BELGIAN ASSOCIATION FOR THE STUDY OF CANCER

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ALKALINE PHOSPHATASE POSITIVE RETICULAR CELL NETWORK RECOVERY AFTER RADIATION-INDUCED MARROW APLASIA IN MICE

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Among bone marrow stromal cells, reticular fibroblasts expressing alkaline phosphatase (ALP) are generally assumed to create microenvironmental « niches » for granulopoiesis. The spatial distribution and the density of this network are profoundly altered in many hematological malignancies. The factors regulating this network are not known : some observations on bone marrow biopsies of post-chemotherapy aplasia during recovery suggest that the recovery of fibroblastic reticular cells, in terms of ALP expression and spatial distribution precedes hemopoietic recovery, which, in turn, seems to exert a negative control on ALP expression. First, we tested this hypothesis; we analyzed the density of ALP positive network during the recovery of murine bone marrow aplasia after a 4 Gy whole-body irradiation. We showed that the ALP positive reticular network has stayed nearly invariable during 24 hours after the irradiation. Thereafter, the density of the network progressively increased to reach a maximum on day 3, when the bone marrow was almost completely empty of hemopoietic cells. Following days, bone marrow cellularity progressively increased in inverse proportion to the decrease of the ALP network density, both parameters reaching normal values on day 14 after irradiation. Then, we wanted to know whether ALP positive reticular network increase is due to reticular fibroblast cells proliferation or to the recruitment of ALP positive cells. To answer this question we did double staining (ALP-BrdU) on mice bone marrow plastic sections after irradiation and BrdU injection. We showed that some ALP positive cells have incorporated the BrdU. These data indicate that the recovery of the bone marrow environment precedes the hemopoietic recovery, partially due to a recruitment of ALP positive cells, and strongly suggest that hemopoietic cells control some features of this microenvironment.

(RE-)INFECTION OF ELDERLY WOMEN WITH HUMAN PAPILLOMAVIRUS. INTERIM RESULTS OF A FOLLOW-UP STUDY

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Background : The implementation of human papillomavirus (HPV) detection in cervical cancer screening is hindered by the high prevalence of HPV positive women with normal cytology results (false-positivity). Previously, we have shown that HPV detection may be a valid tool to reduce overscreening of elderly women, since the prevalence of HPV positive women is very low in this age group (3 – 3.5 %). Furthermore, it has been shown that HPV detection has a high negative predictive value. However, little is known about the frequency of (re-)infection of elderly women with HPV. Therefore, we are currently performing a follow-up study to investigate this frequency.

Methods : DNA was isolated from the residual cells of the liquid-based cytology material by a freeze-thaw cycle followed by boiling. DNA integrity was checked by beta-globin PCR and HPV DNA was detected by the GP5+/6+ general primer PCR, followed by non-radioactive enzyme immunoassay detection.

Results : To date, 367 women, aged between 17 and 80 years, have provided 781 samples, ranging from 2 to 4 smears per woman. Seventeen women had one or more smears that were beta-globin negative, and were therefore excluded from further analysis. 302 women were HPV-negative in all smears, 24 women cleared their initial infection within two years (range 2 – 24 months, median 11 months), 12 women had a persistent infection (same HPV type detected at both/all visits) and eight women (age range 20 – 43 years) acquired a new infection during follow-up. Four women had a more complex HPV pattern.

Discussion : Preliminary results show that (re-)infection did not occur in 119 women of 50 years and older after a follow-up period of one to two years, suggesting that withdrawal of HPV-negative women with normal cytology results from cervical cancer screening does not put these women at an increased risk. However, both the number of women will have to be increased and the duration of follow-up extended.

BOLD CONTRAST WITHIN TUMORS : HOW DOES IT CORRELATE TO PO_2? SIMULTANEOUS AND CO-LOCALIZED MEASUREMENT USING GRADIENT-ECHO FMRI AND OXYLITE-OXYFLO®

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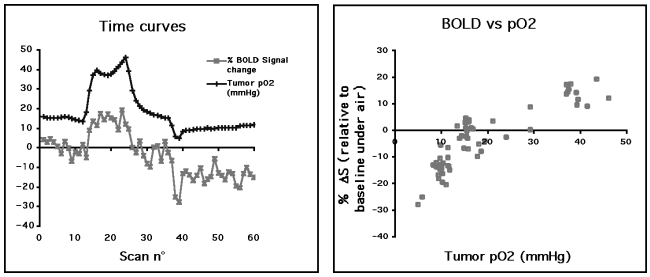
BOLD imaging is often proposed as a tool to evaluate the responsiveness of tumors to oxygen changes. However, the signal is combination of flow and oxygen dependant parameters. Up to-now, the validity of the approach relies only on the comparison of BOLD signal with oxygen measurements carried out on different animals or animals measured at different times and location. We have developed an experimental setup which allows a simultaneous and co-localized measurement of pO2/flow and gradient echo fMRI.
Isoflurane anesthetized mice were imaged at 4.7 Tesla. The slice for imaging was carefully selected such that it was positioned on the extremity of the Oxylite-/Oxyflo probe inserted into the intramuscularly implanted tumor. The spatial resolution for imaging was chosen to get voxels dimensions compatible with the sensitive area of the probe, while keeping a good BOLD contrast. GE images (200/20/45°) were continuously acquired during modification of gas breathing (air-50%O2-air-100%O2-air-Carbogen-air). A set of multi-echo GE images (200/6.1/6/45°) were then acquired following the same protocol. From Multi-echo images, both T2* map and S_0 map were created. We computed (pixel-by-pixel analysis) the correlation of the three time series with the signals coming from OxyLite and OxyFlo.
A typical co-localized and simultaneous recording of the GE signal and tumor pO_2.



In conclusion, BOLD contrast imaging is a powerful tool to appreciate the efficacy of a treatment of O_2 breathing. In carbogen breathing protocols, the variation in signal intensity is higher than using the oxygen protocol; this effect is likely to be due to the combination of an inflow effect together (increase in So) with a higher increase in pO_2 (increase in T2*). BOLD MRI is a non invasive method that allows a mapping of the response, and the variations observed are directly related to pO_2 changes. However, this technique has no value to estimate the basal pO_2 before treatment and BOLD MRI should be considered as a technique complementary to other methods allowing absolute quantification of the pO_2.

EXPRESSION OF VEGF AND ITS RECEPTORS IN HUMAN ILIAC CREST AND FEMORAL BONE MARROW

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The understanding of the mechanisms regulating hematopoiesis in normal bone marrow is required for progressing in the elucidation of the alteration occurring in many hematological malignancies such as myelodysplastic syndromes and leukemias. The aim of this study is to further explore the hypothesis that normal human hematopoiesis may be regulated by paracrine regulatory loops and by cross-talk between hematopoietic cells and their environment. The vascular network is the first candidate, which can be implicated, in these regulatory loops. We investigated by RT-PCR the mRNA level of the vascular endothelial cell growth factor (VEGF), its splice variants and its receptors, the VEGR-1/Flt1, the VEGFR-2/KDR and the specific VEGF165 isoform co-receptor, the neuropilin-1 (NRP-1) as well as its ligand semaphorin-III (SEM-III) known to function as VEGF antagonist. Bone marrows were collected from the iliac crest and the femur from 11 donors. VEGF121 and 165 are the main isoforms expressed at variable levels in iliac crest and femur while the extracellular matrix binding-VEGF189 is present in all samples, although at low level. Neither the 145 nor the 206 variants are detected. The VEGFR-2 is similarly expressed in all samples. Interestingly we observed a higher steady state level of VEGFR-1, NRP-1 and SEM-III in the femoral bone marrow, rich in adipocytes than in the hematopoietic iliac crest. To assess the cellular origin of this overexpression in femoral bone marrow, adipocytes were isolated from collagenase-digested femoral biopsies on the basis of their floating properties. The purified adipocyte population effectively expressed more NRP-1 mRNA than the sedimented cells population (stromal cells and hematopoietic cells). These results will be confirmed by immunostaining and western-blot analysis at the protein level. In conclusion, this preliminary study showed that the expression of NRP-1 in adipocytes is high when hematopoiesis is low, suggesting that these cells may play an active role in regulation of hematopoiesis and angiogenesis.

QUANTIFICATION OF MICROMETASTATIC DISEASE IN BREAST CANCER PATIENTS : OPTIMALISATION OF THE METHOD USING REAL-TIME QUANTITATIVE PCR FOR THE DETECTION OF CK19 MRNA IN BLOOD

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Introduction : Earlier diagnosis has led to stage migration and better prognosis, but a subgroup of patients with early breast cancer continue to succumb to metastatic disease. Nodal status remains the most valuable marker to predict for the presence of occult spread. Morphological and molecular techniques suffer from low sensitivity and a lack of quantification. We have developed a sensitive and quantitative assay to detect breast cancer cells in venous blood using a quantitative RT-PCR identifying transcripts of the CK19 gene

Methods : Total RNA was isolated from full blood samples of patients and healtzy volunteers using the Qiagen Rneasy ® total RNA kit. For generation of first strand cDNA, 2µg of total RNA was reverse transcripted with the high-Capacity cDNA Archive Kit (Applied Biosystems). A cDNA-specific CK19 Taqman ™ primer and probe set was developed using Primer Express ® software. PCR was performed on the ABI Prism 7700 system and the standard thermal cycling protocol. The cycle at witch the emission intensity rises above baseline is referred to as cycle threshold (CT).

Results : The CK19 quantities were analyzed in triplicate, normalized against GAPDH as a control gene and calculated relative to a reference sample. To evaluate the sensitivity of the assay, CK19 mRNA was measured in blood samples spiked with MDA MB 231 cells. The minimum detectable level was 1 tumor cell in 5 000 000 WBC. The correlation coefficient of the standard curve with MDA MB 231 cells was at least 0.99. The within-assay reproducibility for Ct CK19 tested on 33 triplicated samples has a mean CV of 0.7% (min CV 0.2% - max CV 3.1%). CK19 and GAPDH efficiencies are approximately equal, so the delta-delta CT method is used to quantify CK19 expression in blood samples. A total of 33 blood samples of healthy volunteers and 30 samples of patients with progressive breast cancer were tested for CK19 and GAPDH-specific Taqman PCR. Two volunteers and 1 patient are excluded due to an abnormal GAPDH expression. Twelve patients with progressive disease had increased CK19 levels in venous blood.
Conclusion : A sensitive and specific real-time quantitative RT-PCR has been developed. This assay will be used in patients with operable breast cancer for the detection of micrometastatic disease.

A DEFINED ROLE OF ANGIOGENESIS INHIBITION AS PART OF THE OVERALL VASCULAR TARGETING ACTIVITY OF COMBRETASTATIN A-4 PHOSPHATE.

