Original Paper

Gynecologic and Obstetric Investigation

Gynecol Obstet Invest 2002;53:105–111

Received: June 14, 2001
Accepted after revision: December 7, 2001

Human Endometrial Epithelial Cells Modulate the Activation of Gelatinase A by Stromal Cells

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Key Words
Metalloproteinase · Reproduction · Endometrium · Gelatinase A/MMP-2 activation

Abstract
Metalloproteinases (MMPs) are central effectors in endometrial physiology. Their production is tightly regulated by ovarian steroids and cytokines. Using zymography, we investigated MMP-2 production by human endometrial cells treated with estradiol-17β + progesterone (E_2+P) and by various key cytokines in endometrial physiology (IL-1β, LIF, TGF-β, and TNF-α). No gelatinase activity was detected in the culture media of epithelial cells. In basal conditions, stromal cells produced the pro form of MMP-2. MMP-2 production/activation was not directly affected by cytokine treatment. Interestingly, activated MMP-2 was only detected after treatment of stromal cells with culture medium from epithelial cells. Cytokine treatment of epithelial cells increased the capacity of conditioned medium to stimulate stromal cells to activate MMP-2. As the tissue inhibitor of MMP-2 (TIMP-2) is a regulator of gelatinase A activity, its concentration was measured by ELISA. TIMP-2 production by stromal cells was not affected by cytokines or by epithelial cell-conditioned medium. These results strongly suggest that regulation of stromal MMP-2 activation involves soluble factor(s) derived from the epithelial compartment.

Introduction

Human endometrium undergoes unique cyclic tissue remodeling. During each menstrual cycle, ovarian steroids drive drastic tissue modifications during the proliferative and luteal phases, followed by breakdown during menstruation if the cycle is infertile [1]. These tissue modifications involve the synthesis, reorganization and degradation of extracellular matrix (ECM) components. During the luteal phase of the cycle, the endometrial stroma becomes edematous and is infiltrated by numerous inflammatory cells. Stromal cells adopt an epithelial-like phenotype (decidual cells), synthesize basement membrane components (laminin, fibronectin, type-IV collagen, etc.), and become surrounded by the resulting membrane [2].

Metalloproteinases (MMPs) are members of a family of zinc endopeptidases that together degrade the ECM, including both interstitial and basement membrane components [3]. The human MMP family consists of at least 26 enzymes, which can be subdivided on the basis of either their substrates or their molecular structure. Four main classes have been characterized, namely collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMPs-3, -10, -11) and membrane-type MMPs (MMPs-14 to 17). They are secreted as inactive pro-enzymes and are activated extracellularly by serine proteases or other MMPs [4, 5]. The activated

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enzymes are inhibited by peptides known as tissue inhibitors of metalloproteinases (TIMPs) [6]. MMPs are controlled at several levels, including transcription and activation, and also by specific inhibition. In physiologic conditions, they play central roles in processes such as embryo implantation and development, angiogenesis, and tissue turnover and remodeling (e.g. menstruation) [7].

In the endometrium, the control of MMP and TIMP production by ovarian steroids is locally mediated, at least partially, by cytokines secreted by the epithelial, stromal, inflammatory and endothelial cells of the endometrium [8–13]. IL-1 is produced by inflammatory cells, trophoblast and epithelial cells, and increases MMP-1, 3 and 9 production, while it inhibits TIMP-1 and 3 expression [14–16]. Leukemia-inhibitory factor (LIF) is mainly of epithelial and trophoblastic origin, and regulates gelatinase activity [17]. Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine secreted by large granular lymphocytes and macrophages present in the secretory endometrium. TNF-α increases MMP-1, 3 and 9 production. Finally, transforming growth factor-β (TGF-β) can be secreted by epithelial and stromal cells, as well by endothelial endometrial and trophoblastic cells. TGF-β produced by stromal cells mediates the progesterone-induced suppression of MMP-7 [15] and stimulates TIMP-1 and 3 expression [16].

