.10^4, time constant 40.96 ms, conversion time 40.96 ms. The number of scans was 4.*

Furthermore, this experiment clearly demonstrated that the hydroxylated metabolites were more efficient antioxidants than the parent drugs. This finding suggests that the phenolic structure resulting from the drug metabolization played a key role in the antioxidant properties of the NSAIDs.

Effects of NSAIDs on lipid peroxidation induced by iron (II) and ascorbate

In the absence of Fe^{2+}, no signal of lipoperoxidation was observed (Figure 3, spectrum A). The addition of 10 µM Fe^{2+} induced linoleic acid peroxidation resulting in the appearance of a six-line ESR spectrum. This signal is characteristic of the 4-POBN radical adducts with the following coupling constants: a_N = 16.0 G and a_H = 2.6 G (Figure 3, spectrum B). Moreover, a two-line ESR spectrum centered at 3480 G (a_H = 1.7 G), characteristic for the ascorbyl free radical (AFR), was also observed. The addition of NIM or 4-HONIM, at the final concentration of 10 µM, decreased by 50 % the ESR spectrum intensity (Figure 3, spectra C and D). INDO and DICLO also exhibited a good inhibiting effect on the ESR signal intensity, but they were less active than NIM or 4-HONIM (Table 2). 4-HOACLO was also a potent scavenger of the lipid peroxides (45% inhibition of the signal height) whereas ACLO was without effect.

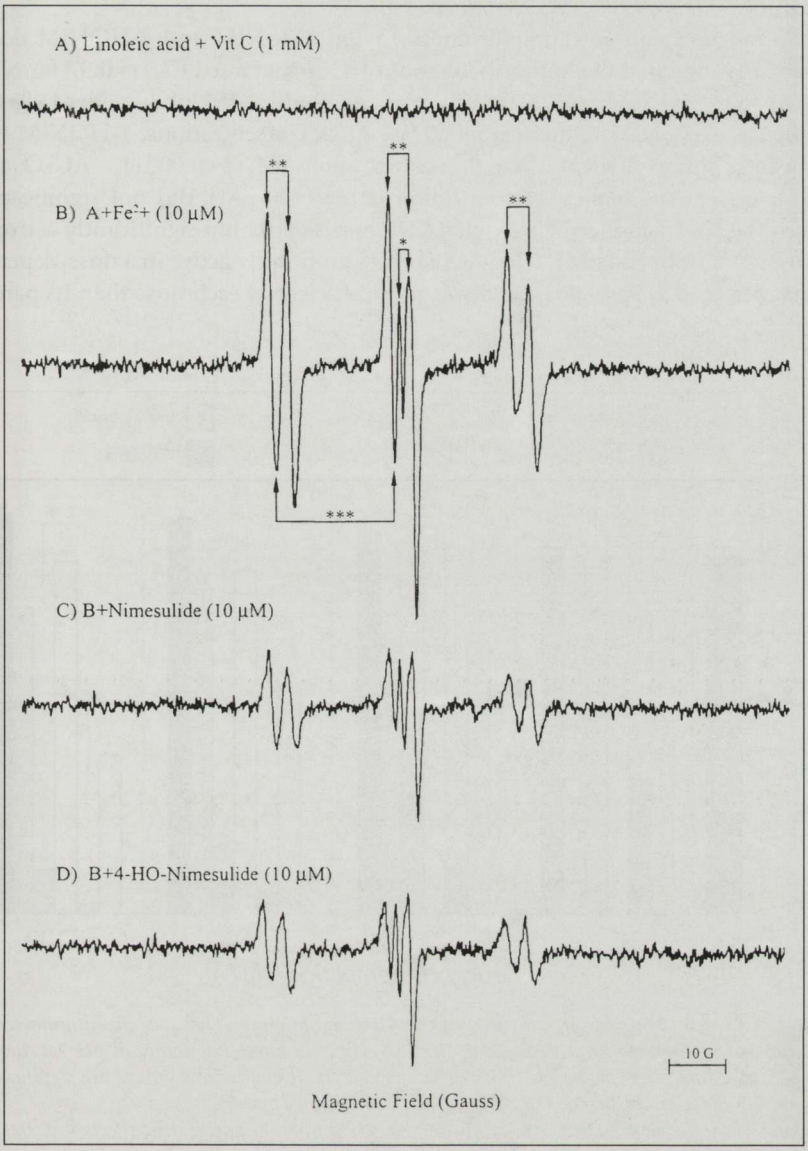


Figure 3. *ESR spectra resulting from the peroxidation of linoleic acid by the Fe^{2+}/ascorbate system. 4-POBN was used as spin trap. A) control: in the absence of Fe^{2+}; B) in the presence of Fe^{2+} (10^{-5} M); C) same as B with 10^{-5} M NIM; D) same as B with 10^{-5} M 4-HONIM.*
** ESR spectrum of ascorbyl free radical (coupling constant a_H = 1.7 G). ** and *** ESR spectrum of 4-POBN spin adduct resulting from the reaction of 4-POBN with ROO• radicals (** a_N = 16.0 G and *** a_H = 2.6 G). The experimental settings were: receiver gain 2.10^4, time constant 40.96 ms, conversion time 40.96 ms. The number of scans was 4.*

Effects of NSAIDs on the NaOCl-induced CL.

In a range of concentrations from 10 to 100 µM, NIM and 4-HONIM dose-dependently inhibited the luminol-enhanced HOCl generated CL (r=0.97 for NIM and 0.88 for 4-HONIM; $p<0.001$) (Figure 4). At 10 µM, NIM inhibited by 15 % the CL whereas 4-HONIM reduced it by 60 %. At all concentrations, 4-HONIM was significantly more efficient than the parent molecule ($p<0.0001$). ACLO and DICLO, even at the highest concentration tested (100 µM), did not significantly reduced the HOCl-induced CL, while INDO was slightly but significantly active at the doses of 50 and 100 µM. 4-HOACLO was significantly active in a dose-dependent manner (r=0.97; $p<0.001$), and was more efficient at each dose than its parent drug.

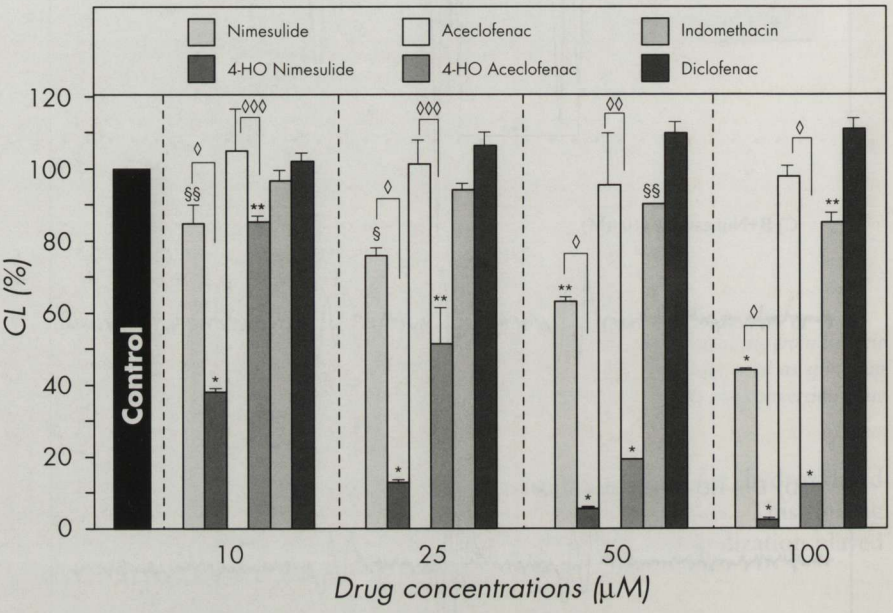


Figure 4. *Effects of increasing amounts of NSAIDs on luminol-enhanced chemiluminescence (CL, expressed in % of control) produced by 10^{-4} M HOCl, in phosphate buffer at pH 7.4. Results are expressed as the percentage of the controls values (n= 5). Drug-treated groups are significantly different from the controls: * $p<0.0001$; ** $p<0.001$; § $p<0.01$; §§ $p<0.05$*
The hydroxylated metabolite effects are significantly different from the parent drug effects: ◊ $p<0.0001$; ◊◊ $p<0.005$; ◊◊◊ $p<0.05$.

Effects of NSAIDs on the kinetic curve of peroxynitrite decomposition.

All the NSAIDs tested were capable to accelerate the decomposition of $ONOO^-$ showing their capacity to scavenge peroxynitrite. The apparent first-order kinetic constant was 3.57 10^{-2} s^{-1} for NIM at the final concentration of 10^{-5} M (Figure 5). The other drugs had a weaker effect on the rate of $ONOO^-$ decomposition, and exhibited similar rate constants: 2.28 10^{-2} s^{-1} for 4-HONIM, 2.24 10^{-2} s^{-1} for ACLO, 2.21 10^{-2} s^{-1} for 4-HOACLO and 2.48 10^{-2} s^{-1} for INDO. Furthermore, metabolites and parent drugs showed similar activity to scavenge $ONOO^-$.

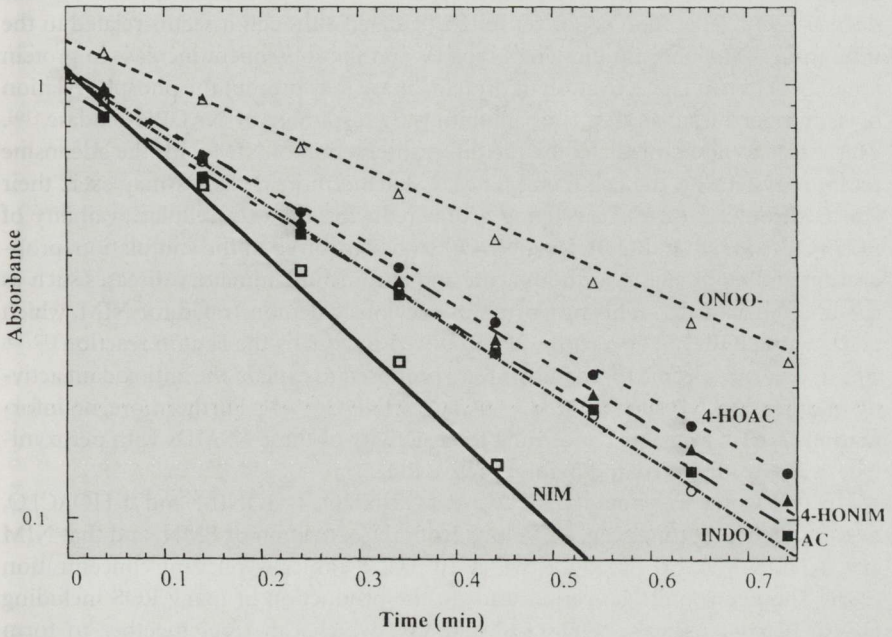


Figure 5. *Rate of decomposition of 0.28 mM peroxynitrite in 50 mM phosphate buffer (pH 8.0), in the absence (ONOO⁻ alone) or in the presence of the drug at 10 μM (n = 4). Absorbance decrease was followed at 302 nm.*

DISCUSSION

Neutrophils, recruited to tissue sites of inflammation, release a variety of oxidants and enzymes, which are responsible for tissue damage. Among the oxidants released are potent chlorinated compounds (such as hypochlorous acid and chloramines), nitric oxide-derived inflammatory oxidants, including peroxynitrite ($ONOO^-$), nitryl chloride (NO_2Cl) and nitrogen dioxide ($^\bullet NO_2$) but also hydroxyl radicals ($^\bullet OH$), superoxide anions ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) which directly induce tissue cell damage but also inactivate α1-antiproteinase, the major inhibitor of elastase [30-34]. We demonstrated herein that many NSAIDs possess

antioxidant activities and therefore, could limit the oxidative stress generated by activated neutrophils [35, 36]. These findings are in agreement with previous papers showing that NSAIDs were capable to inhibit the respiratory burst of neutrophils triggered by various activator agents [8, 10, 11, 23]. The finding that NSAIDs are able to blunt ROS production from activated neutrophils, leads to suggest that the inhibition of oxidative burst may represent an additional mechanism of the antiinflammatory activity of these drugs. This mechanism of action is particularly well documented for NIM, which has been shown to decrease FMLP and PMA-stimulated $O_2^{\bullet}$ production in polymorphonuclear leukocytes [8, 10]. The mechanism whereby NIM decreases $O_2^{\bullet}$ generation is not yet fully elucidated although it seems related to the inhibition of the phosphodiesterase type IV and the subsequent increase in protein kinase A activity. The activation of protein kinase may prompt the phosphorylation of a number of substrates, thus inhibiting the assembly of NADPH-oxidase [10]. This effect could be mediated by the direct interaction of NIM with the adenosine receptor system on the cell membrane [8]. Furthermore, NSAIDs may exert their effects through a direct scavenging action reducing the extracellular avaibility of ROS ($O_2^{\bullet}$, •OH, RO•, ROO•, R•, $ONOO^-$) largely involved in the stimulation, propagation and maintenance of both acute and chronic inflammatory diseases such as rheumatoid arthritis. This property was previously demonstrated for NIM, which reacts with HOCl but also with •OH produced *in vitro* by the Fenton reaction [12, 13, 37]. To date, no specific mechanisms were proposed to explain the antioxidant activity of other NSAIDs such as ACLO, DICLO or INDO [38, 39]. Furthermore, no informations are yet available concerning the reactivity of these NSAIDs with peroxynitrite, a newly identified and highly reactive ROS.

In this work, we show that NIM, ACLO, INDO, 4-HONIM and 4-HOACLO, decreased chemiluminescence resulting from the activation of PMN, and that NIM and 4-HONIM were already active at 10 μM, a clinically relevant concentration (29). The neutrophils activation leads to the production of many ROS including HOCl, peroxyl radicals, •OH , $O_2^{\bullet}$ and •NO which can react together to form $ONOO^-$ [40- 42]. All these ROS may contribute to the enhancement of CL observed when neutrophils are activated. By ESR, we observed that the free radical species produced by the activated neutrophils was mainly the superoxide anion since SOD reduced for more than 90 % the ESR spectrum intensity (data not shown). The DMPO-OH adducts that we observed were thus likely to derive from the decomposition of the DMPO-OOH adducts, but we cannot exclude that a minor part of these adducts derived from a direct scavenging of •OH (produced by a "Fenton-like" reaction or by the decomposition of peroxynitrite). The inhibiting effects of the drugs, especially NIM and 4-OHNIM, on the activation of the neutrophils were obtained at lower concentrations in the CL assays compared to ESR. But in the ESR studies, the free radical species produced by the activated neutrophils is mainly superoxide anion. Higher concentrations of NIM and its metabolite seem to be needed to inhibit this particular radical.

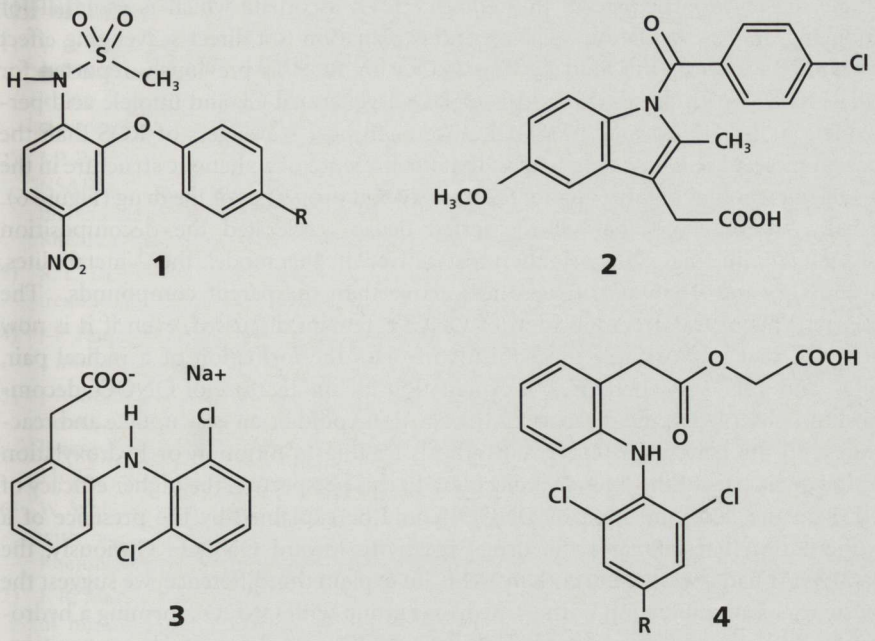


Figure 6. *Chemical structures of the tested drugs (parent drugs and hydroxymetabolites).*
1. with R = H: nimesulide [N-(4-nitro-2-phenoxyphenyl) methanesulfonamide]; with R = OH : 4-hydroxynimesulide. 2. indomethacin [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid] 3. diclofenac [2-[(2,6-dichlorophenyl)amino] benzene acetic acid monosodium salt] 4. with R = H: aceclofenac [2-(2,6-dichlorophenyl)amino acid carboxymethyl ester]; with R = OH: 4'-hydroxy-aceclofenac.

The CL of activated neutrophils results from the total ROS produced by the cells and among these ROS, there are non radical species such as HOCl. As the CL method does not allow the identification of the ROS species targetted by NSAIDs, and to identify which of these ROS were scavenged by the drugs, we used three different *in vitro* methods: chemiluminescence generated by HOCl, the kinetics of peroxynitrite decomposition and the lipoperoxidation of linoleic acid induced by ascorbate/Fe^{2+}, a system which produces ferryl radicals responsible for triggering lipid oxidation.

In the model of CL induced by HOCl, NIM, 4-HONIM and 4-HOACLO showed a dose-dependent inhibiting effect whereas INDO, DICLO and ACLO were without significant effect. This higher reactivity of NIM and 4-OHNIM may be explained by the presence of a sulfonanilide function (Figure 6, structure 1) which is more susceptible to react with HOCl [43]. All NSAIDs, excepted ACLO, were active in the lipid peroxidation model indicating that these drugs interact with the production of the ferryl radical. Two hypotheses can be advanced to explain this inhibitory effect. Firstly, NSAIDs act by chelating Fe^{2+}. This chelating property has been previously described for 4-HONIM [13]. Indeed, it is highly probable that NIM chelates Fe^{2+},

hindering it to participate in the redox cycle of ascorbate which is essential for inducing lipid peroxidation. The second explanation is a direct scavenging effect of NSAIDs towards the lipid radicals (ROO• or RO•) as previously reported for 4-HONIM [13]. In these two models of HOCl-generated CL and linoleic acid peroxidation, 4-OHNIM and 4-HOACLO were stronger scavengers of ROS than the parent molecules. These data suggest that the presence of a phenolic structure in the 4-OH-metabolites highly enhance the antioxydant properties of the drug (Figure 6). Finally, we observed that all the tested drugs accelerated the decomposition of $ONOO^-$, but that NIM was the most active. In this model, the 2 metabolites, 4-HONIM and 4-HOACLO, were less active than the parent compounds. The mechanisms of the decomposition of $ONOO^-$ remain discussed, even if it is now accepted that one of these mechanisms involves the formation of a radical pair, $NO_2^•$ and •OH [44]. Therefore, an explanation for the increase of $ONOO^-$ decomposition observed in the presence of the NSAIDs could be an easy uptake and reaction with the peroxynitrite-derived radicals leading to nitration or hydroxylation of the cyclic part of the NSAID molecules. In this perspective, the higher efficacy of NIM on the decomposition of $ONOO^-$ could be explained by the presence of a sulfur atom that increases the drugs reactivity toward $ONOO^-$. Curiously, the 4-OHNIM had a weaker effect than NIM. To explain this difference, we suggest the existence of an interaction of the 4-hydroxyl group with $ONOO^-$, forming a hydrogen bond that would temporarily stabilize $ONOO^-$ and slower its decomposition. However, to support this hypothesis, more investigations are needed, particularly to identify the products of the reaction of NSAIDs with $ONOO^-$. But, taking into account the involvement of $ONOO^-$ in diverse pathophysiological disorders and joint inflammation diseases [3, 20, 45], our observations demonstrate the "anti-peroxynitrite" properties of NSAIDs.

In conclusion, this study demonstrated that many NSAIDs possess antioxidant properties but that their mechanisms of action can be fully different. For example, NIM is a potent scavenger of $ONOO^-$ and lipid radicals but is ineffective on $O_2^•$. Inversely, ACLO is a potent scavenger of $ONOO^-$ but does not act on lipid radicals and HOCl. We also demonstrated that NSAIDs may scavenge peroxynitrite. Finally, we conclude that hydroxylated metabolites are more potent scavengers of ROS than the parent molecules suggesting that they could be the active molecule in situ. Therefore, modulation of the local metabolization of NSAIDs by joint cells could be an interesting therapeutic approach.

ACKNOWLEDGEMENTS

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ANNEXE 16

Metabolism of Human Articular Chondrocytes Cultured in Alginate Beads.

Long-term Effects of Interleukin 1ß and Nonsteroidal Anti-inflammatory Drugs.

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ABSTRACT

Objectives. To investigate the longterm effects (12 days) of nonsteroidal anti-inflammatory drugs [NSAID: aceclofenac (ACECLO), sodium diclofenac (DICLO), indomethacin (INDO), nimesulide (NIM), rofecoxib (ROFE), celecoxib (CELE), piroxicam (PIROX), and ibuprofen (IBUP)], on the metabolism of human chondrocytes cultured in alginate beads.

Methods. Enzymatically isolated osteoarthritic (OA) chondrocytes were cultured in alginate beads in a well defined culture medium for 12 days. The DNA content was measured according to a fluorimetric method and cell proliferation was determined by the incorporation of ^{3}H-thymidine in the newly synthesized DNA. Interleukin (IL)-6 and IL-8, stromelysin [matrix metalloproteinases-3 (MMP-3)], and aggrecan (AGG) productions were assayed by specific enzyme amplified sensitivity immunoassays, and prostaglandin E_2 (PGE_2) production by specific radioimmunoassay. All NSAID were tested at the mean peak plasma concentration (C_{max}) obtained after oral administration of a therapeutic dose.

Results. In alginate beads, chondrocytes synthesized high amounts of AGG, which were largely (98 %) immobilized in the alginate matrix. A large amount (43 %) of the IL-8 produced was stored in the alginate beads whereas almost all IL-6 production (94 %) was released in the culture supernatant. At the therapeutic concentration, all NSAID tested fully blocked PGE_2 production. ACECLO, DICLO, INDO, NIM significantly inhibited basal and IL-1ß stimulated IL-6 production; CELE and IBUP only inhibited IL-1ß stimulated IL-6 production; and ROFE and PIROX had no significant effects. No NSAID showed significant effects on basal and IL-1ß stimulated IL-8 production, except CELE and IBUP, which slightly increased basal IL-8 production. ACECLO and INDO increased AGG content by 25 % in the alginate beads, while the other NSAID were without significant effect. No NSAID were able to modify the inhibitory effect of IL-1ß on AGG production. Finally, NSAID did not modify MMP-3 production.

Conclusion. The mechanism of action of NSAID seems to be multifactorial and not limited to the inhibition of cyclooxygenases. Furthermore, in our culture conditions, at the C_{max} and by comparison with other NSAID, ACECLO and INDO show an advantageous activity profil. They fully blocked PGE_2 production, inhibited IL-6 synthesis, and increased aggrecan synthesis. These effects appear advantageous for the longterm treatment of chronic joint diseases such as osteoarthritic.

Key Indexing Terms: Chondrocytes, Osteoarthritis Inflammation, Cytokines, Nonsteroidal Anti-inflammatory, Drugs, Cartilage.

INTRODUCTION

Articular cartilage is essentially composed of an extensive hydrated matrix, synthesized and turned over by chondrocytes, the only type of cell found in the cartilage. Cartilage matrix is principally composed of aggrecan (AGG) and type II collagen whose synthesis is controlled by hormones, cytokines and mechanical stimuli. In normal conditions, cartilage matrix remodeling is a slow process consisting of a phase of chondrocyte resorption, directly compensated for by a phase of chondrocyte repair [1-3]. In pathological conditions such as osteoarthritis (OA), cartilage turnover may accelerate, leading to an early repair reaction followed by cell decompensation and cartilage degradation. Repair reaction is characterized by cartilage hypertrophy and increased synthesis of matrix components and metalloproteases [4,5]. This homeostatic reaction is not efficient, is transient, and ultimately fails to repair cartilage defects, although the etiological factors persist. Cartilage resorption is mediated by enzymes and free radicals produced by neighboring tissues, but also by chondrocytes themselves. These processes are induced and controlled by potent autocrine and paracrine mediators, mainly interleukin 1ß (IL-1ß) and tumor necrosis factor-α, and probably other cytokines such as IL-6 and IL-8 [6,7]. *In vitro*, IL-1 is a potent inhibitor of proteoglycans and collagen type II synthesis, and a stimulator of metalloproteases, IL-6, IL-8, prostaglandin E_2 (PGE_2), nitric oxide and reactive oxygen species production [7-11]. For these reasons, IL-1ß is commonly used in culture models to mimic the circumstances leading to *in vivo* cartilage degradation.

Nonsteroidal antiinflammatory drugs (NSAID) are the most commonly used drugs in the treatment of OA [12]. Until now, NSAID were considered to be symptom modifying drugs that acted by inhibiting prostaglandin synthases. There is little doubt that certain NSAID can provide more than symptomatic relief. Several *in vitro* and animal studies have shown that some NSAID may reduce cartilage degradation or stimulate proteoglycans synthesis, whereas others may have a deleterious effect [13-15]. These observations suggest that NSAID could interfere with the cartilage metabolism and modulate the disease progression [16-18]. Further, recent studies have revealed that the mechanism of action of the NSAID is multifactorial and involves pathways other than the inhibition of prostaglandin (PG) synthesis. Indeed, some NSAID exert antioxidant properties [19-21], inhibit cytokine production [22,23], decrease the expression of adhesion molecules by human neutrophils [24,25], modulate enzymatic activity [16,26], or induce tissue inhibitor of metalloproteases (TIMP-1) synthesis [27]. Further, in experimental dog OA, carprofen reduces growth of osteophytes, the severity of cartilage lesions, and the remodeling of subchondral bone, suggesting that this NSAID could prevent cartilage degradation by preventing the abnormal metabolism of subchondral osteoblasts [28]. All together, these observations might suggest modifying recommendations for use of NSAID to protect cartilage in OA.

At this time, most knowledge of the actions of NSAID comes from short term cell or explant cultures [29-32]. While many patients with OA have taken these drugs

for several years, few studies were devoted to the longterm effects of NSAID [33-35]. Further, different NSAID were rarely compared in the same experiment [36]. Longterm studies have faced challenges, because chondrocyte dedifferentiation occurs when these cells are cultivated in monolayer [37, 38]. Chondrocyte cultured in alginate beads permit the chondrocyte phenotype to remain stable for a long period of time [39-43].

We used this culture model to investigate the metabolism of chondrocytes by assessing AGG, IL-6, IL-8, PGE_2 and stromelysin [matrix metalloproteinases (MMP-3)] production and the longterm influence of IL-1ß and several NSAID within these parameters.

MATERIALS AND METHODS

Chondrocytes culture in alginate bead.

Cartilage was obtained from the knees of cadavers immediately after death, being excised from the superficial and medium layers of cartilage and avoiding the calcified layer. Upon dissection, the femoral, patellar, and tibial articular surfaces were evaluated for gross pathological cartilage modifications. Using the scale of Moskowitz *et al.* [44], the severity of pitting was recorded for each donor. Four different grades were considered: 0, normal white cartilage in all areas examined; I, the presence of a yellow-grey area with some superficial fibrillations on one or more articular surfaces; II, irregular surface with deep fibrillations on one or more articular surfaces; and III, ulcers penetrating to the subchondral bone on one or more articular surfaces. Experiments were performed with OA cartilage from 4 donors (3 male, one female) with a mean age of 49 (39-69) years. All donors showed OA cartilage lesions of grade I (n=3) or III (n=1).

Cartilage was cut into small fragments and then subjected to enzymatic digestions sequentially with hyaluronidase, pronase, and collagenase as previously described [9, 10, 23]. The cells were then filtered through a nylon mesh with a pore diameter of 70 µm, and then washed 3 times with sterile water 0.9 % NaCl. Cells viability was estimated by trypan blue exclusion test and in all cases was superior to 95 %. Chondrocytes were suspended in 1.2 % low viscosity alginate (Sigma-Aldrich, Bornem, Belgium) in 0.9 % NaCl solution at a density of 4×10^6 cells/ml, which was slowly passed through a 25 gauge needle in a dropwise fashion into a 102 mM $CaCl_2$ solution (Sigma-Aldrich). After instantaneous gelation the beads were allowed to polymerise further for 10 min in this $CaCl_2$ solution. Then, they were washed with saline solution and 18 beads were cultured in 1 ml of culture medium per well in a 24 well plate. Culture medium was DMEM supplemented with 1% ITS+ (ICN Biomedicals, Asse-Relegem, Belgium), 10 mM HEPES, penicillin (100 U/ml) and streptomycin (100 U/ml), 200 µg/ml glutamine (Biowhittaker Europe, Verviers, Belgium), 50 µg/ml ascorbic acid (Sigma-Aldrich), 2 mM proline (Gibco, Merelbeke, Belgium). ITS+ is a premixed cell growth system containing in one ml: 0.625 mg insulin, 0.625 mg transferrin, 0.625 µg selenious acid, 0.125 g bovine serum albumin and 0.535 mg linoleic acid. Cells remained in this culture medium (washout medium) for 48 h as a precaution, against *in vivo* contamination with

drugs that donors may have taken before death. After this washout period, culture medium was changed and the various drugs added. Culture medium was changed every 3 days and the collected supernatants were kept at -20 °C until analysis. Chondrocytes were cultured in alginate beads for a period of 12 days.

Treatment.

Chondrocytes were cultured for 12 days in the absence or in presence of IL-1ß-10^{-10} M (Boehringer, Mannheim, Germany) and with or without NSAID. NSAID were tested at the concentration corresponding to the mean peak plasmatic concentration (C_{max}) obtained after oral administration of a therapeutic dose. The C_{max} used in this study were 7.4 µg/ml for aceclofenac (ACECLO) [45, 46], 1.4 µg/ml for diclofenac (DICLO) [47], 2 µg/ml for indomethacin (INDO) [48], 3 µg/ml for nimesulide (NIM) [49, 50], 1 µg/ml for rofecocib (ROFE) [51], 0.7 µg/ml for celecoxib (CELE) [52], 7 µg/ml for piroxicam (PIROX)[53,54], and 25 µg/ml for ibuprofen (IBUP) [55, 56]. All the NSAID tested were first dissolved in N,N-dimethylformamide (DMF; Sigma-Aldrich), and then diluted in culture medium to achieve the required final concentration. The final concentration of DMF was 0.1 %. Three wells were used for each concentration of drugs and for the corresponding controls, and the experiments were repeated 3 times with the cartilage of 3 different donors. All NSAID were tested on cartilage specimens coming from the same donors.

