ASSOCIATION BELGE POUR L' ÉTUDE DU CANCER
BELGISCHE VERENIGING VOOR DE STUDIE VAN KANKER
(A.B.E.C./B.V.S.K.)

Abstracts of the annual meeting of the Belgian Association for Cancer Research:
"The tumor micro-environment :
new insights and therapeutic perspectives"
16 February 2008
UCL medical school, avenue Mounier
Bruxelles - Belgium

ADIPOCYTES BLOCK GRANULOPOIESIS THROUGH NEUROPILIN-1 INDUCED G-CSF INHIBITION

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Adipocytes are a part of hematopoietic microenvironnement, but it was usually admitted that they play a passive role in hematopoiesis. We have previously shown that neuropilin-1 (NP-1) is differentially expressed in hematopoietic iliac crest bone marrow (BM) (NP-1low) and femoral BM (NP-1high). We hypothesized that adipocytes could regulate negatively hematopoiesis by a mechanism involving NP-1. We show here that isolated BM adipocytes differentiated into fibroblast-like fat cells (FLFC) displaying the majority of the primitive unilocular fat cell characteristics. Morphological and immunophenotypic analysis of FLFC/CD34+ co-cultures demonstrated that: 1) FLFC induced CD34+ differentiation into macrophages independently of cell-cell contact. Likewise, these cells constitutively produced M-CSF; 2) In contrast, granulopoiesis was poorly represented during cell-cell contact and was restored in transwell experiments in correlation with G-CSF production. Neutralizing NP-1 antibody, in cell-cell contact context, induced G-CSF production and granulopoiesis. Our data may provide a new role for adipocytes that may actively participate to the pathophysiology of BM failure disorders. It remains to determine by which mechanisms NP-1 operates in this process.

EFFECTS OF OSTEOBLAST-LIKE CELLS ON BONE-RELATED AND INVASIVE GENE EXPRESSIONS IN BREAST CANCER CELLS

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Breast cancer cells frequently metastasize to the skeleton, where they induce extensive osteoclast-mediated bone destruction, resulting in hypercalcemia, fractures and pain. Osteoblasts, depending on their maturation stage, play a dual role in mediating the effects of breast cancer cells on osteoclasts and in controlling bone formation. However, data on the influence of osteoblastic cells on tumor cells through direct cell-cell contacts are scarce. Our objective was to investigate the effects of osteoprogenitor-like cells (MG-63) and mature osteoblast-like cells (SaOS-2) on luminal-like (MCF-7) and basal-like (MDA-MB-231) breast cancer cells, evaluating the modulation in tumor cells of (i) osteoclastogenesis factors (RANKL, OPG, PTHrP), (ii) osteoblast regulatory factors (RUNX2, OPN, ON), and (iii) invasion-associated factors (MMP-1, -2, -9).
Breast cancer cells, labelled with CellTrace CFSE Cell Proliferation Kit (Eugene, OR, USA), were cultured with osteoblastic cells in alpha-MEM during four days. Cells were then separated by their differential fluorescence using a sorter Epics Elite (Coulter, USA). The purity degree of the cell populations was evaluated by Epcam expression level (Becton Dickinson, USA), an epithelial-specific marker, using flow cytometry (FACScalibure, Beckman, USA). Of note, only CFSE-labelled cells (tumor cells) were positive for Epcam, excluding fluorochrome contamination of unlabelled cells during the experiments. After sorting, the purity of MCF-7 and MDA-MB-231 cells was 98.9 ± 0.1 % (mean ± SEM) and 96.3 ± 2.5 %, respectively.
RT-PCR analysis showed several modifications in the expression of the tested genes. MG-63 cells weakly decreased the expression of CBFA1 and DLX5, while they strongly increased the expression of ON in the two breast cancer cell lines. In addition, they increased RANKL gene expression only in MCF-7 cells. By contrast, SaOS-2 cells mainly affected MCF-7 cells by markedly increasing gene expression of osteolytic factors (RANKL, PTHrP), and of osteoblast-related factors (CBFA1, DLX5, SOST, OPN). Of note, CBFA1, DLX5 gene expression levels were also increased in MDA-MD-231 cocultured with SaOS-2 cells. A strong increase in MMP-2 gene expression was observed in both tumor cell lines cocultured with MG-63 cells; the effect was weaker with SaOS-2 cells.
In summary, our results suggest that osteoprogenitor-like cells increase the invasive ability of breast cancer cells, while osteoblast-like cells enhance osteoclastogenesis factors expression and markers of the osteoblast phenotype mainly in the luminal-like MCF-7 cells. Thus, cells from the osteoblast lineage might modulate the ability of breast cancer cells to invade the bone matrix and to adapt to the bone microenvironment.