MMP-2 is expressed in the human endometrium throughout the menstrual cycle. Its production in the endometrium appears to be constitutive [14–16, 18, 19], although it was thought until recently to escape progesterone control [20]. This is consistent with the structure of the MMP-2 gene, which contains a housekeeping promoter lacking most response elements (AP-1, PEA-3) [21]. However, the degree of MMP-2 activation appears to be at least as important as its abundance [22–24]. MMP-2 activation is a sophisticated process that takes place at the cell surface and necessitates controlled amounts of TIMP-2 and of MT1-MMP, a membrane-anchored matrix MMP [4, 25]. MMP-2 could play a significant role in endometrial biology, including ECM remodeling associated with the menstrual cycle, decidualization, menstruation [20, 22, 26], endometriosis [27] and embryo implantation [28, 29]. However, the regulatory mechanisms governing MMP-2 activity in endometrial tissue are unknown.

Here, we report evidence of cross-talk between epithelial and stromal cells that affect the activation of MMP-2 by stromal cells, but not its production.

Materials and Methods

Tissue Collection

Human luteal-phase endometrium was obtained from 20 patients free of endometrial disease undergoing hysterectomy for benign disorders. A small portion of each sample was formalin-fixed for cycle dating based on the histological criteria of Noyes et al. [1]. The protocol was approved by the ethics committee of our medical faculty, and written informed consents were obtained from the patients.

Human Endometrial Stromal and Epithelial Cell Isolation and Culture

Epithelial and stromal cells were separated essentially as described by Tseng and Zhu [30]. Cell preparations were depleted of immune cells as previously described [31]. Briefly, epithelial cells were separated from stromal cells by sequential sedimentation, followed by differential adhesion. The isolated cells were cultured for 48 h in DMEM/Ham F12 medium (Gibco, Gaithersburg, Md., USA) supplemented with insulin (25 mU/ml), 10% charcoal-stripped fetal calf serum (FCS) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone). They were then washed with the same medium and subcultured in serum-free medium supplemented with 1 mg/ml bovine serum albumin (BSA; Sigma, St. Louis, Mo., USA) and insulin (25 mU/ml). This medium was renewed every 2 days. Estradiol-17β (E_2) and progesterone (E_2+P; Sigma) were added daily at final concentrations of 10 and 100 n*M*, respectively, from day 0 until extraction. For cytokine treatment, recombinant IL-1β, LIF, TNF-α, and TGF-β (R&D Systems, Minneapolis, Minn., USA) were added individually to the culture medium of confluent stromal or epithelial cells at a final concentration of 1 n*M* in the absence of serum. This concentration was chosen on the basis of previous reports addressing the role of these cytokines in the regulation of MMP expression and activation in endometrial cell cultures [10, 14–16]. According to these data, cytokines used in this range of concentrations are able to affect the regulation of different MMPs in explant culture and isolated cell culture. After 24 h of treatment, the medium was collected, centrifuged (180 *g* for 10 min) and stored at –20°C until analysis. To test for a potential paracrine action of epithelial cells on gelatinase activity, confluent stromal cells pretreated with E_2+P were incubated for 24 h in epithelial cell-conditioned medium from the same patients, diluted twofold with serum-free medium. The conditioned medium was obtained after epithelial cells had been treated with E_2+P, and with or without cytokines (IL-β, LIF, TNF-α or TGF-β) as described above. Figure 1 shows the experimental design.

Immunocytochemistry

Cell purity was assessed immunocytochemically as described by Laird et al. [32] using markers for stromal cells (vimentin, clone V9; Dako, Glostrup, Denmark), epithelial cells (cytokeratin, clone MN 116; Dako), endothelial cells (factor VIII; Dako) and leukocytes (CD-45, clone PD7/26; Dako). Separated cells grown on glass coverslips were fixed and permeabilized in methanol for 10 min at –20°C and washed with PBS; endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS for 10 min. After blocking solution (7% bovine serum albumin in PBS), cells were incubated with the primary antibody (anti-vimentin, anti-cytokeratin, anti-factor VIII or anti-CD-45) at the optimal dilution for 1 h at room temperature. After washing, the streptavidin-biotin-labeled second antibody system was added to the cells for 30 min. The coverslips were then incubated with