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Using various rodent tumour models, several research teams demonstrated the severe intratumoral effects (e.g. vessel collapse, haemorrhagic necrosis) induced by the vascular targeting Combretastatin A-4 phosphate (combreAp). With the syngeneic rat rhabdomyosarcoma model, we demonstrated not only the presence of an important anti-tumour efficacy (Landuyt et al, Eur J Cancer, 2000), but moreover a translation in a significant, yet tumour size dependent, growth delay. With the strategy to introduce a second combreAp injection at a specific stage of tumor regrowth, we showed a clearcut additional tumor growth dalay as compared with the growth delay measured after a single injection .
Elaborating this result, together with some indications in the literature on proliferating endothelial cell (EC) damage, we hypothesized that combreAp, separate from its major vascular targeting activity, may interfere with the angiogenesis. The plausible anti-angiogenic effect was investigated in different ways . Data using the classical *in vitro* HUVEC migration assay clearly show interference with this vessel renewal-reflecting mechanism in a drug concentration-dependent way. Furthermore the data indicate that EC migration was significantly inhibited at a dose that did not arrest the proliferation of the EC's. We also used *in vitro*/*in vivo* colagen-based sprout formation assay , and demonstrated that combreAp also inhibits BCE (bovine capillary EC's) sprout formation .
As it may be suggested that HUVEC's are not fully represenive for the morphological identity of intratumoral blood vessel endothelium, we compared the combreAp effectivity *in vitro* using capillary EC's as well as tumor EC's with the effectivity in the HUVEC system.
Separately, we assessed the possibility of a direct tumour cell cytotoxic effect from combreAp. This thought was partly based on the efficiency of the compound being stronger in larger rat rhabdomyosarcomas, in terms of growth delay, than in smaller ones. These *in vitro* results demonstrate a major direct tumour cell kill in the rodent tumour R1 (rhabdomyosarcoma) cell line. Moreover, using several human tumour cell lines (breast – HS570T and colon – LS174T), the same conclusion could be drawn. In fact, the combreAp-induced cell kill was 10 to 1000 fold stronger with tumour cells as compared with HUVEC's.
Induction of combreAp-induced apoptosis ,which was quantified by measuring subdiploid cells after propidium-iodide staining, was measured in both HUVEC (5%) and BCE (16%) cells. Interestingly, an increase of cells in G2/M in time was observed in HUVEC's but no cell cycle phase change in BCE. These investigations overall indicate the complexity and at the same time the potential use of 'vascular targeting' compounds in anti-cancer therapies, as well as the presence of an anti-angiogenic action part.

DMP-1, A DENTIN GLYCOPROTEIN, PROMOTES ATTACHMENT BUT NOT MIGRATION OF HUMAN ENDOTHELIAL CELLS

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We have recently demonstrated that bone sialoprotein (BSP), a bone extracellular matrix glycoprotein, mediates both attachment and chemotactic migration of human umbilical endothelial cells (HUVEC). We have shown that these interactions involved avb3 integrin receptors on endothelial cells and the RGD domain of BSP. Based on biochemical and genetic features, bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein I (DMP-1) and dentin sialophosphoprotein (DSPP) have been recently classified in a unique family named SIBLING for Small Integrin-Binding Ligand, N-linked Glycoprotein. BSP and DMP-1 are similar in that they are secreted, phosphorylated and sulphated sialoproteins that are acidic in character. As BSP, DMP-1 primary structure contains the integrin-binding RGD tripeptide. Therefore, we have investigated whether DMP-1 could mediate the attachment and the migration of HUVEC. We show that HUVEC are able to bind and spread on purified recombinant DMP-1 coated in plastic wells. This attachment could be prevented by the addition of anti-avb3 but not anti-avb5 antibodies. Next, we tested the chimiotactic properties of DMP-1 using the Boyden chamber assay. We also tested DMP-1 for its ability to stimulate ongoing angiogenesis on the chick chorioallantoic membrane (CAM) assay. Contrarily to what we had previously observed with BSP, DMP-1 failed to promote both the migration of HUVEC even at millimolar concentrations and did not stimulate the formation of new vessels in the CAM assay.
In this study, we present evidence that DMP-1 and BSP promote endothelial cells attachment through their RGD domain. However, they do not affect migration and angiogenesis in the same way in spite of the fact that they share structural and biochemical features.

DMP-1, A DENTIN MATRIX GLYCOPROTEIN, IS EXPRESSED IN HUMAN LUNG CARCINOMA

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Lung cancer belongs to the group of malignant lesions that specifically select bone as secondary implantation site. We have previously shown that bone sialoprotein (BSP) is expressed in various type of cancers that are characterized by a high affinity for bone such as breast, prostate and lung adenocarcinoma. Based on biochemical and genetic features, BSP, osteopontin (OPN), dentin matrix protein I (DMP-1) and dentin sialophosphoprotein (DSPP) have been recently classified in a unique family named SIBLING for Small Integrin-Binding Ligand, N-linked Glycoprotein. Therefore, we decided to investigate whether DMP-1 could also be detected in lung cancer. Using immunophosphatase technique and a specific polyclonal antibody directed against DMP-1, we examined the expression of DMP-1 in 29 squamous carcinoma, 20 adenocarcinoma and 10 bronchioloalveolar carcinoma. Our preliminary results show that DMP-1 is expressed in the majority of the adenocarcinoma and most of the squamous carcinoma. The staining was mainly cytoplasmic.

This study represents the first demonstration of DMP-1 expression in cancer. The role of DMP-1 in lung cancer is largely unknown and its potential involvement in the propensity of lung cancer cells to metastasize to the skeleton remains to be determined.

INVESTIGATION OF THE ROLE OF NUCLEAR GALECTIN-3 IN PROSTATE CANCER PROGRESSION

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Our research project aims to determine the functions played by galectin-3, a b-galactose-binding lectin, in the nucleus of prostate cancer cells. Galectin-3, a 30 kDa protein, is involved in several physiological and pathological events. Extracellular galectin-3 mediates cell-cell and cell-matrix interactions, and cytosolic galectin-3 mediates apoptosis control. Studies conducted in various laboratories, including ours, have demonstrated decreased galectin-3 expression in cancer cells compared with normal cells. Cytoplasmic expression and nuclear exclusion are characteristics of invasive cancer cells from endometrium, colon and prostate carcinomas, a finding associated with bad prognosis in prostate carcinoma. Our project aims to better understand the biological significance of nuclear galectin-3 in cancer cells. We have cloned the galectin-3 ORF cDNA upstream of 3 nuclear localization sequences (NLS) in an expression vector, that was transfected in LNCaP prostate carcinoma cells that do not express endogenous galectin-3, in order to verify the hypothesis according to which the expression of galectin-3 in the nucleus could decrease or prevent the expression of cancer phenotype. After determination of the best transfection conditions in transient transfections experiments and verification of the nuclear localization of galectin-3 by immunohistochemistry, stable transfections have been performed using G418 selection. Screening of the G418-resistant clones revealed that several of them presented with G3NLS fusion protein expression. Immunohistochemical analysis of these clones revealed effective nuclear localization of galectin-3 immunoreactivity. Preliminary characterization of the clones revealed no differences in cell proliferation and adhesion to laminin-1. Zymographic analysis of the conditioned media revealed increased pro-MMP2 (progelatinase A) activity in cytoplasmic galectin-3-expressing clones compared to nuclear galectin-3-expressing clones. Further *in vitro* and *in vivo* experiments are ongoing to better characterize the functions played by nuclear and cytoplasmic galectin-3 in prostate cancer cells.

GENERATION OF TUMOUR CELL – DENDRITIC CELL HYBRIDS VIA THE EXPRESSION OF A VIRAL FUSOGENIC MEMBRANE GLYCOPROTEIN FOR THE INDUCTION OF T-CELL IMMUNITY AGAINST MALIGNANT MELANOMA

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The discovery of tumour specific antigens has led to the development of immunotherapy strategies that could activate the immune system to recognize and eliminate tumour cells. T-cells, playing a central role in cellular immunity, are only able to recognize tumour antigens when presented as peptides in association with MHC molecules. Moreover, expression of co-stimulatory molecules on the tumour cells is required for T-cells to react adequately upon recognition. Most tumour cells have downregulated or are completely lacking the expression of both MHC and co-stimulatory molecules and therefore escape from immune surveillance.
In order to create a powerful tool to stimulate T-cell immunity, the establishment of tumour cell-dendritic cell (TC/DC) hybrids was proposed to combine the characteristics of both cell types, the expression of tumour antigens and the antigen presentation machinery with the co-stimulatory molecules. To date, methods such as poly-ethylene glycol (PEG) and electrofusion have been studied for this purpose, but they are not very efficient. In our laboratory two fusion strategies, using the Gibbon Ape Leukaemia Virus-derived fusogenic membrane glycoprotein (FMG), are being developed.
The first strategy involves the establishment of a stable tumour cell line that expresses FMG. The EB.81 melanoma cell line was chosen because it expresses a large range of tumour antigens. The expression of FMG will be regulated by a tetracycline-inducible (Tet-on) promoter to avoid continuous expression of the FMG, which would lead to extensive, lethal, cell fusion. The Tet-on/FMG plasmid has been generated and is inducible in transient transfection experiments. A stably transfected EB.81 melanoma cell line is being established. This stable cell line will be fused with autologous dendritic cells from melanoma patients for anti-tumour vaccination.
For the second strategy a recombinant adeno-associated virus vector has been constructed and produced. This vector carries the FMG transgene under the regulation of a CMV promoter. After infection, tumour cells express the FMG protein, which results in the formation of syncytia between the tumour cells. Overlaying infected cells with dendritic cells will result in the formation of TC/DC hybrid cells. In the future this strategy will be used to fuse AAV/FMG-transduced autologous tumour cells with autologous dendritic cells from melanoma patients.

ONCOTROPIC VECTORS DERIVED FROM THE AUTONOMOUS PARVOVIRUS MVM(P)

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The preferential expression of autonomous parvoviruses in tumour cells and their oncolytic activity have attracted attention to the potential use of these viruses as vectors for cancer gene therapy. Moreover they are non-pathogenic in adult animals and they seem to be associated with low or no immunogenicity. Other interesting features are their episomal replication and high stability.

MVM/IL2 vectors were elaborated for tumour cell targeted gene transfer to induce an anti-tumour immune response. The original problems encountered with these vectors, low titres and contamination with wild-type virus, have been partially alleviated by integrating helper sequences into a packaging cell line to prevent recombination and to allow for the amplification of vector stocks through serial infections.
Based on the structure of spontaneously occurring defective particles, we have derived second-generation vectors lacking all VP sequences downstream the insertion site for transgenes. Matched helper plasmids that have no homology with the vectors in the right hand part of the genome were also constructed. With this system helper-free vector stocks have been produced, but still at low titres. Several modifications to the vector i.e. the insertion of mouse genomic DNA to increase its size and the addition of an NS1 nicking site to the viral origin of replication, have improved vector yields. These second generation vectors have two significant advantages over first generation vectors : (i) the generation of replication competent virus is greatly reduced (ii) the expression of the IL2 transgene/particle is about 6 fold higher.

With MVM/IL2 stocks obtained in a first generation packaging cell line, we could show that MVM/IL2 infection of K-1735 melanoma cells strongly reduces their ability to form tumours *in vivo*. We could also show that MVM/IL2 infected cells implanted in one flank could prevent the development of non-infected tumours on the opposite flank. In contrast to most other viral vectors, this antitumour effect could be obtained when the vector was used at a very low multiplicity of infection.