PAI-1 IS DISPENSABLE FOR LYMPHANGIOGENESIS BUT IN INFLAMMATORY CONDITIONS

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The plasminogen activating system plays a key role in the cascade of tumor-associated proteolysis leading to extracellular matrix degradation, stromal invasion and blood vessel recruitment and inroad. Changes in expression of components of this system, including plasminogen activator inhibitor-1 (PAI-1), have been associated with a defective angiogenesis. The present study was undertaken to assess the role of PAI-1 in lymphangiogenesis, novel lymphatic vessel formation.
Using a new model of lymphatic endothelial cell culture, the lymphatic ring assay, we observed that lymphangiogenesis was impaired in the absence of PAI-1, in contrast to what has been previously demonstrated for angiogenesis with the aortic ring assay. Mammary cancer cells which over-expressed the lymphangiogenic factor VEGF-C (VEGF-C MCF7) were injected orthotopically in PAI-1 deficient mice (KO) and their littermates (WT). PAI-1 KO or WT mice grafted with VEGF-C MCF7 cells developed more tumors (53% and 22% respectively) with a higher volume than mice grafted with control MCF7 cells (7.5% and 0%). Moreover, less PAI-1 KO (22%) mice developed tumors than WT mice (53%). However, no difference was seen concerning lymph node and lung metastases. Similar results were obtained by crossing PAI-1 KO mice with mice expressing polyoma virus middle T antigen (PyMT) under the MMTV promoter. The lymphangioma model was next used to assess lymphangiogenesis related to inflammation. With this aim, PAI-1 KO and WT mice in C57b/6 genetic background were injected with incomplete Freund adjuvant. Mice developed masses composed of hyperplasic lymphatic vessels and inflammatory cells. These masses were smaller in PAI-1 KO mice than in WT mice (p=0.0079). Histochemistry study demonstrated a higher proportion of inflammatory cells (p=0.0001) and lymphatic vessels (p=0.0025) which seem to be enlarged (p=0.0003) in PAI-1 KO mice.
We conclude that PAI-1 doesn't seem to play a major role in lymphangiogenesis as it does in angiogenesis, at least in physiological and tumoral conditions. However, PAI-1 seems to be important in inflammatory conditions.

3

CLINICAL VALIDATION OF TWELVE DEFORMABLE REGISTRATION STRATEGIES IN ADAPTIVE RADIATION THERAPY FOR HEAD AND NECK CANCER PATIENTS

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Background and purpose : Significant anatomy modifications occur during a head and neck (HN) radiation therapy (RT) course, due to patient weight loss, shrinkage of the tumor, edema ... One possible strategy to take into account those modifications is adaptive radiotherapy (ART) with imaging re-assessment of the patient during the RT course. This technique allows us to take into consideration the shrinkage of the tumor leading to smaller irradiated volumes. On the other hand, recognition of anatomy modifications such as weight loss needs specific image registration tools allowing non-rigid deformation in the image leading to deformable registration (DR). As DR enables either automatic recontouring or possible deformation of dose distribution maps; it is a critical step in the ART process and has to be validated in a clinical environment.
Materials and methods : We elaborated a comparison methodology of 12 voxel-based deformable registration strategies in order to select the best one for HN-ART. Evaluated strategies were: Level-set (LS), Level-set implemented in multi-resolution (LS_{MR}), Demons' algorithm implemented in multi-resolution (D_{MR}), D_{MR} followed by LS (D_{MR}-LS), Fast free-form deformable registration via calculus of variations (F3CV) and F3CV followed by LS (F3CV-LS). We also studied the interest of an edge-preserving denoising tool called "Local M-Smoothers" applied on the registered images and combined to all the aforesaid strategies (fLS; fLS_{MR}, fD_{MR}, fD_{MR}-LS, fF3CV, fF3CV-LS). Moreover, all deformable strategies were compared to a rigid registration based on Mutual Information (MI, fMI). For this purpose, all strategies were evaluated on a dataset of 5 patients' computerized tomography (CT) scans which were acquired before and during RT. We performed chronological and anti-chronological registration and studied the potential effect of the time sequence registration. We used a voxel intensity criteria called cross correlation (CC) and a volume-based criterion called Dice similarity index (DSI) on a total of 18 different manually contoured volumes.
Results : In terms of DSI, the best results were obtained using D_{MR} with a median value of 0.86 and an interquartile range (IQR) of 0.10 followed by fD_{MR}-LS (Median: 0.85; IQR: 0.10) and fD_{MR} (Median: 0.85; IQR: 0.10). In terms of CC, the best strategy is fD_{MR}-LS (Median: 0.97; IQR: 0.11) followed by D_{MR}-LS (Median: 0.96; IQR: 0.09) and D_{MR} (Median: 0.94; IQR: 0.15). Concerning time sequence analysis, anti-chronological time sequence shows better DSI median and IQR than the chronological one (Median: 0.84 vs. 0.83; IQR: 0.11 vs. 0.12). For CC, anti-chronological time sequence has a slightly decreased median (0.910 vs. 0.912) but a better IQR (0.16 vs. 0.21).
Conclusions : Both D_{MR} and fD_{MR}-LS are good compromise for HN-ART as we can expect median DSIs around 0.86 and median CCs around 0.95. Due to contradictory results in terms of time-sequence analysis between DSI and CC analysis, one cannot come to the conclusion that a chronological time sequence is better than the anti-chronological one.