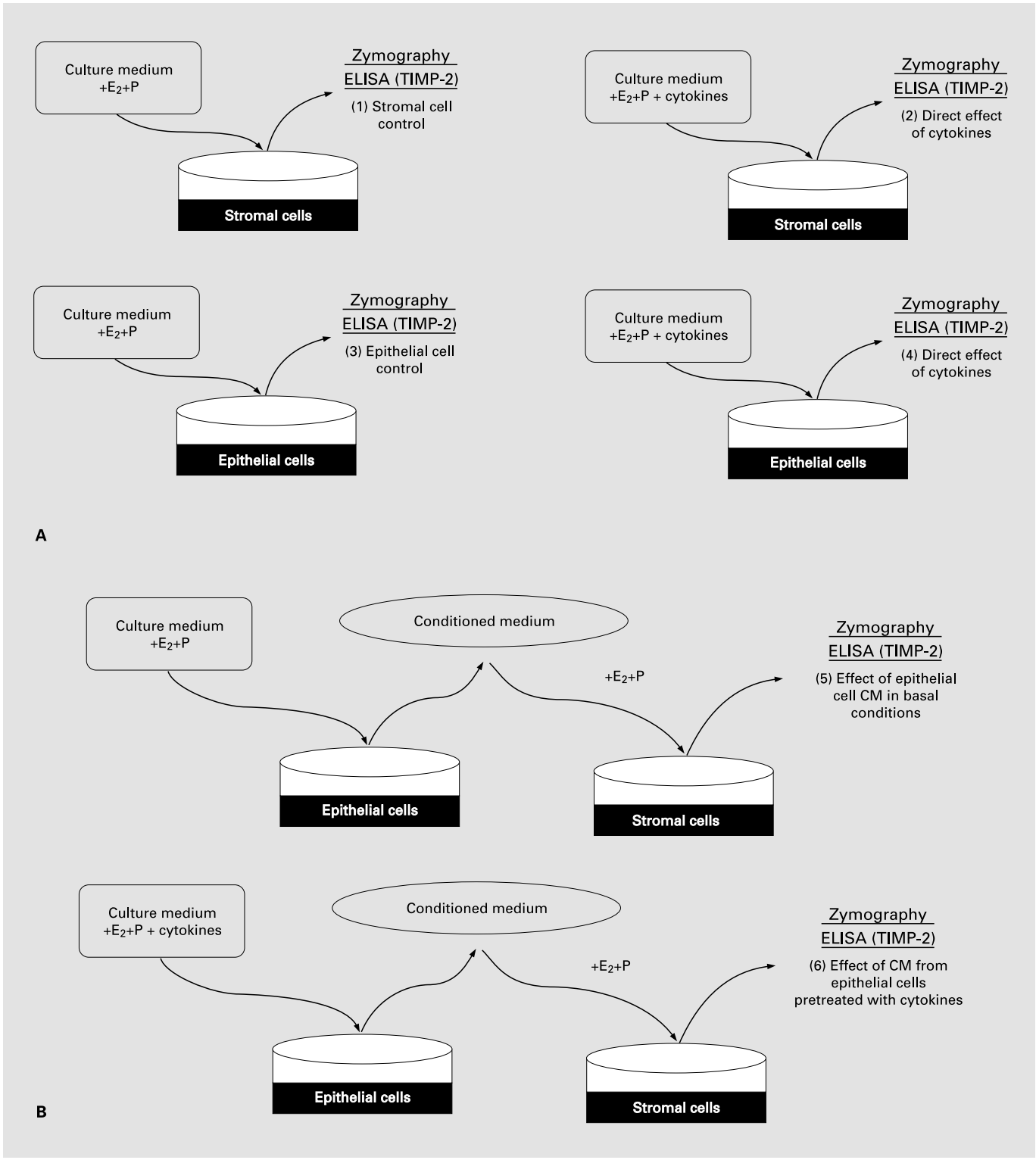

Fig. 1. Experimental design. Assessment of gelatinolytic activity by zymography and of TIMP-2 levels by ELISA in various conditioned media (CM). A Control (1 and 3): medium conditioned by purified stromal or epithelial endometrial cells (with E_2+P), without additional cytokines. Direct effect of cytokines (2 and 4): conditioned medium (pretreatment with E_2+P) obtained after 24 h of culture with cytokine. B Effect of epithelial cell conditioned media (CM) in basal conditions (5). Effect of CM from epithelial cells pretreated with cytokines (6).