EVALUATION OF CAMEL SINGLE-DOMAIN ANTIBODY FRAGMENTS AS MODULAR BUILDING BLOCKS TO MAKE BISPECIFIC CONTRUCTS FOR USE IN IMMUNOTHERAPY

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Size reduction and affinity increase is decisive for the successful *in vivo* use of antibodies in diagnosis and/or therapy. The discovery that functional antibodies comprising heavy-chains only evolved naturally in camelids formed the basis to clone the VH-only repertoire of the peripheral blood lymphocytes of an immunized dromedary and to select antigen-specific single-domain antibodies (V_HH) from these libraries by panning. Two camel single-domain fragments, cAb-Lys2 and cAb-Lys3, recognizing an overlapping epitope of lysozyme, and a bivalent cAb-Lys3 were investigated for their ability to target transgenic tumors expressing lysozyme on their membrane. Biodistribution studies revealed that these monomeric and bivalent camel single-domain antigen binders specifically target lysozyme-expressing tumors and metastatic lesions, but no antigen-negative tumors and metastases. The excess of antibody is rapidly eliminated from the blood circulation and no VHH retention was observed in normal organs.
Having established the tumor targeting potential of V_HH, we went on using cAbs as modular building block in the generation of more complex constructs that have a favourable therapeutic effect. More specifically, recombinant fusion proteins were constructed that contain anti-CEA (human carcino-embryonic antigen) or anti-lysozymeVHH fused to the *Enterobacter cloacae* ß-lactamase (bL), expressed, and purified to homogeneity in an *E. coli* expression system. *In vitro* experiments demonstrated that CEA5-cAb-bL bound to LS174T colon adenocarcinoma cell line expressing the CEA antigen and effected the activation of CCM, a cephalosporin nitrogen mustard prodrug. Further work aims at evaluating these immunoconjugates to detect and eliminate tumor cells upon systemic administration in *in vivo* experimental tumor model systems.

DNMT3L, A MEMBER OF THE DNMT3 DNA METHYLTRANSFERASE, ASSOCIATES WITH HISTONE DEACETYLASE ACTIVITY

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Transcriptional regulation can give rise to cellular dysfunctions, which can lead to cancerogenesis. Recent advances strongly suggest that DNA methylation has a pivotal role in tumorigenesis. Indeed, it seems increasingly clear that methylation is an alternative mechanism to mutation to inactivate tumor supressor genes, thereby contributing to cancer. The molecular mechanisms by which DNA methylation participates to cancer are yet to be established. To elucidate these mechanisms, it is crucial to understand how DNA methylation represses transcription.
We have recently shown that the DNA methyltransferase Dnmt1 and Dnmt3a interact with the HDAC histone deacetylases and repress transcription, at least partly, through histone deacetylase activity (Fuks et al., 2000; Fuks et al. 2001), suggesting that methylation and deacetylation could act together to potentiate the repressed state. Here, we show that Dnmt3L, which shows strong sequence homology with Dnmt3a, interacts both *in vitro* and *in vivo* with the histone deacetylase HDAC1. Consistent with this interaction, we found by means of the histone deacetylase assay that Dnmt3L can purify histone deacetylase activity from nuclear extracts. We find that the link between Dnmt3L and deacetylation is mediated by the cysteine-rich region of Dnmt3L that is closely related to the PHD–like motif. In transfection assays using GAL4-fusion constructs, we identify the PHD-like motif of Dnmt3L as a transcriptional repressor domain. Collectivelly, these data indicate that the connection to deacetylase activity is a conserved and shared feature within the Dnmt3 family. Interestingly, the identification of Dnmt3L, which lacks a methylase domain but retains the potential to associate with deacetylase activity highlights the fact that, in under some circumstances, the HDAC associated functions may be working independently of the methylase functions. In conclusion, this work strongly suggest that the DNA methyltransferases are multi-faceted proteins with their de novo methylase activity beeing only one of their roles in the cell.

THE ROLE OF TRANSCRIPTION FACTOR NF-KAPPAB IN CELLULAR RADIORESPONSE. A NEW HYPOTHESIS!

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Background : Several studies showed that inhibition of the NF-kB pathway enhances radiosensitivity by increasing radiation-induced apoptosis and by decreasing DNA-damage repair (1; 2). NF-kB is therefore thought to protect cells from radiation damage.
Our laboratory recently showed that iNOS (inducible Nitric Oxide Synthase), activated by cytokines in hypoxic conditions is capable of radiosensitizing tumour cells through the production of NO (3). We hypothesize that NF-kB is responsible for this iNOS activation and may thereby support the radioresponse of tumour cells in a hypoxic microenvironment.
Methods : EMT-6 tumour cells were exposed to increasing concentrations of LPS in 1% oxygen and afterwards were analysed for the expression and binding activity of NF-kB, the expression of iNOS and the accumulation of nitrite, an oxidative product of NO. The expression of NF-kB and iNOS were estimated by Western blot, the binding activity of NF-kB by EMSA. Hypoxic cell radiosensitivity was measured by clonogenic survival after irradiation of cell micropellets.
Results : LPS at 0.001 – 0.1 mg/ml induced a dose dependent expression and binding activity of NF-kB. This was associated with an increasing iNOS expression and nitrite production, which were abrogated by the NF-kB inhibitors PAO and TLCK. Treatment of tumour cells with 0.01 – 0.1 mg/ml LPS resulted in respectively a 1.8 and 2.1 fold hypoxic cell radiosensitization, which was inhibited by PAO and TLCK and by the iNOS inhibitor aminoguanidine.
Conclusion : NF-kB plays an important role in the hypoxic up-regulation of iNOS by LPS. In contrast to previous studies, we clearly demonstrate a positive impact of NF-kB on the radioresponse of tumour cells under hypoxic conditions.
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IN VIVO EFFICACY OF SPACER-CONTAINING ANTITUMOR PRODRUGS OF DOXORUBICIN DESIGNED FOR SPECIFIC ACTIVATION BY TUMOR-ASSOCIATED PLASMIN

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Like most chemotherapeutic agents, the anthracycline doxorubicin (DOX) suffers from severe dose-limiting side effects, such as cumulative cardiotoxicity, and in addition resistance mechanisms can develop after treatment with this agent. In order to increase the therapeutic index, low-toxic prodrugs of doxorubicin were synthesized that were designed for selective activation in tumor tissue. Prodrugs 1 and 2 contained a tripeptide specifier to be directly recognized by the tumor-associated enzyme plasmin, which is present in elevated levels in invading tumors. In Prodrug 1 the specifier was connected to DOX via a conventional 1,6-self-elimination spacer, and we previously reported in vitro selective cytotoxicity of 1 against urokinase plasminogen activator (u-PA) transfected MCF-7 cells. In Prodrug 2 a novel elongated self-elimination spacer system was incorporated between tripeptide and parent drug, to increase the efficiency of plasmin activation. Here, we present the first in vivo evidence that indicates that it is feasible to target tumor-associated plasmin. Plasmin-activated prodrugs 1 and 2 were evaluated both in vitro and in vivo on murine EF43.*fgf-4* tumor cells, and compared to DOX. In vivo toxicity (weight loss, death) and antitumor efficacy (tumor volume changes) were evaluated. The two prodrugs showed similar in vitro toxicity towards EF43.*fgf-4* cells than DOX. Furthermore, in the presence of the selective plasmin inhibitor Trasylol, the prodrugs were much less toxic against EF43.*fgf-4* cells, indicating a plasmin mediated drug release. In sharp contrast to DOX, both prodrugs significantly reduced the volume of EF43.fgf-4 tumors in Balb/c mice without discernable systemic toxicity. In large EF43.*fgf-4* tumors, the elongated spacer containing prodrug 2 showed to be significantly more effective in reducing tumor growth than prodrug 1. These data validate the concept of tumor-targeting of chemotherapeutic agents activated by plasmin and the use of elongated spacer systems to improve the efficiency of in vivo drug release.

SECRETORY LEUKOCYTE PROTEASE INHIBITOR (SLPI) ENHANCES METASTATIC POTENTIAL OF LEWIS LUNG CARCINOMA CELLS

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Solid tumors are 'organoids' which consist of malignant cells, host cells and extracellular matrix. Malignant cells and their surrounding environment constantly interact with each other. These interactions profoundly influence the phenotypic profiles of both cancer cells and infiltrating host cells. Identification of genes that are engaged in, and/or affected by, cross-talk between cancer cells and tumor microenvironment is therefore crucial for the development of prognostic, diagnostic and therapeutic approaches.
We report that subcutaneous growth of low-metastatic Lewis Lung Carcinoma (3LL-S) cells coincides with massive infiltration of inflammatory cells and increases the lung-colonizing potential of these cancer cells significantly. Through a subtracted cDNA library between *in vitro* cultured and subcutaneous tumor-derived 3LL-S cells, among several genes showing differential expression, the gene encoding murine Secretory Leukocyte Protease Inhibitor (mSLPI), a serine protease inhibitor with pleiotropic anti-inflammatory properties, was identified as up-regulated in 3LL-S cells upon subcutaneous growth. Furthermore, using transfection experiments, we demonstrate that *mSLPI* plays a causative role in the metastatic behaviour of these cells. We also show that *mSLPI* expression is induced by TNF-a and IL-1b, suggesting that the effect of the tumor microenvironment on *mSLPI* expression, and eventually on metastasis can in part be exerted by these inflammatory cytokines. Moreover, although the proteins exhibit different characteristics, transfection of the human homologue of *mSLPI* in 3LL-S cells, also enhances lung-colonizing potential of these cells.

IN VIVO EFFICACY OF THERAPEUTIC DC VACCINES IS INCREASED BY CHEMOTHERAPY TREATMENT OF TUMOR CELLS

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Recent developments in immunotherapy allow to envisage new treatments of cancer. The most promising are cytokine expression at tumor site and dendritic cell (DC) vaccination. DC are the only antigen presenting cells capable to activate naive CD4+ and CD8+ T lymphocytes and initiate regulatory and cytotoxic immune responses.
It has been shown recently by *in vitro* studies that DC, not macrophages, can phagocyte apoptotic cells, present derived peptides on MHC class I molecules and stimulate therefore cytotoxic T CD8+ specific responses.
The aim of our work was to test *in vivo* the efficacy of vaccination with DC pulsed by apoptotic tumor cells to induce therapeutic immune responses, within a rat tumor model (9L gliosarcoma).
Apoptosis is a programmed cell death whose major consequences are : caspase activation, phosphatidylserin translocation, chromatin condensation and DNA fragmentation. We first compared and quantified *in vitro* the apoptosis induced in 9L or 9L mGM-CSF cells following irradiation or treatment with chemotherapy molecules (mitomycin C or cisplatin).
Results showed that all agents induced apoptosis of 9L and 9L mGM-CSF cells whatever the test used to measure it. Irradiation and mitomycin C, even at 1µg/ml, were the best inducers of apoptosis *in vitro*. Cisplatin at 3µg/ml was a bad inducer.
We then tested *in vivo* the efficacy of therapeutic vaccinations with DC pulsed by apoptotic tumor cells. As experimental model, 10^5 9L cells were implanted s.c. at day 0 in one flank of Fisher 344 rats. At day 4, 11, 18, therapeutic vaccines were delivered s.c. in the other flank. These vaccines were 3.10^6 naive bone-marrow derived DC injected in combination with 5.10^6 9L mGM-CSF cells pre-treated by irradiation or mitomycin C or cisplatin. These vaccines led to cure respectively 50%, 75% and 87% of the rats. Comparatively, all the control rats died around day 40.
More detailed studies could help to investigate precisely the differences between irradiation versus chemotherapy molecules as concerns the induction of apoptosis and the induction of anti-tumor immune responses.