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VASCULAR ARCJOTECTURE OF BREAST CANCER XENOGRAFTS OVER-EXPRESSING MT4-MMP

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Our previous data show that MT4-MMP, a membrane-anchored MMP essentially expressed by breast tumor cells, increases primary breast cancer growth with frequent enlargements of intra-tumor blood vessels and promotes lung metastases[1]. In order to understand the way by which MT4-MMP affects tumor progression, we have performed ultrastructural (Fig. 1), immunohistological (Fig. 2) and gene expression profile (Fig. 3) studies of MDA-MB-231 breast cancer xenografts expressing or not MT4-MMP in mice.
Transmission electron microscopy (Fig. 1) and immunohistological analyses (Fig. 2A) show that most of the intra-tumoral blood vessels are covered by pericytes in both conditions. However, in the presence of MT4-MMP, mural cells (α-SMA+) are more frequently detached from the endothelium with irregular shapes and extend irregular cytoplasmic processes in contact or not with endothelial cells. Moreover, basement membranes of these vessels are more often degraded or absent in MT4-MMP condition.
Automatic computer-assisted quantifications (Fig. 2C) performed on 150 thick sections confirmed an enlargement of blood vessels (Fig. 2D) and a difference in pericytes distribution around the blood vessels (Fig. 2E) in xenografts expressing MT4-MMP. Indeed, pericytes are more dispersed and distant from endothelial cells. In MT4-MMP condition, the ratio between pericytes area and endothelial cells area, reflecting the pericyte coverage, is also increased (Fig. 2E).
In conclusion, MT4-MMP could facilitate tumor growth and metastasis by disturbing the vascular integrity. Further studies are however required to assess the vascular leakage in xenografts and to determine whether MT4-MMP is directly or not implicated in the lack of pericytes / endothelial cells interaction. Validations of a global gene expression analysis (Fig. 3) performed on breast cancer xenografts expressing or not MT4-MMP will also give precious information about the MT4-MMP mechanism of action in tumor progression.

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MOLECULAR ELECTRON PARAMAGNETIC RESONANCE IMAGING OF MELANIN IN MELANOMAS : A PROOF-OF-CONCEPT