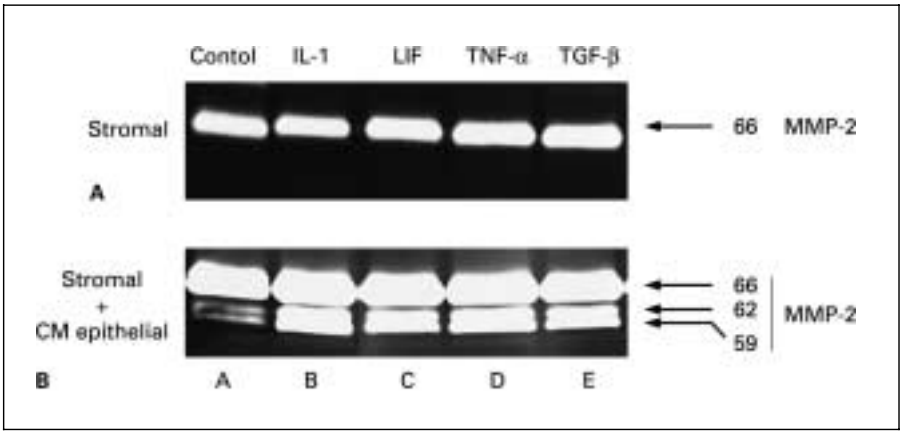

Fig. 2. Gelatin zymography of culture medium from: (A) endometrial stromal cells in basal conditions (control) and after cytokine treatment (IL-1β, LIF, TGF-β or TNF-α), and (B) endometrial stromal cells treated with epithelial cell conditioned media (CM) in basal conditions (control) and after treatment with the same cytokines. The figure is representative of three independent experiments.

diaminobenzidine (DAB, 0.02% in PBS). The slides were washed in running water and counterstained with hematoxylin.

Zymography

Proteinase activity in conditioned medium and cell extracts was analyzed by means of zymography. Sample loading was normalized to the protein concentration determined using the DC Protein Assay Kit (BioRad Laboratories, Hercules, Calif., USA). Samples were denatured in nonreducing conditions and resolved by SDS-PAGE in 10% gels containing 0.1% gelatin (w/v) as described elsewhere [33]. Semiquantitative densitometric analysis of gelatinolytic bands was used to assess the relative activities of the latent and active forms of MMP-2, using an Imaging Densitometer model GS-700 (Bio-Rad, Life Science).

TIMP-2 Enzyme-Linked Immunosorbent Assay (ELISA)

TIMP-2 concentrations in conditioned medium were determined by using a sandwich ELISA described in detail by Remacle et al. [34]. This ELISA detects free active TIMP-2 and TIMP-2/MMP complexes, and does not react with other TIMPs. The detection limit is 0.3 ng/ml, and the concentration/absorbance curve is linear in the 0.3–20 ng/ml range. Results are expressed as nanograms per milligram of total protein determined using the DC Protein Assay Kit (BioRad Laboratories).

Results

Cell Culture and Treatment

The purity of cells isolated from secretory endometrium (late luteal phase, day 24–26) was first assessed by immunocytochemistry. All cultures and experiments were performed in triplicate, with less than 10% variation between specimens. Epithelial fractions were found to be enriched by more than 95%, and stromal cells were contaminated by less than 1% of epithelial cells, as determined with anti-cytokeratin and anti-vimentin antibodies. Staining with an antibody against factor VIII-VW excluded contamination by endothelial cells. The absence of CD45-positive cells in epithelial and stromal cell cultures confirmed the efficient depletion of immune cells.

Gelatinase A Production and Activation by Epithelial and Stromal Cells

Zymographic analysis showed that gelatinolytic activity was essentially undetectable in the culture medium of endometrial epithelial cells (not shown). In contrast, medium conditioned by stromal cells (in basal conditions with E_2+P) yielded a gelatinolytic band corresponding to the inactive pro form (66 kD) of MMP-2 (fig. 2A, lane A).