[1] FUSING HPV16 E7 PROTEIN TO HIV-1-TAT PEPTIDE 49-86 ELICITS POTENT THERAPEUTIC ANTI-TUMOR IMMUNITY

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Background : In cervical cancers induced by HPV infections, the E7 protein is required for transformation process and for persistence of the transformed phenotype. Moreover, E7 possesses epitopes recognized by human and mice T cells suggesting that E7 could be an appropriate target for immunotherapy of HPV-positive lesions. We constructed a histidine-tagged E7 protein (His_6-E7) and a fusion protein His_6-E7-HIV-1TatD1-48 (His_6-E7-Tat), in order to benefit from the capacity of the Tat peptide to introduce the chimerical protein into cells, which should enhance E7's epitopes presentation on MHC-1 molecules. Using CAT assay experiments, we have already shown that E7-Tat enters into cells and activates transcription of an E7-responsive promoter. In this study, we tested the efficiency of His_6-E7-Tat/QuilA immunization to protect (or cure) C57BL/6 mice (pre)injected with HPV16+ tumor cells and compared it with the one previously reported for the His_6-E7/QuilA vaccine.

Methods : To test protective anti-tumor immunity, C57BL/6 mice were injected subcutaenously (s.c.) with 10µg of His_6-E7 or 13µg of His_6-E7-Tat mixed or not to 15 µg of QuilA. Two weeks later, mice were challenged s.c. with $5X10^5$ C3 HPV16+ tumor cells. Mice were bled before and after immunization. Sera were harvested and used for anti-E7 and anti-Tat immunoglobulin ELISA detection tests. For evaluation of therapeutic anti-tumor potential, mice were challenged with $5X10^5$ C3 cells, then injected with His_6-E7/QuilA or His_6-E7-Tat/QuilA 5 and 10 days later.

Results : Prophylactic immunization with His_6-E7/QuilA or His_6-E7-Tat/QuilA has protected 57.1 to 62.5% and 75 to 100% of the mice, respectively, whereas all of the mice treated with QuilA, His_6E7 or His_6-E7-Tat showed tumor growth. Regarding the tumor therapy experiments, we observed complete tumor rejection in 62.5% of the mice injected with His_6-E7-Tat/QuilA (3 independent experiments). In comparison, 40, 0, 62.5 and 0% of the His_6-E7/QuilA injected mice eliminated pre-established tumor while it grew in all of the control animals. Comparable levels of anti-E7 immunoglobulins (IgG and IgG1) were produced after immunization with His_6-E7/QuilA or His_6-E7-Tat/QuilA. A low level of Tat-specific antibodies were detected in sera of mice injected with His_6-E7-Tat/QuilA .

Conclusions : After a single vaccination, His_6-E7-Tat/QuilA protected a higher percentage of animals against tumor growth than His_6-E7/QuilA. The former was also more potent to cure pre-established tumor, giving more reproductible results than the latter.
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ATTEMPT TO ENHANCE SUICIDE GENE THERAPY AGAINST BREAST CANCER CELLS BY USING CONNEXIN 43 GENE

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Suicide gene therapy mediated by the *Herpes simplex* thymidine kinase/ganciclovir (HSV*tk*/GCV) system has been shown to treat efficiently various types of tumors in animal models. It has been demonstrated that connexin (Cx)-dependent gap junctions between cells facilitate the intercellular spread of TK-activated GCV, thereby creating a bystander effect that improves tumor cell killing. However, the expression of Cxs in human cancer cells is often greatly reduced or undetectable, resulting in a low bystander killing which affects the efficacy of the HSV*tk*/GCV strategy.
In the case of human breast tumors, we have previously demonstrated that the HSV*tk*/GCV system is able to eradicate 50% of tumors (Grignet-Debrus *et al*., 2000, Cancer Gene Ther., 7 :215-23), despite the fact that breast cancer cells do not express Cx43 and thereby have lost their ability to communicate through gap junctions. We have also previously shown that the introduction of Cx43 gene in breast cancer cells led to a restauration of gap junctional intercellular communication and moreover, was correlated with a reduction of cell growth rate and tumorigenicity. Interestingly, we observed that when Cx43-expressing cells (Cx^+ /HSVtk^-) are present within a non-communicating population of HSVtk^+ breast cancer cells (Cx^-/HSVtk^+), there is an enhancement of the *in vitro* bystander effect. These results suggest a possible dual effect of Cx43 in breast cancer cells as tumor suppressor and mediator of bystander effect.
To pursue this study and in order to improve the suicide gene therapy efficiency against breast tumors, we investigated whether the co-expression of HSV*tk* and Cx43 genes in MDA-MB-435 cells could potentiate the HSV*tk*/GCV strategy. A clone of Cx43 expressing MDA-MB-435 cells previously generated (Grignet-Debrus *et al*., 2000, Cancer Gene Ther., 7 :1456-68) and selected for its high gap junctional intercellular communication was transfected with a vector carrying the HSV*tk*. These transfected cells demonstrated a sensitivity to GCV 900-fold higher than the parental cells, while their capacity remained similar to that of parental cells. We are currently investigating *in vivo* in nude mice, whether the co-expression of HSV*tk* and Cx43 genes can enhance the antitumoral activity of HSV*tk*/GCV gene therapy.

MONITORING OF THE CELLULAR IMMUNE RESPONSE TO A HUMAN PAPILLOMAVIRUS TYPE 16 (HPV16) E7-DERIVED VACCINE IN WOMEN WITH HPV16 POSITIVE GRADE 3 CERVICAL INTRAEPITHELIAL NEOPLASIA

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Background : Presently, there is a need to use more sensitive and quantitative assays to monitor ongoing HPV-specific cellular immunity. The characterization of this response, known to be crucial in controlling HPV infection, might be indicative of either efficient natural immunity or vaccine-induced response. We have optimized a protocol based on cytokine flow cytometry (CFC) to monitor HPV16 E7-directed cellular immunity in CIN3 patients enrolled in an HPV16 E7 protein-based vaccination trial.
Methods : Patients with biopsy-proven CIN3 and high HPV16 DNA copy number are receiving 3 injections, at 2 weeks intervals, of a recombinant HPV16 E7 fusion protein (D16E7, mutated E7 protein fused to one third of the *Haemophilus influenzae* protein D) mixed with the GSK adjuvant AS02B. Blood is obtained at each vaccination date and 4 weeks after the last vaccine injection. Plasma is stored for detection of anti-E7 IgG (ELISA) and fresh Ficoll/Hypaque-separated PBMCs are incubated overnight with or without antigens (D16E7 or His_6E7). Supernatants are frozen to test cytokines production (ELISA) and cells are stained with antibodies directed at cell surface molecules, fixed, permeabilized and stained for intracellular cytokines. Cells are analyzed by flow cytometry to enumerate IFN-g-producing $CD4^+$/$CD8^-$, $CD4^-$/$CD8^+$ and $CD3^-$/CD16-56^+ cells, and IL-5-producing $CD3^+$/$CD4^+$ cells.
Results : Out of 6 patients enrolled to date, 5 (P1-2, P4-6) have completed the vaccination program. A pre-existing D16E7-directed CD8 T-cell response was detected in 3 women (P2, P5-6); two (P5-6) also had a CD4 T-cell response, characterized by IFN-g, not IL-5 production. Only P6 had His_6E7-directed pre-vaccine responses (CD4 and CD8). The percentage of CD8- (4/5) and CD4-cells (3/5) producing IFN-g in response to D16E7 was increased/induced after vaccination. Two patients (P1, P5) developed CD4- and CD8-cells responses to His_6E7. Of note, they also showed a partial colposcopically measured regression of their lesions. Vaccine has not induced specific IL-5-producing cells. CFC and ELISA results were concordant, with CFC detection being more sensitive. Vaccine has elicited the production of anti-E7 IgG (4/4) undetectable in pre-immune plasma. DTH to D16E7 was not detected, neither before nor after vaccination. Interestingly, massive infiltration with lymphocytes was present in P5 post-vaccination cone biopsy. Such cells were barely detectable in the pre-vaccine CIN3 biopsies. Treatment was also associated to HPV16 DNA clearance (P5) or decrease (P2) from cervical smears and biopsies.
Conclusions : This report demonstrates that a single short *in vitro* stimulation of PBMCs from CIN3 patients with HPV16 E7-derived proteins, followed by CFC, allow to quantify and characterize pre-existing and vaccine-induced specific cellular immunity. We are currently following up additional patients in order to confirm vaccine immunogenicity and to assess correlation between immunological, virological and clinical responses.
We thank GlaxoSmithKline (GSK) Biologicals Belgium for providing the study vaccine. This work was supported by the Yvonne Böel Foundation, the Fonds de la Recherche Scientifique Médicale and the Région Wallonne.

DIFFERENTIAL PRODUCTION OF CYTOKINES AND ACTIVATION OF NF-KB IN HPV-TRANSFORMED KERATINOCYTES

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The chronic infection of keratinocytes of the uterine cervix by oncogenic human papillomaviruses (HPV) is associated with the development of (pre)-cancerous lesions of the uterine cervix. We have proposed that chronic infection of keratinocytes by HPV modifies the expression of cytokines potentially important for the anti-tumoral response by interfering with the NF-kB signal pathway. We evaluated the constitutive and IL-1b-induced expression of GM-CSF and TNF-a and the expression/activity of NF-kB in HPV^+ and HPV^- cell lines. Despite the enhanced expression of the functional components of the NF-kB signaling pathway (p50, p65, c-Rel and IkBa) in HPV^+ cell lines, several of which are due to the expression of E6, the constitutive activity of NF-kB and expression of GM-CSF/TNF-a were significantly reduced relative to the HPV^- cell line and normal keratinocytes. In contrast, we observed a superactivation of NF-kB activity after IL-1b stimulation, a strong and transient induction of GM-CSF/TNF-a mRNA, but undetectable levels of secreted proteins in HPV^+ cell lines. Our data demonstrate that the HPV oncoprotein E6 modulates the NF-kB signaling pathway and suggest that other HPV proteins also interfere with GM-CSF/TNF-a expression by transcriptional and/or post-transcriptional mechanisms.

DIFFERENTIATION OF MURINE STROMAL AND HEMOPOIETIC CELLS ENGRAFTED UNDER KIDNEY'S CAPSULE

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Attempts were made to establish models to study interactions between marrow stromal cells and hematopoietic cells *in vivo*. We implanted a murine stromal cell line (MS-5) and/or hematopoietic cells beneath the kidney's capsule of syngeneic mice. A morphological analysis shows that several days postimplantation MS-5 cells form adipocytes as shown by morphological observations, by S100 immunological staining and by Red oil staining. These adipocytes do not express alkaline phosphatase, an enzyme thought to play a role in the myelopoiesis. Hematopoietic cells injected alone give rise to fibroblastic-like cells that do not express alkaline phosphatases. Stromal cells and hematopoietic cells coimplanted display a different morphological aspect : some cells are elongated resembling to fibroblastic cells; others possess small empty vacuoles or display the morphology of mature adipocytes. Many clusters are observed wherein a network of alkaline phosphatase-positive reticular cells surrounds clusters of granulopoiesis identified by the expression of chloroacetate esterases.
In conclusion, our results indicate that these grafts are viable and that adipocytic differentiation of MS-5 stromal cells is inhibited by the presence of hematopoietic cells in the graft. Moreover, MS-5 stromal cells are able to sustain the differentiation of granulocytic colonies whereas hematopoietic cells implanted alone do not form any structure resembling normal bone marrow. These results show that this model is suitable to analyze adult stromal - hematopoietic cells interactions *in vivo*. They support the fact that interactions between stromal cells and hematopoietic cells are necessary for hematopoiesis. This model will be further used to test the homing of hematopoietic precursors after intravenous injection and to test the reversibility of adipocyte phenotype. Finally it will be used to analyze the influence of human hematopoietic cells on human stromal cells from healthy patients or from various hematological diseases after implantation into NOD-SCID mice.