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Introduction and Objectives of the study : The incidence of malignant melanoma is increasing at alarming rates. Prevention, early detection, appropriate clinical and histological diagnoses are critical to favourable outcomes. If a lesion is suspicious for melanoma, adequate biopsy is necessary for staging and management. Thicker melanomas show greater risk of metastatic disease. In case of suspicious melanoma, there is a need to conduct a sentinel lymph node biopsy technique. Finally, the early detection of metastasis (in liver or lungs) may improve the long-term survival of patients. Newer diagnostic techniques should be evaluated, as they may reduce the need for biopsies, and may help in the early detection of distant melanoma metastases.
Our work is dealing with the development of new methods allowing the selective high resolution imaging of melanomas. Melanins are amorphous, irregular, polymeric pigments that contain organic free radicals of semiquinone type. This stable free radical can be easily detected at room temperature using Electron Paramagnetic Resonance (EPR) spectroscopy. We hypothesized that the most recent developments in EPR imaging could make this technique a suitable method to map these free radicals with high sensitivity and high resolution, and render 3D-images of these malignant tissues.
Methods and Materials : Melanoma B16 implanted in C57Bl6 mice and grown subcutaneously were surgically removed and freeze-dried along with metastatic lungs. A variety of spatial and spectral-spatial (2D and 3D) EPR images have been performed on whole intact melanoma samples and metastatic lungs using a X band system.
To assess the relevance of our technique for human melanomas, we applied the method to paraffin-embedded human melanomas excised from patients.
To exclude a possible contribution of freeze-drying or histological process induced radicals, EPRI was also applied ex vivo and in vivo. In vivo EPR techniques, 1.1 GHz, using advanced surface and/or whole body coils were used to provide a signal from melanin and for imaging of melanoma.
Results : Melanin complexes in melanomas present at room temperature an EPR signal with a g value of 2.005, and a line width of around 9-10 Gauss. 2D and 3D EPR imaging give us unprecedented quantitative and qualitative information concerning the spatial distribution of paramagnetic melanin radicals in tumours or metastatic lung. The lung melanoma metastases, seen as brown spots of melanin pigments, can be visualised by 3D EPR images.
As an illustrative example, we image human melanoma metastasis with melanocytes irregular clusters. We have observed that the EPR image is comparable with the histological section.
For the first time we were able to detect and image in vivo these endogenous radicals of mice SC melanoma. The EPR spectrum was unambiguously ascribed to melanin and using an appropriate field gradient we could record in vivo an EPR 2D image of subcutaneous implanted melanoma.
Conclusions and Perspectives : EPR imaging can provide unique information concerning the spatial distribution of the free radicals (in special melanin complexes) in melanoma and melanoma metastases. Developments in EPRI represents may open a new way for the early diagnostic of melanomas.

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GLYCOME BIOMARKER TO DETECT EARLY STAGE OF LIVER CANCER

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Hepatocellular carcinoma (HCC), also known as primary liver cancer, is the fifth most common cancer worldwide. As is the case for most cancers, diagnosis is also difficult because the effects of some liver tumors may resemble those of other medical conditions, and because liver cancer can't be diagnosed using a standard panel of liver tests. No completely accurate screening test for liver cancer exists. Doctors sometimes use a blood test that checks for the presence of alpha-fetoprotein (AFP) — a type of protein not normally found in adults — to screen people at high risk of the disease. Unfortunately, an elevated level of AFP is detectable in only about two-thirds of patients with early-stage HCC, and high mortality from HCC is due to the inability to detect these cancers in their earliest stages. Thus, to detect small tumors, a very sensitive biomarker is needed, with an intensity that should be proportional to the size of the tumor.
Our group took advantage of a recently developed technique to study the total glycosylation pattern of protein mixtures, e.g. serum proteins. Sugar trees are cut from the proteins, collected and separated. We have been able to analyze the serum samples of over 500 cirrhosis patients. It was clear that the changes in serum glycosylation correlated with the presence of tumors and with tumor size (Liu. et al, 2007). This study included 450 HBV-infected patients with liver fibrosis, cirrhosis and HCC. We found that a branch alpha(1,3)-fucosylated triantennary glycan (NA3Fb) was more abundant in HCC patients than in cirrhosis patients, fibrosis patients and healthy blood donors, whereas a bisecting core alpha (1,6)-fucosylated biantennary glycan (NA2FB) was elevated in cirrhosis patients. The ratio of these values forms the basis of the new blood test (the log ratio of peak9/peak7, renamed as GlycoHCCTest). The increased levels of protein linked branch fucose sugar in serum could be caused by changing biosynthetic characteristics. This hypothesis is confirmed in the our group using diethyl nitrosamine (DENA)-induced HCC mouse model. Nevertheless, using GlycoHCCTest, we were able to make the correct diagnosis in 70% of the cases, a success rate that equals that of the AFP tumor marker currently used in the clinic. Promisingly, when the AFP test was used in combination with the new GlycoHCCTest, the accuracy of HCC diagnosis increased dramatically. The new glycomic test succeeded in detecting the liver cancer in more than half of the patients with cirrhosis and cancer of the liver for whom the AFP test was inconclusive.