Effect of Epithelial Cell-Conditioned Medium on Stromal Gelatinase A Activity

To detect a potential paracrine action of epithelial cells on stromal cell MMP-2 production, stromal cells pretreated with E_2+P until confluency (5–7 days) were incubated for 24 h with culture medium conditioned by epithelial cells pretreated with E_2+P. Stromal cell production of MMP-2 was not affected, as shown by zymography, but 10% of MMP-2 was composed of the 62- and 59-kD activated forms (fig. 2B, lane A).

Direct Effect of Cytokines

The above results suggested that gelatinase A activation by stromal cells was modulated by soluble factor(s) produced by epithelial cells. Consequently, the effect of four major endometrial cytokines (IL-1, LIF, TNF-α, TGF-β) potentially able to regulate MMP production and activation [12, 14–16, 35] was tested on stromal cells. None of these cytokines directly affected MMP-2 production or activation by stromal cells, as shown in figure 2A

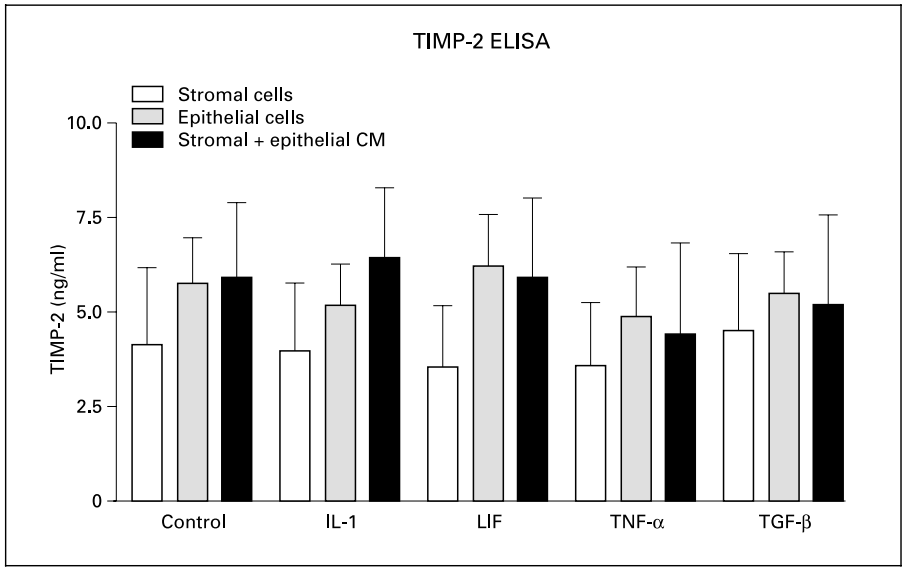

Fig. 3. TIMP-2 ELISA. No significant modulation of TIMP-2 production was found in the conditioned media (CM) by stromal, epithelial or stromal cells incubated in epithelial CM. CM from epithelial cells was added to stromal cell cultures after epithelial cells were treated with estrogen and progesterone alone (control), or after epithelial cells were treated with estrogen and progesterone plus cytokines (IL-1β, LIF, TGF-β or TNF-α). Values are means ± SD.

(lanes B–E). The same cytokines failed to induce MMP-2 production by epithelial cells (data not shown).

Indirect Effect of Cytokines

Combined treatment of epithelial cells with E_2+P from day 0 until extraction, and with one of the above cytokines for 24 h before extraction, markedly enhanced the capacity of the resulting conditioned medium to activate MMP-2 produced by stromal cells (fig. 2B, lanes B–E): up to 40% of the MMP-2 produced by stromal cells was activated in these conditions.

TIMP-2 Production

To determine if this increased MMP-2 activation by stromal cells resulted from reduced levels of the specific inhibitor of MMP-2 activation, TIMP-2, we used a specific ELISA. TIMP-2 production by epithelial and stromal cells is shown in figure 3. Neither the cytokines (IL-1, LIF, TNF-α, TGF-β) nor culture medium conditioned by epithelial cells affected TIMP-2 production by either cell type. Each measurement was made in triplicate and experiments were repeated three times.