End of the culture.

The beads of each well were dissolved in 1 ml 0.1 M citrate for 10 min. The resulting suspension was centrifuged at 1200 rpm for 10 min. With this method, 2 fractions were collected: the supernatant containing macromolecules from the further-removed matrix (FRM) compartment, and a pellet containing cells with their associated cellular matrix (CM). These 2 fractions were studied separately. The cell pellets were washed with phosphate buffer saline (PBS; Biowhittaker Europe), and then homogenized in 1 ml of PBS by ultrasonic dissociation at 4 °C for DNA assay. CM and FRM were kept at -20 °C until analysis.

DNA assay.

The DNA content of the cultures was measured according to the fluorimetric method of Labarca and Paigen [57].

^{3}H-Thymidine incorporation analysis

At the end of different culture periods (0, 24 h, 48 h, 5, 8, 11 days), 1 µCi of methyl-^{3}H-thymidine (50 Ci/mmole, ICN Biomedicals) was added to the culture supernatant for 24 h. Afterward, the beads were dissolved in a citrate solution as described in the previous paragraph. Cells pellets obtained by centrifugation were washed with PBS and dissolved in 1 ml of 10 % trichloroacetic acid for 15 min at 4 °C. After ultrasonic dissociation and centrifugation at 2500 rpm, the remaining pellet containing chromatin is homogenized in 500 µl NaOH 1N. After 30 min, the volume was adjusted to 1 ml by addition of HCl and the pH was neutralized.

Incorporated radioactivity was then counted twice by scintillation with a beta counter.

PGE_2 radioimmunoassay.

PGE_2 was assayed in the different compartments (culture supernatants, FRM, and CM) without previous extraction according to a radioimmunoassay [58]. In this assay, polyclonal antiserum obtained from rabbit does not cross reacts with other prostanoids (TxB_2, 6-keto-PGF1α, PGA_2) or fatty acids (arachidonic, linoleic or oleic acids). ^{3}H-labeled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule (PGE_2) from Sigma-Aldrich. Intra- and interassay coefficients of variation were 6 and 10 %, respectively. The limit of detection of the RIA was 20 pg/ml.

Immunoassays for AGG, IL-6, IL-8 and MMP-3.

AGG, IL-6, IL-8 and MMP-3 were measured in the different compartments (culture supernatants, FRM, CM) by specific enzyme amplified sensitivity immunoassays (Biosource Europe, Fleurus, Belgium). The assays were based on the oligonal system in which several monoclonal antibodies were directed against distinct epitopes of the molecules. In the case of MMP-3, the antibodies are directed against pro-MMP-3, activated-MMP-3 and MMP-3 bound to TIMP-1 and TIMP-2. The intra and interassay coefficients of variation were less than 5 % for all immunoassays.

Histology.

At the end of the 12 day period, alginate beads were processed for light microscopy by the method described by Petit *et al.* [43]. The alginate beads were fixed for 4 h at 4 °C with 4% paraformaldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) to which 10 mM $CaCl_2$ was added to prevent disintegration of the beads. After washing overnight at 4 °C in 0.1 M sodium-cacodylate buffer containing 50 mM $BaCl_2$, the beads were dehydrated in a series of graded methanol washes followed by a xylene wash and finally embedded in paraffin. Five micrometers sections were cut with a microtome, rehydrated and stained with hematoxylin and eosin.

Calculation and statistical analysis.

The results (mean ± SD) were expressed as the concentration of AGG, MMP-3, IL-6, IL-8 and PGE_2 in the culture supernatants, CM and FRM per μg of DNA. The total amount AGG, MMP-3 or cytokines present in alginate bead was obtained by the addition of the quantities found in the CM and in the FRM. Comparison of mean values was performed culture using the Mann-Whitney U-test with a limit of significance at $p < 0.05$.

RESULTS

Effects of IL-1ß and NSAID on cell proliferation and viability.

As observed by light microscopy, cells retain their spherical shape (Figure 1).

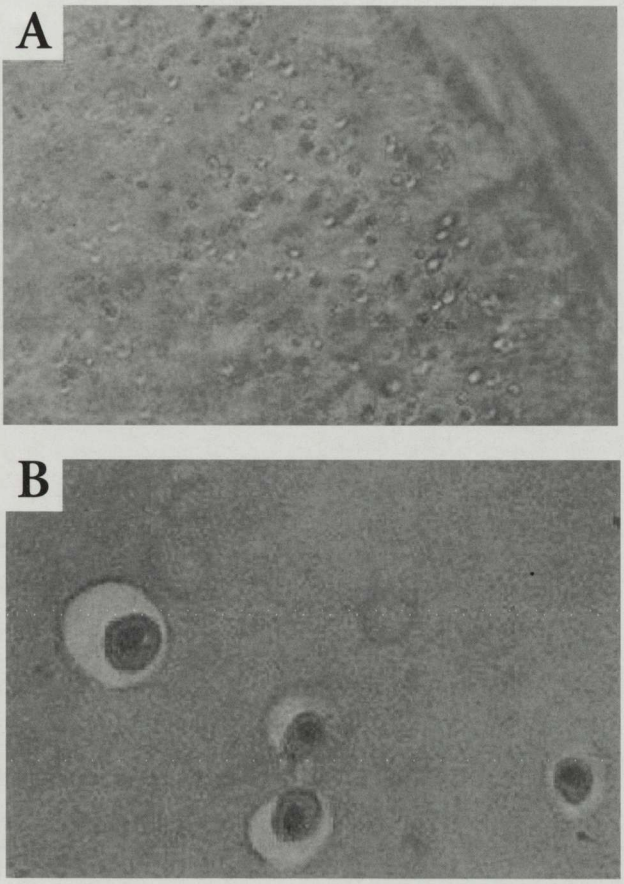


Figure 1: *(A): Microphotography of an alginate bead after 12 days of culture in the control conditions (magnification 200X). (B): Hematoxylin-eosin staining of alginate bead slices (magnification 1200X).*

We noted that ^{3}H-thymidine incorporation reached a maximum the first day and then decreased as a function of culture duration (Figure 2A). Cell proliferation resulted in an enhancement of DNA content during the first 3 days of the culture, followed by a slow but progressive increase between the day 3 and the day 12 (Figure 2B). The viability of the cells was > 90 % as estimated by trypan blue exclusion after their release from alginate beads. Cell viability and DNA content were not significantly affected by drug or IL-1ß treatments.

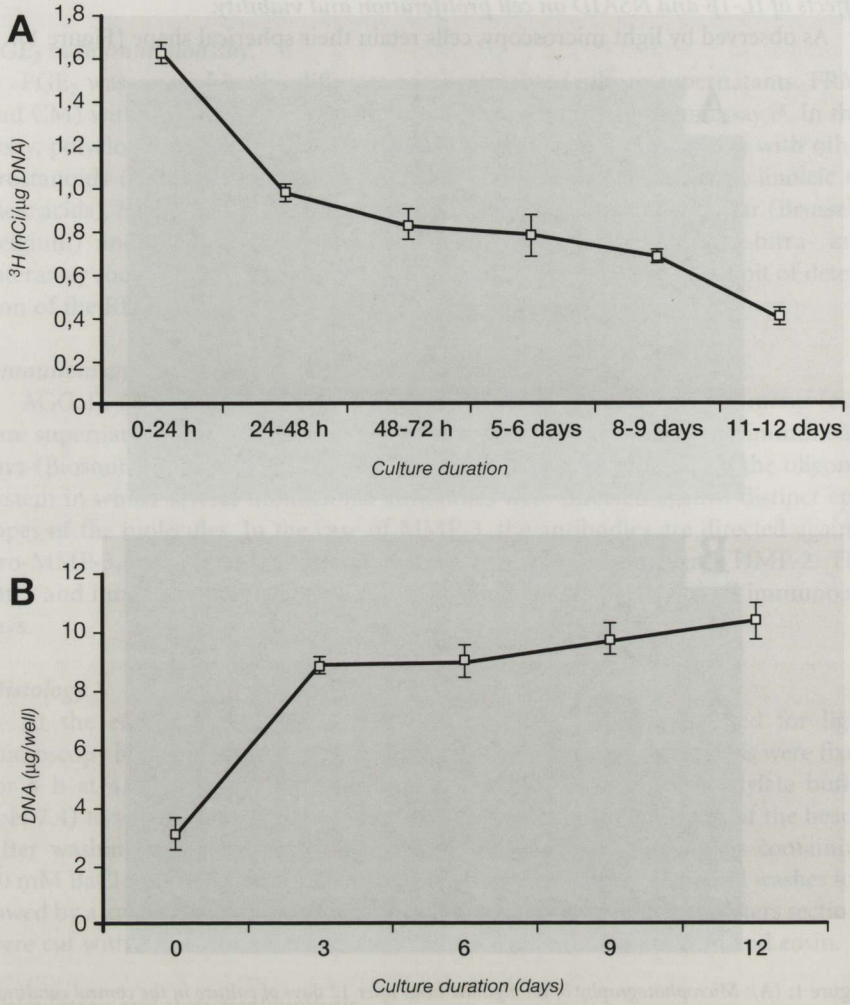


Figure 2: *Cell proliferation. A. ^{3}H-thymidine incorporation according to the culture duration. Results are expressed in nCi /µg DNA/24h. The means ± SD values of triplicate wells are represented. B. Evolution of DNA content present per well based on culture duration. Results are the mean +/- SD values of 4 independent experiments.*

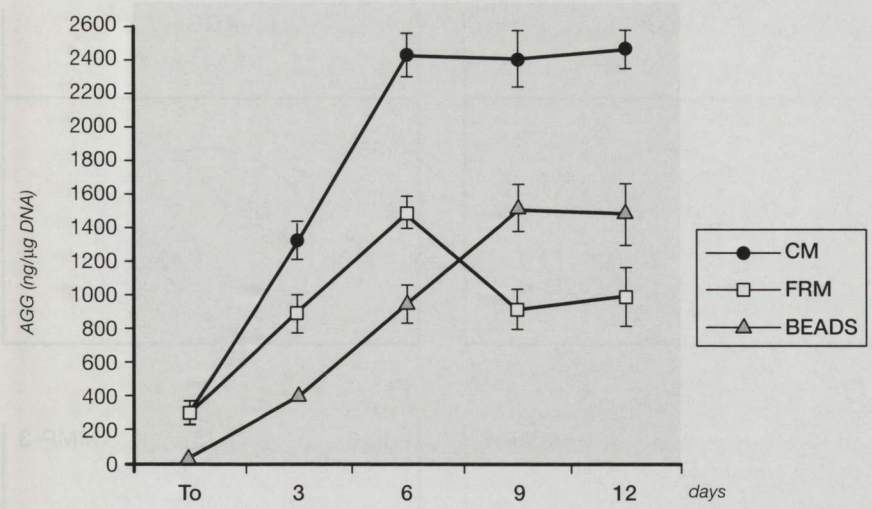


Figure 3. *Kinetic curves of aggrecan (AGG) contained in cellular matrix (CM), further-removed matrix (FRM) and in the beads. Bead content corresponds to the total production resulting from the addition of the amount of AGG contained in the CM and in the FRM. Results expressed in means ± SD of 4 independent experiments.*

Effect of IL-1ß and NSAID on AGG production and distribution.

AGG were quantified in the CM, FRM, and culture supernatants. We observed that the total amounts of AGG contained in the beads (sum of AGG contained in the CM and the FRM) increased essentially during the first six days of the culture and then remained stable until day 12 (Figure 3). During the first 6 days of culture, the AGG manifested mainly in the CM and then slowly distributed themselves through the FRM (Figure 3). At day 6, AGG content increased in FRM but decreased in the CM suggesting that newly synthesized AGG progressively migrated from the CM to the FRM. After 12 days of culture, only 2 % of the AGG production was released in the culture supernatants (Figure 4). The amount of AGG accumulated in the beads at the end of the culture was drastically decreased by IL-1ß 10^{-10} M (Figure 5). This inhibitory effect was observed both in the CM and the FRM compartments (Figure 5). The amount of AGG found in the culture supernatant was also reduced by IL-1ß in comparison with the controls; however, the ratio of AGG released into the culture supernatant was doubled (Figure 4).

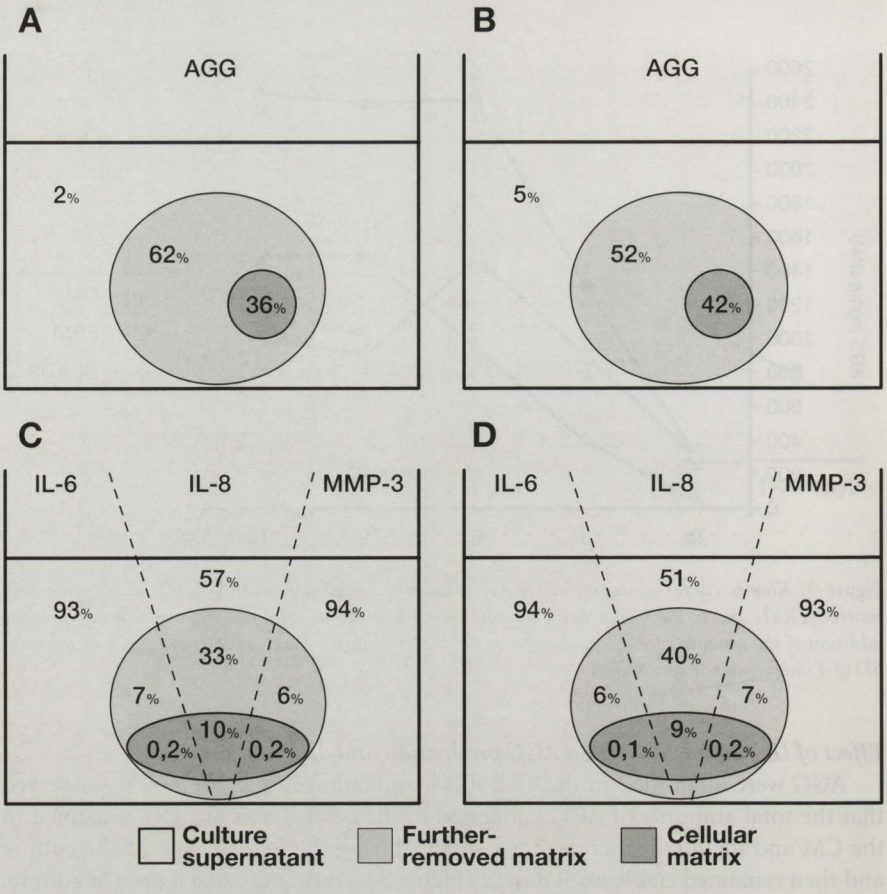


Figure 4. *Distribution through the different culture compartments of aggrecan (AGG), interleukin-6 (IL-6), interleukin-8 (IL-8), and stromelysin [matrix metalloproteinase-3 (MMP-3)] in the absence (A,C) or in the presence (B, D) of IL-1ß (10^{-10} M). C and D. The production percentage present in the culture supernatant and in the beads (further-removed matrix and cellular matrix) for IL-6, IL-8, and MMP-3. A and B. Percentage present in culture supernatant, further-removed matrix and cellular matrix for aggrecan.*

In absence of IL-1ß 10^{-10} M, ACECLO and INDO increased the quantity of AGG contained in the beads at the end of the culture by 22.3 % and 25.9 %, respectively. The other NSAID did not show any significant effects at the tested doses (Figure 6A). In the presence of IL-1ß 10^{-10} M, none of the NSAID significantly affected AGG production (Figure 6A).

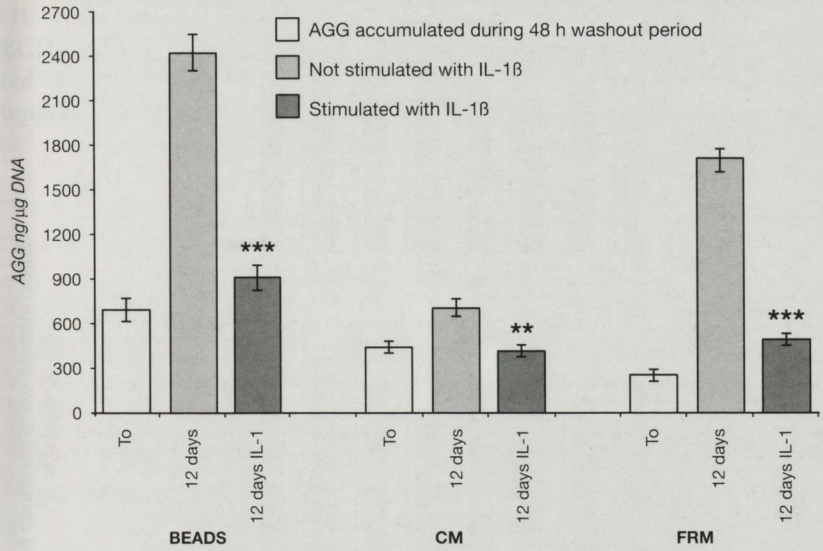


Figure 5: *AGG accumulated in the beads after 12 days of culture in the absence or in the presence of IL-1ß (10^{-10} M). Effect on cellular matrix (CM) and further-removed matrix (FRM) is represented, and the total amount present in the beads (FRM + CM). Time 0 (To, white bars) represents the amount of AGG accumulated during the 48 h wash out period before IL-1 treatment. Results are expressed as means ± SD values of 3 experiments performed with cartilage coming from 3 different donors. ** p<0.01 vs not stimulated with IL-1ß; *** p<0.001 vs not stimulated with IL-1ß.*

Effects of IL-1ß and NSAID on IL-6 and IL-8 production and distribution

OA chondrocytes spontaneously produced IL-6 and IL-8. These productions increased as a function of the culture duration. In the control conditions, the majority of IL-6 production (94 %) was found in the culture supernatant while, a high proportion (43 %) of the IL-8 production remained in the alginate beads (Figure 4).

In the presence of IL-1ß 10^{-10} M, the production of these 2 cytokines was markedly increased to 7.5 times for IL-6 and 10 times for IL-8. The distribution of IL-6 between the different compartments was identical to that observed in the controls. In contrast, the amount of IL-8 immobilized in the alginate beads was slightly, but not significantly, increased in the IL-1 treated culture and reached 50 % of the total IL-8 production (Figure 4).

At the plasma concentrations, NSAID did not modified the IL-8 production, except CELE and IBUP which increased basal IL-8 production by 25 to 30 % (Figure 6B). However, CELE and PIROX modified IL-8 distribution. Indeed, in the basal conditions, CELE increased the IL-8 level found in the culture supernatant, but did not modify IL-8 content in the alginate bead. Therefore, the basal proportion of IL-8 contained in alginate decreased from 43 % to 31 % (Figure 7). PIROX had no significant effect on the distribution of the basal IL-8 production, but significantly modified the distribution of IL-8 in the IL-1ß stimulated conditions. Indeed, while the ratio of IL-8 present in the alginate bead decreased from 50 % to 25 %, 75 % of

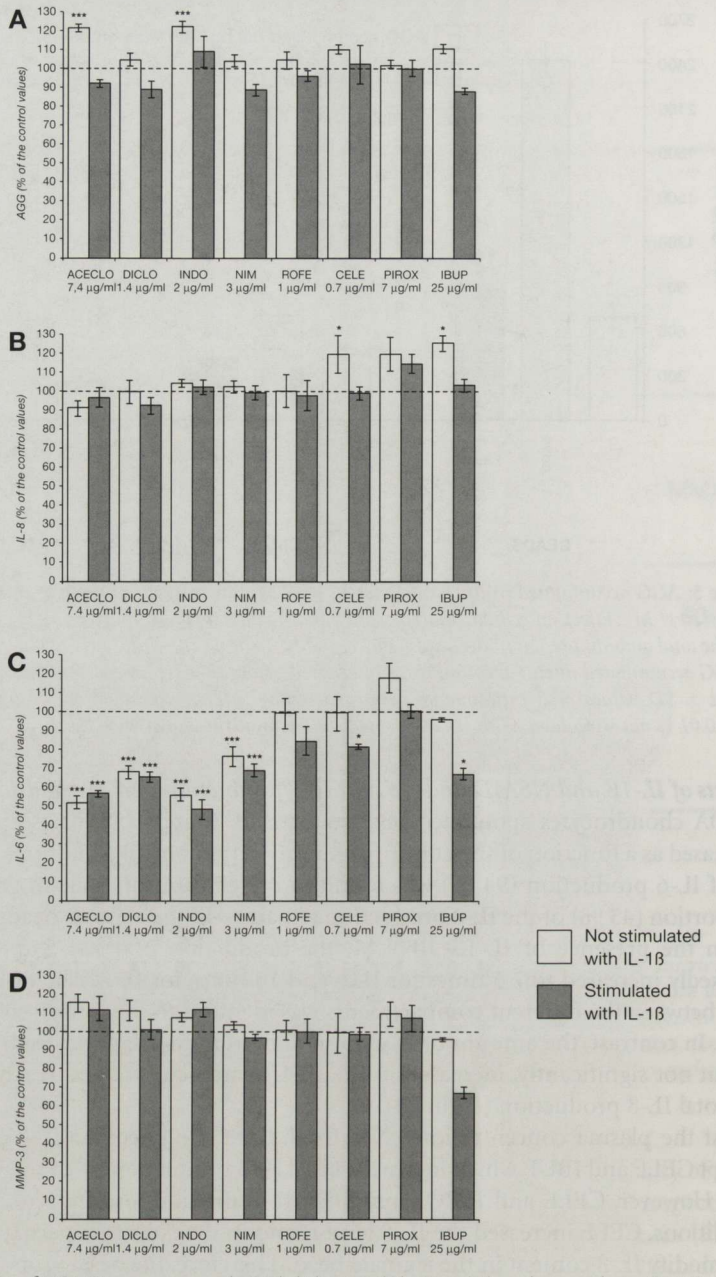


Figure 6: *Effect of NSAID on aggrecan (AGG) (A), IL-8 (B), IL-6 (C) and stromelysin (MMP-3) (D) total production (cellular matrix + further-removed matrix + culture supernatant) after 12 day culture in the presence or absence of IL-1ß 10^{-10} M. Results are expressed as a percentage of control values. NSAID were added at the following doses: ACECLO 7.4 µg/ml, DICLO 1.4 µg/ml, INDO 2 µg/ml, MIN 3 µg/ml, ROFE 1 µg/ml, CELE 0.7 µg/ml, PIROX 7 µg/ml, IBUP 25 µg/ml. Results are expressed in means ± SD values of 3 experiments performed separately with cartilage from 3 different donors. * $p<0.05$, *** $p<0.001$.*

the IL-1ß-stimulated IL-8 production was released in the supernatant (Figure 7). ACECLO, DICLO, INDO and NIM significantly reduced both basal and IL-1ß stimulated IL-6 production, while CELE and IBUP decreased only IL-1ß stimulated IL-6 production (Figure 6C).

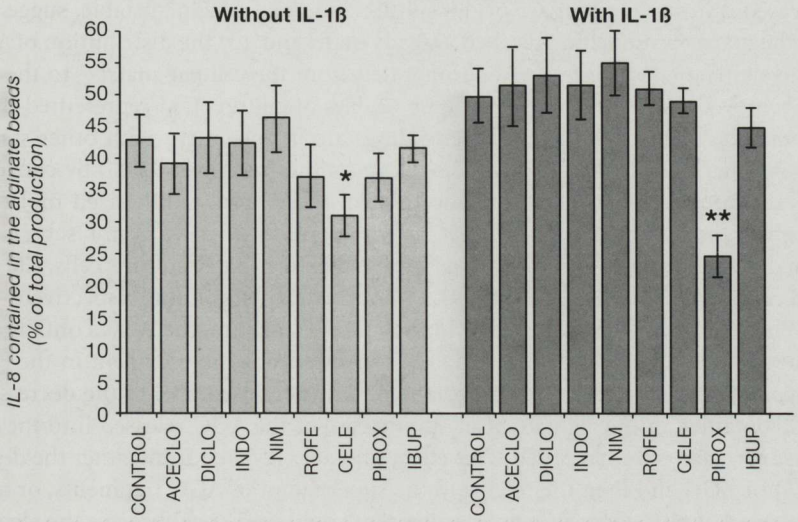


Figure 7. *Effect of NSAID on IL-8 distribution through the different culture compartments after 12 day culture period. Results are expressed as percentage of the total IL-8 production contained in the beads (cellular matrix + further-removed matrix) in the presence or in the absence of IL-1ß 10^{-10} M. Results are expressed in means ± SD of 3 experiments performed with cartilage from 3 different donors. * $p<0.05$, ** $p<0.01$.*

Effects of IL-1ß and NSAID on MMP-3 production and distribution.

At each culture period, more than 90% of the MMP-3 spontaneously produced by human OA chondrocytes were released in the culture supernatant. IL-1ß stimulated MMP-3 production without affecting its distribution (data not shown). In our experimental conditions, NSAID did not significantly affect MMP-3 synthesis and distribution even in the presence of IL-1ß (Figure 6D).

Effects of IL-1ß and NSAID on PGE_2 production.

In basal conditions, the production of PGE_2 was very low overall during the culture period. In the presence of IL-1ß 10^{-10} M, PGE_2 synthesis increased markedly. When chondrocytes were incubated with a therapeutic dose of each NSAID, production became undetectable even in the presence of IL-1ß.

DISCUSSION

In normal articular cartilage, chondrocytes express a specific phenotype charac-

terized by low rate of proliferation, spherical shape of the cells, and by the synthesis of highly specialized macromolecules, mainly aggrecan and type II collagen, which form the extracellular matrix (ECM)[1-3]. In our study, the quantitative analysis of the AGG synthesis showed that (1) the majority (98 %) of the newly synthesized AGG were immobilized in the alginate matrix; (2) AGG content in the alginate bead increased during the first days of the culture and then remained stable, suggesting that the matrix remodelling reached a steady-state; and (3) the distribution of AGG in the alginate was progressing and migrated from the cellular matrix to the further-removed matrix. Nevertheless, after 12 days of culture, CM represented about 35 % of the total production. These findings are in agreement with other studies showing that AGG is the major proteoglycan synthesized (over 95 %) by chondrocytes in alginate culture, and that one-third of these newly synthesized molecules remain in the CM beyond the 3 week culture period[40]. In 1998, Van Osch *et al.* [42] evaluated the relative volume occupied in alginate. In a 13 day culture, cells, CM and FRM represented 3.5 %, 5.2 % and 91.3 % of the relative volume, respectively.

When IL-1ß 10^{-10} M was added to the culture medium, the AGG contained in the beads were significantly reduced. The reduction was more evident in the FRM compartment than in the CM compartment. Further, in parallel to the decrease of AGG contained in the alginate bead, an increase of the AGG released into the culture supernatants was recorded. This effect probably resulted from either the degradation of AGG and then the release to the supernatant of AGG fragments, or from an increase of non-aggregating molecules that could more readily leave the alginate beads.

For the first time, we have shown that IL-6 and IL-8 were diversely distributed between the various matrix compartments. In the normal conditions, 43 % of IL-8 production remained in the FRM, while IL-6 production was essentially (94 %) released in the culture supernatant. Further, IL-1ß widely stimulated production of IL-6 and IL-8, but while the IL-6 distribution was unaffected by IL-1 stimulation, IL-8 was accumulated in the FRM. The role played by IL-8 in the pathophysiology of OA remains obscure. However, in rheumatoid arthritis (RA), IL-8 is an important chemoattractant factor for neutrophils and T lymphocytes towards the inflammatory center [59]. Recently, it was reported that IL-8 in the cartilaginous matrix *in vivo*, creating a gradient of concentration in the matrix, promotes the migration of neutrophils through the degraded cartilage towards the subchondral bone [60-62]. At this level, IL-8 can induce neutrophils activation and degranulation, triggering the release of free radicals and lysosomal enzymes [63, 64] by these cells, which are mediators of cartilage degradation.

In this model, we have also studied the effects of different NSAID (Table 1). Until now, no study has investigated the effects of NSAID on chondrocyte metabolism using the alginate culture model. Except ACECLO and INDO, which slightly enhanced AGG production by chondrocytes, the other NSAID showed a neutral effect on AGG synthesis. These findings must be compared with results of Dingle [14,65] which classified NSAID into three groups according to their effects on sulfated glycosaminoglycan (S-GAG) synthesis by human OA explants cultured for 6 days in

the presence of fetal calf serum or insulin-like growth factor-I. The first group, included NSAID that stimulated GAG synthesis such as ACECLO. The second group, including DICLO and PIROX, exerted neutral effects. The third group was composed of NSAID that reduced GAG synthesis, including INDO, NIM and IBUP. Concerning aceclofenac, our results corroborate Dingle's conclusions. On the other hand, we are in disagreement regarding the effects of INDO, NIM and IBUP. In the Dingle study, these NSAID were classified as inhibitors of proteoglycan synthesis. In our study, INDO was shown to increase PG synthesis whereas NIM and IBUP were without significant effects. This discrepancy can be explained by the drug concentrations used in the both studies. Indeed, in Dingle's study, INDO and NIM were tested at 30 µg/ml and 50 µg/ml, respectively, doses largely superior to therapeutic concentrations used in our study. It was more surprising to find that INDO increased AGG synthesis. This finding contradicts *in vivo* studies that describe a deleterious effect of INDO von cartilage [16, 17]. Other *in vitro* studies have shown a depressive action of INDO on GAG synthesis [66-68]. On the other hand, other authors have shown a neutral effect of therapeutic concentrations of INDO in canine [13], pig [31] or human explants [32]. Another explanation is the difference in the culture medium composition. Dingle and collaborators used fetal calf serum, whereas our culture medium was serum-free. Serum contains hormones and growth factors that create different patterns of cells stimulation and then modify the response of cells to the drugs.