Xue-en Liu, Liesbeth Desmyter, Chun-fang Gao, Wouter Laroy, Sylviane Dewaele, Valerie Vanhooren, Ling Wang, Hui Zhuang, Nico Callewaert, Claude Libert, Roland Contreras and Cuiying Chen (2007) N-glycomics changes in hepatocellular carcinoma patients with liver cirrhosis induced by hepatitis B virus. Hepatology, in press. (Epub 2007 Aug 7)

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THE USE OF PET IMAGING FOR ADAPTIVE RADIOTHERAPY: STRENGTH AND LIMITATION ASSESSED IN ANIMAL MODEL

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Purpose : Adaptive image-guided radiotherapy aims at refining dose distribution as a function of target volume modification during the course of treatment. In this framework, functional imaging and in particular PET imaging combined with various tracers may help to identify relevant biological sub-volumes, which might require additional adaptive dose delivery. A prerequisite for this approach is that PET imaging should be sensitive and specific enough to trace spatial changes and modification in the signal intensity in the relevant biological processes. A validation of the use of PET imaging for adaptive radiotherapy was undertaken in an animal model by comparing small animal PET images (2.5 mm resolution) with autoradiography (AR) (100 µm resolution) in various tumor models and physiological situations.
Material and methods : A special template for tumor-bearing mouse imaging was designed. The caudal part of the animal was immobilized in gelatin, surrounded by Teflon® tubes filled with a contrast agent or a radioactive tracer. The whole device was then imaged with MRI (Biospec, Brücker) and, after ^{18}F-FDG injection, with small animal PET (Mosaic, Philips). Directly after the PET, the animal was euthanized in the device, frozen and sliced. The slices were imaged with autoradiography, revealing the microscopic distribution of the tracer. The slices images were then realigned and transformed in an ECAT volume. The three modalities (MRI, PET, AR reconstructed) were registered on the basis of the tubes with sub-millimetric accuracy. The tumor was delineated on the MRI images and the corresponding volume transferred to the two other modalities. On the PET and AR images, the tumor was then segmented using a threshold-based method. The thresholds were selected to obtain absolute equal volumes in the PET and AR images, corresponding to the same fraction of the overall tumor volume. Matching index were then calculated between the various volumes. FSAII tumors (n=5), SCCVII (n=5) and irradiated (35 Gy) FSAII tumors (n=5) were studied with this device.
Results : Considering small but highly active subvolumes, we found low matching values (around 40%) between the volumes delineated on PET compared to those delineated on AR. These values progressively increased when bigger volumes were compared. Small and highly active regions scattered within the tumors were invisible in PET (false negative). On contrary, some regions in the PET images were depicted as containing activity while being negative in autoradiography (false positive regions). These findings were independent of the tumor type, irradiation or tumor size.
Conclusion : Discrepancies were found between the in vivo PET images and the underlying microscopic reality. These differences were very important if considering small therefore highly active regions of the tumors. Dose painting based on PET images should therefore be carefully considered and these limitations should be taken into account.

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HYPOXIA PROMOTES RESISTANCE TO ETOPOSIDE BY REGULATING P53 STABILITY AND C-JUN DNA-BINDING ACTIVITY