QUANTIFICATION OF CK19 MRNA IN PERIPHERAL BLOOD (PB) AND BONE MARROW (BM) FROM PRIMARY OPERABLE BREAST CANCER (BC) PATIENTS PRE- AND POSTOPERATIVELY TO INVESTIGATE POSSIBLE SHEDDING OF CK 19+ CELLS DURING THE OPERATION

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Introduction : We previously reported the development of a sensitive and quantitative assay to detect BC cells in PB of BC patients using real-time quantitative RT-PCR (ABI Prism 7700, Taqman ") identifying transcripts of the cytokeratin-19 (CK19) gene (Aerts J. ,et al, Ann Oncol 2001; 12 : 39-46). In stage IV - BC patients we found a statistically significant number of transcripts of CK19 compared to healthy volunteers. We further analysed CK 19+ transcripts in bone marrow in a control population and primary operable BC patients. Pre- and postoperatively PB samples of these patients were further analysed to investigate possible shedding of CK 19+ cells during the operation.

Methods : Bone marrow samples of 22 patients with haematological malignancies were taken as control population. In 54 primary operable BC patients (pathological stage I (18pts.), stage II (28pts.), stage III (8pts.), we analysed 50 BM samples taken preoperatively and 297 PB samples. The PB samples were collected before surgery(-1), immediately after surgery(0), on first(+1), second(+2), fifth(+5) days and one month (+1m) postoperatively.

Results : In the BM of the control population and the BC patients, we detected a median of 28.4 (95% CI [16; 67]) and 568 (95% CI [266; 1573]) CK19 positive cells/5x10^6 leukocytes respectively (Mann-Whitney $p < 0.001$). In PB(-1) samples we measured a median of 109 (95% CI [58; 298]) CK positive cells/5x10^6 leukocytes. Using the upper limit of the 95% CI of the control groups as cut-off, 74% and 52% of BM and PB(-1) samples respectively were considered as showing CK 19 positivity. The relationship between presence of CK 19+ cells in BM and PB(-1) and classical prognostic factors showed no significant correlation with pathological tumour size, nodal involvement, stage, differentiation grade and receptor status. The possible shedding of CK 19+ cells during the operation was investigated and no significant difference between the time points with respect to the average CK 19+ was detected ($F=1.21$, $p= 0.32$).

Overview : In primary BC patients, we detected high numbers of CK 19+ cells in BM and PB-1 samples compared to the control population. However, in this study population, no significant correlation between presence of CK 19+ cells in BM and PB(-1) and the classical prognostic factors was found. With this technique, we detected no statistically significant influence of surgical manipulation on the amount of CK 19 positivity.

INSULIN INCREASES THE SENSITIVITY OF TUMORS TO IRRADIATION : INVOLVEMENT OF AN INCREASE IN TUMOR OXYGENATION MEDIATED BY AN NO DEPENDENT DECREASE OF THE TUMOR CELLS OXYGEN CONSUMPTION

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Introduction : In recent years, several studies have shown that hyperinsulinemia can increase blood flow and pO_2 in human skeletal muscle in vivo. Until now, the potential effect of insulin on tumor perfusion and oxygenation has not been evaluated. As the partial pressure of oxygen of the tumor is a key factor for the response to radiotherapy, our purpose was to explore the potential effect of insulin on tumor using EPR oximetry, MRI and probes combining Laser Doppler flowmetry, oximetry and thermocouple (OxyLite/OxyFlo, Oxford-Optronix). In addition, we tested the potential radiosensitizing effect of insulin.

Materials and Methods : A transplantable mouse liver tumor model (TLT) and the FSAII tumor model were implanted in the thigh of mice. Insulin was infused IV at a rate of 16 mU/kg/min. We performed EPR oximetry using a low-frequency EPR spectrometer to quantify tumor and muscle pO_2 before and after insulin infusion. We also used the Oxylite/Oxyflo technique for monitoring tissue blood flow and oxygenation. In addition, tumors were imaged using magnetic resonance at 4.7 Tesla with dynamic Gd-DTPA (FLASH imaging) contrast enhanced imaging after IV administration of insulin. We also measured tumor cell oxygen consumption using a high frequency EPR spectrometer and further identified by immunoblotting that eNOS was activated by phosphorylation after in vivo insulin infusion. Finally, a 16 Gy (RX) irradiation was selected in order to test the radiosensitizing properties of insulin.

Results and discussion : An increase in tumor pO_2 during and after insulin infusion was demonstrated by EPR oximetry and Oxylite experiments. Signal enhancement on FLASH Gd-DTPA contrast-enhanced images was less important after insulin administration than after a single bolus of the contrast agent. This result suggests a decrease in tumor perfusion due to insulin (the increase in normal tissue blood flow due to vasodilation shunted away the blood flow from the tumor to the muscle ("steal effect")). We observed an important decrease in tumor oxygen consumption after insulin infusion. This is probably the origin of increase in tumor pO_2 as the contribution of flow to this improvement in oxygenation has been excluded using MRI. We found that the insulin-induced increase of tumor pO_2 is inhibited by a NO synthase inhibitor and demonstrated that the NO pathway involves a phosphorylation of eNOS. In addition, we demonstrated the radiosensitizing properties of insulin on the FSAII tumor model. We observed a regrowth delay of 9.1±1.1 days for insulin + RX, 7.3±0.8 days for carbogen + RX used as positive control and 4.8±0.6 days for RX alone.

EFFECT OF INTRACELLULAR GLUTATHIONE AND CYSTEINE DEPLETION ON RADIOSENSITIVITY OF HUMAN MELANOMA CELLS

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Glutathione (GSH) and its precursor, the cysteine (CYS), are both known to react within any cell, with oxidative species and thus play an important role in cellular defense mechanisms against oxidative stress. In melanocytes, these are also important precursors of melanogenenesis by reacting non-enzymatically with L-dopaquinone to form the sulfur-containing pheomelanin. The later may also act as a free radical donor.

Our aim was to assess their role in cellular radioprotection mechanism in a human melanoma cell model. We first compared cell survival of Cys deprived and glutathione depleted cells to control cells. Cys deprivation was achieved by decreasing Cys concentration in culture medium for 24H. In this condition no toxicity was observed, Cys and GSH level decreased melanogenesis switched to higher eumelanin synthesis and cells were significantly more resistant to 10 Gy of X-ray than control cells. Glutathione depletion was achieved with the "-glutamylcystein synthetase inhibitor Buthionine-S-sulfoximine (BSO) for 24 H at a concentration yielding no measurable toxicity. In this condition, intracellular GSH level decreased but no change in pigmentation was observed and cells were slightly more sensitive to radiation than the control.

Secondly, for a dose of 10 Gy, we compared DNA radio-induced damages by comet assay in control cells, cells treated as above and cells with stimulated pigmentation.

Results showed that when intracellular eumelanin content increased by a factor of at least 1.8, DNA damages substantially decreased. Pigmentation was enhanced by two ways : 1) by increasing Tyr medium concentration and 2) eumelanin switch by Cys deprivation. By contrast, DNA damages increased in cells treated with BSO.

We may conclude that increasing intracellular eumelanin content is able to compensate the loss of the two intracellular radioprotectors that are glutathione and cysteine.

VIRAL LOAD AND CELLULAR IMMUNE RESPONSE IN PATIENTS WITH CERVICAL HPV-ASSOCIATED PRENEOPLASTIC LESIONS

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Background : Cellular immune defects have been observed in patients with human papillomavirus (HPV)-asociated lesion of the uterine cervix. These data suggest that the restoration of a cellular immune response could be beneficial for these patients. We have therefore established a vaccination protocol with a mutated E7 protein of HPV16 in patients with low grade cervical lesion. The goal of this study was to set up protocols to follow up the HPV16 viral load and the systemic immune response against E7 during the clinical trial.

Methods : Real time PCR was chosen to quantify the viral DNA on cervical brush specimens whereas the immune response against HPV is evaluated using an IFNg intracellular staining on PBMC stimulated in vitro with the vaccine protein. To set up these techniques, cervical specimens and blood of patients with low grade and high grade lesions were used.

Results : In our group of 13 patients, 7 were HPV positive by classical PCR. We performed real time PCR on HPV+ patients using HPV16 specific primers and probe (1). In these HPV+ patients, 4 were positive for HPV16 with one with very high amount of HPV16 copies.

We performed IFNg intracellular staining on PBMC of patients and normal donors after an overnight stimulation. As positive control, cells were activated with PHA and for all patients and normal donors we observed cells producing IFNg. Surprisingly, the percentage of IFNg+ cells seemed to be higher in HPV16+ patients. After stimulation with mutated E7 protein, we observed a response against this protein in some HPV+ patients with the highest response in the patient showing the highest copy number of HPV16.

Conclusion : We have developed techniques allowing to determine the HPV16 viral load and the immune response against HPV 16 during the vaccination protocol.

DETECTION OF MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) AND GALECTIN-3 IN HUMAN COLORECTAL CANCERS AND THEIR INVOLVEMENT IN TUMOR PROGNOSIS.

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This study aims to investigate whether the macrophage migration inhibitory factor (MIF) is detectable in human colorectal tumors and relates to the levels of expression galectin-3 and p53, and finally to analyze the involvement of these 3 factors in the progression of malignancy in these tumors. We collected 75 colorectal tumors (19 Dukes A, 21 B, 17 C and 18 metastatic tumors), 11 hepatic metastases from colorectal cancers and 20 normal specimens (biopsies). The immunohistochemical levels of expression of MIF and galectin-3 were quantified on routine histological slides by means of computer-assisted microscopy. The percentage of cells with p53 nuclear staining was also evaluated. Separate analyses were performed in epithelial and
connective tissue.
Our results show that the levels of expression of both MIF ($p = 0.002$) and galectin-3 ($p = 0.0002$) increased in tumor tissue if compared with normal specimens. In the tumor specimens, we observed a very higher level of expression of MIF and galectin-3 in the epithelial tissue than in the peritumoral connective tissue ($p < 10\text{-}6$). A positive correlation was established between the expression of MIF and galectin-3 in peritumoral connective tissue (Kendall tau = 0.31, $p = 0.0001$). In the case of Dukes A and B tumors, we established a negative and significant correlation between the patients' survival times and the levels of expression of MIF in peritumoral connective tissue (Kendall tau = -0.35, $p = 0.006$). MIF expression in tumoral tissue exhibited a similar, but not significant, relation with patients' survival times (Kendall tau = -0.24, $p = 0.06$), as do galectin-3 expression (Kendall tau = -0.26, $p = 0.04$). In contrast, in the case of advanced tumors (Dukes C, metastatic tumors and metastases), we observed a strong positive correlation between the patients' survival times and the levels of expression of MIF in peritumoral connective tissue (Kendall tau = 0.40, $p = 0.0005$). All these survival data have been confirmed by means of Kaplan-Meyer analyses.
In conclusion, MIF and galectin-3 expression levels in colorectal tumors are related to the levels of biologic aggressiveness reflected in the patient survival status. However, this relation with survival is different in the cases of Dukes A and B tumors than for more advanced ones. More particularly, in the cases of Dukes A and B tumors, MIF and galectin-3 seem to cooperate to form an anti-apoptotic feedback loop in accordance to the hypothesis that these two factors allow cells to bypass apoptosis mediated by p53.