Our data reveal that, at therapeutic plasma concentration, ACECLO, DICLO, INDO and NIM significantly inhibited baseline and IL-1ß stimulated chondrocyte IL-6 production, whereas CELE and IBUP inhibited only IL-1ß stimulated production. These results confirm our previous report demonstrating that NIM and DICLO are potent inhibitors of IL-6 synthesis by human OA chondrocytes in short term cultures[23]. Depending of the exact role of IL-6, the clinical relevance of this observation remains unclear. Indeed, IL-6 is a cytokine with multiple and varied effects, some proinflammatory and others antiinflammatory. Interestingly, ROFE and CELE which both preferentially inhibit cyclooxygenase-2 (COX-2) at therapeutic dose, were ineffective on IL-6 production compared with classical NSAID. The inefficiency of COX-2 specific inhibitors on IL-6 production can be explained by the low dose of the drug tested. In our study, NSAID were tested at concentration corresponding to the mean plasma concentration (C_{max}) obtained after oral administration of a therapeutic dose. For ROFE and CELE, these concentrations were very low and correspond to 1.0 µg/ml and 0.7 µg/ml, respectively. At these concentrations, they selectively inhibit COX-2 but weakly inhibit IL-6 [69]. On the other hand, PIROX which at the concentration of 7 µg/ml inhibits both COX-1 and COX-2, is also inefficient on IL-6 production. Taken together, these results suggest that the effect of NSAID on IL-6 production is not PGE_2 dependent and probably related to the structure of the NSAID. Interestingly, we observed that no NSAID reduce IL-8 production by chondrocytes. Nevertheless, PIROX reduced the proportion of IL-8 accumulated in the presence of IL-1.

We conclude that alginate beads constitute an interesting model for studying effects of drugs on production of aggrecan and cytokines by chondrocytes, but also on their distribution in the extracellular matrix. We have also demonstrated *in vitro* that the effects of NSAID on chondrocytes are differ according their chemical structure and that the mechanism of action of NSAID is multifactorial (Table 1).

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Table 1 : Summary of the results, expressed in significant variations of total amount of AGG, IL-6, IL-8, MMP-3 and PGE_2 produced in 12 days of culture compared to control conditions.

Drug tested	AGG	IL-6	IL-8	MMP-3	PGE_2
Aceclofenac 7.4 µg/ml					
without IL-1ß	↗↗↗	↘↘↘	0	0	↘↘↘
with IL-1ß	0	↘↘↘	0	0	↘↘↘
Diclofenac 1.4 µg/ml					
without IL-1ß	0	↘↘↘	0	0	↘↘↘
with IL-1ß	0	↘↘↘	0	0	↘↘↘
Indomethacin 2 µg/ml					
without IL-1ß	↗↗↗	↘↘↘	0	0	↘↘↘
with IL-1ß	0	↘↘↘	0	0	↘↘↘
Nimesulide 3 µg/ml					
without IL-1ß	0	↘↘↘	0	0	↘↘↘
with IL-1ß	0	↘↘↘	0	0	↘↘↘
Rofecoxib 1 µg/ml					
without IL-1ß	0	0	0	0	↘↘↘
with IL-1ß	0	0	0	0	↘↘↘
Celecoxib 0.7 µg/ml					
without IL-1ß	0	0	↗	0	↘↘↘
with IL-1ß	0	↘	0	0	↘↘↘
Piroxicam 7 µg/ml					
without IL-1ß	0	0	0	0	↘↘↘
with IL-1ß	0	0	0	0	↘↘↘
Ibuprofen 25 µg/ml					
without IL-1ß	0	0	↗	0	↘↘↘
with IL-1ß	0	↘	0	0	↘↘↘

0 : no significant effect, ↗ ($p<0.05$), ↗↗ ($p<0.01$), ↗↗↗ ($p < 0,001$): significant increase, ↘($p<0.05$), ↘↘ ($p<0.01$) and ↘↘↘ ($p<0.001$) : significant decrease. AGG : aggrecan; IL: interleukin; MMP-3: stromelysin (matrix metalloproteinase-3); PGE_2: prostaglandin E_2.

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ANNEXE 17

Effects of three avocado/soybean unsaponifiable mixtures on metalloproteinases, cytokines and prostaglandin E_2 production by human articular chondrocytes

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Abstract: The *in-vitro* effects of avocado and soybean unsaponifiable residues on neutral metalloproteinase activity, cytokines and prostaglandin E_2 (PGE_2) production by human articular chondrocytes were investigated. Avocado and soybean unsaponifiable residues were mixed in three ratios: 1:2 (A1S2), 2:1 (A2S1) or 1:1 (A2S2). Freshly isolated human chondrocytes were cultured for 72 h in the absence or presence of interleukin-1ß (IL-1ß) (17 ng/ml), with or without unsaponifiable residue mixtures at a concentration of 10 µg/ml. A/S unsaponifiable residues were also tested separately at concentrations of 3.3, 6.6 and 10 µg/ml. All A/S unsaponifiable mixtures reduced the spontaneous production of stromelysin, interleukin-6 (IL-6), interleukin- 8 (IL-8) and prostaglandin E_2 (PGE_2) by chrondrocytes. At concentrations of 3.3 and 6.6 µg/ml, A/S residues, tested separately, were potent inhibitors of the production of IL-8 and PGE_2. Nevertheless, only avocado residue inhibited IL-6 production at these concentrations. A/S unsaponifiable mixtures had a more pronounced inhibitory effect on cytokine pro-duction than avocado or soybean residues added alone. As anticipated, IL-lß induced a marked release of collagenase, stromelysin, IL-6, IL-8 and PGE_2. A/S unsaponifiable mixtures partially reversed the IL-1 effects on chrondrocytcs. These findings suggest a potential role for A/S unsaponifiable extracts in mitigating the deleterious effects of IL-lß on cartilage.

Keywords: Avocado; Chondrocytes; Cytokines; Osteoarthritis; Soja; Unsaponifiables.

Introduction

The unsaponifiable part of avocado (A) and soybean (S) oils mixed in a ratio of 1:2 (A1S2, Piascledine, Laboratories Pharmascience, Courbevoie, France) has been used to treat connective tissue diseases for several years [1-7]. After concentration by molecular distillation and saponification by hydroxide in ethanol, the unsaponifiable part of avocado and soybean oils is extracted on a counter-current column using ethylene dichloride. Eight of the nine components identified in the main fraction of avocado oil unsaponifiables (the H or A fraction), accounting for up to 50 %, have been characterised as having a homogeneous structure [1]. A furyl nucleus is substituted in position 2 by an aliphatic, mono- or polyunsaturated chain. The chain length varies from 13 to 17 carbon atoms, always an odd number [2]. This fraction is highly specific for the avocado oil unsaponifiables and can be linked with its pharmacological activities. Squalen (2 %) long-chain saturated hydrocarbons (up to 5 %), polyols (up to 15 %), tocopherols (trace amounts) and sterols (between 4 and 20 %), mainly as ß-sitosterols, are the other components of the avocado unsaponifiables. On the other hand, the soybean oil unsaponifiables, almost totally identified, include saturated hydrocarbons (about 1 %), squalen (about 4 %), tocopherols (around 10 %), sterols (between 40 and 65 %), terpene alcohols (about 1-10 %), aliphatic alcohols (less than 1%) and methyl sterols·(less than 5%) [3]. This mixture was demonstrated to be efficient in the treatment of connective tissue diseases [4]. Studies have shown favourable results for these substances in the treatment of scleroderma [5], pyorrhoea [6] and other periodontal diseases [7]. It has been suggested that soya and avocado extracts also provide some beneficial effects for joint diseases. Two recent studies [8,9] have reported that Piascledine (A1S2) is a symptomatic slow acting drug for osteoarthritis (SYSADOA) with possible structural effects on hip osteoarthritis. A1S2 was reported to decrease fibronectin biosynthesis and increase proteoglycan and collagen production by rabbit chondrocytes [10-12]. AlS2 exhibited an inhibitory effect on the production of collagenase and elastase activity by rabbit chondrocytes [10, 13].

In vitro, interleukin-1 (IL-1) was widely recognised as a promoting factor for cartilage degradation [14]. Indeed, in response to IL-l, chondrocytes secrete neutral metalloproteinases and active oxygen species that are directly implicated in the destruction of cartilage matrix [15, 16]. Moreover, this cytokine is a potent inhibitor of proteoglycan and collagen synthesis [17, 18]. It was suggested that A1S2 inhibits IL-1 effects. As demonstrated on human synoviocyte cultures, A1S2 partially reversed the inhibitory effect of IL-1 on collagen synthesis [11]. However, the optimal ratio between avocado and soybean (A/S) unsaponifiables was not clearly eva-luated in terms of prevention of cartilage breakdown. On the other hand, to the best of our knowledge, no information is yet available concerning the effect of these substances on IL-6 and IL-8 production. The purpose of this study is to compare the effect of A1S2 (Piascledine) and two other unsaponifiable residue mixtures, A2S2 and A2S1, on neutral metalloproteinase activity, proinflammatory cytokines (IL-6 and IL-8) and PGE_2 production by human chondrocytes.

Materials and Methods

Short-term Culture of Human Chondrocytes

Chondrocytes were cultured for a short-term period to maintain their phenotype. Cartilage obtained from the knee joints of normal young adults shortly after death was used for this study. Avoiding the calcilied layer, cartilage slices were excised from the superficial and medium layers of cartilage, cut into small fragments and submitted to enzymatic digestion. The fragments were treated sequentially with hyaluronidase, pronase and collagenase. Firstly, cartilage pieces were incubated with hyaluronidase, previously dissolved in Dulbecco's Modified Eagle's Medium (DMEM, Biowhittaker, Brussels, Belgium) (0.5 mg/ml, 3 g of cartilage per 10 ml of enzyme solution) for 30 min at 37 °C and constantly agitated (100 r.p.m.). Cartilage fragments were then placed into pronase solution (1 mg/ml DMEM, 3 g per 10 ml of enzymatic solution) and incubated for 1 h at 37 °C maintaining constant agitation (100 r.p.m.). The fragments were reincubated with collagenase (0.5 mg/ml, 3 g per 10 ml of enzymatic solution) dissolved in DMEM containing 1 % Ultroser G (Gibco, Gent, Belgium) for 20 h at 37 °C. Cells were then filtered through 25 μM nylon mesh, washed three times, counted (range between 1 and 1.5 x 10^6 cells/ml) and suspended in DMEM containing 1 % ITS+ (Flow Laboratories, Irvine, Scotland). ITS+ is a premixed cell growth system containing, in 1 ml, 0.625 mg of insulin, 0.625 mg of transferrin, 0.625 μg of selenium acid, 0.125 g of bovine serum albumin and 0.535 mg of linoleic acid. Cells were maintained in suspension by constant agitation on a giratory shaker (100 r.p.m.) in an atmosphere of 95 % air and 5 % CO_2. Cells and supernatant were separated by centrifugation (160 g, 5 min). Cell viability, measured by trypan blue incorporation, was 95 %. Culture media were directly assayed for stromelysin, collagenase, IL-6, IL-8 and PGE_2.

Treatments

Three different A/S unsaponifiable mixtures termed A1S2 (1 part A and 2 parts S), A2S1 (2 parts A and 1 part S) and A2S2 (2 parts A and 2 parts S) were tested (Pharmascience Laboratories, Courbevoie, France). Chondrocytes were cultured for 72 h in the presence or absence of 2 and 10 μg/ml of A/S unsaponifiable mixture, with or without human recombinant IL-lß. Avocado and soybean unsaponifiable residues were also tested separately at concentrations of 3.3, 6.6 and 10 μg/ml. The lack of toxicity of unsaponifiable mixtures was demonstrated by two different methods. Short-term incubation (5 h) of chondrocytes with A/S unsaponifiable mixtures did not modify the release of ^{51}Cr from the cells. Moreover, cell viability estimated by trypan blue incorporation was not affected by a long-term (72 h) exposure to A/S unsaponifiable mixtures. Human recombinant IL-lß (Boehringer, Mannheim, Germany) was added at a concentration of 17 ng/ml, corresponding to 850 U/ml. One unit was defined by the manufacturer as the amount of recombinant IL-lß required to support half-maximal [^3H]thymidine incorporation into mouse C3H/HeJ thymocytes in the presence of IL-2 (50 U/ml). For each concentration and corresponding control, three culture flasks containing ± 1.5 x 106 cells were used.

Collagenase Assay

The collagenase assay was performed on chondrocyte conditioned medium using the modified method of Cawston and Barrett [19]. Acid-soluble collagen purified from rat skin was labelled by ^{3}H-acetylation as previously described by Gisslow and McBridge [20]. The specific activity of the tracer was 5.4 x 10^5 cpm/mg of type I collagen. The native state of the collagen was assessed by trypsin digestion, which released a maximum of 10 % radioactivity. The activation of latent collagenase was performed by incubating 1 ml of culture medium with 100 µg of trypsin (Sigma Chemicals, St Louis, USA: 14900 U/mg protein) for 10 min at 37 °C. The chondrocyte medium (100 µl) was incubated for 16 h at 25 °C with 6500 cpm of [^{3}H]collagen in the presence of 0.4 mM N-ethylmaleimide (NEM) and 0.5 mM phenylmethanesulphonyl fluoride (PMSF). After being set aside for 16 h at 25 °C, the enzymatic reaction was stopped by adding 35 mM EDTA. The reaction mixture was then incubated with trypsin (0.1 mg/ml) for 90 min at 34 °C. The enzymatic reaction was interrupted when soybean trypsin inhibitor (SBTI) (0.4 mg/ml) and ethanol at a final concentration of 17 % (v/v) were added. The samples were kept for 30 min at 4 °C and then centrifuged at 9000 g for 20 min. Under these conditions, uncleaved substrate was totally precipitated, whereas more than 95 % of the cleaved fragments remained in the supernatant. Measurements of the radioactivity in the supernatant was performed with a liquid scintillation beta counter (Beckman Instruments, USA). Parallel samples containing 35 mM EDTA added before incubation were similarly measured, providing blank values that never exceeded 10 % of the total radioactivity in the test.

Stromelysin Assay

Casein-degrading activity was assayed on resorufin-labelled casein from cow's milk (Boehringer, Man-nheim, Germany). Latent stromelysin was activated by adding 0.5 mM APMA (P-aminophenylmercuric acetate, Sigma Chemie, Deisenhofen, Germany) and incubating the culture medium for 4 h at 37 °C. APMA-activated cultured media (100 µl) were incubated with 20 µg of resorufin-labelled casein for 18 h at 37 °C in a standard buffer (Tris-HCl 0.2 mol/l, pH 7.5) containing 0.02 mol/l $CaCl_2$. The enzymatic reaction was then halted by adding trichloroacetic acid at a final concentration of 1.8 % and 20 mM EDTA. The samples were centrifuged at 7000g for 15 min. Cleaved casein was then measured fluorimetrically in 600 µl supernatant aliquots previously buffered with 900 µl (Tris-HCl 0.5 M, pH 8.8). The excitation wavelength was 574 nm and the emission wavelength was 584 nm. Under these conditions, the uncleaved casein was totally precipitated whereas more than 95 % of the cleaved casein remained in the supernatant. Parallel samples containing 20 mM EDTA added before incubation were similarly measured, providing blank values that were subtracted from the values obtained for the active medium. The standard curve obtained by incubation of increased quantities of purified stromelysin allowed conversion of the measured fluorescence values into a stromelysin concentration equivalent. The stromelysin was a generous gift of

J.-M. Jaspar (Medgenix Diagnostic, Fleurus, Belgium). The molecular weight of the detected casein-degrading proteinase was assessed by electrophoresis on a casein-containing gel (Zymogram), as described by Pardo *et al.* [21].

Prostaglandin E_2 Radioimmunoassay

Prostaglandin E_2 (PGE_2) was assayed in conditioned culture medium according to a previously described radioimmunoassay (RIA) [22]. PGE_2 was directly measured in the culture medium without previous extraction. Polyclonal antiserum, obtained from the rabbit, did not cross-react with other prostanoids (thromboxane B2, 6-keto-PGF-1α, PGA2) or with fatty acids (arachidonic, linoleic and oleic acids). The ^3H-labelled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecular PGE_2 from Sigma (St Louis, USA). PGE_2 was measured in duplicate on 100 µl aliquots of each sample. After a 48 h incubation at 4 °C, free antigen was separated from antibody-bound antigen by charcoal precipitation and centrifugation (20 min at 1000 g, 4 °C). The supernatant was counted by liquid scintillation. Intra- and interassay coefficients of variation were, respectively, 6 and 10 %. The recovery of known amounts of PGE_2 added to the culture medium was satisfactory (90 - 97 % for amounts of PGE_2 ranging from 2500 to 312 pg/ml). The limit of detection of the RIA was 20 pg/ml.

Cytokine Assays

IL-6 and IL-8 produced and released by chondrocytes into the culture media were assayed by two enzyme amplilied sensitivity immunoassays (EASIAs) set up by Medgenix (Fleurus, Belgium) [23]. They were based on the oligonal system in which several monoclonal antibodies (MAbs) directed against distinct epitopes of the cytokines were used. The assays were performed directly in the culture media without any previous extraction.

DNA Assay

Chondrocyte DNA content was measured according to the fluorimetric method of Labarca and Paigen [24].

Calculations and Statistical Analysis

The results were expressed as the amount of stromelysin, cytokine and PGE_2 measured in the culture medium per microgram of DNA. Each unsaponifiable mixture was tested separately on two different cartilage samples from two donors. The mean ± standard deviation (SD) of each variable was calculated. Comparison of mean values was performed using the unpaired Student's t-test with a significance level of $p < 0.05$.

Results

Effects of Avocado/Soybean Unsaponifiable Mixtures on Collagenase Activity Production

In the absence of IL-lß, A1S2 and A2S2 at 10 µg/ml induced a significant decrease in the stromelysin activity observed in the culture medium ($p < 0.05$). A2S1 did not affect this parameter (Fig. 1 a). When IL-1ß was added, stromelysin activity in the culture medium increased strongly (up to 200%). The IL-1ß effect was inhibited by A1S2 and A2S2 at a concentration of 10 µg/ml. No significant effect was observed with A2S1.

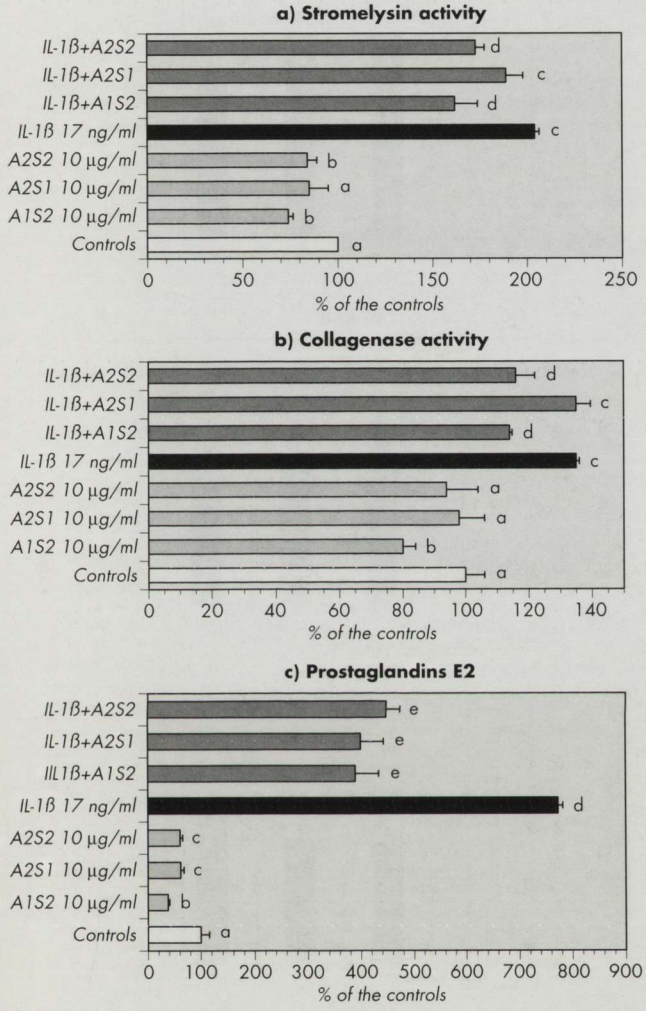


Fig. 1. *Three avocado/soybean unsaponifiable mixtures were compared on the same cartilage sample for their effects on production of stromelysin activity, (a) collagenase activity (b) and PGE_2 (c) . ResuIts are expressed as per cent of control value. Letters a to e indicate the difference between groups (a≠b≠c≠d≠e, $p<0.05$).*

Effects of Avocado/Soybean Unsaponifiable Mixtures on Stromelysin Activity Production

Collagenase activity produced by unstimulated chondrocytes was significantly decreased in the presence of A1S2 (10 µg/ml) ($p<0.05$). A2S1 and A2S2 had no significant effect (Fig. 1b). IL-1ß enhanced the amount of collagenase released into the culture medium. This stimulating effect of IL-1ß was partially inhibited by A1S2 and A2S2 (10 µg/ml). A2S1 did not significantly affect collagenase activity production.

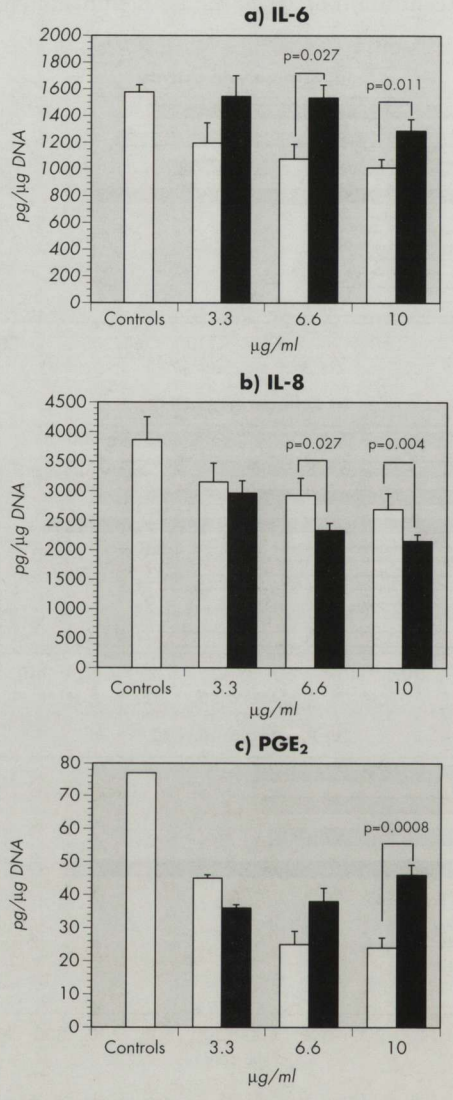


Fig. 2. *Effects of avocado (solid bars) and soybean (hatched bars) unsaponifiable residues on production of IL-6 (a), IL-8 (b) and PGE_2 (c) by unstimulated chondrocytes. Values shown are means ± SD (n=3).*

Effects of Avocado/Soybean Unsaponifiable Mixtures on PGE_2 Production

Unstimulated chondrocytes produced low levels of PGE_2 and this was significantly inhibited ($p<0.05$) by the three A/S unsaponifiable mixtures at a concentration of 10 µg/ml. Both the avocado and the soybean unsaponifiables added at concentrations of 3.3, 6.6 or 10 µg/ml were effective and significantly reduced PGE_2 produc-

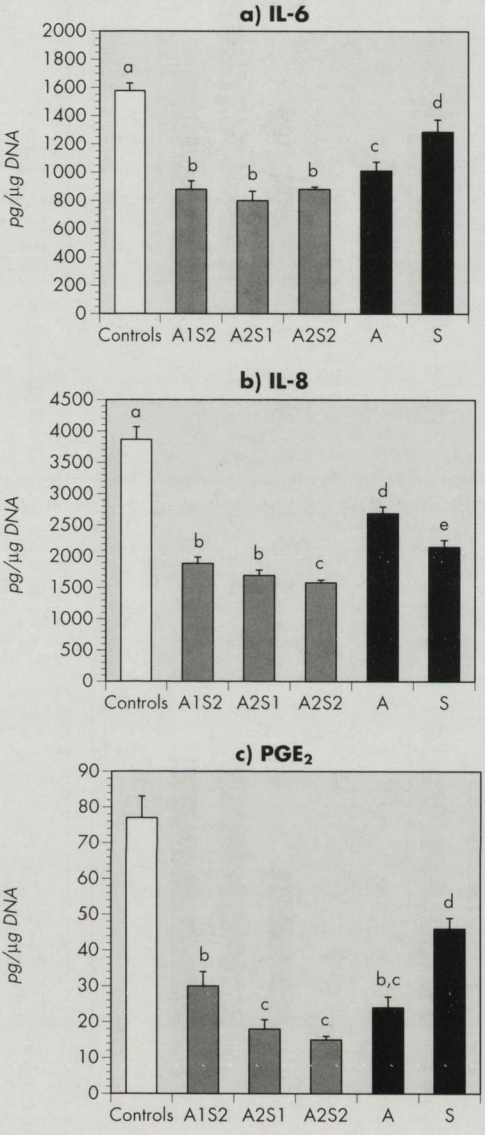


Fig. 3. *Effects of avocado (A) and soybean (S) unsaponifiable residues added separately or as a mixture on production of IL-6 (a), IL-8 (b) and PGE_2 (c) by unstimulated chondrocytes. Unsaponifiable residues or mixtures were tested at a concentration of 10 µg/ml. Values are means ± SD (n=3). Letters a to e indicate the differences between groups ($a \neq b \neq c \neq d \neq e$, $p<0.05$).*

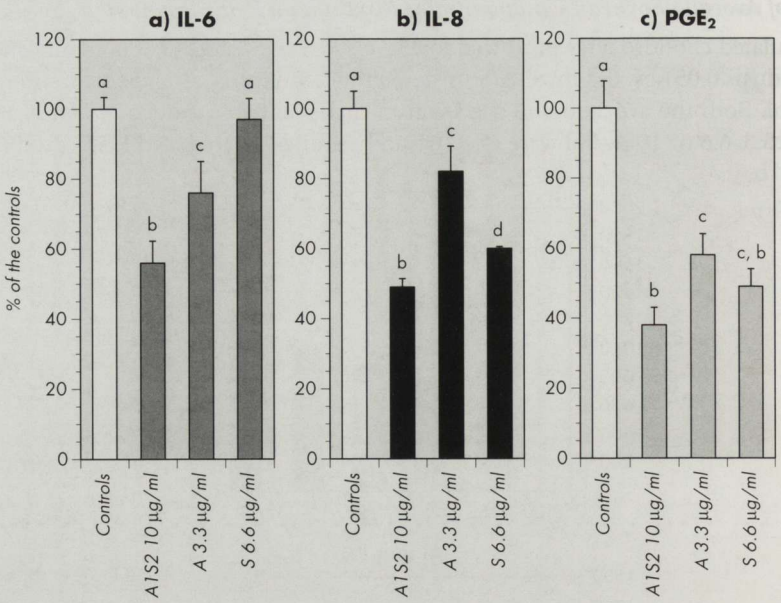


Fig. 4. *Effects of A1S2 (10 µg/ml), avocado (A, 3.3 µg/ml) and soybean (S, 6.6 µg/ml) residues on production of IL-6 (a), IL-8 (b) and PGE_2 (c) by unstimulated chondrocytes. Values are means ± SD (n=3). Letters a to c indicate the differences between groups ($a \neq b \neq c$, $p < 0.05$).*

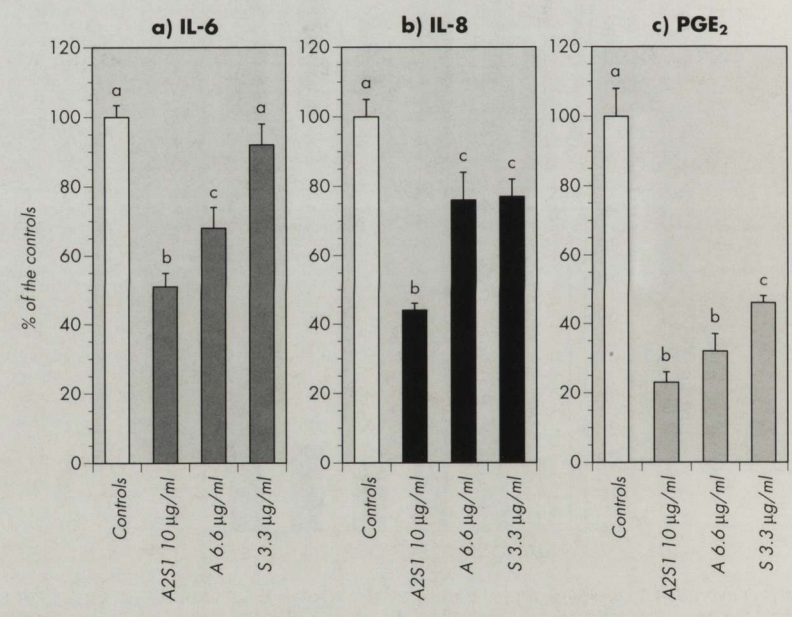


Fig. 5. *Effects of A2S1 (10 µg/ml), avocado (A, 6.6 µg/ml) and soybean (S, 3.3 µg/ml) residues on production of IL-6 (a), IL-8 (b) and PGE_2 (c) by unstimulated chondrocytes. Letters a to c indicate the differences between groups ($a \neq b \neq c$, $p<0.05$).*

tion ($0.01< p<0.001$) (Fig. 2c). At a concentration of 10 µg/ml, the inhibitory effect of avocado was significantly more pronounced ($p < 0.001$) than soybean. Moreover, it appeared that avocado unsaponifiable residue (10 µg/ml) alone was as effective as A/S unsaponifiable mixtures in inhibiting PGE_2 production (Fig. 3c). As shown in Fig. 1c, IL-lß greatly stimulated PGE_2 production. At the concentrations tested, A1S2, A2S1 and A2S2 significantly reduced IL-lß-stimulated PGE_2 production ($p<0.01$). The inhibitory effects on IL-lß-stimulated PGE_2 production were similar for all mixtures. The inhibitory value was measured as being between 40 and 50 % (Fig. 1c).