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Hypoxia is a common feature of the microenvironment of most solid tumors which contributes to radio- and chemoresistance. Hypoxia can be transient or chronic. If transient (acute) hypoxia is a consequence of abnormal vasculature and high interstitial pressure that cause inadequate blood flow, chronic hypoxia is caused by increased oxygen diffusion distance due to tumor expansion. Chronic hypoxia affects tumor area at distance greater than 70-100 µm from the nearest capillary and is associated with poor outcome for patients. The presence of hypoxia contributes to aggressive behaviour of cancer cells and to resistance to therapy due to poor distribution of chemotherapeutic drugs in this area and attenuation of the cytotoxicity of these drugs. Moreover, tumor cells under hypoxic condition present adaptation which increases survival and drug resistance. These adaptations are mainly driven by post-translational modifications or gene expression regulation. Hypoxia promotes angiogenesis, glucose transport and anaerobic metabolism, invasion, resistance and survival particularly by modulating the apoptotic process. The main transcription factor involved in adaptation to hypoxia is hypoxia-inducible factor-1 (HIF-1) but other transcription factors can also be involved in response to hypoxia.
Previous studies have shown that hypoxia protected human hepatoma cell line HepG2 from apoptosis induced by etoposide, an inhibitor of Topoisomerase II, through an HIF-1-independent pathway (1). In this study, we investigated the involvement of several hypoxia- or DNA-damage-responsive transcription factors in the hypoxia-induced resistance to etoposide. DNA-binding assays were performed to detect changes in DNA-binding activity of c-jun, p53, NF-κB and c-myc in response to etoposide and/or hypoxia.
We observed that hypoxia inhibited the etoposide-induced stabilization and DNA binding activity of p53. Involvement of p53 in hypoxia-induced resistance to etoposide has therefore been envisaged using a knock down approach. As described previously, hypoxia inhibited the activation of caspase-3 induced by etoposide and the cleavage of its main substrate PARP-1. Here, we observed that the knock down of p53 reduced the activity of caspase-3 and the cleavage of PARP-1, when cells were incubated with etoposide under normoxia. These results show that p53 is required for the activation of caspase-3 by etoposide and thus the resistance observed in hypoxia may be a consequence of the downregulating effect of hypoxia on the abundance of p53.
The involvement of another transcription factor, c-jun was also studied because hypoxia in the presence of etoposide enhanced its DNA-binding activity. Knock down of c-jun/AP-1 strongly increased the activity of caspase-3 and the cleavage of PARP-1 in the presence of etoposide under hypoxia but had no effect under normoxia. These results indicate that c-jun is involved in protecting cells under hypoxia. Contrary to what had been observed by Eferl et al (2), the inhibition of the expression of c-jun had no effect on the abundance of p53.
In conclusion, these results show that hypoxia negatively regulates the abundance of p53 and positively the DNA-binding activity of c-jun to promote the resistance of cells to etoposide-induced apoptosis.

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HIF-1 IS INVOLVED IN THE HYPOXIA-INDUCED PROTECTION AGAINST APOPTOSIS

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The hypoxia-inducible factor-1 (HIF-1) is a transcription factor which is activated in response to low oxygen tension, hypoxia. It mediates cell survival and adaptation to the hypoxic environment. Most solid tumors contain hypoxic regions. Those tumors are associated with a malignant phenotype and resistance to radiotherapy and chemotherapy leading to poor patient prognosis. However, the mechanisms underlying this resistance to treatments are not yet well known.
We have shown that, under hypoxic conditions, HepG2 cells were protected from etoposide-induced apoptosis. Indeed, in those conditions, caspase activity, PARP cleavage and DNA fragmentation were reduced. The aim of this study was to investigate the role of HIF-1 in this protection. For that, we inactivated HIF-1 using RNA interference, and showed that the inhibition of HIF-1 prevented the apoptotic protection under hypoxic condition. Indeed, the inactivation of HIF-1 by anti-HIF-1-α siRNA under hypoxia-etoposide conditions increased PARP cleavage and caspase activity.
To understand how HIF-1 intervenes in the apoptotic protection, we performed a transcriptomic analysis using low density DNA microarrays. The data obtained indicate that the expression of several pro- and anti-apoptotic genes was modified. The expression of Bak, a p53 target gene, was detected in normoxia-etoposide, but not under hypoxic conditions. However, when HIF-1 was downregulated by RNA interference, under hypoxia and in the presence of etoposide, the expression of Bak was higher. These results have been confirmed by real-time RT-PCR experiments. Thus, it is probably, at least in part, by regulating Bak expression that HIF-1 induces cell resistance to apoptosis.
In conclusion, we have shown that downregulation of HIF-1 prevents the apoptotic protection under hypoxia. In addition, HIF-1 is responsible for decreasing Bak expression. This process could be part of the mechanisms by which HIF-1 protects HepG2 cells from the etoposide-induced apoptosis under hypoxia.

Acknowledgement : A. Sermeus and J.-P. Cosse are recipients of a FRIA fellowship (Fonds pour la Recherche dans l'Industrie et l'Agriculture, Belgium) and Carine Michiels is Research Director of FNRS (Fonds National de la Recherche Scientifique, Belgium).[1]

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MODIFICATION OF THE TUMOR MICROENVIRONMENT BY A PLASMID ENCODING AN ANTIANGIOGENIC FACTOR

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Gene therapy with antiangiogenic factor delivered by electrotransfer in tumor could be a promising method for the treatment of cancer in combination with other anticancer treatment. Hence, the aims of our study were i) to check how electrotransfer of a plasmid encoding an antiangiogenic factor (AP) affects tumor growth and tumor microenvironment ii) to determine the optimal therapeutic window for combination with radiotherapy.