Discussion

Tissue changes during the menstrual cycle involve complex processes, including cross-talk between various cell types. Gelatinase A (MMP-2) is widely considered to be a key effector of ECM remodeling, by direct proteolysis of ECM proteins and subsequent activation of other MMPs. It is also involved in cell invasion mechanisms and tumor progression [36, 37]. Its overall activity depends not only on its abundance but also on the balance between activated MMP-2 forms and the physiological inhibitor TIMP-2 [22, 23].

In this work we examined interactions between endometrial epithelial and stromal cells that might affect the overall activity of MMP-2. In our experimental conditions, cultured epithelial cells did not produce gelatinase A, in keeping with data obtained by in situ hybridization [8, 37] and with cultured cells [38, 39]. According to previous reports, gelatinase A is only produced by cultured stromal cells [12, 14, 31, 39]. Our data show that stromal cells produce the proform of MMP-2, and do not activate MMP-2 in basal conditions (fig. 2A). About 10% of total MMP-2 produced by stromal cells became activated, being converted into the 62- and 59-kD forms, within 24 h of incubation in the presence of medium conditioned by epithelial cells (fig. 2B). This indicates that epithelial cells secrete a soluble factor that induces MMP-2 activation by stromal cells. This epithelial soluble factor is unlikely to be IL-1, LIF, TNF-α or TGF-β, as none of these cytokines was able to reproduce the effect of epithelial cell-conditioned medium when directly added to cultured stromal cells. In contrast, treatment of epithelial cells with each of these cytokines markedly increased the effectiveness of conditioned media in stimulating stromal cells to activate MMP-2. This epithelial factor increased the proportion of activated MMP-2 (40%), while TIMP-2 concentrations

remained essentially unaffected. Thus, the balance between MMP-2 proteolytic activity and its inhibition appeared to be displaced in favor of the former.

MMP-2 is activated in various tissues, including endometrium, through a process occurring at the cell surface and requiring the membrane-anchored metalloproteinase MT1-MMP and controlled amounts of TIMP-2 [20, 40, 41]. In the field of cancer biology, previous reports have suggested the existence of soluble factor(s) that promote MMP-2 activation by MT1-MMP [42, 43]. During the menstrual cycle, MMP-2 expression and activation may be regulated [8, 22]. Data obtained with explants and purified stromal cell cultures suggest that MMP-2 regulation differs from that of other MMPs [14], but these studies did not examine potential cooperation among the different endometrial cell populations. Our data shed some light on the mechanism of MMP-2 activation in endometrial tissue. It is plausible that the uncharacterized epithelial soluble factor able to stimulate MMP-2 activation but not its production increases MT1-MMP expression at the surface of stromal cells. We are currently attempting to purify this factor and to verify its molecular mechanism of action.

In the endometrium, the regulation of other MMPs, such as MMP-1, MMP-3, MMP-7 and MMP-9, depends on paracrine loops involving cytokines (IL-1, LIF, TGF-β, TNF-α). The cytokines tested in the present study were unable, alone, to stimulate MMP-2 activation; however, pretreatment of epithelial cells by these cytokines increased the level of the putative soluble factor able to induce MMP-2 activation by stromal cells incubated with this conditioned medium. This further strengthens the hypothesis that a cytokine network linking all endometrial cells culminates in epithelial control of stromal-mediated proteolysis, as previously demonstrated for MMP-1, 3, 7 and 9. As MMP-2 is able to degrade various matrix components, including epithelial and subendothelial or decidual basement membrane material [7, 44], this apparent paracrine regulation of MMP-2 activation could be important during cellular phenotype transitions linked to decidualization, implantation and/or menstruation.

These preliminary results show that endometrial epithelial cells produce a soluble factor that stimulates stromal cells to activate MMP-2. Further studies are required to identify this factor, and to determine its regulation and effect on MT1-MMP expression.

Acknowledgements

This work was supported by grants from the Fond National de la Recherche Scientifique (FNRS, Brussels, Belgium), the Fondation Léon Frédéricq (University of Liège, Belgium), the Communauté Française de Belgique (Actions de Recherche concertée), and the Fonds d'Investissements de Recherche Scientifique (FIRS, CHU, Liège, Belgium).

F.G. and S.P. are research assistants of the FNRS of Belgium.

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