ACTIVE SUPPRESSION OF ANTI-TUMOR CTL RESPONSE IS MEDIATED BY ALTERNATIVELY ACTIVATED CD11B+ MYELOID CELLS LEADING TO TUMOR PROGRESSION

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The $CD8^+$ cytotoxic T cell mediated immune response is responsible for the spontaneous tumor regression observed in the murine BW-Sp3 T cell lymphoma. However, this anti-tumor response is inadequate to eliminate the tumor cells in some of the recipients resulting in an aggressive tumor progression. This is due to a drastic reduction of anti-tumor CTL activity in these animals. Furthermore, immunotherapy with the BW-Sp3 (B7-1) transfectants favours the progression of original tumor. In the present study, we demonstrated that the impaired T cell function in tumor progressors was due to active inhibition by a splenic adherent population. Removal of the adherent cells from CTL cultures of progressors considerably restored the tumor specific lymphocytotoxicity. Furthermore, co-culture with the suppressive adherent splenocytes abolished the CTL activity in regressors. The suppressive activity was strictly dependent on a direct cell-cell contact. Further investigation revealed that tumor progression was accompanied by a dramatic expansion of $CD11b^+$ monocytes in the spleen. Depleting the $CD11b^+$ cells also reversed the T cell suppression in progressors. Moreover, the suppressive monocytes were characterised by a high arginase activity indicating their alternative activation status. In a contrary, the tumor regressors exhibited no significant expansion of $CD11b^+$ monocytes but predominantly developed the accessory cells capable of inducing tumor specific CTL in naïve splenocytes. Additional provision of the B7-1 transfectants as immunoyherapy facilitated a rapid expansion of alternatively activated myeloid cells in progressors. This was correlated to the elevated IL-4 and IL-10 production by the splenocytes of these animals. Thus, the subtle balance between the effective antigen presenting cells and the alternatively activated suppressive monocytes determined the fate of a subcutaneously inoculated BW-Sp3 tumor. Counteracting the alternative activation of myeloid cells may provide a potential mean to overcome the T cell suppression in cancer patients.

ANALYSIS OF GENE EXPRESSION IN HUMAN THYROID TUMORS BY MICROARRAY TECHNOLOGY

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The identification of genes involved in cellular proliferation and the changes occurring in cancer cells is essential for the understanding of molecular mechanisms that govern cell development. The behavior and biology of any cell type is defined and conditioned by the genes that this cell expresses as RNA and as proteins. Recent technological advances have made possible the simultaneous study of the quantitative expression for thousands of genes. In the present study we have used the newly developed DNA microarray technology as a means of identifying genes involved in proliferation and tumorigenesis. We analyzed the expression profiles of 2400 genes in solitary thyroid autonomous adenomas and their corresponding quiescent tissue using the MICROMAX‰ cDNA microarray system (NEN, Life Science Products) that allows to perform the experiments with very small quantities of starting material due to their efficient system of signal amplification (TSA : tyramide signal amplification). Comparison of gene expression in the quiescent tissue and the adenomas demonstrates a close correspondence for the great majority of expressed genes. Approximately 25 different genes were found to be systematically overexpressed (> 2 fold) in mRNA from autonomous adenoma tissue compared with similar amounts of mRNA from normal tissue. These overexpressed genes included receptors, small G proteins, and structural proteins. 29 genes were downregulated in all our adenomas. They are mainly genes involved in apoptosis, thyroid function genes and growth factors. Studies are currently ongoing to confirm these regulations by Northern blotting or RT-PCR and to extend these results to a larger number of adenomas.
Thyroid carcinoma, despite its low mortality rate, remains a significant medical problem because of the cost of establishing an accurate diagnosis, the need for prolonged follow-up and a relatively important rate of recurrence. The identification and characterization of genes responsible for abnormal cellular proliferation and aggressiveness of malignancy, as well as the proteins they code for, is one of the primordial goals of fundamental research in cancer. The results of such research could potentially be used in the diagnostic interventions (by providing new histochemical markers), allow a better understanding of the pathophysiology of the tumors and provide ways by which the targeted therapeutic interventions can be improved.

PATHWAYS FOR THE REGULATION OF ICAM-1 EXPRESSION IN HUMAN MELANOMA CELLS

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ICAM-1 is a cell adhesion molecule belonging to the Immunoglobulin Superfamilly. It is expressed by many cells types. It has been found in the melanocyte lineage, with a preferential distribution on those which have undergone malignant transformation, and is thought to be involved in tumor invasiveness and metastasis. Numerous authors have already reported ICAM-1 expression as directly related to tumor aggressiveness. Actually, ICAM-1 expression in primary lesions was found associated with a reduction of disease-free interval and survival and patients with elevated serum ICAM-1 levels had significantly shorter survival. ICAM-1 expression is higher in metastatic than in primary lesions, suggesting that melanoma cells that are able to metastasise express higher ICAM-1 levels. Understanding the mechanisms controlling its expression became therefore of a major interest.
Inflammation may occur during tumor growth in depth also promoted by protease synthesis by the tumor itself leading to cytokine release like TNF and IFN-". TNF triggers numerous important events in many cells including activation of nuclear transcription factors. Many of them can lead to the overexpression of ICAM-1. On the other hand, the promoter gene of ICAM-1 possesses consensus sequences for many transcription factors.
Our work focused on the regulation of ICAM-1 expression in melanoma cells by activating different relevant pathways with different cytokines : TNF, IL-1", IL-6 and IFN-".
Our results show that ICAM-1 overexpression triggered by each of the 4 cytokines can be significantly but differently inhibited in terms of potency, by cAMP and reversed by the use of a speciific PKA inhibitor. The same results could be obtained when the phosphorylation of IB was inhibited.
On the other hand, superoxide anion (also after TNF challenge) was able to stimulate ICAM-1 expression and SODs can very significantly limit the effect.
Our data clearly point to many cytokine pathways leading to ICAM-1 overexpression at a large part through NFB activation. However, other transcription factors seem to be also involved such as STAT-1 and AP-1.

SCHEDULE DEPENDENCY OF THE RADIOSENSITIZING EFFECT OF GEMCITABINE IN VITRO

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Gemcitabine (dFdC) is an active antitumor agent with radiosensitizing properties. Since the radiosensitizing effect results in increased antitumor activity, but also in a severe increase in toxicity, there is an urgent need for optimization of this combined therapy. Therefore, we investigated the schedule dependency of the radiosensitizing effect of dFdC in vitro, by varying the incubation time and the time interval between the dFdC and radiotherapy (RT).
ECV304, an epidermoid bladder carcinoma cell line and H292, a mucoepidermoid lung cancer cell line were used in this study. Cells were treated with radiosensitizing concentrations of dFdC, i.e. 2 nM in ECV304 cells and 4 nM in H292 cells. To investigate the influence of the incubation time, cells were treated during 32, 24 or 8 hrs prior to RT. The influence of the time interval was tested by a 24 hrs incubation with dFdC 0, 4, 8 or 24 hrs before RT. To investigate whether the start of dFdC treatment in relation to RT was crucial, we also tested an incubation period of 16, 20 or 24 hrs with an interval of 8, 4 and 0 hrs before RT, respectively (resulting in 24 hrs between start of dFdC treatment and RT). Cell survival was determined 7 days after RT by the sulforhodamine B test. Experiments were performed at least 3 times. ID50, radiation dose resulting in 50% cell kill, was calculated from the survival curves, fitted according to the linear-quadratic model model : survival=exp(-αD-ßD^2). The radiosensitizing effect can be represented by the dose enhancement factor (DEF) : $ID50_{control}/ID50_{pretreated}$.
The radiosensitizing effect increased with a longer incubation period : the DEFs were 1.30, 1.97 and 2.50 in ECV304 and 1.04, 1.45 and 1.78 in H292 cells after treatment during 8, 24 and 32 hrs, respectively. When the cells were treated for 24 hrs but with an interval between the radiation and the dFdC incubation, the radiosensitizing effect of dFdC decreased with an increasing interval : DEFs were 2.26, 1.95, 1.77 and 1.49 in ECV304 and 1.45, 1.36, 1.31 and 1.11 in H292 with an interval of 0, 4, 8, and 24 hrs, respectively. The start of dFdC treatment in relation to RT did not seem to play an important role because treatment during 16, 20 and 24 hrs with intervals of 8, 4 and 0 hrs, respectively resulted in DEFs of 1.51, 1.89 and 2.21 in ECV304 and 1.18, 1.48 and 1.62 in H292 cells.
In conclusion, the radiosensitizing effect of dFdC increases with a longer incubation time and decreases with a longer time interval between dFdC treatment and RT in vitro. These results are the basis for more extensive in vitro research on the mechanism of the radiosensitizing effect of dFdC.

Influence of sex hormones on the production of cytokines/chemokines by normal and HPV-transformed cervical keratinocytes

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The impaired interactions between keratinocytes and Langherans/Dendritic cells (LC/DC) in cervical epithelium may contribute to the progression of human papillomavirus (HPV)-related (pre)neoplastic lesions. It has been previously demonstrated that this mechanism is associated with the regulation of local cytokines/chemokines production by keratinocytes. Since the development of cervix cancer is dependent on the hormonal status, the aim of our study was to evaluate the influence of estrogen and progesterone at physiological concentrations on the *in vitro* production of cytokines/chemokines by normal and HPV-transformed cervical keratinocytes. Whereas no effect was observed for TNF-a, IFN-g and IL-10 production, female sex hormones inhibited the secretion of GM-CSF and MIP3a by HPV-immortalized keratinocytes. However, this effect was dependent of the steroid and of its concentration. A dose-dependent inhibition of GM-CSF production was observed in keratinocytes treated by estradiol (10^{-11} to 10^{-6} M) whereas only the highest concentration of progesterone (10^{-4} M) inhibited the GM-CSF secretion. In contrast, a dose-dependent inhibition of MIP3a production was observed after treatment with progesterone (10^{-8} to 10^{-4} M) whereas estradiol had no influence. Preliminary results indicated that similar steroid-dependent effect on GM-CSF and MIP3a production was detected in normal keratinocytes treated by sex hormones. In contrast to the observation in HPV-immortalized keratinocytes, estradiol and progesterone could induce the IL-10 secretion by normal keratinocytes. Taken together, these data support the notion that estrogen and progesterone could be independent co-factors in the development of HPV-associated cervix neoplasia, by regulating the production of cytokines/chemokines involved in keratinocytes-LC/DC interactions.