AP electrotransfer in the tumor significantly delayed the growth of TLT murine liver tumor as previously described on melanoma model. Tumor microenvironment parameters were studied after AP electrotransfer. Tumor pO_2, measured using electron paramagnetic resonance, was increased significantly after the treatment with a maximal value observed three days after the treatment. In order to determine the mechanisms of this oxygenation, tumor perfusion and oxygen consumption rate were assessed. Tumor perfusion measured by patent blue staining and by laser Doppler imaging, was not modified for the 3 days after treatment, indicating that the increase in pO_2 was not caused by an increase in oxygen supply The oxygen consumption rate of tumor cells after AP electrotransfer was measured using high frequency EPR. Finally, the combination of this gene therapy with AP and radiotherapy was assessed. Irradiation (6 Gy) was applied when the oxygenation was maximal after AP electrotransfer, and the tumor radiosensitivity was significantly enhanced by the combination treatment. These results demonstrate the potential of antiangiogenic gene therapy for radiosensitizing tumors.

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PACLITAXEL-LOADED PEGYLATED NANOCARRIERS: PREPARATION, PHYSICOCHEMICAL CHARACTERIZATION AND IN VITRO ANTI-TUMORAL ACTIVITY

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The final aim of the work is to determine the optimal strategy to deliver anticancer drug encapsulated in nanocarriers to tumor by comparing the passive targeting of polymeric micelles by Enhanced Permeation and Retention effect and the active targeting of polymeric nanoparticles by a specific ligand of the tumor endothelium. Hence, we developed PEGylated polymeric micelles and polymeric nanoparticles loaded with a poorly soluble anticancer drug, Paclitaxel. Both nanocarriers were designed to be intravenously administered and devoid of Cremophor® EL side effects (Taxol® vehicle).

Polymeric micelles formed by a copolymer of poly-caprolactone and trimethylenecarbonate ($mmePEG_{750}P$(CL-co-TMC)) had a size of 24 +/- 0.1 nm and a ζ potential of -3.1+/- 3.4 mV. The solubility of Paclitaxel in micelles was increased from approximately 1µg/ml to 1.500 +/- 200 µg/ml.

Paclitaxel-loaded PEGylated PLGA-based nanoparticles were prepared by nanoprecipitation method. Nanoparticles size and ζ potential were respectively 112 +/- 4 nm and – 22.2 +/- 2.9 mV. The encapsulation efficiency reached 70 +/- 4 %.

Nanoparticles targeting the tumor endothelium (integrins $\alpha_v\beta_3$) were prepared by incorporating PEGylated poly-caprolactone grafted with GRGDS to PLGA.

The anti-tumoral activity of both nanocarriers loaded with Paclitaxel was assessed in vitro on Human Cervic Carcinoma cells (HeLa) and compared to the anti-tumoral activity of the commercial formulation Taxol®. When exposed to 25, 12.5 and 2.5 µg/ml of Paclitaxel, the HeLa viability was much lower for the Paclitaxel-loaded $mmePEG_{750}P$(CL-co-TMC) micelles and for Paclitaxel-loaded PEGylated PLGA-based nanoparticles than for Taxol®. In contrast, Cremophor® El alone was much more cytotoxic than drug-free micelles or nanoparticles.

In conclusion, the encapsulation of Paclitaxel into polymeric micelles and nanoparticles enhanced its solubility and its cytotoxic effect in vitro compared to Taxol®. In vivo studies, using SKOV-3 human ovarian cancer cells injected on nude athymic mice are ongoing.

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^{18}F-FLT AND ^{18}F-FDG PET TO MEASURE RESPONSE TO TREATMENT IN A COLORECTAL XENOGRAFT MODEL

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This project is part of the European FP6 BIOCARE project (WP3)

A major issue in biological targeting is predicting response early so as to be able to modify treatment. A non-invasive method such as PET scanning may have the potential to achieve this aim. The aim of this study is to determine if PET scanning can be used to measure response to radiation with or without the COX-2 inhibitor celecoxib (Celebrex ®) in a colorectal xenograft model.