Table 1: *Effects of avocado/soybean unsaponifiable mixtures on IL-6 production (pg/µg DNA) by human chondrocytes.*

	Without IL-1ß			With IL-1ß		
	A1S2	A2S1	A2S2	A1S2	A2S1	A2S2
0 µg/ml	24 ± 1.2	24 ± 1.2	24 ± 1.2	23817 ± 1562	23817 ± 1562	23817 ± 1562
10 µg/ml	19 ± 0.6	18.6 ± 1.5	19.7 ± 0.7	17047 ± 2555	19346 ± 1594	19005 ± 1045
% of inhibition	-21%	-22%	-18%	-27%	-19%	-20%

There was a significant difference between 0 and 10 µg/ml; $p < 0.05$

Table 2: *Effects of avocado/soybean unsaponifiable mixtures on IL-8 production (pg/µg DNA) by human chondrocytes.*

	Without IL-1ß			With IL-1ß		
	A1S2	A2S1	A2S2	A1S2	A2S1	A2S2
0 µg/ml	25.3 ± 0.7	25.3 ± 0.7	25.3 ± 0.7	31878 ± 2788	31878 ± 2788	31878 ± 2788
10 µg/ml	21.9 ± 1.8	17.8 ± 0.5	18.3 ± 2	18017 ± 695	22273 ± 1488	22470 ± 565
% of inhibition	-14%	-28%	-18%	-43%	-30%	-30%

There was a significant difference between 0 and 10 µg/ml; $p < 0.05$

Effects of Avocado/Soybean Unsaponifiable Mixtures on Cytokine Production

In our culture conditions, chondrocytes spontaneously produced IL-6 and IL-8. The avocado residue significantly inhibited IL-6 production at concentrations of 3.3, 6.6 and 10 µg/ml ($0.05<p<0.01$). Interestingly, the soybean residue was only effective at 10 µg/ml (Fig. 2a). Furthermore, the inhibitory effects obtained with 6.4 and 10 µg/ml of avocado were signilicantly more marked ($p <0.05$) than the inhibition observed with similar concentrations of soybean (Fig. 2a). As shown in Figs 4a and 5a, it was the avocado component of the A1S2 and A2S1 mixtures that affected IL-6 production. Nevertheless, the avocado effect was potentiated by the soybean unsaponifiable. Both the avocado and soybean unsaponifiable residues significantly decreased IL-8 production at concentrations of 6.6 and 10 µg/ml (Fig. 2b). Soybean unsaponifiable was a significantly more potent inhibitor of IL-8 production than avocado unsaponifiable (Fig. 2b). All A/S unsaponifiable mixtures induced a more marked inhibition of IL-8 production than avocado and soybean residues added alone (Fig. 3b). As expected, IL-lß greatly increased IL-6 and IL-8 synthesis (Tables

1 and 2). A1S2, A2S1 and A2S2, respectively, inhibited IL-l-stimulated IL-6 production by 27, 19 and 20% and IL-lß-stimulated IL-S production by 43, 30 and 30 % (Tables 1 and 2).

Discussion

Currently, there are three therapeutic pathways for the management of cartilage destruction in osteoarthritis: administration of drugs for symptomatic relief; drugs that stimulate production of cartilage matrix components; and drugs that inhibit cartilage destruction. Some *in-vivo* studies have reported that an A/S unsaponifiable mixture (A1S2) induced relief of osteoarthritic symptoms (mobility and pain) [25] and decreased the collagenolytic activity reported in osteoarthritic cartilage [26]. Other studies [13, 14] have demonstrated that an A1S2 unsaponifiable mixture enhanced the production of collagen and proteoglycan by human and rabbit chondrocytes cultured in a monolayer. Moreover, osteoarthritic chondrocytes appeared to be more sensitive to A1S2 than normal chondrocytes [27]. The present study suggests that A/S unsaponifiable mixtures are also potent inhibitors of the production of basic and IL-1ß-induced PGE_2, IL-6, IL-8 and metalloproteinases, suggesting that A/S unsaponifiables may act on the catabolic processes to limit or delay cartilage degradation.

All the A/S unsaponifiable mixtures tested decreased the stromelysin activity in the culture medium of IL-lß-stimulated and unstimulated chondrocyte cultures. However, it was found that only the unsaponifiable mixtures containing two parts of soybean residue (A1S2 and A2S2) were effective in decreasing the production of collagenolytic activity. These findings are important for several reasons. These metalloproteinases play animportant role in producing tissue damage, as observe din rheumatic diseases. Metalloproteinase-3/stromelysin digests various components of the extracellular matrix, including proteoglycans, fibronectin, collagen IX and X, and collagen II telopeptides [28-30]. Collagenase degrades interstitial collagens, including type II collagen [31]. Moreover, our findings show that A/S unsaponifi-able mixtures are capable of interfering with, and thereby limiting, IL-1-induced effects. *ln vitro*, IL-lß can inhibit synthesis of cartilage matrix components and sti-mulate metalloproteinases production. These *in vitro* observations suggest that IL-lß could be involved in the development of cartilage lesions that occur in osteoarthritis and rheumatoid arthritis. Our results provide new information compared with previously published results [12, 13, 32]. It appears that the effects of unsaponifiable residue mixtures on enzyme production depend on the type of cell studied. Indeed, Pujol and collaborators [13] found that Piascledine (A1S2) at a concentration of 10 µg/ml increased production of collagenase activity by human rheumatoid synovial cells but had no effect on unstimulated rabbit chondrocytes. Nevertheless, A1S2 was capable of partially reversing the IL-1ß stimulating effect. Another study showed that acid and neutral protease activities were significantly elevated in the carrageenin-induced granulomas of animals treated with the combined avocado/soybean extract (2:1 v/v) [32].

The unsaponifiable part of avocado/soybean oils (A1S2) also possesses several anti-inflammatory properties. A1S2 has been shown to inhibit neovascularisation and decrease polymorphonuclear neutrophil migration in palatal inflammation induced experimentally by carrageenin injection [33]. In our work, we have shown that A/S mixtures greatly inhibit production of basic and IL-1ß-stimulated PGE_2, IL-6 and IL-8. As previously reported, stimulation of chondrocytes with IL-1 leads to increased PGE_2 production [34]. PGE_2 derived from chondrocytes could alter the vascular permeability of adjacent tissues or influence pannus proliferation [35]. PGE_2 may also act on several metabolic pathways, such as protein synthesis, by activating adenylate cyclase, leading to an increase in intracellular cyclic adenosine monophosphate (cAMP) [36]. Recently, enhanced protease synthesis via this pathway was suggested [37], which may partially explain the inhibition of protease production registered in the A/S mixtures. Nevertheless, we have also observed this inhibitory effect of A/S mixtures in the unstimulated cultures in which the PGE_2 content was very low. Interestingly, A/S unsaponifiable mixtures inhibited IL-6 and IL-8 production by chondrocytes. These cytokines are directly involved in inflammatory processes. In fact, IL-8 is a neutrophil chemoattractant and stimulates neutrophils to produce superoxide anions and lysosomal enzymes [38]. IL-6 can act as an endocrine at sites distant from the cells of origin and induce synthesis of protective acute phase proteins (mostly α-l-antitrypsin) by hepatocytes [39]. Moreover, IL-6 may also act as an autocrine or paracrine and stimulate both the catabolic and anabolic functions of the chondrocytes. IL-6 can induce the production of tissue inhibitor of metalloproteinases and not metalloproteinases themselves, indicating that this cytokine plays a part in the feedback mechanism, limiting enzyme damage [40]. On the other hand, IL-6 is necessary for the IL-l-induced inhibition of proteoglycan synthesis in human articular cartilage [41]. Finally, we have also demonstrated that IL-6 is able to inhibit partially the spontaneous production of IL-8 by chondrocytes [42]. Consequently, we must be cautious in considering the real physiopathological implication of the inhibition of IL-6 production by A/S unsaponifiable mixtures.

At the concentrations in the A/S unsaponifiable mixtures, avocado and soybean residues can inhibit PGE_2 and IL-8 production. Nevertheless, the inhibitory effect is more marked when avocado and soybean residues are mixed. This finding suggests that avocado and soybean act in synergy to decrease PGE_2 and IL-8 production. Interestingly, only avocado affects IL-6 production; the greater effect of unsaponifiable avocado on this parameter could be linked with the presence of a large quantity of furanic derived, which are potent inhibitors.

In conclusion, avocado/soybean unsaponifiable mixtures are potent inhibitors of neutral protease activity, proinflammatory cytokines and PGE_2 production by human articular chondrocytes. These substances also act as functional IL-1ß antagonists by partially inhibiting *in-vitro* responses to IL-lß stimulation. The mixture containing two parts soybean unsaponifiable and one part avocado (A1S2) appears to be the most effective substance. These findings provide a preclinical basis for *in-vivo* testing of A/S unsaponifiable mixtures as a new approach to joint diseases.

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ANNEXE 18

Avocado/Soybean Unsaponifiables Increase Aggrecan synthesis and Reduce Catabolic and Pro-inflammatory Mediators Production by Human Osteoarthritic Chondrocytes

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ABSTRACT

Objectives. To investigate the effects (12 days) of avocado/soybean unsaponifiables (ASU) on the metabolism of human osteoarthritic (OA) chondrocytes cultured in alginate beads.

Methods. Enzymatically isolated OA chondrocytes were cultured in alginate beads in a well-defined culture medium for 12 days, in the presence or not of 10^{-10} M IL-1ß. The DNA content was measured according to a fluorimetric method. Aggrecan (AGG), stromelysin-1 (MMP-3), tissue inhibitor of metalloproteinases-1 (TIMP-1), macrophage inflammatory protein-1 beta (MIP-1ß), interleukin (IL)-6 and -8 production were assayed by specific enzyme amplified sensitivity immunoassays (EASIA). Prostaglandin (PG) E_2 was measured by a specific radioimmunoassay and nitrite by a spectrophotometric method based upon the Griess reaction. Avocado and soybean unsaponifiable residues were mixed in the ratio 1:2 (A1S2) or tested separately in a range of concentrations comprised between 0.625 and 40 µg/ml.

Results. After 12 days of incubation, A1S2 increased in a dose- and time-dependent manner AGG synthesis and accumulation in alginate beads. Furthermore, A1S2 promoted the recovery of aggrecan synthesis after a three days period of IL-1ß treatment. ASU were potent inhibitors of both basal and IL-1ß-stimulated MMP-3 productions. They also weakly reversed the inhibitory effect of IL-1ß on TIMP-1 production. Furthermore, ASU inhibited MIP-1ß, IL-6, IL-8, •NO and PGE_2 basal production by OA chondrocytes and partially counteracted the IL-1-stimulating effect on PGE_2. In A1S2 mixture, A and S had an additive effect on AGG synthesis and acted in synergy to inhibit NO production.

Conclusions. From this work, we can conclude that ASU stimulated aggrecan production and recovered aggrecan production after IL-1ß treatment. In parallel, ASU decreased MMP-3 production and stimulated TIMP-1 production. Taken together, these results support the concept that ASU could have structure-modifying effects in OA by inhibiting cartilage degradation and by promoting cartilage repair.

Key-words: Avocado, Soybean, Cartilage, Cytokines, Osteoarthritis.

INTRODUCTION

Osteoarthritis (OA) is a complex disease characterized by bone remodelling, synovium inflammation and cartilage loss. The progression of cartilage degradation is not linear but interrupted by attempts at repair suggesting that chondrocytes keep potential synthetic capacities even at end-stage of the disease[1, 2, 3]. The mechanisms that conduct to cartilage failure have been largely investigated. They primarily involved an excessive production of metalloproteinases, including collagenases (MMP-1, -8, -13)[4, 5, 6], aggrecanases (ADAM-TS4 and -TS5)[7], stromelysin-1 (MMP-3)[8, 9] and gelatinases (MMP-2, -9)[10, 11], by the chondrocytes and the neighboring cells. The metalloproteinases production is upregulated by interleukin-1ß (IL-1ß), a cytokine found to be elevated in OA cartilage[12, 13, 14]. IL-1ß also contributes to cartilage degradation by inhibiting matrix components synthesis (aggrecan and type II collagen)[15, 16]. It also stimulates the synthesis of pro-inflammatory cytokines (IL-6, IL-8, MIPs) and reactive oxygen species (•NO, $O_2^{\bullet}$, H_2O_2), which both contribute to the chronicity of synovium inflammation[17, 18, 19].

To promote cartilage repair reaction and/or to reduce metalloproteinases synthesis/activity constitute important research objectives for drugs used in the treatment of OA. The unsaponifiables part of avocado (A) and soybean (S) oils mixed in a ratio 1:2 (A1S2, Piascledine, Laboratories Expanscience, Courbevoie, France) has been used to treat OA for several years[20, 21, 22]. After concentration by molecular distillation and saponification by hydroxide in ethanol, the unsaponifiable part of avocado and soybean oils is extracted on a counter-current column using ethylene dichloride. Eight of the nine components identified in the main fraction of avocado oil unsaponifiables (the H or A fraction), accounting for up to 50 %, have been characterized as having a homogeneous stucture. A furyl nucleus is substituted in position 2 by an aliphatic, mono- or polyunsaturated chain. The chain length varies from 13 to 17 carbon atoms, always an odd number. This fraction is highly specific for avocado oil unsaponifiables and can be linked with its pharmacological activities. Squalen (2 %), long-chain saturated hydrocarbons (up to 5 %), polyols (up to 15 %), tocopherols (trace amount) and sterols (between 4 and 20 %), mainly as b-sitosterols, are the other components of the avocado unsaponifiables. On the other hand, the soybean oil unsaponifiables, almost totally identified, include saturated hydrocarbons (about 1 %), squalen (about 4%), tocopherols (around 10 %), sterols (between 40 and 65 %), terpene alcohols (about 1-10 %), aliphatic alcohols (less than 1 %) and methyl sterols (less than 5 %)[23].

At present, some clinical studies have demonstrated the beneficial effects of avocado/soybean unsaponifiables (ASU) on OA symptoms[20, 21, 22]. In a prospective clinical trial, ASU treatment showed significant symptomatic efficacy over placebo in the treatment of hip OA, acting from month 2 and showing a persistent effect for at least 2 months after a 6-months period of treatment[21]. The authors concluded that ASU was a slow acting symptomatic drug with a persistent effect and an excel-

lent safety. Further, ASU have shown potential structure modifying effects in animal and clinical studies. Indeed, a 2 years placebo controlled trial has recently reported evidence of reduced joint space narrowing (JSN) in OA hip in ASU treated group, but only in the sub-group of patients with severe JSN (under the median value of joint space width) at baseline[24].

In animal models, oral ASU treatment following menisectomy appeared to confer a subtle but statistically significant effect of the drugs, in maintaining articular cartilage proteoglycan content and thickness, and reducing subchondral bone sclerosis[25]. There is also *in vitro* evidences that ASU are capable of stimulating matrix production and reducing the deleterious effect of IL-1, possibly via the production of transforming growth factors beta (TGFßs), which appear to be one of the most important family of growth factors in terms of cartilage homeostasis[26]. We have also reported that ASU possess anti-inflammatory properties by inhibiting interleukin (IL)-6 and -8 production by human chondrocytes[27]. However, no study concerning the effect of ASU on aggrecan synthesis and deposition have been published so far. Therefore, to study ASU on chondrocytes cultured in alginate beads would be helpful to assess their potential "structure modifying" effects. This model is particularly suitable to investigate matrix deposition and organization and allows keeping stable the chondrocytes phenotype for at least 12 days[28]. This study was designed to investigate the long-term (12 days) effect of ASU on human articular OA chondrocytes.

MATERIALS AND METHODS

Chondrocytes culture in alginate beads

Cartilage was obtained from the knees of cadavers with primary OA immediately after death, being excised from the superficial and medium layers of cartilage and avoiding the calcified layer. Upon dissection, the femoral, patellar and tibial articular surfaces were evaluated for gross pathological cartilage modifications according to a personal scale. The severity of pitting was recorded for each donor. Four different grades were considered: 0, normal white cartilage in all areas examined; I, the presence of a yellow-grey area with some superficial fibrillations on one or more articular surfaces; II, irregular surface with deep fibrillations on one or more articular surfaces; and III, ulcers penetrating to the subchondral bone on one or more articular surfaces. Experiments were performed with osteoarthritic cartilage coming from six different donors with a mean age of 42 (35-52) years old. All donors showed OA cartilage lesions of grade I or III.

Cartilage was cut into small fragments and then subjected to enzymatic digestions sequentially with hyaluronidase, pronase and collagenase (3 g of cartilage per 10 ml of enzyme solution) as previously described[29]. Cells viability was estimated by trypan blue exclusion test and in all cases was superior to 95 %. Chondrocytes were suspended in alginate beads as previously described[28] and maintained in culture for 12 days in DMEM supplemented with 1 % ITS+ (ICN Biomedicals, Asse-

Rele̲em, Belgium), 10 mM HEPES, penicillin (100 U/ml) and streptomycin (100 µg/ml), 200 µg/ml glutamine (Biowhittaker Europe, Verviers, Belgium), 50 µg/ml asco:bic acid (Sigma-Aldrich, Bornem, Belgium), 2 mM proline (Invitrogen, Merelbeke, Belgium). ITS+ is a premixed cell growth system containing in one ml: 0.62̲ mg insulin, 0.625 mg transferrin, 0.625 µg selenious acid, 0.125 g bovine serum albumin and 0.535 mg linoleic acid. Cells remained in this culture medium (wash-out medium) for 48h as a precaution against *in vivo* contamination with drugs that donors might have taken before death. After this wash out period, culture medium was changed and the various drugs added. Culture medium was changed every three days and the collected supernatants were kept at -20°C until analysis.

Treatments

Chondrocytes were cultured for twelve days in the absence or in presence of 10^{-10} M IL-1ß (1.7 ng/ml or 85 U/ml; Boehringer, Mannheim, Germany) and with or without avocado or soybean mixed in a ratio 1:2 (A1S2, Piasclédine®, Laboratoires Expanscience™, Courbevoie, France) or added separately. A1S2 was tested at concentrations ranged among 0.625 to 40 µg/ml. A and S unsaponifiable residues were also tested separately at the concentrations of 3.3, 6.6 or 10 µg/ml. All the drugs tested were first dissolved in tetrahydrofurane (THF, Sigma-Aldrich, Bornem, Belgium), and then diluted in culture medium to achieve the required final concentration. The final concentration of THF was 0.1 % in all culture conditions, including the controls (i.e. IL-1ß). For the recovery experiment, chondrocytes were treated for three days with IL-1ß (pre-incubation), washed and then cultured for the next 15 days with or without A1S2 10 µg/ml. In the same experiment, each culture condition was performed in triplicate and each experiment was repeated three times using three different donors.

End of the culture

The culture medium (S) was carefully discarded and the beads of each well were dissolved in 1 ml 0.1 M citrate for 10 min. The resulting suspension was centrifuged at 1200 rpm for 10 min. With this method, two fractions were collected: the supernatant containing macromolecules originated from the further-removed matrix (FRM) compartment, and a pellet, containing cells with their associated matrix (CM). These two fractions were studied separately. The cell pellets were washed with PBS (Biowhittaker Europe, Verviers, Belgium), and then homogenized in 1 ml of PBS by ultrasonic dissociation at 4°C for DNA assay. CM and FRM were kept at -20°C until analysis.

DNA assay

The DNA content of the cultures was measured according to the fluorimetric method of Labarca and Paigen[30].

LDH release assay

The cell viability was estimated by the release of lactate deshydrogenase (LDH) in the culture supernatant. In a 96-wells plate, 50 ml of tris 0.01 M buffer, pH 8.5, containing 0.1 % BSA and lactate 800 mM and 50 ml of a solution 1.6 mg/ml INT (p-iodonitrotetrazolium violet, Sigma-Aldrich, Bornem, Belgium), 4 mg/ml NAD (Roche pharmaceuticals, Brussels, Belgium) and 0.4 mg/ml phenazine methosulfate (Sigma-Aldrich, Bornem, Belgium) were added to 100 µl of culture supernatant (before any freezing). The absorbance at 492 nm was read after 5 min of incubation at room temperature. The calibration was performed with LDH isolated from rabbit muscle. The cells death score was obtained by calculating the ratio: (LDH supernatants/total LDH) x 100. Total LDH corresponded to the sum of the LDH measured in the CM, the FRM and the culture supernatants (3 to 12 days).

Prostaglandin (PG) E_2 radioimmunoassay

PGE_2 was assayed in the different compartments (culture supernatants, FRM and CM) without previous extraction according to a previously described radioimmunoassay[31]. In this assay, polyclonal antiserum obtained from rabbit cross-reacts neither with other prostanoids (TxB_2, 6-keto-PGF1α, PGA_2) nor with any fatty acids (arachidonic, linoleic or oleic acids). ^{3}H-labeled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule (PGE_2) from Sigma-Aldrich (Bornem, Belgium). Intra- and inter-assay coefficients of variation were 6 and 10 %, respectively. The limit of detection of the RIA was 20 pg/ml.

Immunoassays for AGG, MIP-1ß, IL-6, IL-8, MMP-3 and TIMP-1

Aggrecan (AGG), macrophage inflammatory protein (MIP-1ß, interleukin (IL)-6 and -8, tissue inhibitor of metalloproteinases-1 (TIMP-1) and stromelysin-1 (MMP-3) were measured in the different culture compartments (S, FRM and CM), by specific EASIA (Enzyme Amplified Sensitivity ImmunoAssays, Biosource Europe, Fleurus, Belgium). The limit of detection of the AGG, MIP-1ß, IL-6, IL-8, TIMP-1 and MMP-3 immunoassays were 5.5 ng/ml, 12.5 pg/ml, 8 pg/ml, 4 pg/ml, 325 pg/ml and 1.25 ng/ml, respectively.

The assays were based on the oligonal system in which several monoclonal antibodies were directed against distinct epitopes of the molecules. In the case of MMP-3, the antibodies were directed against pro-MMP-3, activated-MMP-3 and MMP-3 bound to TIMP-1 and TIMP-2. Aggrecan synthesis was quantified by an EASIA using a monoclonal antibody raised against the keratan sulphate region of AGG as a capture antibody, and a monoclonal antibody to the hyaluronic acid binding region as a detector. The intra- and inter-assay coefficients of variation were less than 5 % for all immunoassays.

Nitric oxide (•NO) assay

Nitrite and nitrate are stable end products of nitric oxide. Nitrate is reduced to nitrite by the addition in the supernatant of nitrate reductase (0.25 U/ml, Roche

Pharmaceuticals, Brussels, Belgium) for 20 min. at 37°C. Nitrite concentrations in conditioned culture supernatants were determined by a spectrophotometric method based upon the Griess reaction[32]. The absorption was measured at 540 nm. Sodium nitrite ($NaNO_2$) was used for calibration.

Calculation and statistical analysis

The results (mean ± SEM) were expressed as the concentration of AGG, MMP-3, TIMP-1, MIP-1ß, NO_2 and PGE_2 in the culture supernatants (3 to 12 days), CM and FRM (or the sum of these values for the total production) per µg of DNA. A multivariate repeated measures ANOVA model with calculation of contrasts was performed on all the experiments (MANOVA; SAS system; General Linear Models).

RESULTS

Effect of ASU on cell viability and DNA content

The DNA content (3.7 +/- 0.3 µg per well after 12 days of culture) increased by 38 % over the culture duration, and was not significantly affected by the presence of IL-1ß (10^{-10} M). The cell death, appreciated by the release of LDH from the cells during the twelve days of culture, was ranged between 5 to 8 %. IL-1ß, even after twelve days of incubation, did not modify LDH release. At concentrations ranged between 0.625 to 40 µg/ml, A1S2 did not significantly affect cell death score or DNA content, even in IL-1ß-treated culture. At 10 µg/ml, neither A nor S added separately significantly affected cells viability (data not shown).

Effects of ASU on AGG production

AGG production was essentially immobilized in the alginate bead. Seventy percents of the newly synthesized AGG were found in the FRM, 28 % in the CM and only 2 % in the supernatant. After twelve days of incubation, the total production of AGG was significantly enhanced by A1S2 at the doses of 10 and 40 µg/ml ($p < 0.001$) (Figure 1A). In the basal conditions, AGG production increased in a time-dependent manner until the sixth day, and then reached a steady state. In the presence of A1S2 (10 µg/ml), AGG synthesis was sustained over the sixth day of culture, and continued to increase until the twelfth day. The stimulating effect of A1S2 on AGG production was already significant after nine days of incubation (Figure 1B). When the different compartments (FRM, CM, S) of the culture were individually analysed for their content in AGG, it appeared that the increase in AGG content, primarily occurred in the FRM (Figure 1C). We have also compared the effect of A1S2 (10 µg/ml) with those of A or S unsaponifiables added separately in the culture medium at the dose of 3.3 and 6.6 µg/ml, respectively. This analysis demonstrated that in the A1S2 mixture, A and S had an additive effect (Table 1). At 10 µg/ml, A and S added separately were as efficient as A1S2 on aggrecan production.

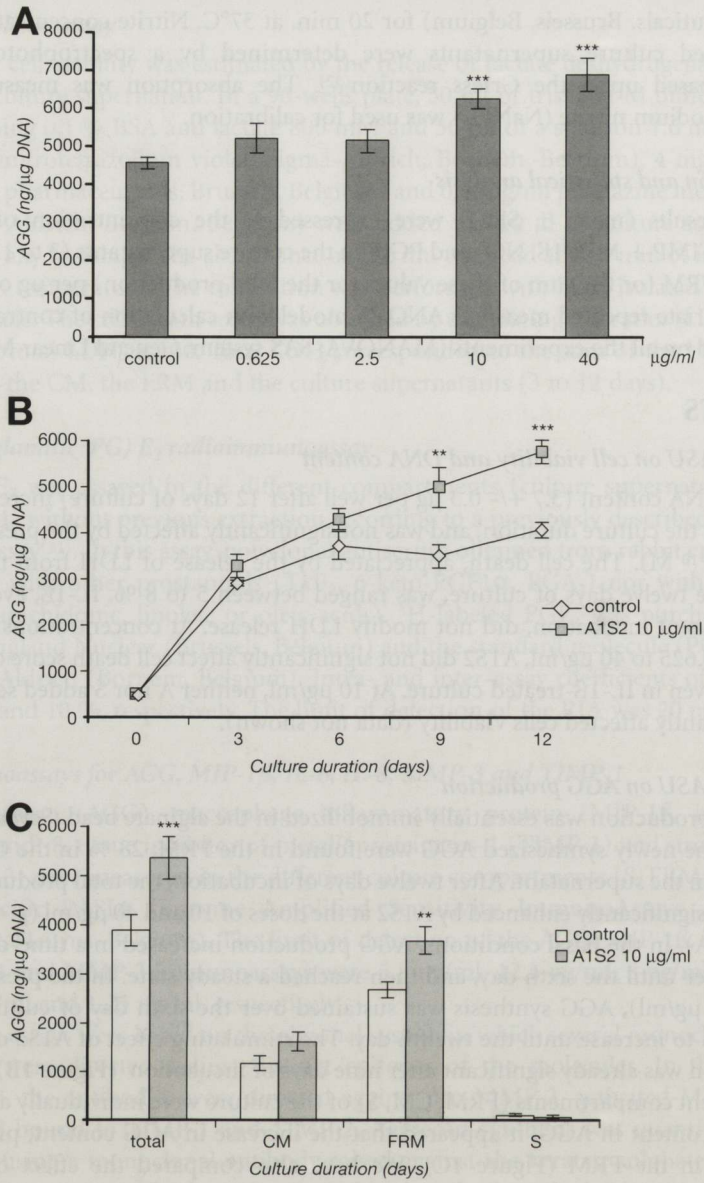


Figure 1: *(A) Effect of increased concentrations of A1S2 on the basal production of aggrecan by human OA chondrocytes. Values correspond to the total amounts (sum of cumulated amounts found in S, CM and FRM) of AGG produced by chondrocytes during 12 days of culture; (B) Kinetic curves of aggrecan production in the absence or in the presence of A1S2 10 µg/ml; (C) Distribution of AGG in the different culture compartment (S = Supernatant (3 to 12 days), CM = Cell-associated matrix, FRM = Further Removed Matrix) at the end of the 12 days of culture. Values are means ± SEM (n = 9) of three independent cultures performed with cartilage specimens coming from three different donors (a 52 years-old man, grade I; a 38 years-old woman, grade I and a 35 years-old woman, grade I). Statistical significance in comparison to the controls: ** $p<0.01$, *** $p<0.001$.*

IL-1ß (10^{-10} M) drastically decreased total AGG production (sum of AGG found in S, FRM and CM) by 73 % and modified AGG distribution through the different culture compartments. The ratio of AGG found in the supernatants increased from 2 to 8 % in the presence of IL-1ß , suggesting that IL-1ß induced AGG degradation. Neither A nor S mixed or added separately, were capable to reverse the effect of IL-1 on AGG synthesis (data not shown).

Finally, we have tested the effect of A1S2 on the recovery of AGG synthesis after a treatment with IL-1ß. In this experiment, chondrocyte cultures were incubated three days with IL-1ß, washed, and then cultured for the next 15 days with or without A1S2 (10 µg/ml). As shown in the figure 2, pre-treatment with IL-1ß fully blocked the AGG synthesis throughout the culture period, whereas A1S2 recovered AGG synthesis after nine days of incubation. After 15 days, AGG production was recovered by 88 %. These results clearly demonstrate that ASU restore aggrecan synthesis after IL-1ß blockage, a cytokine involved in OA physiopathology.

	Controls	A1S2 10 µg/ml	A 3.33 µg/ml	A 10 µg/ml	S 6.6 µg/ml	S 10 µg/ml
AGG ng/µg DNA	4,542 ± 143	6,261 ± 217 *	5,242 ± 168 *#	5,728 ± 505 *	5,641 ± 156 *	5,752 ± 208 *
MMP-3 ng/µg DNA	6,233 ± 185	4,246 ± 286 *	6,803 ± 326 #	4,463 ± 308 *	4,147 ± 88 *	4,225 ± 434 *
TIMP-1 ng/µg DNA	307.8 ±11.7	295.1± 26.1	274.6 ±23.6	272.6 ± 10.2	354.6 ± 15.5 *¶	378.0 ± 17 *¶
IL-6 pg/µg DNA	5162 ± 139	734 ± 113 *	3287 ± 57 *#	1718 ± 53 *#	607 ± 19 *	625 ± 24 *
IL-8 pg/µg DNA	3234 ± 103	893 ± 83 *	2636 ± 112 *#	1719 ± 49 *#	1162 ± 68 *¶	1287 ± 33 *¶
MIP-1ß pg/µg DNA	54.77 ±0.15	25.54 ±0.60 *	53.70± 2.10 #	39.28 ±1.34 *#	28.10 ±1.90 *	23.7 ±1.74 *
NO_2 nmol/µg DNA	4.03 ±0.49	0.75 ±0.10 *	3.90 ±0.41 #	1.29 ±0.01 *#	1.38 ±0.04 *¶	1.32± 0.5*¶

Table 1: *Effect of A1S2, A and S on the basal production of aggrecan (AGG), stromelysin-1 (MMP-3), tissue inhibitor of metalloproteinases-1 (TIMP-1), interleukin-6 (IL-6), IL-8, macrophage inflammatory protein-1ß (MIP-1ß) and nitrite (NO_2). Values are means ± SEM (n=9) of three independent cultures performed with cartilage specimens coming from three different donors. The symbol * means a significant difference with $p < 0.05$ between the treated groups and the controls. The symbol # means a significant difference between A treated group and A1S2 treated group and finally the symbol ¶ means a significant difference between S treated group and A1S2 treated group.*

Effects of ASU on MMP-3 and TIMP-1 production

In the basal conditions, A1S2 dose-dependently inhibited stromelysin-1 (MMP-3) production by human OA chondrocytes ($0.01 \leq p \leq 0.001$) (Figure 3A). By comparison with the control group, the inhibitory effect of 10 µg/ml ASU was already significant at day 3 of culture ($p \leq 0.01$) and its magnitude increased with incubation time (Figure 3B). After 12 days, basal MMP-3 synthesis was decreased by

30 %. A1S2 (10 µg/ml) and S (6.6 µg/ml) showed a similar effect on this parameter, while A (3.3 µg/ml) did not modified MMP-3 synthesis, suggesting that S was the active fraction (Table 1). At 10 µg/ml, A1S2, S and A inhibited similarly MMP-3 synthesis.