A MORPHOLOGICAL AND FUNCTIONAL ANALYSIS OF PLATELET VEGF IN CANCER PATIENTS

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The growth of primary tumours is dependent on angiogenesis. The switch to the angiogenic phenotype involves changes in the local equilibrium of cytokines with either pro- or anti-angiogenic properties. Vascular Endothelial Growth Factor (VEGF) is one of the major positive regulators of tumour angiogenesis. Recent evidence suggests that platelets are the main contributors of serum VEGF. In this study we analysed ultrastructurally and with immunofluorescence techniques, the topographical localisation of VEGF in platelets. An alpha-granule and membranous VEGF-localisation was identified. In 34 untreated metastatic breast cancer patients, 21 patients with locoregional breast cancer and 24 healthy individuals we analysed serum VEGF (SVEGF) and plasma VEGF (PVEGF) levels. The mean circulating SVEGF levels for the healthy controls, localised breast cancer group and patients with metastasis were 97.20 pg/ml (med. : 77 pg/ml; SD= 71.23), 330.50 pg/ml (med. : 279.3; SD= 249.10) and 405.5 0 pg/ml (med. : 331.8; SD= 249.1) resp. (P<0.0001; Kruskal-Wallis test). Correlation analysis between SVEGF and platelets reached borderline significance in both the untreated (r= 0.42, P= 0.07) as well as in the metastatic breast cancer group (r= 0.32; P= 0.07). The calculated SVEGF load per platelet rose from 0.39 pg/ 10e6 platelets in the control population, 1.22 pg/ 10e6 platelets till 1.79 pg/10e6 for platelets in patients with metastasis (P<0.0001; Kruskal-Wallis test). Mean PVEGF levels were 13.7 pg/ml (med. : 12.55 pg/ml; SD= 9.2) for the control group, 32.12 pg / ml (med. : 30.25 pg/ml; SD=16.25) for the localised breast cancer group and 54.78 pg/ml (med. : 43.75 pg/ml; SD= 35.98) for the metastasis group (P<0.0001; Kruskal-Wallis test). The correlation coefficients between PVEGF and the VEGF load/platelet were 0.57 (P=0.004), 0.60 (P=0.005) and 0.57 (P=0.0001) for the healthy control population, for the localised and advanced breast cancer group. In 12 patients with colon cancer we analysed with immunohistochemistry for the platelet membrane glycocalicin platelet adherence and aggregation on tumoural endothelium. Our results suggest that platelets are important in the storage of circulating VEGF. The progressive accumulation of VEGF in platelets according to tumoural progression may be due to endocytosis of - tumour cell produced-VEGF and/or enhancement of VEGF synthesis in platelet precursors, the megakaryocytes. Platelets may subsequently adhere on tumoural endothelium and release, concomitant with aggregation, the higher VEGF load and thereby provide an additional stimulus for ongoing intra-tumoural angiogenesis.

IN VITRO INTERACTION BETWEEN ET-743 AND RADIOTHERAPY

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Introduction : Since chemoradiation combinations are used more frequently, it is important to investigate possible interactions between new cytostatic agents and radiotherapy. In recent years, a family of structurally related compounds, ecteinascidins, is isolated from the Caribbean tunicate Ecteinascidia turbinata, of which ET-743 is selected for further development on its potent antitumor activity. The aim of the present study is to investigate a possible interaction between ET-743 and radiotherapy *in vitro*.
Materials and methods : Three human tumor cell lines were used : ECV304, a bladder cancer cell line and A549 and H292, both lung cancer cell lines. Cells were first treated with a range of different concentrations of ET-743 alone during 24 hours to determine the IC50 in each cell line. To investigate a possible interaction between ET-743 and radiotherapy, cells were incubated for 24h with ET-743 and then irradiated (Co-60g rays, 0-8 Gy). Cell survival was determined by the sulforhodamine B assay 7 days after radiation. The dose-survival curves were fitted according to the linear quadratic model to calculate the ID50 (= radiation dose required to kill 50% of the cells). A possible radiosensitising effect was represented by the Dose Enhancement Factor (DEF = ID50 controle / ID50 ET-743 treated). All experiments were performed at least three times.
Results : All three cell lines were very sensitive to ET-743, with IC50 values of 1.81 ± 0.13 nM, 1.29 ± 0.10 nM and 1.61 ± 0.19 nM for ECV304, H292 and A549 cells, respectively. A 24h incubation with ET-743 prior to radiotherapy slightly increased the radiosensitivity of ECV304 and H292 cells. DEFs in ECV304 cells were 1.46 ± 0.09 (n=3) and 1.94 ± 0.22 (n=5) for 1.5 and 2.0 nM ET-743, respectively. In H292 cells DEFs were 1.34 ± 0.22 (n=8) and 1.91 ± 0.26 (n=4) for 0.5 and 1.0 nM ET-743. In A549, the radiosensitising effect was less pronounced with DEFs of 1.11 ± 0.08 (n=6) and 1.29 ± 0.15 (n=3) for 0.8 and 1.8 nM ET-743, respectively.
Conclusion : In our study, ET-743 showed moderate radiosensitisation in ECV304 and H292 cells, but only when cytotoxic concentrations were used. In A549, the radiosensitising effect was less pronounced. The clinical relevance of this concentration and cell line dependent interaction between ET-743 and radiotherapy still has to be investigated.

EXPRESSION OF KAISO, A P120 CATENIN INTERACTION PARTNER, IN NORMAL AND CANCEROUS HUMAN TISSUES

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Kaiso, a member of the BTB/POZ zinc finger superfamily, was originally identified as an interaction partner of the p120 catenin (Daniel and Reynolds, 1999). Since this protein has been localized in the nucleus of different cell lines and has been described recently to act as a DNA methylation-dependent transcriptional repressor (Prokhortchouk et al., 2001), Kaiso is generally considered a nuclear protein. A link between cell surface signaling and control of gene expression was suggested on the basis of its interaction with p120ctn, the latter being nuclear in particular in E-cadherin-lacking cells (van Hengel et al., 1999).
In this study we compared the expression of Kaiso in two human tumor cell lines *in vitro* and *in vivo* by analyzing xenografts of these cell lines in athymic mice. The antibodies used were kind gifts of J. Daniel and A. Reynolds. The injected tumor cells showed Kaiso-positive nuclei, but growing tumors showed heterogeneity with Kaiso negativity or staining outside the nuclei.
We also performed an immunohistochemical study on normal and tumor tissues of different human organs. Tissues analyzed were from oesophagus, bronchus, stomach, colon, breast, testis, ovarium and heart. Also in the latter experiment our findings differed from those on the *in vitro* stainings. Some cells showed a positive cytoplasmic staining whereas only few cell types showed positive staining in the nucleus. When comparing normal, tumor and metastatic tissues, we noticed changes in quantity and location of Kaiso expression.
Further studies will focus on the differential functions of Kaiso in the cytoplasm vs. the nucleus, and on the influence of the *in vivo* environment on Kaiso expression and location.

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MT1-MMP EXPRESSION PROMOTES TUMOR GROWTH AND ANGIOGENESIS THROUGH AN UP REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION

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Membrane-type-1 metalloprotease (MT1-MMP) is a transmembrane metalloprotease which plays a major role in the extracellular matrix remodeling, directly by degrading several of its components, and indirectly by activating pro-MMP2. We investigated the effects of MT1-MMP overexpression on in vitro and in vivo properties of human breast adenocarcinoma MCF7 cells which do not express neither MT1-MMP, nor MMP-2. MT1-MMP and MMP-2 cDNA were either transfected alone or co-transfected. All clones overexpressing MT1-MMP (i) were able to activate endogenous or exogenous pro-MMP-2, (ii) displayed an enhanced in vitro invasiveness through matrigel-coated filters, independently of MMP-2 transfection, (iii) induced the rapid development of highly vascularized tumors when injected sub cutanously in nude mice, and (iv) promoted blood vessels sprouting in the rat aortic assay, an *in vitro* model of angiogenesis. These effects were observed in all clones overexpressing MT1-MMP, regardless to their MMP-2 expression levels, suggesting that the production of MMP2 by tumor cells themselves does not play a critical role in these events. Interestingly, the angiogenic phenotype of MT1-MMP-producing cells was associated with an up-regulation of VEGF expression.
In conclusion, while MMP-2 overexpression by tumor cells did not affect tumor progression, MT1-MMP overproduction promoted tumor growth, tumoral angiogenesis and VEGF expression. These results open new opportunities for the development of anti-angiogenic strategies combining inhibitors of MT1-MMP and VEGF antagonists.

LUNG METASTASES OF RENAL CELL CARCINOMAS AND OF COLORECTAL ADENOCARCINOMAS ARE DISTINCT IN GROWTH PATTERN AND IN ANGIOGENESIS

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The hypothesis of this study was that lung metastases are a heterogeneous group consisting of different growth patterns with distinct angiogenic profiles. Although a considerable fraction of tumours probably needs angiogenesis to grow, alternative mechanisms of tumour vascularization have been described which are not based on endothelial cell proliferation. One of these mechanisms is the co-option of pre-existing capillaries in normal tissue adjacent to the tumour. The hypoxic drive of angiogenesis might be reduced or absent at the tumour-lung interphase, given the dense vascularization of the lung parenchyma, and provided the metastases do not change the stromal architecture of the lung.
To test whether some metastases are capable of preserving the vasculature of the alveolar walls, tissue sections of 39 metastases (15 of colorectal cancer (CRC) and 24 of renal cell cancer (RCC)) obtained after elective surgery were analysed. One metastasis was analysed per patient. A haematoxylin-eosin stain and a reticulin stain were made to evaluate the growth pattern. An immunohistochemical staining with anti-CD34 antibodies (clone QBEnd/10, Biogenex) was used to perform a Chalkley morphometric analysis in the vascular hot spots and along a line drawn from one margin through the centre to the other margin. A double immunostaining (anti-CD34 - anti-PCNA) was used to measure the fraction of proliferating endothelial cells (ECP).
Four growth patterns were found. In the alveolar, the bronchiolo-alveolar and the interstitial growth pattern ("putative non-angiogenic" patterns), the architecture of the lung parenchyma was conserved within the tumour, at least at the tumour-lung interphase. In the desmoplastic growth pattern, a rim of connective tissue separated the tumour from the lung parenchyma. The architecture of the lung was not conserved within the tumour. Eighty percent of the CRC metastases and only 39% of the RCC metastases had a putative non-angiogenic growth pattern (p=0,01). No differences were found of Chalkley count in vascular hot spots between the growth patterns or the histiotypes (mean 10,1, standard deviation 3,4). "Dilution" of the vascular density towards the centre of the metastasis (at least 2 Chalkley dots) was present in 66% of the putative non-angiogenic growth patterns and in 12% of the angiogenic growth pattern (p=0,002). ECP ranged from 8,7% to 35,9% in the desmoplastic growth pattern. In the alveolar growth pattern, ECP ranged from 1,0% to 1,7%.
The hypothesis of the study is confirmed. The non-angiogenic growth patterns might be less sensitive to cytotoxic anti-cancer treatment and to anti-angiogenic treatment directed at proliferating endothelial cells. The growth patterns are likely to reflect underlying biological differences (protease activity, pattern of adhesion molecule expression, growth factor production, hypoxia-sensing capacity, angiopoietin-1,2 expression pattern). Non-invasive imaging techniques detecting the different patterns might be necessary for future decision-making in the treatment of lung metastases.