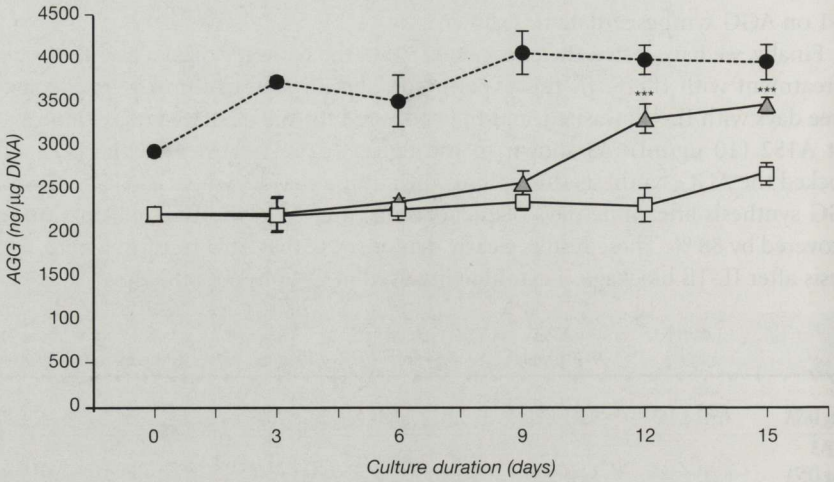


Figure 2: *Effect of A1S2 10 µg/ml on AGG synthesis recovery after a 3-day treatment with IL-1ß 10^{-10} M. The curves represent the total production, which correspond to the sum of AGG found in S, CM and FRM. In this set of experiments, chondrocyte cultures were pre-treated or not (dotted line, --● --) for 3 days with 10^{-10} M IL-1ß , washed and then cultured for the next 15 days (between day 0 to day 15) in the absence (-□ -) or in the presence (-△ -) of A1S2 10 µg/ml. Values are means ± SEM (n= 9) of three independent cultures performed with cartilage specimens coming from three different donors (a 51 years-old man, grade III; a 55 years-old man, grade I and a 44 years-old woman, grade II). Statistical significance in comparison to the controls (treated by IL-1ß): *** p<0.001.*

In the presence of IL-1ß, MMP-3 highly increased over the control values during the first three days of culture, reached a maximum after the third day, and then remained stable until the end of the culture. In contrast, in the basal condition, MMP-3 synthesis increased throughout the culture period. A1S2 weakly, but significantly inhibited the IL-1ß induced MMP-3 over-production observed after 3 days of incubation (Figure 3B).

In the basal conditions, MMP-3 production by OA chondrocytes largely exceeded TIMP-1 production (Table 1). After 12 days of culture, the ratio MMP-3/TIMP-1 was equal to 22. At 10 µg/ml, A1S2 and A did not significantly modify the basal TIMP-1 production, whereas S increased it by 23 %. As primarily a consequence of the MMP-3 decrease, the ratio MMP-3/TIMP-1 was reduced by half in the presence of A1S2 (10 µg/ml). In the presence of IL-1ß (10^{-10} M), the production of TIMP-1 was decreased by 82 %. This IL-1ß-inhibitory effect was weakly reversed by A1S2 , A and S at 10 µg/ml (Figure 3C).

In our experimental conditions, ASU decreased MMP-3/TIMP-1 ratio even in the presence of IL-1ß indicating that ASU administrated therapeutically could slow-down enzymatic degradation of cartilage in OA.

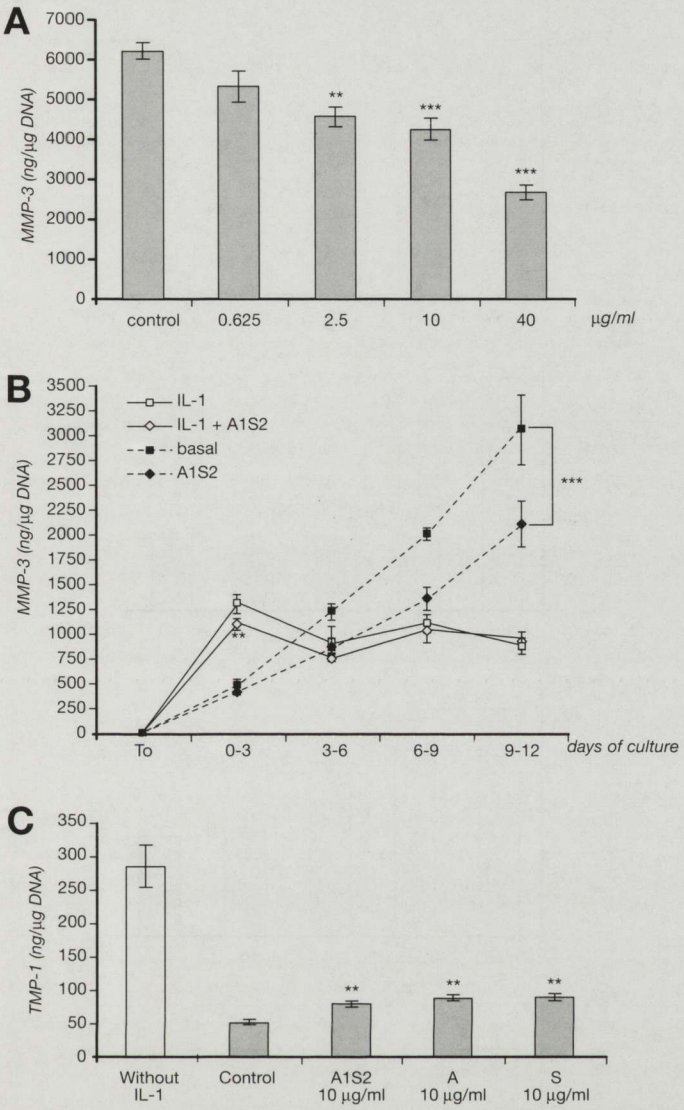


Figure 3: *Effect of A1S2 on stromelysin-1 (MMP-3) and tissue inhibitor of metalloproteinases (TIMP-1) synthesis by human OA chondrocyte. (A) Dose effect of A1S2 on the basal production of MMP-3. Values correspond to the amounts of MMP-3 released in the culture supernatant by chondrocytes during a 3-days period of culture (0 to 3, 4 to 6, 7 to 9 and 10 to 12); (B) Kinetic curves of MMP-3 synthesis in the absence (dotted lines) or in the presence of 10^{-10} M IL-1ß (continuous line) and without (-□ -) or with (-◇ -) A1S2 10 µg/ml; (C) Effect of A1S2, A and S at 10 µg/ml on the inhibitory effect of IL-1ß on TIMP-1 production. Values correspond to the total amounts (sum of the cumulated amounts found in S, CM and FRM) of MMP-3 produced by chondrocytes during 12 days of culture. Values are represented by means ± SEM (n = 9) of three independent cultures performed with cartilage specimens coming from three different donors (a 52 years-old man, grade I; a 38 years-old woman, grade I and a 35 years-old woman, grade I). Statistical significance in comparison to the controls: ** $p<0.01$, *** $p < 0.001$ (MANOVA test).*

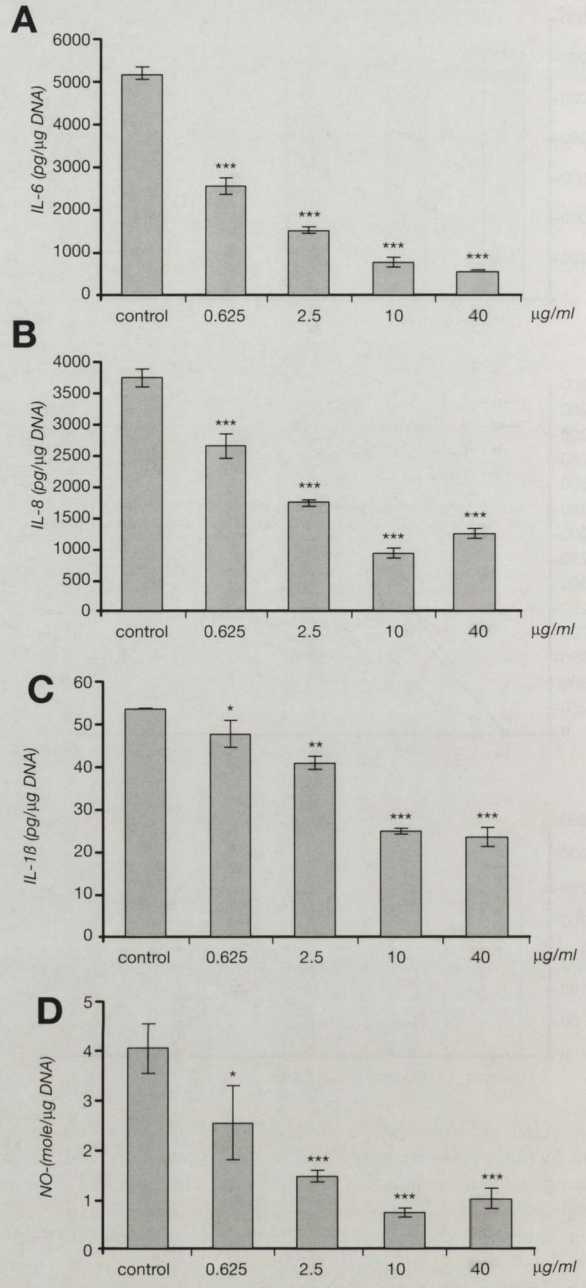


Figure 4: *Effects of increased doses of A1S2 on the basal IL-6 (A), IL-8 (B), MIP-1ß (C), and NO_2 (D) production by human OA chondrocytes cultured for 12 days in alginate beads. Values are means ± SEM (n = 9) of three independent cultures performed with cartilage specimens coming from three different donors (a 52 years-old man, grade I; a 38 years-old woman, grade I and a 35 years-old woman, grade I). Statistical significance in comparison to the controls: * p<0.05, ** p<0.01, *** p<0.001.*

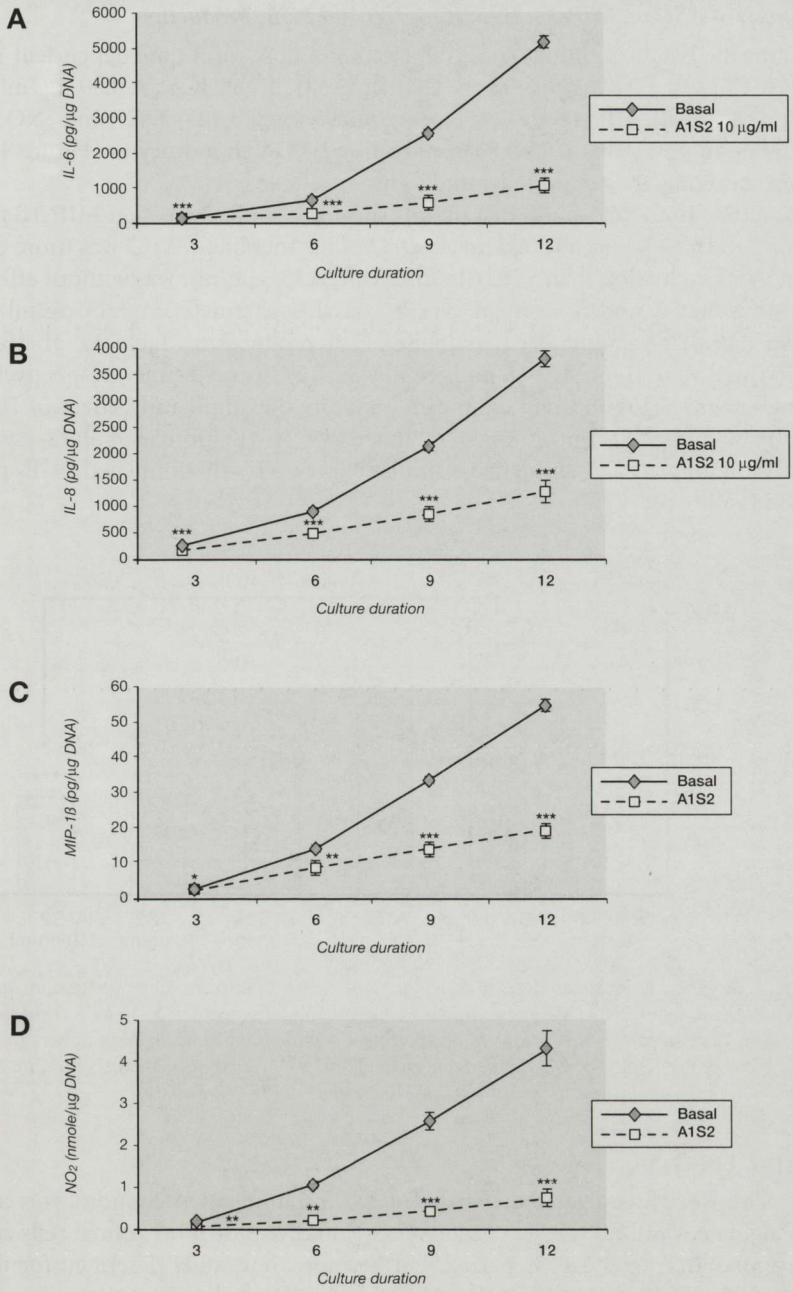


Figure 5: *Time effect of A1S2 on the basal IL-6 (A), IL-8 (B), MIP-1ß (C), and •NO (D) production by human OA chondrocytes cultured for 12 days in alginate beads. Values are means ± SEM (n = 9) of three independent cultures performed with cartilage specimens coming from three different donors (a 52 years-old man, grade I; a 38 years-old woman, grade I and a 35 years-old woman, grade I). Statistical significance in comparison to the controls: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.*

Effects of ASU on MIP-1ß, IL-6, IL-8, NO and PGE_2 production

In the basal condition, A1S2 inhibited in a dose- and time dependent manner IL-6 (IC_{50} = 0.50 µg/ml; 95 % CI 0.46-0.54), IL-8 (IC_{50} = 0.89 µg/ml; 95 % CI 0.80 - 0.98), MIP-1ß (IC_{50} = 3.22 µg/ml; 95 % CI 3.12 – 3.32), and •NO (IC_{50} = 0.69 µg/ml; 95 % CI 0.54-0.84) production by OA chondrocytes (Figure 4 and 5) whereas PGE_2 was found to be under the detectable level.

A1S2 (10 µg/ml) and S (6,6 µg/ml) showed a similar effect on MIP-1ß production, while A (3.3 µg/ml) had no effect. On the other hand, A1S2 was more efficient on •NO production than S (6.6 µg/ml), while A (3.3 µg/ml) was without effect, suggesting that A and S acted in synergy on this parameter. A (3.3 µg/ml) and S (6,6 µg/ml) also synergized to inhibit IL-8 production (Table 1). IL-1ß highly increased IL-6, IL-8, MIP-1ß and •NO by 20, 12, 40 and 2 times, respectively (data not shown). A1S2 did not significantly modify the stimulating effect of IL-1ß on MIP-1ß and •NO, but strongly inhibited the IL-1ß-stimulated PGE_2 synthesis. At 10 µg/ml, A1S2, A and S had a similar effect on IL-1ß-stimulated PGE_2 production (Figure 6).

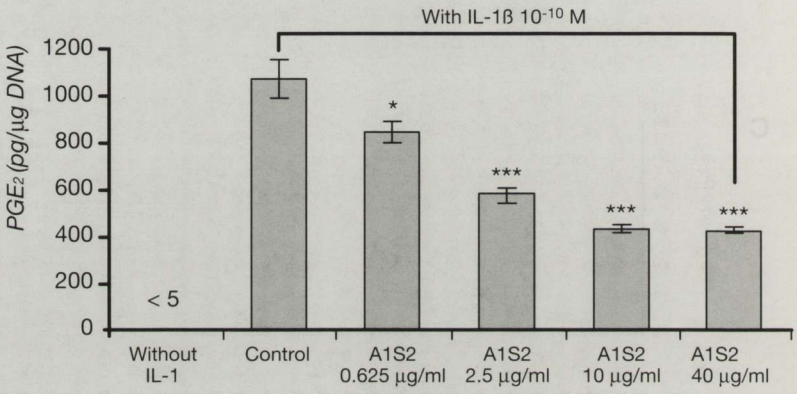


Figure 6: *Effects of increased doses of A1S2 on the IL-1ß-stimulated PGE_2 production by human OA chondrocytes cultured for 12 days in alginate beads. Values are means ± SEM (n = 9) of three independent cultures performed with cartilage specimens coming from three different donors (a 52 years-old man, grade I; a 38 years-old woman, grade I and a 35 years-old woman, grade I). Statistical significance in comparison to the controls (IL-1ß treated group): *** p<0.001.*

DISCUSSION

This work investigated the effects of ASU on human OA chondrocytes cultured in alginate. Until now, ASU effects were primarily studied on animal cells cultured in monolayer for a 3-days period. Furthermore, our study described for the first time the effects of ASU on AGG, MIP-1ß and •NO production.

Drugs for the treatment of OA have been distinguished according to their symptom and/or structure-modifying effects. ASU have showed a delayed and persistent

symptomatic effect in the treatment of knee and hip OA[21]. Nevertheless, we do not yet know whether ASU have also structure-modifying properties, as was suggested in a subgroup of patients with advanced joint space narrowing of the hip (≤ 2.45 mm at baseline) in a radiological pilot study with a 2-year follow-up[24]. Our study supports the hypothesis that ASU could interfere with the structural changes of the disease. In fact, ASU was demonstrated to promote cartilage matrix formation and to recover aggrecan synthesis after IL-1 treatment. Furthermore, ASU decreased MMP-3 production by OA chondrocytes and partially reversed the IL-1ß-inhibitory effect on TIMP-1 synthesis. MMP-3 is particularly effective in cartilage degradation, since it cleaves a wide variety of matrix macromolecules, e.g., proteoglycans, fibronectin, and several collagens[33, 34]. Furthermore, MMP-3 has been found to be capable of activating other metalloproteinases (collagenase-1, collagenase-3, and 92-kd gelatinase)[35]. From these findings, we can conclude that ASU down-regulate the synthesis by chondrocytes of some catabolic factors that contribute to the onset and development of cartilage lesion in OA. In parallel, ASU promote the production of aggrecan, one of the major matrix components, and restore its synthesis after IL-1ß treatment. Taken together, these findings suggest that ASU could be helpful for promoting cartilage repair in OA. Nevertheless, these *in vitro* observations must be extrapolated with caution to clinical situations since *in vitro/ in vivo* conformity has not yet been demonstrated.

Interestingly, the effect of ASU on AGG was delayed and became significant only after 6 to 9 days of incubation. This observation suggests that ASU effect could be secondary and mediated by regulatory peptides secreted by chondrocyte themselves. Transforming Growth Factors (TGFs) are potential candidates since TGF-ß1 and -ß2 expression has been demonstrated to be up-regulated by ASU in bovine chondrocyte cultured in monolayer[26]. Interestingly, TGF-ß1 and -ß2 are growth factors that exhibit similar effects on cartilage that those described for ASU. TGFßs can increase aggrecan synthesis, decrease metalloproteinases production by chondrocytes and counteract some deleterious effects of IL-1ß on cartilage[36, 37, 38].

Another important feature in OA is the inflammation of the synovium. Inflammatory synovium changes in OA are visible to areas near the cartilage lesions[39]. Among these factors, cytokines released by chondrocytes play an important role. In this study, we have demonstrated that OA chondrocytes produced levels of MIP-1ß similar to that found in synovial fluid of patients with OA[40, 41] and that ASU were potent inhibitors of this production. Furthermore, and confirming a previous study[27], we have shown that ASU inhibited IL-6 and IL-8 production, two cytokines which are recognized to promote synovium inflammation. All together, these findings contribute to explain the anti-inflammatory action of ASU observed in connective tissue diseases[42]. Indeed, all these cytokines possess pro-inflammatory properties. MIP-1ß is a C-C chemokine that is found to be elevated in the synovial fluid of OA compared to normal or RA patients[40]. This cytokine is primarily active in the recruitment of monocytes/macrophages at the inflammatory site[43]. IL-8 is probably the most potent naturally occurring chemoattractant for poly-

morphs. It also activates polymorphs to release degradative enzymes and reactive oxygen species[44, 45]. In addition, it is a chemotactic for T lymphocytes, which were found to be elevated in the synovial fluid of OA patients[46]. IL-8 increased in a synergic manner the TNFα induced PGE_2 released by human OA synovial fibroblasts[47]. IL-6 is a pleiotropic cytokine that plays important role both in stimulating B-cell differentiation to antibody forming plasma cells and in regulating proliferation and differentiation of T lymphocytes into cytotoxic T cells[48, 49]. Apart from its role in immunity, IL-6 is involved in propagation and chronicity of joint inflammation[50, 51]. Furthermore, these cytokines have been implicated in the local destructive processes that accompany arthritis[52, 53, 54, 55]. Taken together, these data suggest that the inhibitory effect of ASU on IL-6, IL-8 and MIP-1ß synthesis could have beneficial effects on synovitis and cartilage degradation in OA. Furthermore, the inhibition of these cytokines could be responsible for the inhibition of MMP-3 by ASU.

IL-1 plays a key role in cartilage degradation and is found to be elevated in OA tissues. Surprisingly, in our experimental model, IL-1ß increased MMP-3 synthesis during the first three days of incubation, and thereafter failed to increase MMP-3 synthesis. This finding can be related to the effect of IL-1ß on •NO synthesis. Indeed, IL-1ß stimulates •NO production during the first 6 days of incubation, and thereafter this stimulating effect disappears (personal communication). Considering that •NO is a mediator involved in the stimulating effect of IL-1ß on MMP-3 synthesis[56], we can hypothesize that the absence of •NO overproduction during the 6 last days of culture is responsible for the disappearance of the IL-1ß- stimulating effect on MMP-3. ASU partially reversed some of the IL-1ß effects such the inhibition of TIMP-1 synthesis or the stimulation of MMP-3, PGE_2, IL-6, or IL-8 production. These data indicate that ASU can be also efficient in the presence of IL-1 and can counteract some of its deleterious effects. When ASU and IL-1 were added simultaneously, ASU had no significant effect on the IL-1ß-induced inhibition of AGG synthesis, but they promoted AGG synthesis recovery when IL-1ß was withdrawn from the medium. This observation suggests that inflammation relieving before ASU administration could be beneficial for the stimulating action of ASU on cartilage matrix synthesis.

	- IL-1ß	+IL-1ß
AGG	↑	↔
MMP-3	↓	↓
TIMP-1	↔	↑
IL-6	↓	↓
IL-8	↓	↓
MIP-1ß	↓	↔
NO	↓	↔
PGE_2	ND	↓

Table 2 : *Summary of the in vitro effects of A1S2 at the concentration of 10 µg/ml, on human OA chondrocytes cultured in the absence or in the presence of IL-1ß.*

In our knowledge, the effective component(s) in ASU mixture is or are not yet clearly identified. In a previous study, we have demonstrated that A1S2 had a significant more marked effect on IL-6 and IL-8 production than A or S added alone at the concentration in the A1S2 mixture, suggesting that A and S compounds act in synergy[27]. In this work, we have compared the effects of A1S2 administrated at the recommended and currently used *in vitro* concentration of 10 µg/ml, with those of A and S added separately at the concentration found in the mixture. By this way, we have shown that A and S had an additive effect on aggrecan synthesis but acted in synergy to inhibit IL-8 and •NO synthesis. On the other hand, when it was added alone at the concentration found in the mixture, S fraction was more efficient on TIMP-1 production than A1S2 suggesting that A counteracted the effect of S on this parameter. All together, these observations contribute to justify the use of avocado/soybean mixture to treat osteoarthritis even if we are aware that our study fails to demonstrate that the net result of A1S2 treatment on cartilage degradation is superior to that of A or S added alone. Further, the superiority of this formulation to another should be demonstrated *in vivo*.

In conclusion, ASU stimulate aggrecan synthesis and accumulation in extracellular matrix and inhibit stromelysin-1 synthesis by OA chondrocytes, suggesting that ASU may correct the imbalance between catabolic and anabolic processes in OA. Furthermore, ASU counteract some IL-1ß deleterious effects and inhibit pro-inflammatory cytokines synthesis by chondrocytes, indicating that these drugs may also control synovitis in OA (Table 2). These findings lend further support to the rationale behind the use of ASU in osteoarthritis.

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ANNEXE 19

Effects of Rhein on Human Articular Chondrocytes in Alginate Beads

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ABSTRACT

This study was designed to investigate the effects of rhein, the active metabolite of diacerhein, on the metabolic functions of human chondrocytes cultured in alginate beads.

Enzymatically isolated osteoarthritic (OA) chondrocytes were cultured in alginate beads in a well-defined culture medium for 12 days. Rhein was tested in a range of concentrations comprised between 10^{-7} and 4 x 10^{-5} M, in the presence or absence of 10^{-10} M IL-1ß. Interleukin (IL)-6 and -8, macrophage inflammatory protein (MIP-1ß), stromelysin-1 (MMP-3), aggrecan (AGG), tissue inhibitor of metalloproteinases-1 (TIMP-1), prostaglandin E_2 (PGE_2) and nitric oxide (NO) productions were assayed. Cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) mRNA steady-state levels were also quantified. In the basal condition, 10^{-5} M rhein increased by 46.5 % the production of AGG, decreased by 17-30 % the production of IL-6, MMP-3, NO and MIP-1ß but enhanced by 50 % the production of PGE_2. IL-1ß increased IL-6, IL-8, MIP-1ß, NO, PGE_2 and MMP-3 productions, but inhibited AGG and TIMP-1 synthesis. Rhein partially reversed the effect of IL-1ß on TIMP-1 and NO production, had no effect on AGG, IL-6 and MIP-1ß production, but up-regulated the IL-1ß stimulated PGE_2 production. The COX-2 and iNOS mRNA levels and IL-8 production were not modified by rhein.

Overall, these results contribute to explain the clinical efficiency of rhein and give new information on its mechanisms of action.

Key words: Cartilage, Chondrocytes, Cytokines, Osteoarthritis, Rhein.

INTRODUCTION

Osteoarthritis (OA) is a complex disease characterized by bone remodelling, synovium inflammation and cartilage loss. The mechanisms that lead to cartilage degradation primarily involve an excessive production of matrix metalloproteinases (MMPs), including collagenases and stromelysins[1]. Their levels are found to be elevated in the synovial fluid and serum of OA patients. Chondrocytes, but also synovium cells, largely contribute to this enhancement, by secreting high levels of MMPs in response to cytokines, primarily IL-1ß, TNFα and oncostatin M. These cytokines act synergically, not only to enhance pro-MMPs secretion, but also to modulate the activation/inhibition system, leading to an increase of the proteolytic activity in cartilage[2]. Among these cytokines, IL-1ß is considered as the most active in OA. *In vitro*, IL-1ß modifies the normal metabolic functions of chondrocytes, and provokes an imbalance between the catabolic and anabolic events, leading to an excess of cartilage resorption[2, 3].

The main objectives in the management of OA are to reduce symptoms, to minimize functional disability and to limit disease progression. Until now, several drugs used in the treatment of OA have been demonstrated to be symptom-modifying drugs that induce symptomatic relief, but few of these drugs have been investigated for their effects on the structural changes of the tissue. Diacerhein is a symptom-modifying drug, which has been recently reported to possess structure-modifying effects[4]. However, little information is available about the mechanisms of action that sustain these effects. Diacerhein is a low molecular weight heterocyclic compound that is entirely transformed by desacetylation during the absorption process in rhein, the active metabolite found in plasma and synovial fluid[5, 6].

In vitro, short-term treatment (72 hours) with high concentrations of rhein or diacerhein (3.33×10^{-5} and 6.66×10^{-5} M) down regulated, at transcriptional and/or post-transcriptional levels, NO production by human OA chondrocytes in monolayer[7]. Further, rhein upregulated IL-1ß stimulated PGE_2 and COX-2 protein synthesis. After 6 days of incubation, synthesis of glycosaminoglycan (i.e. ^{35}S-suphate incorporation) and collagens (i.e. ^{3}H-proline labelling) by rabbit chondrocytes in monolayer culture was increased by 10^{-6} M diacerhein, whereas collagenolytic activity released into the culture medium was concentration-dependently decreased in the range of 10^{-7} to 10^{-4} M[8]. The positive effect of diacerhein on cartilage matrix formation was explained by its stimulating effect on TGF-ß1 and TGF-ß2 gene expression and by its inhibiting action on IL-1ß secretion[9-14].

In an accelerated canine model of OA in which sensory input from the ipsilateral hind limb was interrupted by L4-S1 dorsal root ganglionectomy prior to anterior cruciate ligament (ACL) transection, diacerhein (15-20 mg/kg once daily for 8 weeks) did not significantly modify the severity of cartilage lesions, the collagenolytic activity and the proteoglycan level contained in OA cartilage[15]. In a less rapidly progressive canine model, in which OA was induced by ACL only, diacerhein (40 mg/kg for 8 months) has been shown to slow down the progression of cartilage

lesions as estimated by an arthroscopic grading scale[16]. However, 32 weeks after ACL transection, the mean proteoglycan concentration of the OA cartilage and the level of collagenolytic activity in cartilage extracts were not significantly different in the diacerhein group compared to the placebo. In an ovine model of OA induced by menisectomy, diacerhein (25 to 50 mg/kg daily for 9 months) failed to significantly improve the gross morphological and histological severity of cartilage lesions[17].

In several randomised, double blind, placebo-controlled clinical trials of 2-6 duration months, diacerhein significantly reduced the pain and functional impairment in patients with hip or knee OA[18-22]. Recently, a three-year, placebo-controlled trial (ECHODIAH) including 507 patients with primary OA of the hip, has demonstrated that the occurrence of radiographic progression (i.e. joint space narrowing measured by a graduated magnifying lens) of at least 0.5 mm during the study was significantly lower and occurred later in the diacerhein (100 mg daily) group as compared with the placebo group[4].

For the first time, this study investigated the 12 days effect of rhein on the metabolism of chondrocytes cultured in alginate beads in which, chondrocytes keep their phenotype stable overall the culture duration. This work provided new mechanisms of action of rhein, and contributed to explain the clinical observations.

MATERIALS AND METHODS

Chondrocytes culture in alginate beads

Cartilage was obtained from the knees (femoral condyles, tibial plateau and patellar cap) of cadavers immediately after death being excised from the superficial and medium layers of cartilage and avoiding the calcified layer. The experiments were performed with osteoarthritic chondrocytes isolated from cartilage specimens coming from six different donors (two males and four females) with a mean age of 48 (38-59) years old.

Cartilage was cut into small fragments and then subjected to sequential enzymatic digestions with hyaluronidase, pronase and collagenase as described previously[23]. The cells were then filtered through a nylon mesh with a pore diameter of 70 mm, and then washed three times with sterile water 0.9 % NaCl. Cells viability was estimated by trypan blue exclusion test and in all cases was superior to 95%. Chondrocytes were suspended in 1.2 % low viscosity alginate (Sigma-Aldrich) in 0.155 M NaCl solution at a density of 4 x 10^6 cells/ml, which was slowly passed through a 25 gauges needle in a dropwise fashion into a 102 mM $CaCl_2$ solution (Sigma-Aldrich). After instantaneous gelation, the beads were allowed to polymerise further for 10 min in this solution. Thereafter, they were washed with a saline solution and nine beads containing each approximately 50,000 cells were cultured in 1 ml of culture medium per well in a 24-well plate. Culture medium was DMEM supplemented with 1 % ITS+ (ICN Biomedicals), 10 mM HEPES (Biowhittaker Europe), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Biowhittaker Europe, Verviers, Belgium), 200 µg/ml glutamine (Biowhittaker Europe), 50 µg/ml

ascorbic acid (Sigma-Aldrich), and 2 mM proline (Invitrogen). ITS+ is a premixed cell growth system containing in 1 ml: 0.625 mg insulin, 0.625 mg transferrin, 0.625 µg selenious acid, 0.125 g bovine serum albumin (BSA) and 0.535 mg linoleic acid. Cells were maintained in this culture medium (wash-out medium) for 48h as a precaution against *in vivo* contamination with drugs that donors might have taken before death. After this wash-out period, culture medium was changed and the drugs added. Culture medium was then changed every three days and the collected supernatants were kept at -20°C until analysis. Chondrocytes were cultured in alginate beads for a period of 12 days following the washout.

At the end of the culture, alginate beads were dissolved in 1 ml 0.1 M citrate for 10 min, and centrifuged at 1200 g for 10 min. The supernatant containing macromolecules originated from the further-removed matrix (FRM) compartment, and the pellet, containing cells with their associated matrix (CM), were then collected and separately analysed. These two fractions were studied separately. The cell pellets were washed with PBS (Biowhittaker Europe, Verviers, Belgium), and then homogenized in 1 ml of PBS by ultrasonic dissociation (3 pulses of 10 s, 50 watts/cm^2) at 4°C for DNA assay. CM and FRM were kept at -20°C until analysis.

Treatments

Chondrocytes were cultured for 12 days in the absence or in presence of 10^{-10} M human IL-1ß (Roche Pharmaceuticals) and with or without rhein (Negma Laboratories). Fresh IL-1ß and rhein were added at each medium change every 3 days. Rhein was tested at concentrations ranging among 10^{-7} M to 4 x 10^{-5} M, was first dissolved in NaOH 0.1 M, and then diluted in culture medium to achieve the required final concentration. After an oral administration of diacerhein in healthy volunteers, the peak concentration (C_{max}) of rhein found in the plasma was 10^{-5} M. Following daily administration of oral diacerhein 50 mg every 12 hr for a month, a pilot study conducted in three arthritic patients revealed that rhein could reach the synovial fluid at concentrations in a range (10^{-6} to 10^{-5} M) very close to that observed in plasma[5, 6]. Chondrocytes coming from 6 different donors have been included. Four out the six have been used for the analysis of AGG, cytokines, PGE_2, MMP-3, TIMP-1 and NO synthesis and the two other for the measurement of COX-2 and iNOS mRNA levels. Three wells were used for each concentration of the drug and for the corresponding controls.

DNA assay

The DNA content was measured using a fluorimetric method[24]. This measurement ensures elimination of result variations caused by the different number of chondrocytes in each culture.

LDH release assay

The cell viability was estimated by the ratio of lactate deshydrogenase (LDH) released in the culture supernatant. In a 96-wells plate, 50 µl of tris 0.01 M buffer,

pH 8.5, containing 0.1 % BSA and lactate 800 mM and 50 µl of a solution 1.6 mg/ml INT (p-iodonitrotetrazolium violet, Sigma-Aldrich, Bornem, Belgium), 4 mg/ml NAD (Roche pharmaceuticals, Brussels, Belgium) and 0.4 mg/ml phenazine methosulfate (Sigma-Aldrich, Bornem, Belgium) were added to 100 µl of culture supernatant, FRM or CM (before any freezing). The absorbance at 492 nm was red after 5 min of incubation at room temperature. The calibration was performed with LDH isolated from rabbit muscle. The cells death score was obtained by calculating the ratio: 100 - (100 / (LDH CM + FRM + supernatant) x LDH CM).

Quantitative Real-time RT PCR

RNA was isolated by the Promega SV Total RNA Isolation System, after 72h of treatment. Cells (3 x 10^6) were lysed and the samples were processed following the manufacturer's protocol. RNA (2 µg) was reverse transcribed in a volume of 30 µl containing 1X RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT), 10 mM DTT, 1 mM dNTPs, 500 ng oligo$(dT)_{15}$ primer, 140 U M-MLV Reverse Transcriptase and 40 U RNaseOUT (Invitrogen, Merelbeke, Belgium) for 60 min at 37 °C. The reaction was halted by incubation at 95 °C for 5 min and by adding 100 µl of H_2O.

PCR reaction was performed using the LightCycler-FastStart DNA master Sybr Green I (Roche Diagnostics, Brussels, Belgium). This reaction mixture was provided as a 10-fold stock solution containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix, 10 mM $MgCl_2$ and SYBR Green I dye. For each LightCycler PCR a mastermix of the following reaction components was prepared in a final volume of 18 µl: 2 µl LightCycler-FastStart DNA master Sybr Green I, 1.6 µl of 25 mM MgCl2, 0.5 µM sense and antisense primers (GADPH sense TTGGTATCGTGGAAGGACTCA, antisense TGTCATCATATTTGGCAGGTTT; iNOS sense CCATGGAACATCCCAAATAC, antisense TCTGCATGTACTTCATGAAGG; COX-2 sense TTCAAATGAGATTGTGGGAAAA, antisense AGATCATCTCTGCCTGAGTATCTT).

The mastermix was loaded into glass capillary (Roche Diagnostics, Brussels, Belgium) and 2 µl of PCR template was added. The template source was either 3 ng first-strand cDNA or purified DNA standard.

Amplification was performed with a spectrofluorometric thermal cycler (LightCycler, Roche Diagnostics, Brussels, Belgium). After an initial denaturation step at 95 °C for 10 min, amplification was performed using 40 cycles of denaturation (95 °C for 15 s), annealing (temperature gradient: 68 °C to 58 °C with a decrement of 0.5 °C per cycle during 20 cycles, continued by 20 cycles at 58 °C, for 5 s) and extension (72 °C for 15 s). For each run, a standard curve was generated from purified DNA ranging from 10^6 to 10 copies of the cDNA of interest. To standardize mRNA levels, we amplified glyceraldehyde-3-phosphate deshydrogenase (GAPDH), a house-keeping gene as internal control. Normalized gene expression was calculated as the ratio between sample and GAPDH cDNA copy number.

After amplification, a final melting curve was recorded by cooling the PCR mixture to 65 °C for 30 s and then slowly heating it to 95 °C at 0.1 °C/s. Fluorescence

was measured continuously during the slow temperature rise to monitor the dissociation of the double strands DNA. Specificity of the expected COX-2 and iNOS products was demonstrated by melting curves analysis. Amplification products performed in the LightCycler were checked by electrophoresis on 1.5 % ethidium bromide stained agarose gel. The estimated size of the amplified fragments matched the calculated size.

Prostaglandin (PG) E_2 radioimmunoassay

PGE_2 was assayed in the different compartments (culture supernatants, FRM and CM) without previous extraction according to a previously described radioimmunoassay[25]. In this assay, polyclonal antiserum obtained from rabbit does not cross-reacts with others prostanoids (TxB_2, 6-keto-PGF1α, PGA_2) or fatty acids (arachidonic, linoleic or oleic acids). ^{3}H-labeled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule (PGE_2) from Sigma-Aldrich (Bornem, Belgium). Intra and interassay coefficients of variation were 6 and 10 %, respectively. The limit of detection of the RIA was 20 pg/ml.

Immunoassays for AGG, IL-6, IL-8, MIP-1ß, MMP-3 and TIMP-1

Aggrecans (AGG), Interleukin (IL)-6, -8, macrophage inflammatory protein (MIP-1ß), tissue inhibitor of metalloproteinases-1 (TIMP-1) and stromelysin-1 (MMP-3) were measured in the different culture compartments (culture supernatants, FRM and CM), by specific EASIA (Enzyme Amplified Sensitivity ImmunoAssays, Biosource Europe, Fleurus, Belgium). The assays were based on the oligonal system in which several monoclonal antibodies were directed against distinct epitopes of the molecules. In the case of MMP-3, the antibodies were directed against pro-MMP-3, activated-MMP-3 and MMP-3 bound to TIMP-1 and TIMP-2. The intra and interassay coefficients of variation were less than 5 % for all immunoassays.

Nitric oxide (•NO) assay

Nitrite and nitrate are stable end products of nitric oxide. Nitrate is reduced to nitrite by the addition in the supernatant of nitrate reductase (0.25 U/ml, Roche pharmaceuticals, Brussels, Belgium) for 20 min at 37 °C. Nitrite concentrations in conditioned culture supernatants were determined by a spectrophotometric method based upon the Griess reaction[26]. The absorption was measured at 540 nm. Sodium nitrite ($NaNO_2$) was used for calibration.

Calculation and statistical analysis

The results (mean ± SEM) were expressed as the concentration of AGG, MMP-3, TIMP-1, IL-6, IL-8, MIP-1ß, NO and PGE_2 in the culture supernatants, CM and FRM per microgram of DNA. The total production was obtained by the addition of the quantities found in the culture supernatant, in the CM and the FRM. The total quantities found in the CM and the FRM correspond to the cumulative amounts over 12 days. Mean values were compared for each culture condition using

the U-test of Mann-Whitney with a limit of significance at $p < 0.05$. The concentration-dependency was demonstrated by calculating the Spearman's correlation coefficient (r).

RESULTS

Effects of rhein on DNA content and LDH release

The content of DNA (about 3.5 µg per well) increased by 38 % over the culture duration and was not affected by the presence of IL-1ß (10^{-10} M) or rhein (10^{-7} to 2 x 10^{-5} M) (Table 1).

Table 1 : Content of DNA (µg) per well after a 12 days culture period, in presence or not of 10^{-10} M IL-1ß.

	Basal	*+ IL-1ß*
Control	3.78 +/- 0.46	3.16 +/- 0.60
10^{-7} M rhein	3.61 +/- 0.37	3.12 +/- 0.34
3.33 x 10^{-7} M rhein	3.63 +/- 0.22	3.34 +/- 0.17
10^{-6} M rhein	3.58 +/- 0.19	3.65 +/- 0.50
3.33 x 10^{-6} M rhein	3.72 +/- 0.34	3.11 +/- 0.12
10^{-5} M rhein	3.70 +/- 0.10	3.08 +/- 0.22
2 x 10^{-5} M rhein	3.45 +/- 0.12	2.67 +/- 0.48
4 x 10^{-5} M rhein	3.17 +/- 0.23	2.23 +/- 0.07 *

* = $p < 0.05$.

In the basal condition and in the presence of 10^{-10} M IL-1ß, the cell death score, estimated by the cumulated amount of LDH released from the cells during 12 days of culture, was below 3 %. At concentrations ranging between 10^{-7} and 10^{-5} M, rhein did not significantly affect the cell death score or the DNA content (Fig.1). In the presence of IL-1ß, 10^{-5} M of rhein slightly increased cell death score, but this score remained below 10 %. However, at concentrations superior to 10^{-5} M, rhein showed a cytotoxicity characterized by a concentration-dependent release of LDH ($r = 0.985$, $p < 0.0001$ without IL-1ß; $r = 0.993$, $p < 0.0001$ with IL-1ß). A decrease of DNA content (Fig. 1 and Table 1) is also observed at the concentration of 4 x 10^{-5} M of rhein.

Effects of rhein on AGG production

In alginate culture model, AGG production was primarily immobilized in the alginate matrix. Seventy percent of the newly synthesized AGG were found in the FRM, 28 % in the CM and only 2 % in the supernatant. After 12 days of culture, rhein at the plasmatic concentration (Cmax; 10^{-5} M) enhanced by 46.5 % the total AGG production. This effect was concentration-dependent in the range of concentrations comprised between 10^{-7} and 3.33 x 10^{-6} M ($r = 0.94$, $p = 0.0165$), reached a maximum at 3.33 x 10^{-6} M, and then slightly decreased at the highest concentration (Fig. 2A). The effect of rhein was primarily observed in the FRM compartment (Fig.

2B). IL-1ß drastically depressed AGG production (basal production 5,877 +/- 317 ng/µg DNA vs IL-1 treated group 939 +/- 46 ng/µg DNA). Rhein had no significant effect on the IL-1ß inhibitory effect whatever the concentration tested (data not shown).

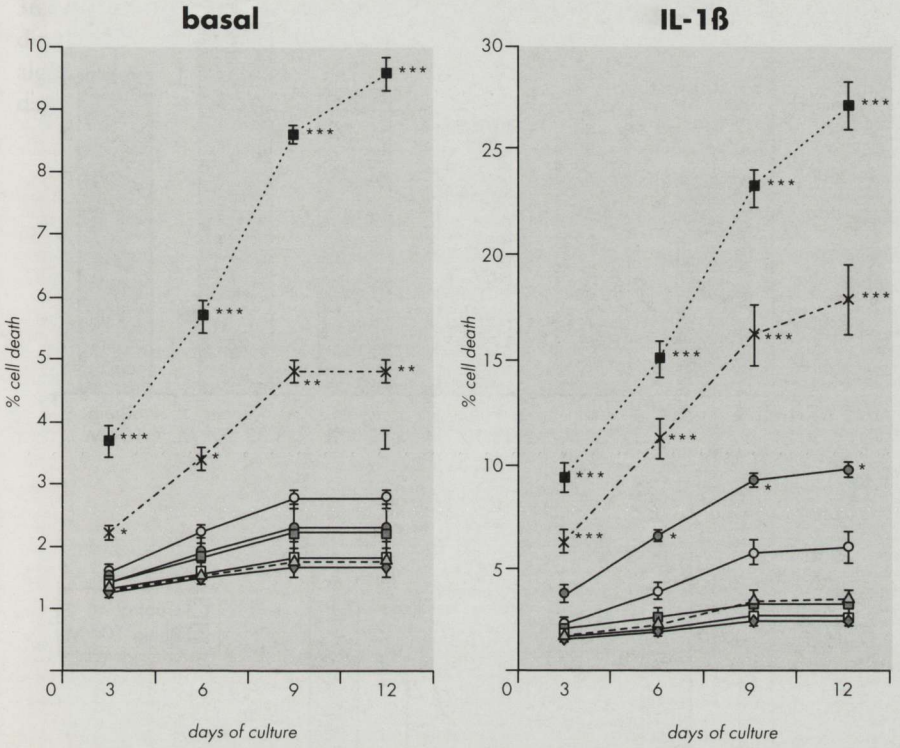


Fig. 1: *Cumulative percentage (sum of the number of cell death at the end of a 3-day culture period) of cell death in alginate chondrocyte culture maintained for 12 days in the absence or in the presence of 10^{-10} M IL-1ß and with or without increased concentrations of rhein (◆ = control, ■ = rhein 4 x 10^{-5} M, × = 2 x 10^{-5} M, ● = rhein 10^{-5} M, ○ = rhein 3.33 x 10^{-6} M, △ = rhein 10^{-6} M, ● = 3.33 x 10^{-7} M, □ = rhein 10^{-7} M). Results are represented by the means ± SEM of four independent cultures performed with chondrocytes coming from different donors. *p<0.05, **p<0.01 and *** p<0.001.*

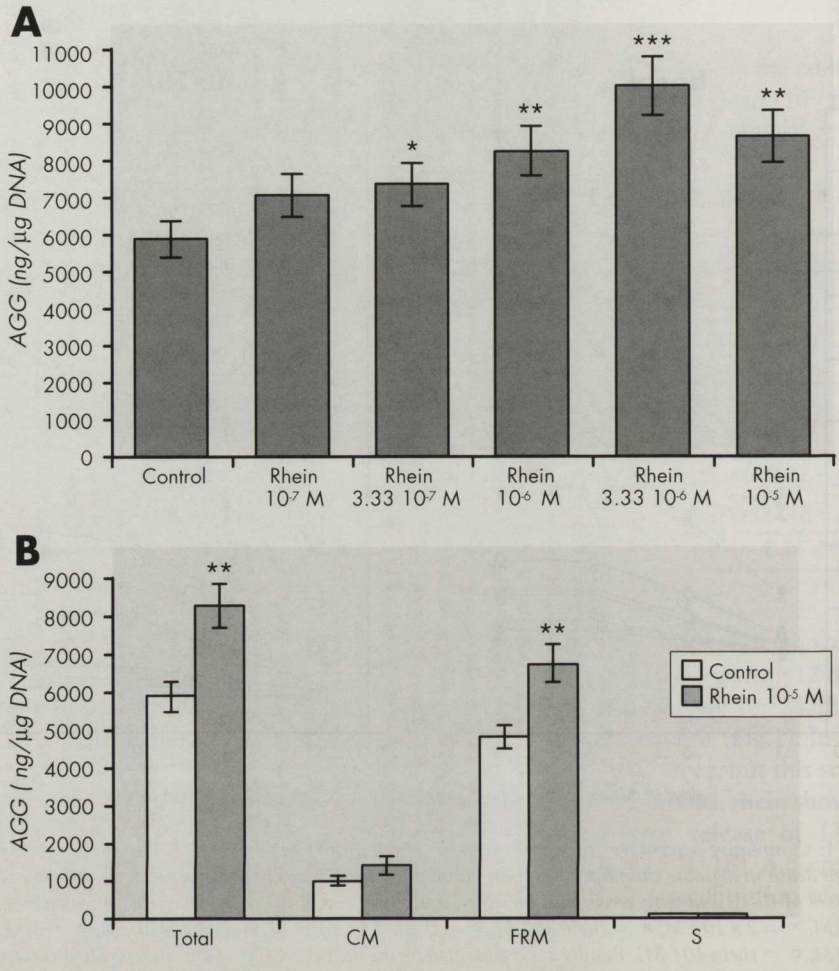


Fig. 2: *(A) Effect of rhein on the total AGG production at the end of 12 days of culture under basal condition (in the absence of IL-1ß). (B) Effect of 10^{-5} M rhein on the amount of AGG accumulated in the supernatant (S), the pericellular (CM) and further removed (FRM) matrix after 12 days of culture in alginate beads. Results are expressed as the percentage of the control, and represented by the means ± SEM of four independent cultures performed with chondrocytes coming from different donors. Here ** indicate $p<0.01$.*

Effects of rhein on TIMP-1 and MMP-3 production

In the basal condition, human chondrocytes released 225 ± 3.5 ng/µg DNA/12 days of TIMP-1. When 10^{-10} M IL-1ß was added to the culture medium, TIMP-1 production was reduced by 83 %. At the concentrations tested, rhein did not modify the basal TIMP-1 production, but partially reversed the IL-1ß inhibitory effect. In the presence of rhein 10^{-5} M, TIMP-1 production by IL-1 treated chondrocytes was doubled. The basal production of MMP-3 by human OA chondrocytes was 6,349 ± 362 ng/µg DNA/12 days. At 10^{-5} M, rhein reduced by 17 % the basal MMP-3 production. No significant effect was observed at the lowest concentrations of rhein. In alginate beads model, IL-1ß increased MMP-3 production only during the first 3 days of treatment (551 +/- 16 ng/µg DNA in basal and 1,270 +/- 126 ng/µg DNA

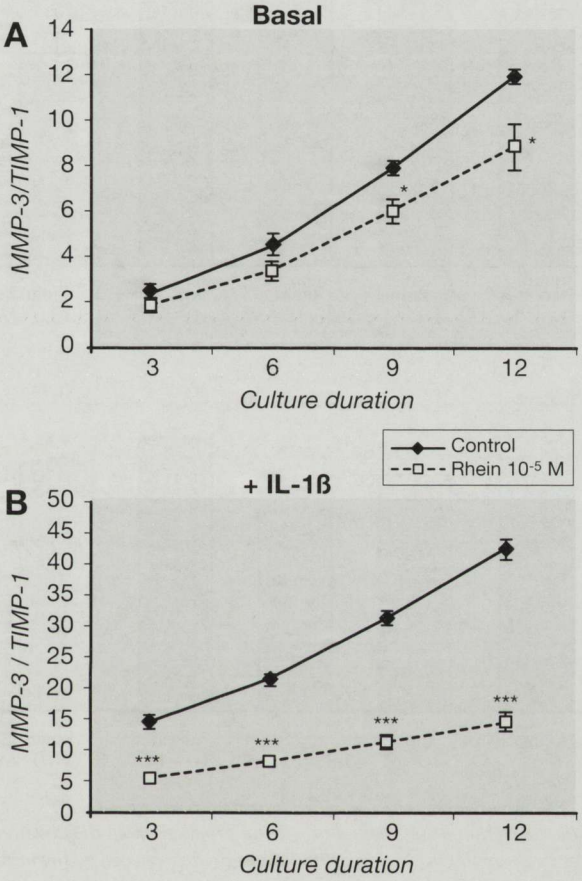


Fig. 3: *Effect of 10^{-5} M rhein on MMP-3/TIMP-1 ratio obtained after 3, 6, 9 or 12 days of culture in the absence (A) or in the presence (B) of 10^{-10} M IL-1ß. The MMP-3/TIMP-1 ratios were calculated on the base of cumulated amounts of MMP-3 and TIMP-1 produced by human chondrocytes after 3, 6, 9 or 12 days of culture in alginate beads. The values are the means and SEM of four independent cultures performed with chondrocytes coming from different donors. Here * indicates $p<0.05$ and *** indicate $p<0.001$*

with IL-1ß), and after the IL-1ß-stimulating effect disappeared. At day 3, rhein 10^{-5} M significantly decreased this IL-1ß-simulated production by 34 % ($p<0.01$). Consequently, the presence of IL-1ß profoundly increased the MMP-3/TIMP-1 ratio (43.5 compared to 12 in basal conditions, Fig. 3). Rhein (10^{-5} M) significantly decreased the MMP-3/TIMP-1 ratio in the basal conditions and in the presence of IL-1ß (Fig. 3).

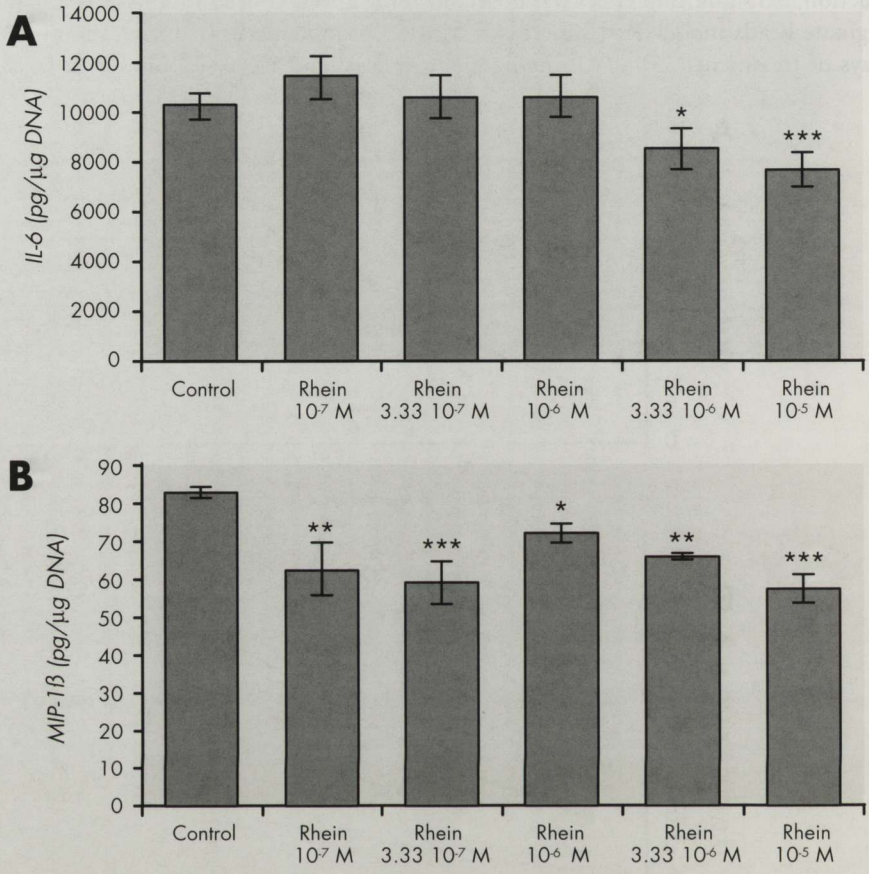


Fig. 4: . *Effects of increase amounts of rhein on the basal production (in the absence of IL-1ß) of IL-6 (A) and MIP-1ß (B) by human OA chondrocytes. Results are represented by the means ± SEM of four independent cultures performed with chondrocytes coming from different donors. Here* indicates $p<0.05$, ** indicate $p<0.01$ and *** indicate $p<0.001$.*

Effects of rhein on IL-6, IL-8 and MIP-1ß production

In the basal condition, OA chondrocytes constitutively produced in average 10.3 ± 0.5 ng/µg DNA of IL-6, 26.7 ± ng/µg DNA of IL-8 and 81.4 ± 1.4 ng/µg DNA of MIP-1ß. Most of the newly synthesized IL-6 and MIP-1ß (96.7 % for IL-6, and 96.3 % for MIP-1ß) was released in the culture supernatant whereas, 23 % of the IL-8 produced was retained in the alginate beads. IL-1ß stimulated IL-6, IL-8 and MIP-1ß production by 13, 11 and 39 times, respectively. In the basal conditions, rhein significantly decreased IL-6 production at the concentration of 3.33×10^{-6} and 10^{-5} M ($r = -0.91$, $p = 0.0015$) (Fig. 4A) but had no effect on IL-8. MIP-1ß production was also inhibited by rhein at concentrations ranging between 10^{-7} to 10^{-5} M (Fig. 4B). In the presence of IL-1ß, rhein did not significantly modified IL-6, IL-8 and MIP-1ß production.

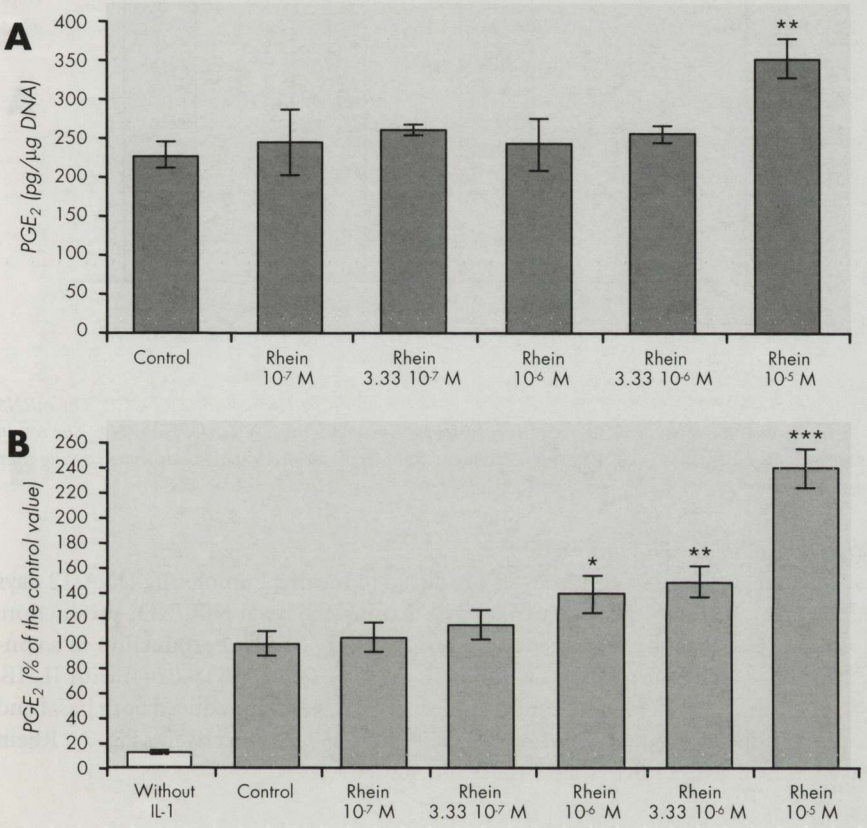


Fig. 5: *Effect of rhein on the total production of PGE_2 by human OA chondrocytes cultured for 12 days in alginate beads with (B) or without (A) 10^{-10} M IL-1ß. The results are represented by means ± SEM of four independent cultures performed with chondrocytes coming from different donors. Here * indicates $p<0.05$, ** indicate $p<0.01$ and *** indicate $p<0.001$.*

Effects of rhein on PGE_2 production

In the basal condition, PGE_2 was produced in low levels (227 ± 16.9 pg/µg DNA), and was all released in the culture supernatants. This production was significantly enhanced by rhein at the concentration of 10^{-5} M (Fig. 5A). The stimulating effect of rhein on PGE_2 production was more marked and concentration-dependent in the presence of 10^{-10} M IL-1ß ($r = 0.984$, $p = 0.0004$). At 10^{-5} M, rhein increased by three times the IL-1ß stimulated PGE_2 production (Fig. 5B) but did not modulate COX-2 mRNA level (Fig. 6) suggesting that rhein regulated PGE_2 production at a post-transcriptional level.