CHARACTERIZATION OF A NEW MELANOMA EPITOPE DERIVED FROM A POINT MUTATION IN THE UBIQUITOUSLY EXPRESSED GENE OS-9

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A cytotoxic T lymphocyte clone (CTL) was generated from the blood T lymphocytes of a melanoma patient, by *in vitro* stimulations with autologous tumor cells. This CTL clone specifically lysed several autologous tumor cells, but not autologous EBV-transformed-B cells nor the NK sensitive K562 cell line. In the present study, we report the identification of a new HLA-B*44.03 restricted melanoma antigen that is derived from a point mutation in the ubiquitously expressed gene OS-9. This gene was originally identified as being frequently amplified in human sarcomas. The mutation (C to T transition) identified in our tumor cell line resulted in a change from a proline to a leucine residue in position 446 of the OS-9 protein. The mutation was found in all autologous melanoma cell lines tested, but not in normal autologous cells such as fibroblasts or EBV-transformed-B cells. Moreover, two cDNA sequences registered in the EST database contained an identical mutation. This indicates that the mutation we identified is not unique and may be shared by several other tumors of different histological type. We now try to investigate different allogeneic tumors for the presence of the mutation.

MELPHALAN AVAILABILITY IN HYPOXIA-INDUCIBLE FACTOR-1**A**+/+ AND FACTOR-1**A**-/- TUMORS IS INDEPENDENT OF TUMOR VESSEL DENSITY, AND IS CORRELATED WITH MELPHALAN ERYTHROCYTE TRANSPORT

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Many tumors are impervious to anticancer agents. Resistance due to lack of cytotoxic penetration into the tumor is often overlooked, but can play a significant role in undermining therapy. Insufficient and irregular vascularisation of tumors is known to be one barrier to drug delivery, but there are others such as impaired vessel permeability or poor interstitial transport. In this study we evaluated the importance of tumor vessel density in the availability of melphalan to tumors. A nude mouse tumor model with different vascularisation due to a single gene deletion of hypoxia-inducible factor 1a (HIF-1a+/+ and HIF-1a-/- embryonic stem cell derived tumors) was used. The availability of melphalan to HIF-1a+/+ (n=20) and HIF-1a-/- (n=23) tumors was similar (p 0.12). Furthermore, in different subgroup analyses accentuating the difference in vessel density, no correlation between vessel density and melphalan availability was found. In the second part of the study, we show that melphalan is transported in blood in mice with a distribution of 24% in erythrocytes vs. 76% in plasma. A strong correlation (r 0.93, $p < 0.000001$) between melphalan concentrations in plasma and erythrocytes was found, indicating an equilibrium between these two compartments. Plasma and erythrocyte concentrations of melphalan are correlated with the tumor availability of melphalan (r 0.66 and 0.64 respectively, p both < 0.001). These data suggest that tumor vessel density is not an important predictor of the tumor availability of small cytotoxic drugs such as melphalan, and indicate the importance of erythrocytes in the transport of melphalan.

THE HEREGULIN-ERBB SYSTEM : A NEW AUTOCRINE LOOP IN HUMAN MELANOMA CELLS

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The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases is frequently overexpressed in various carcinomas. To date, four members of this receptor family have been identified, EGFR/erbB1/HER1, neu/erbB2/HER2, erbB3/HER3 and erbB4/HER4. Based on their receptor binding capacities, the EGF-like ligands can be divided into four classes : binding only EGFR, only erbB4, EGFR and erbB4, or erbB3 plus erbB4. The latter class comprises neuregulins 1 (human : heregulins, HRG) and neuregulins 2.
Growth regulation by a variety of cytokines and other regulatory polypeptides has been described for melanomas, often leading to uncontrolled growth. However, controversy exists concerning the role of erbB receptors in melanomas.
While looking at hyperphosphorylated proteins in the Bowes melanoma cell line, we found the presence of a heavily phosphorylated band at 185 kDa. This phosphorylated band corresponds to activated erbB2 and erbB3 and is lost when the cells are treated with an irreversible erbB-inhibitor. Moreover, conditioned medium (CM) taken from the melanoma cells is able to induce erbB-phosphorylation and spreading of other cell lines, which can also be blocked using the erbB-inhibitor, meaning that an erbB phosphorylating factor is secreted/released by the melanoma cell line. The presence in Bowes melanoma cells of heregulins, ligands of erbB3 and erbB4, could be shown by both RT-PCR, western blotting and immunocytochemistry. Upon depleting the CM of heparin binding factors using heparin beads, we obtained two fractions : the depleted conditioned medium, devoid of activity, and the beads, retaining spreading and phosphorylating activity. To verify if the HRG/erbB is implicated in growth stimulation of Bowes melanoma cells, we performed a growth assay and found that blocking erbB phosphorylation leads to significant growth inhibition.
To extend these findings, we checked for the presence of the HRG/erbB system in a panel of 12 additional melanoma cell lines and found expression of heregulins in two other cell lines. However, in these cell lines no constitutive activation of the erbB receptors was present.
In conclusion, we were the first to show the secretion of heregulins by melanoma cells. These may use the HRG/erbB system to stimulate their own growth (autocrine) or to act on other, adjacent cells (paracrine). Interfering with this erbB-signaling might be a future target of interest for therapy.
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THE TWO-HANDED E-BOX-BINDING ZINC FINGER PROTEIN SIP1 DOWNREGULATES E-CADHERIN AND INDUCES INVASION

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Malignancy of carcinoma cells is characterized by loss of both cell-cell adhesion and cellular differentiation. The epithelial cell-cell adhesion protein E-cadherin is a genuine tumor suppressor as well as an established invasion suppressor. Transcriptional downregulation of E-cadherin in various epithelial tumors appears to be a main event during tumor progression. SIP1 (ZEB-2) is a Smad-interacting, multi-zinc finger protein, which is expressed in several E-cadherin-negative human carcinoma cell lines and is induced by TGF-b treatment of mouse NMe epithelial cells. The expression of wild type but not mutated SIP1 was shown by us to downregulate mammalian E-cadherin transcription via binding to both conserved E2-boxes of the minimal E-cadherin promoter (Comijn et al., 2001). Conditional expression of SIP1 in E-cadherin-positive MDCK cells abrogated E-cadherin-mediated intercellular adhesion and simultaneously induced invasion and loss of cell aggregation, a process that is not correlated with induction of either Snail or soluble b-catenin. SIP1 appears to be a new and potent invasion promoter protein in malignant tumors from epithelial origin.
The role of SIP1 expression in epithelial tumor formation will be further examined by performing in situ hybridizations and by immunohistochemistry on various tumors and tumor cell lines. Differential gene expression analysis of SIP1-expressing vs. non-expressing cell lines may reveal additional genes whose expression is regulated by SIP1 and that are possibly involved in the invasion process. cDNA-AFLP analysis is currently performed on the inducible MDCK-Tetoff-SIP1 cell line and micro-array analysis will be performed for differential gene expression analysis of stably transfected mouse and human SIP1-expressing cells vs. non-expressing cells.

Reference :
Comijn, J., Berx, G., Vermassen, P., Verschueren, K., van Grunsven, L., Bruyneel, E., Mareel, M., Huylebroeck, D., and van Roy, F. (2001) *Mol. Cell* 7 : 1267-1278.

DETECTION OF BONE SIALOPROTEIN IN HUMAN (PRE)NEOPLASTIC LESIONS OF THE UTERINE CERVIX

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Bone sialoprotein (BSP) is a secreted glycoprotein primarily found in the mineral compartment of developing bones. BSP can be detected in a variety of human cancers and particularly those that metastasize to the skeleton. We have shown that BSP expression in breast and prostate cancer is associated with bad prognosis and the development of bone metastases. These data prompted us to investigate whether BSP might also be expressed in human cervical (pre)neoplastic lesions which often metastasize to bone.
In this study, we examined BSP expression in cervix epithelial tissue samples from 54 patients including : normal tissue, squamous intraepithelial lesions (SIL) of low and high grade and invasive cervix cancers. BSP expression was evaluated by immunophosphatase technique using a BSP polyclonal antibody. All the invasive cancers examined expressed high levels of BSP while normal tissues showed no or low BSP immunoreactivity. High grade SIL were significantly more positive for BSP than low grade lesions. Expression of BSP was generally higher in invasive cancer when compared to SIL. We also examined whether BSP expression was detectable in SiHa and CaSki cervix cancer cell lines using real-time PCR but no BSP RNA was detectable in both cell lines suggesting that microenvironmental factors might be important for the induction of BSP expression in these lesions.
Our study demonstates for the first time that ectopic BSP expression is a common feature in high grade SIL and invasive squamous carcinoma of the uterine cervix. The prognostic value of BSP detection in these lesions and the potential role of BSP in the affinity of this type of cancer for bone are currently under investigation.

BISPHOSPHONATES ANTAGONIZE THE EFFECTS OF BONE-DERIVED GROWTH FACTORS ON MCF-7 BREAST CANCER CELLS

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The high concentrations of growth factors in bone matrix provide a fertile soil in which metastatic breast cancer cells grow easily. Bisphosphonates are successfully used as powerful inhibitors of osteoclast-mediated bone resorption in tumor-induced osteolysis. We recently showed that bisphosphonates also induce human breast cancer cell death in vitro (apoptosis and/ or necrosis) which could contribute to their beneficial clinical effects (Fromigué et al., J Bone Miner Res 2000). In this study, we have investigated the effects of the co-addition of bisphosphonates (ibandronate, pamidronate, zoledronate and clodronate) and bone-derived growth factors on human breast cancer cells (MCF-7) growth and apoptosis.

We first tested the effects of bisphosphonates in combination with growth factors on MCF-7 cell viability, as estimated by the colorimetric microassay described by Mosmann (MTT test). Exposure of MCF-7 cells for 24 hrs to IGF-I (0.1-100 ng/ml), IGF-II (1-1000 ng/ml), bFGF (0.05-50 ng/ml) dose-dependently increased cell viability. IGF-I, IGF-II and bFGF were then used at concentrations exhibiting optimal stimulatory effects, i.e. 10 ng/ml for IGF-I and bFGF, and 100 ng/ml for IGF-II, whereas the concentration of bisphosphonates was 10^{-6} M. We found that the stimulatory effects of growth factors on MCF-7 cells were partly inhibited by bisphosphonates. The mean decrease was 22% for bFGF, 10% for IGF-II and 8% for IGF-I.

We next investigated the effects of growth factors alone or in combination with a bisphosphonate on Bax (pro-apoptotic factor) and Bcl-2 (apoptotic inhibitor) expression levels. Using Western blot analysis, we found that IGF-I (5ng/ml) or bFGF (5ng/ml), alone, increased Bcl-2 protein level and only slightly affected Bax protein level. In contrast, IGF-II (50 ng/ml) decreased Bax protein level but only slightly modify Bcl-2 expression. Growth factors thus decreased the Bax/Bcl-2 ratio by 22% for IGF-I, 4% for IGF-2 and by 8% for bFGF. When bisphosphonates were combined with IGF-I, the Bax/Bcl-2 ratio was increased by 8-37% according to the bisphophonate used. The IGF-II induced changes in the Bax/Bcl-2 ratio were only influenced by pamidronate (increase of 12%). Lastly, bisphosphonates modulated bFGF effects on the Bax/Bcl-2 ratio which was increased by 5-13%.

In conclusion, bisphosphonates decreased the stimulatory effects of bone-derived growth factors on breast cancer cells growth and reduced the protective effects of growth factors on MCF-7 cells apoptosis. In addition to our previously demonstrated direct "antitumor" effects of bisphosphonates, the present data further indicate that their beneficial effects in the process of tumor-induced osteolysis are much more complex than a "simple" antiosteoclast activity.