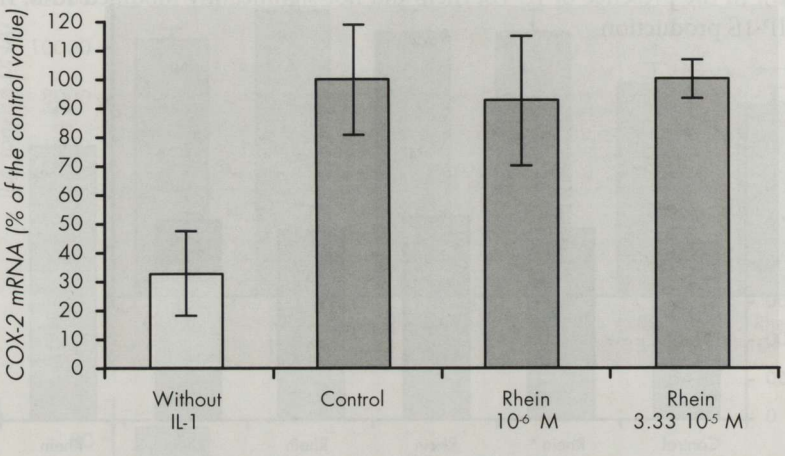


Fig. 6: *Effect of rhein on IL-1ß stimulated COX-2 mRNA quantified by real time RT-PCR. The mRNA copy numbers were normalized against the corresponding copy number of GADPH mRNA. The results are represented by means ± SD of two independent cultures performed with chondrocytes coming from different donors.*

Effects of rhein on •NO production

In the basal condition, chondrocytes produced in mean 32 nmoles/µg DNA/12 days of NO_2/NO_3. IL-1ß (10^{-10} M) increased by 3 times the basal NO_2/NO_3 production. In the absence or in the presence of IL-1, rhein decreased NO production in a concentration-dependent manner (without IL-1ß, $r = -0.972$, $p = 0.0244$; with IL-1ß, $r = -0.966$, $p = 0.0017$). At the concentration of 10^{-5} M, rhein reduced both basal and IL-1ß stimulated production of NO by 50 % and 45 %, respectively (Fig. 7). Rhein did not affect iNOS mRNA level (data not shown).

DISCUSSION

Until now, *in vitro* observations were limited to the 3 first days of treatment with rhein using human or animal chondrocytes cultured in monolayer. In this work, chondrocytes were cultured in alginate beads in order to keep stable their phenotype, for at least 12 days of culture[27]. The alginate bead model is highly recommended for studying cartilage matrix formation, because it allows the separate analysis of the pericellular and the interterritorial matrix (or further removed matrix). Interestingly, we have demonstrated that rhein strongly increased in a concentration-dependent manner the amount of aggrecan contained in the alginate bead, supporting the concept that rhein could promote cartilage matrix formation. As previously suggested by Felisaz and collaborators[9], this stimulating effect of rhein on aggrecan synthesis could be secondary to the over expression of TGF-ß1 and -ß2 by chondrocytes themselves. *In vitro*, TGF-ßs enhance the expression of type II collagen and aggrecan by isolated chondrocytes as well as by cartilage

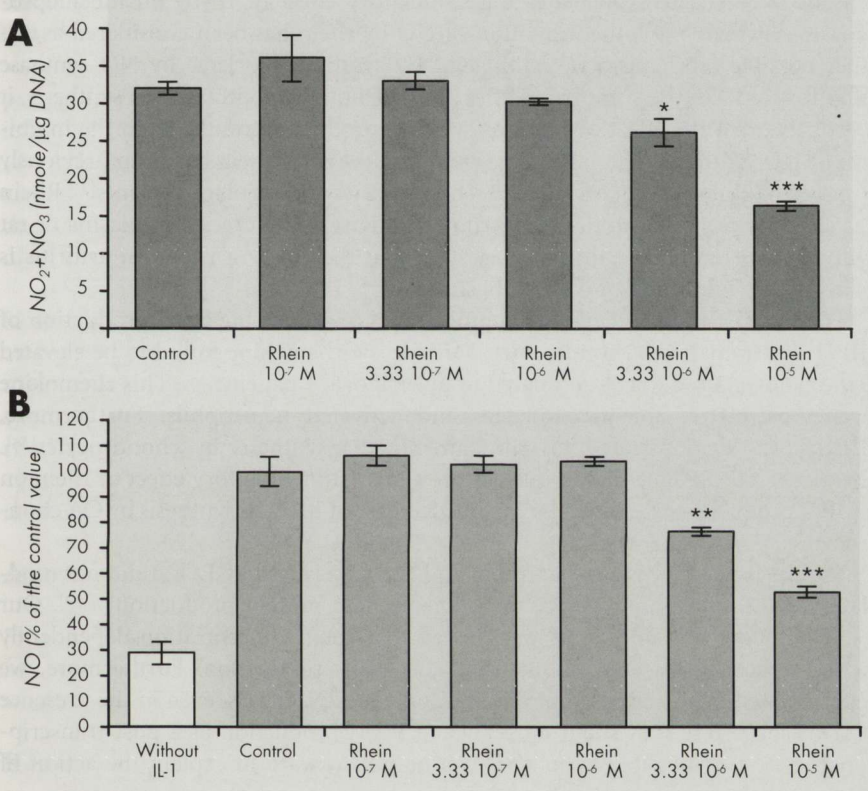


Fig. 7: *Effect of rhein on the total production of NO_2/NO_3 by human OA chondrocytes cultured for 12 days in alginate beads with (B) or without (A) 10^{-10} M IL-1ß. The results are represented by means ± SEM of four independent cultures performed with chondrocytes coming from different donors. Here * indicates $p<0.05$, ** indicate $p<0.01$ and *** indicate $p<0.001$.*

explants[28, 29]. In parallel, rhein depressed MMP-3 synthesis and increased TIMP-1 production by IL-1ß treated chondrocytes suggesting that rhein could counteract some of the IL-1 effects on cartilage catabolism. These results corroborate the Tamura's study demonstrating that rhein suppressed the IL-1α-induced production of proMMPs-1, -3, -9 and -13 but increased TIMP-1 synthesis by rabbit chondrocytes in monolayer[30]. In parallel, Tamura and collaborators have shown that rhein decreased the total activity of MMPs in the culture supernatants and reduced proteoglycan degradation[31]. Taken together, these observations suggest that rhein might have a protective effect on proteoglycan degradation via the suppressive effect of rhein on the production and on the activity of proMMPs. This duality leads to a strong decrease of the MMP-3/TIMP-1 ratio showing that rhein can also counteract catabolic events. The antagonism of rhein towards IL-1 can be explained by its capacity to decrease the number of IL-1 receptors on OA chondrocytes[12]. Nevertheless, rhein was not efficient against the overall IL-1 effects suggesting that other more specific regulatory features are involved in the rhein activity. Since it was demonstrated that NO mediates the stimulatory effect of IL-1b on metalloproteinases synthesis[32, 33], the inhibition of NO by rhein has been considered as another possible mechanism of action. As NO synthesis blockage by NO synthase inhibitors reduces the stimulating effect of IL-1 on metalloproteinases synthesis, it is probable that the effect of rhein on MMP-3 production results from the inhibition of NO synthesis. The inhibition of NO synthesis by rhein has been previously observed by Pelletier et al on both OA chondrocytes and cartilage explants[7]. Rhein was also demonstrated to decrease arthritis activity and NO level in plasma of rat with adjuvant-induced arthritis, suggesting that the effect of rhein on arthritis is related to the inhibition of NO synthesis[34].

For the first time, a drug was demonstrated to reduce the basal production of MIP-1ß by human OA chondrocytes. MIP-1b is a chemokine found to be elevated in the synovial fluid of OA compared to normal or RA patients[35]. This chemokine is chemoattractive for macrophages and activated neutrophils. Furthermore, MIP-1ß was demonstrated to stimulate MMP-3 synthesis by chondrocytes[36]. Therefore, it is possible that in our culture system, the inhibitory effect of rhein on MMP-3 production was secondary to the decrease of MIP-1ß synthesis by OA chondrocytes.

Studies have shown that diacerhein and rhein increased PGE_2 but did not modulate COX-2, phospholipase A_2, or 5-lipoxygenase protein production[37-39]. Our results support this finding as we showed that rhein concentration-dependently increased both basal and IL-1ß-stimulated PGE_2 production. Furthermore, we demonstrated that rhein did not modify COX-2 mRNA levels, even in the presence of IL-1, suggesting that rhein up-regulated PGE_2 production at a post-transcriptional level. Two possible hypothesis can be put forward to explain the action of rhein on PGE_2 synthesis:

1. Rhein acts as an antioxidant and this prolongs the cyclooxygenase lifetime. Some arguments support this hypothesis. Firstly, rhein structure possesses scavenger groups, such as phenolic function, for reactive oxygen species. Secondly, COX-2 is an endoperoxide synthase forming a tyrosyl radical, which regulates its activity. To

scavenge this radical could prolong its activity and then increase the PGE_2 production.

2. We have reported that rhein is a potent inhibitor of NO. NO was demonstrated to down-regulate PGE_2 synthesis by chondrocytes. Therefore, the overproduction of PGE_2 could be secondary to the inhibition of NO synthesis[40].

It is of note that, in contrast and as previously reported[27], nonsteroidal anti-inflammatory drugs (NSAIDs) fully block PGE_2 synthesis by chondrocytes cultured in similar conditions. This finding clearly indicates that these two classes of drugs act by different pathways on pain and inflammation in OA. Nevertheless, some questions remain on the clinical impact of PGE_2 over expression. It is now well accepted that the release of high levels of PGE_2 at the inflammatory site contributes to inflammatory pain. Nevertheless, rhein has been shown to relieve chronic pain in knee and hip OA[18-22]. On the other hand, PGE_2 could act as a regulator of the inflammatory reaction, by decreasing IL-1 secretion and activity[41]. Furthermore, PGE_2 has been reported to enhance proteoglycans and collagen type II synthesis, by stimulating IGF-1 release by chondrocytes[42, 43], and so could exert cartilage protective properties.

Finally, we have observed that rhein at concentrations over 10^{-5} M and in the presence of a high concentration of IL-1ß, induced cytotoxicity. Never, this cytotoxicity has never been reported in humans. One explanation could be that rhein *in vivo* is metabolized to glucurono and sulfo-conjugate[44]. Furthermore, the cytotoxic effect reported herein, has been observed at concentrations of rhein superior to those found in the synovial fluid of arthritic patients after daily administration of diacerein 50 mg.

From this work, we can conclude that rhein may act on different biochemical factors involved in OA pathophysiology. This drug stimulates aggrecan production and promotes matrix formation, decreases the production of some pro-inflammatory mediators and correct the MMPs/TIMPs imbalance by stimulating TIMP-1 synthesis and decreasing MMP-3 production by OA chondrocytes. Furthermore, rhein counteracts some of the deleterious effects of IL-1ß, one of the most active cytokine in OA. Overall, these results contribute to explain the clinical efficiency of rhein[18-22] and give new informations on its mechanisms of action.

Acknowledgments

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ANNEXE 20

Strontium ranelate increases cartilage matrix formation

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Abstract

Based on previous studies showing that strontium ranelate (S12911) modulates bone loss in osteoporosis, it could be hypothesized that this drug also is effective on cartilage degradation in osteoarthritis (OA). This was investigated *in vitro* on normal and OA human chondrocytes treated or not treated with interleukin-1 (IL-1β). This model mimicks *in vitro* the imbalance between chondroformation and chondroresorption processes observed *in vivo* in OA cartilage. Chondrocytes were isolated from cartilage by enzymatic digestion and cultured for 24-72h with 10^{-4}-10^{-3} M strontium ranelate, 10^{-3} M calcium ranelate, or $2{\cdot}10^{-3}$ M $SrCl_2$ with or without IL-1β or insulin-like growth factor-I (IGF-I). Stromelysin activity and stromelysin quantitation were assayed by spectrofluorometry and enzyme amplified sensitivity immunoassay (EASIA), respectively. Proteoglycans (PG) were quantified using a radioimmunoassay. Newly synthesized glycosaminoglycans (GAG) were quantified by labeled sulfate ($Na_2{}^{35}SO_4$) incorporation. This method allowed the PG size after exclusion chromatography to be determined. Strontium ranelate, calcium ranelate, and $SrCl_2$ did not modify stromelysin synthesis even in the presence of IL-1β. Calcium ranelate induced stromelysin activation whereas strontium compounds were ineffective. Strontium ranelate and $SrCl_2$ both strongly stimulated PG production suggesting an ionic effect of strontium independent of the organic moiety. Moreover, 10^{-3} M strontium ranelate increased the stimulatory effect of IGF-I (10^{-9} M) on PG synthesis but did not reverse the inhibitory effect of IL-1β. Strontium ranelate strongly stimulates human cartilage matrix formation *in vitro* by a direct ionic effect without stimulating the chondroresorption processes. This finding provides a preclinical basis for *in vivo* testing of strontium ranelate in OA.

Key words: strontium ranelate, chondrocytes, proteoglycans, stromelysin, osteoarthritis

Introduction

Cartilage is constantly being turned over in the balance between extracellular matrix synthesis and degradation. Chondroformation is stimulated by growth factors such as insulin-like growth factor-I (IGF-I).[1] Chondroresorption, which involves metalloproteinases like stromelysin,[2, 3] is stimulated by cytokines such as interleukin-1β (IL-1β). [4] In osteoarthritis, this delicate equilibrium is progressively disrupted leading to an excess of tissue degradation and, finally, tissue disappearance. The imbalance between stromelysin and proteoglycans (PG_2) constitutes an important pathophysiological process. The levels and activity of stromelysin were found to be high in OA cartilage, synovia, and synovial fluid,[5-7] whereas PG synthesis and content decreased with lesion severity.[8] The metabolic disruption is characterized by PG release, fixed charge density, and cation concentration decrease.[9] As the rate of PG synthesis decreases when the ionic strength is below the physiological strength, cation administration could have beneficial effects on OA chondrocyte metabolism.[10, 11] Strontium, a divalent cation, has shown evidence of beneficial effects on bone metabolism [12] and interference in cartilage metabolism.[13] Strontium ranelate (S12911), an uncoupling agent developed as a preventive and curative treatment of postmenopausal osteoporosis,[12, 14-16] prevents bone loss induced by estrogen deficiency by inhibiting bone resorption and stimulating bone formation.[17]

The present study investigated the effects of strontium ranelate on stromelysin and PG synthesis in human chondrocytes cultured with or without IL-1β or IGF-I. In chondrocyte culture, IL-1β reproduces the decoupling effect leading to a decrease in PG and an increase in stromelysin synthesis [4, 18] whereas IGF-I stimulates PG production.[1] The effects of strontium ranelate were compared with the effects of calcium ranelate and strontium chloride ($SrCl_2$), which were used as controls.

Materials and Methods

Human chondrocyte culture

Freshly isolated human cartilage chondrocytes were cultured at a high density for a short-term period (24 to 72 h) in order to retain their phenotype. Cartilage samples were obtained from the knee of 13 cadavers (23-83 years old) immediately after death. They were excised from the superficial and medium layer of cartilage, avoiding the calcified layer. Pathological cartilage modifications were evaluated on femoral and patellar articular surfaces using the Moskowitz scale. [8] Each culture was run with chondrocytes from a single patient. The cartilage was cut into small fragments. Chondrocytes were isolated from their extracellular matrix by enzymatic digestion. Cartilage fragments (3 g) were first treated (30 minutes, 37 °C, 200 rpm) with 10 ml hyaluronidase solution (Sigma Chemie, Bornem, Belgium) previously dissolved (0.5 mg/ml) in Dubbelcco's Modified Eagle's Medium (DMEM, Biowhittaker, Brussels, Belgium). Cartilage fragments were then treated (1 h at 37 °C) with 10 ml pronase solution (1 mg/ml in DMEM; Merck-Belgolabo, Overijse, Belgium). Finally they were incubated (20 h, 37 °C, 200 rpm) with 10 ml

collagenase (Sigma Chemie) solution (1 mg/ml in DMEM containing 1 % Ultroser G, a serum substitute supplied by Gibco, Gent, Belgium). The cells were filtered through a nylon mesh (70 μm), washed three times, counted (range 1 to $2 \cdot 10^6$ cells/ml) and resuspended in 1 ml of adequate culture medium (DMEM supplemented with 1 % ITS+ or TS+, 2 mM glutamine and 50 μg/ml ascorbate). ITS+ is a culture supplement containing 0.625 mg/ml insulin, 0.625 mg/ml transferrin, 0.625 μg/ml selenious acid, 125 mg/ml bovine serum albumin, and 0.530 mg/ml linoleic acid. When chondrocytes were incubated with IGF-I, ITS+ was replaced by TS+ (TS+ is ITS+ without insulin). DMEM contained 1.8 mM calcium.

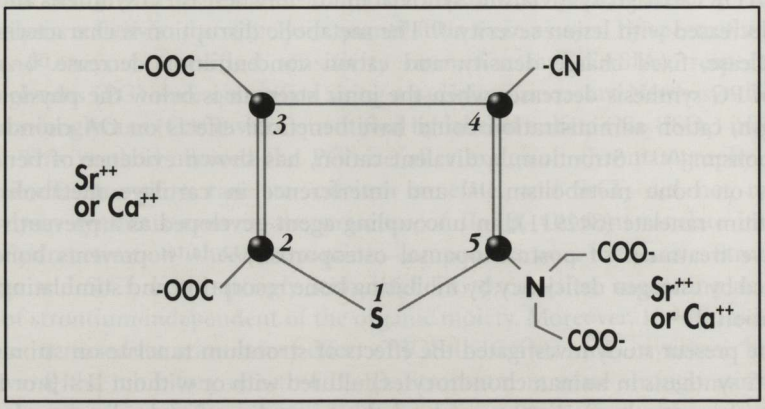


Fig. 1: *Structure of strontium ranelate and calcium ranelate.*

Cells were kept in this culture medium for 48h to eliminate *in vivo* contamination with drugs that the donors may have taken before death. After this washout period, cells were seeded in 10 ml polypropylene Falcon tubes ($1\text{-}2 \cdot 10^6$ cells/ml) and maintained under constant agitation (100 rpm) in a 95 % air/5 % CO_2 atmosphere. At the end of each culture period (1, 2 or 3 days), cells (chondrocyte pellet = [CP]) and supernatant (Culture medium = [CM]) were separated by centrifugation (1000 rpm for 5 min). The chondrocytes recovered after collagenase digestion of the tissue were 95% viable (trypan blue test). CPs were extracted before PG and DNA analysis (CP extract). They were washed twice with Hank's Buffered Saline Solution) (HBSS Gibco) and homogenized by ultrasonic dissociation (10 s, 50 W/cm^2) in phosphate-buffered saline, pH 7.5, containing proteinase inhibitors. All the chemicals for which the source was not mentioned were of the purest grade commercially available.

Culture treatment

Human chondrocytes ($1\text{-}2 \cdot 10^6$ cells/ml) were cultured for 24-72 h in the absence or presence of strontium ranelate ($5 \cdot 10^{-4}\text{-}10^{-3}$ M), $SrCl_2$ ($2 \cdot 10^{-3}$ M) or calcium ranelate (10^{-3} M) with or without IL-1β (10^{-11} and 10^{-10} M) or IGF-I (10^{-10} and 10^{-9} M). The tested compounds were directly dissolved in the CM which was then sterilized by filtration before cellular culture. Strontium ranelate (S12911;

Servier, Courbevoie, France) is made up of two stable strontium atoms and ranelic acid as carrier (5-{bis(carboxymethyl)amino}-2-carboxy-4-cyano-3-thiophenacetic acid). In calcium ranelate (S12911-0), strontium atoms are replaced by two calcium atoms (Fig. 1). Three flasks were used for each concentration of the drug and for the corresponding controls.

DNA assay

Chondrocyte DNA content is correlated directly to the cell number of each culture. DNA content was measured in the CP extract using a fluorimetric method. [19] This measurement ensures elimination of result variations due to the different number of chondrocytes in each culture.

Stromelysin activity measurement

Stromelysin activity released in the CM was measured on resorufin-labeled casein from cow's milk (Boehringer, Mannheim, Germany) using a spectrofluorimetric method. Latent stromelysin (prostromelysin) was activated or not activated *p*- aminophenylmercuric acetate [20] (APMA; Sigma Chemie) treatment of the CM (0.5 mM, 4h at 37 °C). APMA-activated CM (100 µl) were then incubated with 20 µg resorufin-labelled casein for 18 h at 37 °C in a standard buffer (Tris-HCl 0.2 M, pH 7.5) containing 0.02 M $CaCl_2$. The enzymatic reaction was stopped by adding trichloroacetic acid at a final concentration of 1.8% and 20 mM EDTA. The samples were centrifuged at 7000 g for 15 min. Cleaved casein was then measured fluorimetrically in 600 µl of supernatant previously buffered with 900 µl Tris-HCl (0.5 M, pH=8.8). Excitation and emission wavelengths were 574 nm and 584 nm, respectively. Parallel samples containing 20 mM EDTA added before incubation were similarly measured, providing blank values. A standard curve obtained by incubation of increased quantities of purified stromelysin (Biosource Europe, Nivelle, Belgium) allowed the conversion of the measured fluorescence values into the stromelysin concentration equivalent (µg Eq strom/ml). The molecular weight of the detected proteinase was assessed by electrophoresis on a casein-containing gel (Zymogram) as previously described. [21]

Stromelysin quantitation

Total stromelysin released in the CM was assayed using an Enzyme Amplified Sensitivity Immunoassay (EASIA; Biosource Europe). In this immunoassay, monoclonal antobodies are directed against pro-stromelysin, activated-stromelysin and stromelysin bound to TIMP-1 and TIMP-2. Total stromelysin corresponds to the level of stromelysin synthesized in the CM including prostromelysin, activated stromelysin, and stromelysin bound to TIMP-1 and TIMP-2. The method was linear between 5 and 20 ng/ml with a limit of detection of 2.5 ng/ml.

PG quantitation

PGs were quantified in CM and CP extracts by radioimmunoassay (RIA) as previously described. [22, 23] Rabbit polyclonal antibodies used are specific to the antigenic determinant of core protein. No cross-reaction was observed with glycosaminoglycans (GAGs) such as chondroitin sulphate or keratan sulphate, or with PG treated with various enzymes. Only treatment with proteolytic enzymes

(papain, trypsin, and chymotrypsin) abolished or diminished immunoreactivity as opposed to treatment with chondrotinase ABC and neuraminidase. Antiserum did not cross-react with other cartilage matrix constituents such as type II, type IX, and type XI collagens. The lack of cross-reaction with bone PG or skin, cornea, liver, heart, and lung tissue extracts, showed that the antiserum used in the study contains only specific chondrocyte-aggrecan antibodies.

Sulphated GAG quantitation

PG monomers are made up of sufated GAG bound on a linear protein core. The quantitation of the newly synthesized PG was carried out by determination of radioactivity incorporated in sulfated GAG (^{35}S-GAG). $Na_2{}^{35}SO_4$ was added to the CM (5 μCi/ml) during the last 24 h of the culture. In order to eliminate unlabelled $Na_2{}^{35}SO_4$, CM was dialyzed three times against Tris-HCl 0.05 M (pH 9) containing protease inhibitors. The protease inhibitors used throughout the study were $5 \cdot 10^{-8}$ M 6-aminohexanoic acid, $5 \cdot 10^{-8}$ M trypsin inhibitor, 0.01 M EDTA, and $6.7 \cdot 10^{-3}$ M sodium azide. CP previously washed three times with HBSS was extracted (24 h at 4 °C, 100 rpm) by 500 μl guanidine hydrochloride (GuHCl at 4 M in 0.5 M sodium acetate solution containing protease inhibitors, pH 5.8). Three CP extracts treated in the same conditions were pooled and dialyzed against distilled water and Tris-HCl, 0.05 M (pH 9). Radioactivity was measured in the samples using an LKB 1214 Rackbeta liquid scintillation counter (Amersham International, Brussels, Belgium).

Determination of the PG size

The size of the PG produced by the chondrocytes was determined by chromatography. CM and CP extract were eluted on gel filtration chromatography under associative conditions. These conditions protect PG complexes from hydrolysis and allow PG separation according to their molecular weight. For analytical purposes, a column (0.8 x 60 cm) was packed with sepharose CL2B (Pharmacia, Uppsala, Sweden). The column was equilibrated and eluted with Tris-HCl 0.05 M (pH 9), containing enzymatic inhibitors. The flow rate was 6 ml/h and a 1 ml fraction was collected. The column was calibrated under the same conditions with a 1 ml sample adjusted to 20,000 cpm ($Na_2{}^{35}SO_4$). The chromatogram was established by determining the radioactivity in 0.5 ml of each fraction. The chromatographic profile of the ^{35}S-GAG was characterized by a distribution coefficient [$Kd = (Ve\text{-}V_o)/(V_s\text{-}V_o)$], where V_o is the void volume of the column, V_s the total volume of the column, and V_e the elution volume of the molecule under consideration. The peak fraction containing ^{35}S-PG aggregates and $Na_2{}^{35}SO_4$ were used to determine V_o and V_s, respectively. Radioactivity allowed repartition of PG into three groups: PG-hyaluronic acid complexes ($K_d \leq 0.13$), PG monomers and small complexes ($0.13 < K_d < 0.7$), and small PG and PG fragments ($K_d \geq 0.7$).

Expression of the results and statistical analysis

The results were expressed as the rate of stromelysin or PG released into the CM or present inside the CP per microgram of DNA. The values presented are the cumulative amounts of stromelysin or PG found in the culture media at the end

of the specified culture period. Total PG production (TPG) was calculated by adding the results measured for the CM and the corresponding CP. Cumulative release rates of ^{35}S-GAG were obtained by adding the amounts found in the CM at the end of each incubation period. The mean ± standard deviation (SD) of each variable was calculated. Comparison of mean values was performed for each culture individually using the unpaired Student's t-test. Production curves were compared for each experimental condition by means of Zerbe's randomization test. [24] Values were treated with a generalized linear mixed model to compare the effects of strontium ranelate, calcium ranelate, and strontium chloride on young normal and old OA cartilage groups: treated groups were compared between themselves and with the controls. Correlations between the variables were investigated by a linear regression analysis and a one-way analysis of variance (ANOVA) was performed.

Results

Chondrocyte viability

Preliminary tests (trypan blue, ^{51}Cr release) showed the absence of cytotoxicity of the tested compounds. Cell viability was always superior to 90 % whatever the culture treatment. The DNA content remained stable during the culture period (no significant difference between 0 and 72 hours) and was not significantly affected by the presence of strontium ranelate (10^{-4}-10^{-3} M), calcium ranelate (10^{-3} M), or $SrCl_2$ ($2 \cdot 10^{-3}$ M, Table 1).

Table 1. DNA Contentts

	Controls		S12911			S12911-0	$SrCl_2$
	T_{0h}	T_{72h}	10^{-4} M	$5 \cdot 10^{-4}$ M	10^{-3} M	10^{-3} M	$2 \cdot 10^{-3}$ M
Without IL-1β	12.5 ± 1.3	13.0 ± 0.8	12.9 ± 1.1	12.3 ± 0.3	12.1 ± 0.7	12.3 ± 0.2	11.1 ± 0.7
With IL-1β	12.9 ± 2.0	13.1 ± 1.0	12.7 ± 1.3	13.9 ± 0.3	13.4 ± 0.4	11.5 ± 0.7	13.0 ± 1.2

Effects of strontium ranelate (S12911), calcium ranelate (S12911-0) or $SrCl_2$ on DNA (μg) contained in CP after 72 h of normal human chondrocyte culture. The DNA content at the begining (T_{0h}) and the end of the culture (T_{72h}) were also compared for the controls. Results are mean values and SD of triplicate primary chondrocyte cultures in a representative experiment.

Stromelysin synthesis and activity

In basal conditions (no IL-1β stimulation), stromelysin activity measured in the CM of unactivated chondrocytes was very low (Fig. 2) or undetectable (Fig. 3), depending on the donor. However, stromelysin synthesis increased linearly with culture duration. Strontium ranelate and $SrCl_2$ did not activate stromelysin whereas calcium ranelate induced enzyme activation in the culture where stromelysin activity was detectable ($p < 0.001$; Fig. 2).

APMA-activated stromelysin activity measured in the CM increased as a function of culture duration but was not affected by the presence of calcium or strontium compounds (Fig. 2). Furthermore, strontium ranelate, calcium ranelate, and $SrCl_2$ did not increased stromelysin protein synthesis (Fig. 2).

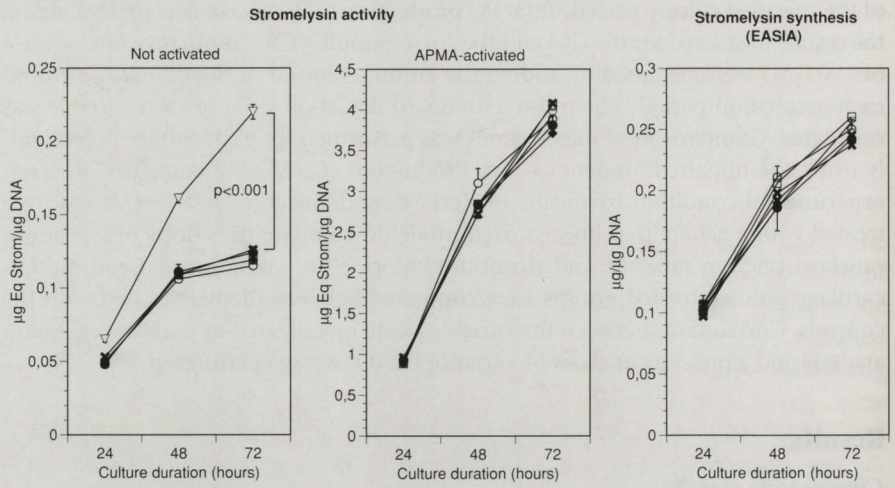


Fig. 2: *Effect of increased amounts of strontium ranelate, $SrCl_2$, or calcium ranelate on stromelysin activity and synthesis by normal human chondrocytes. Primary chondrocytes were cultured for 72h in the absence (-○-) or presence of 10^{-4} M (- X -), $5 \cdot 10^{-4}$ M (-♦-) or 10^{-3} M (-□-) strontium ranelate, $2 \cdot 10^{-3}$ M $SrCl_2$ (-●-), or 10^{-3} M calcium ranelate (-△-). Results are the mean values of triplicate primary chondrocyte cultures in a representative experiment and error bars represent SD. The values represent the cumulative amounts of stromelysin over the specified culture periods. Differences between curves are tested by the Zerbe test.*

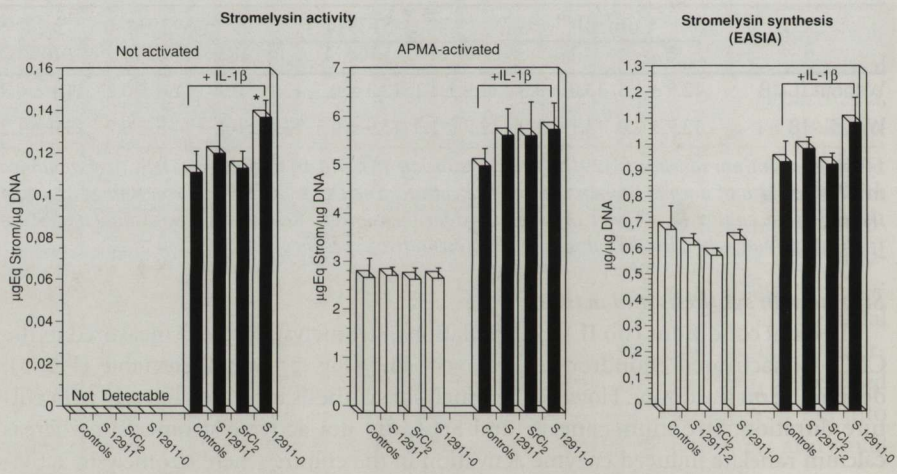


Fig. 3: *Stromelysin activity and synthesis in the CM by unstimulated or IL-1ß-stimulated (10^{-11} M) normal human chondrocytes were measured after 72 h of treatment with 10^{-3} M strontium ranelate (S12911), $2 \cdot 10^{-3}$ M $SrCl_2$, and 10^{-3} M calcium ranelate (S12911-0). A significant increase in the stromelysin activity was observed in the presence of 10^{-3} M calcium ranelate in comparison with unactivated stromelysin. Response is significant with $p < 0.05$.*

IL-1β stimulated stromelysin synthesis and activity with a maximal effect after 72h of incubation ($p < 0.01$; Figure 3). The 72 h incubation period was retained to test the compounds with IL-1β stimulation. Strontium compounds did not significantly modify IL-1β-stimulated stromelysin synthesis and activity whereas calcium ranelate increased IL-1β induced stromelysin activity ($p < 0.05$; Fig. 3).

PG production

In control conditions and in strontium ranelate-treated cultures, the PG that were released in the CM (Fig. 4A) and contained in the CP (Fig. 4B) of normal chondrocyte cultures increased as a function of the culture duration. After 24 h of culture, strontium ranelate did not significantly modify PG production by normal chondrocytes whatever the concentration used. After 48h of culture, strontium ranelate significantly increased the amount of PG released in the CM only at the concentration of 10^{-3} M and the PG contained in the CP at the concentrations of $5 \cdot 10^{-4}$ and 10^{-3} M. After 72 h of culture, strontium ranelate dose-dependently increased the PG released in the CM ($r = 0.92$, $p < 0.01$). This increase was significant at concentrations of $5 \cdot 10^{-4}$ and 10^{-3} M in the CP ($p < 0.05$). TPG was strongly and significantly increased (approximately 20 %-35 %) by $5 \cdot 10^{-4}$ or 10^{-3} M strontium ranelate after 48 h and 72 h of culture (data not shown).

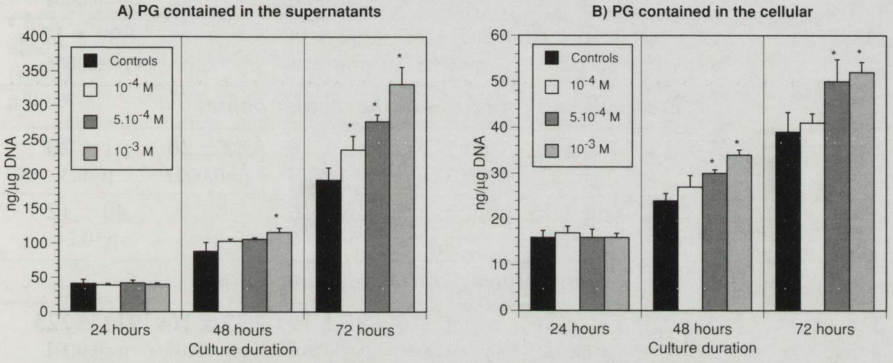


Fig. 4: *Effect of strontium ranelate on human articular chondrocyte cultures. Normal human chondrocytes were incubated for the time specified in the absence (control condition) or presence of varying concentrations of strontium ranelate. At the end of the culture period, CM and CP were separated by centrifugation and were assayed for PG by a specific RIA. The data are expressed as amounts of PG found in the CM or in the CP per micrograms of DNA and presented as the mean and SD of triplicate cultures. Statistical significance in comparison to the controls: * $p < 0.05$.*

The effects of strontium ranelate were then compared between normal and OA chondrocyte cultures. Six different cartilage samples were used. They were excised from 3 normal young donors (< 40 years old) and 3 OA donors (> 60 years old). OA samples showed one or more lesions scored III on the Moskowitz scale. [8] As expected, chondrocytes isolated from the cartilage of normal donors always produced higher amounts of PG than OA chondrocytes; (Table 2). The PG amount released into the CM and contained in the CP after 72h of culture with or without

strontium ranelate was significantly lower in the OA group than in the normal group ($p < 0.001$; generalized linear mixed model) (Table 2). Strontium ranelate (10^{-4} M-10^{-3} M) significantly increased TPG ($0.0005 < p < 0.0001$) in normal chondrocyte cultures. No significant increase was observed at the concentration of 10^{-4} M when strontium ranelate was tested on OA cartilage, whereas higher concentrations ($5 \cdot 10^{-4}$ and 10^{-3} M) induced a significant increase in total proteoglycan production (TPG) ($0.002 < p < 0.02$). $SrCl_2$ ($2 \cdot 10^{-3}$ M) is as efficient as strontium ranelate in increasing TPG produced by normal and OA chondrocytes (Table 2). Nevertheless, strontium ranelate increased PG contained in the CP of normal chondrocyte cultures whereas $2 \cdot 10^{-3}$ M $SrCl_2$ did not significantly modify this parameter. Calcium ranelate (10^{-3} M) also increased TPG in normal chondrocyte cultures to the same extent as strontium ranelate (Table 2).

Table 2. PG production measured by RIA

	Controls	S12911			S12911-0	$SrCl_2$
		10^{-4} M	$5 \cdot 10^{-4}$ M	10^{-3} M	10^{-3} M	$2 \cdot 10^{-3}$ M
			Proteoglycans contained in the culture supernatants			
Normal	622 ± 184	**707 ± 146** p=0.0012	**814 ± 191** p=0.0001	**800 ± 176** p=0.0001	**752 ± 145** p=0.0001	**844 ± 206** p=0.0001
OA	212 ± 68	216 ± 62 p=0.86	**267 ± 91** p=0.03	**292 ± 90** p=0.0088	-	**302 ± 12** p=0.0006
			Proteoglycans contained in the cellular phases			
Normal	68 ± 25	73 ± 24 p=0.13	**82 ± 20** p=0.0003	**85 ± 18** p=0.0001	**82 ± 26** p=0.0001	71 ± 20 p=0.3
OA	36 ± 15	36 ± 15 p=0.9	41 ± 17 p=0.13	45 ± 18 p=0.27	-	40 ± 12 p=0.24
			Total proteoglycan production			
Normal	686 ± 210	**780 ± 167** p=0.0005	**894 ± 210** p=0.0001	**885 ± 194** p=0.0001	**833 ± 169** p=0.0001	**914 ± 223** p=0.0001
OA	248 ± 81	253 ± 75 p=0.85	**309 ± 105** p=0.02	**362 ± 156** p=0.002	-	**340 ± 115** p=0.0007

Effects of strontium ranelate (S12911), calcium ranelate (S12911-0), or $SrCl_2$ on PG production (ng/µg DNA) by normal or OA human chondrocytes at 72h. Values are the means and SD of three separate chondrocyte cultures. Values with $p < 0.05$ are significantly different from the corresponding controls (in bold).

Chondrocyte responses to IGF-I and IL-1β

The effects of strontium ranelate on chondrocyte responses to IGF-I and IL-1ß were studied in normal human chondrocyte cultures. As expected, IGF-I dose-dependently stimulated TPG ($p = 0.02$) whereas IL-1ß drastically inhibited PG synthesis after 72 h of culture. IGF-I (10^{-9}-10^{-8} M) increased TPG by 15 % and 40 %, respectively. When IGF-I (10^{-9} M) and strontium ranelate ($5 \cdot 10^{-4}$ M or 10^{-3} M) were added simultaneously, the stimulatory effect of IGF-I was higher than

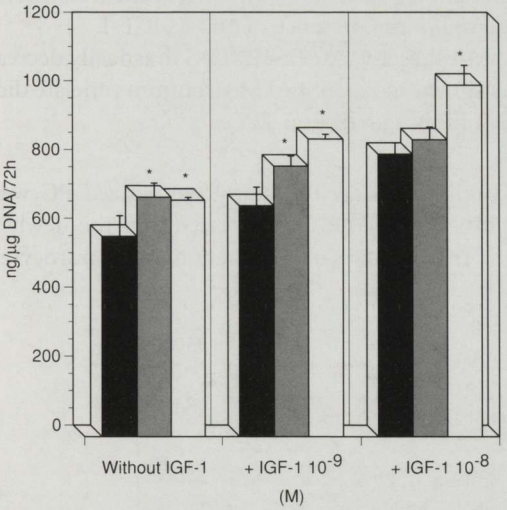


Fig. 5: *Effect of strontium ranelate on normal human chondrocyte response to IGF-I. At the concentrations of 10^{-9} and 10^{-8} M, IGF-I significantly stimulated TPG (sum of the PG amount released in the CM and contained in the corresponding CP) of normal human chondrocytes cultured for 72h in the absence (black column) or in the presence of 10^{-3} M strontium ranelate (grey column) or $2 \cdot 10^{-3}$ M $SrCl_2$ (white column). Values are mean and SD (n=3). Strontium increased the IGF-I stimulating effect with $p < 0.05$.*

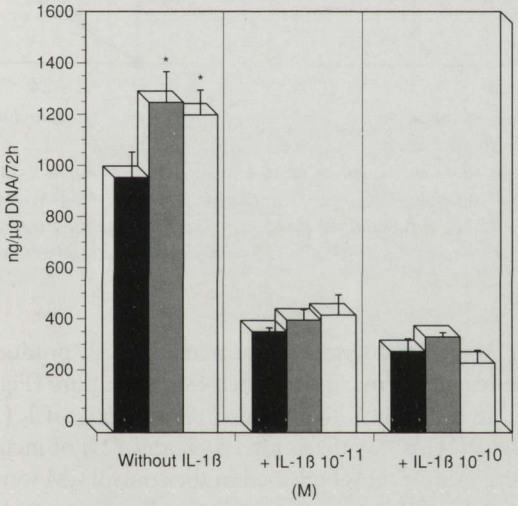


Fig. 6: *Effects of strontium ranelate on normal human chondrocyte response to IL-1ß: TPG produced by human chondrocytes cultured for 72 h in the absence (black column) or in the presence of 10^{-3} M strontium ranelate (grey column) or $2 \cdot 10^{-3}$ M $SrCl_2$ (white column).*

the effect of IGF-I alone ($0.03<p<0.004$; unpaired Student's t-test) (Fig. 5). No similar effects were observed in the presence of 10^{-8} M IGF-I.

In the presence of 10^{-11} or 10^{-10} M IL-1β, TPG drastically decreased by 60 % and 68%, respectively. As shown in Fig. 6, 10^{-3} M strontium ranelate did not significantly modify the IL-1β-inhibiting effect on TPG.

PG size

In the culture conditions used, the newly synthesized PG were built up with ^{35}S-GAG. The amounts of ^{35}S-GAG incorporated into the newly synthesized PGs during the last 24 h of 1, 2, or 3 days of normal human chondrocyte cultures are presented in Fig. 7.

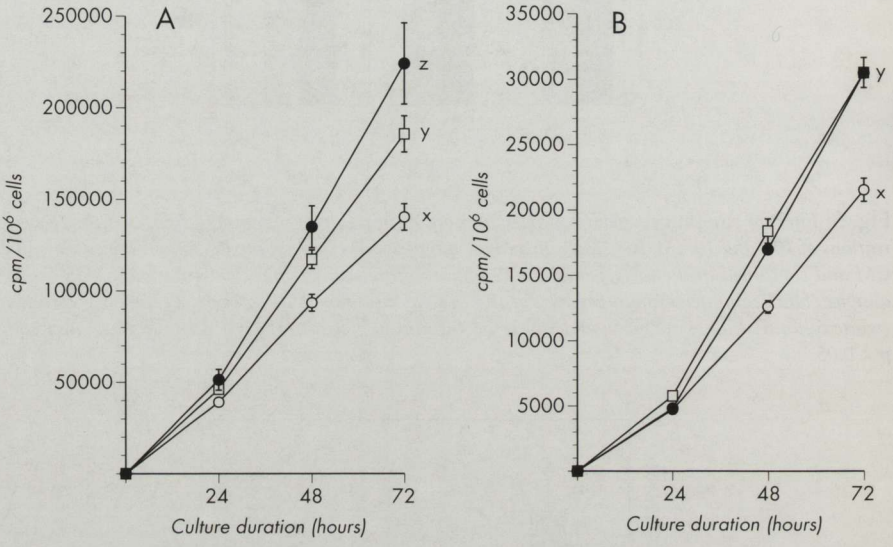


Fig. 7: *Normal human chondrocyte effects of 10^{-3} M strontium ranelate (-□-) or $2{\cdot}10^{-3}$M $SrCl_2$ (-●-) compared with the controls (-○-) on the cumulative amount of ^{35}S-GAG released in the CM (A) or contained in the CP (B) as a function of the culture duration (cumulative values; means + SD). Statistical significance: in the 72 h CM (A): x vs y, $p< 0.025$; x vs z, $p < 0.01$, and in the 72 h CP (B): x vs y; $p < 0.01$.*

PG released in the CM: In the absence of treatment, the daily production of ^{35}S-GAG released in the CM reached a maximum after 48 h of culture (Fig. 7A, same slope after 24 h and after 48 h). Strontium ranelate (10^{-3} M) and $SrCl_2$ ($2{\cdot}10^{-3}$ M) significantly increased the ^{35}S-GAG synthesis after 48 h and 72 h of incubation.

The newly synthesized ^{35}S-GAG released in the control CM were eluted through a CL2B chromatographic column in two major peaks (Figure 8A). At each culture period, a large part of the newly synthesized ^{35}S-GAG was eluted with a $K_d \leq 0.13$ (Table 3). The percentage of ^{35}S-GAG eluted with a $K_d \leq 0.13$ (high-molecular-weight PG complexes) increased with the culture duration (36 %-49 %) whereas the

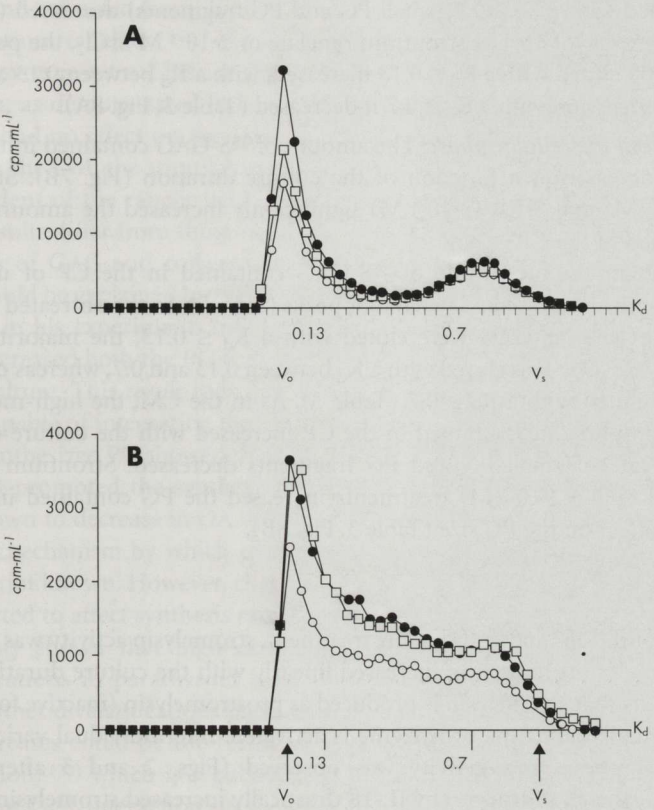


Fig. 8: *Associative size exclusion chromatograms of ^{35}S-labelled PG produced by normal chondrocytes cultured for 72 h in the absence (-○-) or in the presence of 10^{-3} M strontium ranelate (-□-) or $2 \cdot 10^{-3}$ M $SrCl_2$ (-●-). (A) Culture media, (B) Cellular phases.*

Table 3. Physicochemical form of the newly synthsized PG

	$K_d \leq 0.13$			$0.13 < K_d < 0.7$			$K_d \geq 0.7$		
	Controls	S12911	$SrCl_2$	Controls	S12911	$SrCl_2$	Controls	S12911	$SrCl_2$
^{35}GAG released in the culture supernatants (%)									
0 – 24 hours	36	42	46	27	30	26	37	28	28
24 – 48 hours	45	45	49	25	26	26	30	29	25
48 – 72 hours	49	55	61	26	24	20	25	21	19
^{35}GAG contained in the cellular phases (%)									
0 – 24 hours	29	29	28	43	40	41	28	31	31
24 – 48 hours	29	36	29	48	44	50	23	20	21
48 – 72 hours	37	34	37	52	52	51	11	14	12

Effects of strontium compounds on the physicochemical form of the newly synthesized PG by normal human chondrocytes. The chomatogram was established by determining the radioactivity in each fraction and dividing into 3 groups according to a distribution coefficient. The values are the percentage of radioactivity contained in 3 groups.

^{35}S-GAG eluted with a $K_d \geq 0.7$ (small PG and PG fragments) decreased (37 %-25 %). In the presence of 10^{-3} M strontium ranelate or $2 \cdot 10^{-3}$ M $SrCl_2$, the percentage of the ^{35}S-GAG eluted with a $K_d \leq 0.13$ increased, with a K_d between 0.13 and 0.7 it was not modified, and with a $K_d \geq 0.7$ it decreased (Table 3, Fig. 8A).

PG contained in the cellular phase: The amount of ^{35}S-GAG contained in the CP of the culture increased as a function of the culture duration (Fig. 7B). Strontium ranelate (10^{-3} M) and $SrCl_2$ ($2 \cdot 10^{-3}$ M) significantly increased the amount of ^{35}S-GAG contained in the CP.

The chromatographic profile of ^{35}S-GAG contained in the CP of untreated chondrocyte cultures presented two major peaks (Fig. 8B). In the untreated cultures, 29 %-37 % of the ^{35}S-GAG were eluted with a $K_d \leq 0.13$, the majority of the ^{35}S-GAG (43 %-52 %) was eluted with a K_d between 0.13 and 0.7, whereas only 11% to 28 % was eluted with a $K_d \geq 0.7$ (Table 3). As in the CM, the high-molecular-weight PG complex rate contained in the CP increased with the culture duration whereas the rate of small PG and PG fragments decreased. Strontium ranelate (10^{-3} M) and $SrCl_2$ ($2 \cdot 10^{-3}$ M) treatments increased the PG contained in the CP without any effect on the PG size (Table 3, Fig. 8B).

Discussion

In basal conditions and without any treatment, stromelysin activity was very low whereas stromelysin production increased linearly with the culture duration. This finding suggests that stromelysin is produced as prostromelysin (inactive form) and that only a small part of the proenzyme is activated. Interindividual variability in stromelysin synthesis and activity was observed (Figs. 2 and 3 after 72 h). Stimulation of the chondrocytes by IL-1ß drastically increased stromelysin activity released in the CM. The mechanism involved may be the plasminogen activator/plasmin system which can transform the proenzyme to the active form of stromelysin.[25] This hypothesis agrees with previous studies where IL-1ß was demonstrated to increase plasminogen activator synthesis by the main cells of the joint, including human chondrocytes.[26,27]

Strontium ranelate and $SrCl_2$ did not activate stromelysin whereas calcium ranelate increased enzyme activity only in basal conditions. Calcium ranelate must act on the proenzyme activation process, since it did not increase stromelysin synthesis and did not modify the activity of APMA-activated stromelysin. This hypothesis agrees with a previous study which reported that Ca^{2+} is required for prostromelysin activation and stability.[20]

In the condition used, chondrocytes cultured without any treatment produced spontaneously aggregating PG and type II collagen. The TPG measured in the cultures from the different chondrocyte donors shows interindividual variation (Table 2, Figs. 4-6 after 72 h without any treatment). A large part of the newly synthesized PG (10-20 %) was contained in the CP of the culture suggesting the formation of a new matrix surrounding the chondrocytes. The qualitative analysis of the newly synthesized PG showed that the majority of the PG released in the CM

(56 %) and extracted from the CP (57 %) is high-molecular-weight PG complexes.

Strontium ranelate (10^{-3} M) strongly stimulated the synthesis of PG by human chondrocytes *in vitro*. This cartilage matrix synthesis was not the result of cell proliferation, as demonstrated in bone formation,[28] since the addition of strontium ranelate had no effect on the DNA level. Because $SrCl_2$ ($2 \cdot 10^{-3}$ M) showed similar effects in the CM, the stimulatory effect of strontium ranelate on PG production is independent of the ranelic acid and strontium must be responsible for this effect. These results differ from those of Svensson [29] who showed that $SrCl_2$ inhibited the synthesis of GAG and collagen in freshly isolated rat chondrocytes. This discrepancy could be explained by the higher strontium concentrations used ($3 \cdot 2 \cdot 10^{-3}$ and 10^{-2} M) in his experiment. Interestingly, strontium and calcium ranelate, but not $SrCl_2$, increased both the PG that were released in the CM and contained in the CP of the culture. This result indicates that strontium ranelate promotes the synthesis of PG capable of integrating the extracellular matrix. The qualitative analysis of the newly synthesized PG showed that strontium ranelate (10^{-3} M) and calcium ranelate (10^{-3} M) promoted the synthesis of high-molecular-weight PG complexes, which were shown to decrease in OA.[30]

The mechanism by which extracellular strontium ranelate influenced PG synthesis is not known. However, changes in the extracellular ionic environment would be expected to affect synthesis rates by altering the intracellular composition. It was previously shown that high extracellular Ca^{2+} increases intracellular free Ca^{2+} concentrations in parathyroid cells [31] and that this effect can be mimicked by several other divalent cations including strontium. [32] Intracellular Ca^{2+} concentration increases could be the result of inositol 1,4,5-triphophate receptor (InsP3R) stimulation, [33] which is regulated by multiple calcium binding sites and at least 4 different Ca^{2+}-interaction sites. [31] The ionic strength of the aqueous environment also can profoundly affect gating calcium channels. Sr^{2+} and Ca^{2+} modulate the opening and closing of Ca^{2+} channels and in this way may affect cellular functions.[34]

Strontium could be hypothesized to be effective in reversing the IGF-I stimulating effect, because factors that lead to increase PG synthesis were reported as causing an initial decrease in cytosolic free Ca^{2+} concentration.[35, 36] The effect of strontium ranelate and IGF-I added simultaneously were tested on PG synthesis by normal human chondrocytes. Surprisingly, IGF-I stimulation of PG synthesis was increased by 10^{-3} M strontium ranelate treatment. This effect could be consequent to chondrocyte proliferation because IGF-I is required for cell proliferation.[37, 38] However, in the culture conditions used, neither [^{3}H] thymidine incorporation nor DNA content was elevated in the presence of IGF-I added alone or in combination with strontium ranelate (data not shown). Autocrine regulation of chondrocyte metabolism by IGF-I produced in response to a high amount of strontium also might be responsible for the PG increase. Indeed, recently, it was reported that a high extracellular Ca^{2+} concentration significantly increased the secretion of IGF-I as well as the expression of IGF-I messenger RNA (mRNA) by osteoblastic MC3T3-E1 cells.[39] This finding could explain why strontium ranelate increased

the IGF-I effect when it was added at a low concentration (10^{-9} M) but not at a high concentration (10^{-8} M).

Strontium ranelate, calcium ranelate, and $SrCl_2$ had similar stimulating effects on TPG. Nevertheless, $SrCl_2$ showed no significant effect on the PG contained in the CP, and calcium ranelate (10^{-3} M) increased the stromelysin activity measured in the CM. Because strontium ranelate also stimulated the production of type II collagen synthesis, [40] it can be concluded that 10^{-3} M strontium ranelate is more efficient in promoting cartilage formation than the other compounds tested. Moreover, 10^{-3} M strontium ranelate increased TPG in OA chondrocyte cultures and could have beneficial effects in the prevention and the treatment of OA lesions. This hypothesis agrees with a previous study that showed that in strontium-induced rachitic rats the size of the side chains of PG monomers was considerably increased in the lower part of the growth plate.[13]

In paralell, previous *in vitro* and *in vivo* works have shown that strontium ranelate reduced bone resorption and stimulates preosteoblastic cell replication in rat calvaria cell and organ culture.[17, 28, 41] Furthermore, administration of 2 g/day of strontium ranelate to postmenopausal osteoporotic women for a 2 year period resulted in uncoupling between bone resorption (decrease of collagen cross-links excretion) and bone formation (increase in bone-specific alkaline phosphatase) which led to increase in the lumbar spine bone density and a significant of 44 % reduction in the number of patients experiencing a new vertebral fracture during the second year of treatment [15, 42]. In a prevention 2-year study, 1 g/day of strontium anelate given to early postmenopausal women without prevalent osteoporosis, induced a significant increase in spine bone mineral density (BMD). Frequency and distribution of adverse reactions were similar in strontium ranelate and placebo-treated patients [16]. These data are consistent with the very good tolerance observed in toxicology studies carried out with strontium ranelate. Taken together, these data suggest that strontium ranelate has beneficial effects on both bone and cartilage remodeling.

In conclusion, 10^{-3} M strontium ranelate strongly increases cartilage matrix formation by ionic stimulation of chondrocyte anabolism without affecting cartilage resorption. In this way, it could restore the imbalance between chondroformation and chondroresorption observed during OA. This finding provides a preclinical basis for *in vivo* testing of strontium ranelate in prevention and treatment of OA.

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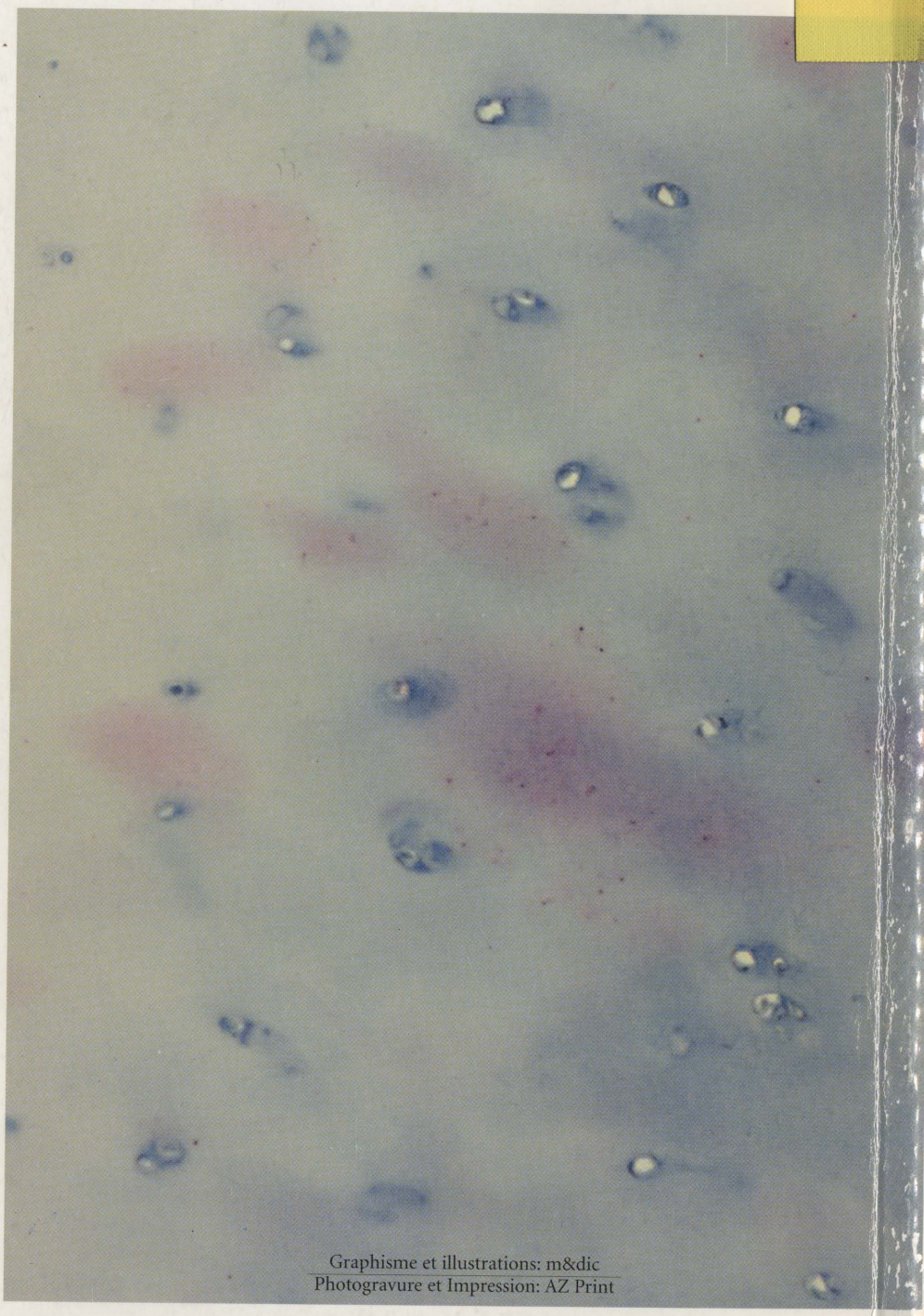
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