For the development of the xenograft model, a colorectal cell line (HCT116) was injected subcutaneously in both flanks of nude mice. The mice were treated for 6 days with a dose of 5mg/kg celecoxib twice daily by gavage with or without a single dose of radiotherapy (25 Gy). MicroPET scans for ^{18}F-Fluorodeoxyglucose (^{18}F-FDG) and ^{18}F- 3'-deoxy-3-fluorothymidine (^{18}F-FLT) were performed before and after treatment. The PET images were reconstructed with OSEM3D and the tumour volume and the mean SUV tumor/liver ratio was calculated.

Until now, ^{18}F-FLT and ^{18}F-FDG scans have been performed with mice treated with a single dose of radiotherapy (25Gy). PET scans were performed before radiotherapy, one day after radiotherapy and 1 week after radiotherapy. The mean tumour volume measured with ^{18}F-FDG was slightly larger than the volume measured by ^{18}F-FLT (p=0.05). However, both the ^{18}F-FDG and ^{18}F-FLT volume correlated significantly with the tumour volume as measured by the caliper (p<0.0001). The analysis revealed so far that both FDG and FLT uptake is decreased already one day after a single dose irradiation of 25 Gy. The ^{18}F-FDG signal decreased from a mean SUV tumor/liver ratio of 2.4 to 2.1 and for ^{18}F-FLT the mean SUV tumor/liver ratio changed form 1.4 to 1.1. One week later, while FDG was still low (1.7), FLT activity was already normalizing (1.3).

We can conclude that both ^{18}F-FDG and ^{18}F-FLT are trustful markers to detect tumours in this xenograft model due to the good correlation with the caliper volume. The single dose radiotherapy experiment implies that proliferation of the tumor cells seems to be restored more rapidly after irradiation in comparison with the glucose metabolism. The results of the combination therapy with the COX-2 inhibitor will be presented at time of congress.

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DOES INFILTRATION OF LYMPHOCYTES PLAY A ROLE IN THE SURVIVAL OF COLORECTAL CANCER PATIENTS?

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Colorectal cancer (CRC) generally arises from precancerous lesions by accumulation of genetic mutations which can give rise to abnormal proteins with aberrant or lost functions. Provided adequate stimulation, a change in amino acid sequence of a protein can be recognized by and stimulate the immune response. However, the immune response against cancer cells is complex, involving the interaction of several cell types and cell products. Additionally, tumor cells have several potential methods of evading the immune response. Nevertheless, it is well recognized that cytotoxic-T-lymphocytes (CTLs) constitute one of the most important effector mechanisms of anti-tumor immunity by releasing lytic components and direct cell-cell interaction. Perforin and enzymatic proteases (such as granzyme B) are released and cause cell death by disruption of the cell membrane and activation of the apoptotic pathway respectively.

Pronounced lymphocytic infiltration in CRC has been described and is more marked in MSI tumors with a proximal location. They are also reported to have a better clinical course. One possible explanation for the favorable prognosis of these MSI CRCs is the continuous production of abnormal peptides that, by acting as neoantigens, could elicit specific antitumor immune responses potentially effective in limiting tumor growth and spread. Several studies showed that the inflammatory response consisted mainly of activated $CD3^+$ and $CD8^+$T lymphocytes.

The current study aimed at describing the location and activation of $CD3^+$ and $CD8^+$ T cells that infiltrate CRC and at correlating these results to the clinical features and survival in order to determine the prognostic potential of CTLs.

Ninety-eight surgically resected cases of CRC were selected from the Universal Hospital of Antwerp and the St.-Augustinus Hospital. These cases have previously been analyzed for microsatellite instability (MSI) status. By immunohistochemistry the $CD3^+$, $CD8^+$ and Granzyme B^+ lymphocytes were classified according to Naito et al. [1] Possible relationships between MSI status, immunophenotypic markers and clinicopathological parameters of CRC were investigated using computerized statistical analyses. All levels of significance were set at p< 0.05. High-frequency MSI tumors were significantly correlated with proximal location, poor differentiation, female gender and lower stage. MSI-H tumors also carried a higher number of $CD8^+$ and $CD3^+$ lymphocytes in all examined areas of the tumor compared to MSS tumors. Granzyme B^+ lymphocytes were significantly more frequently detected within cancer cell nests in MSI-H tumors. Univariate analysis revealed that stage, and the presence of $CD8^+$ and $CD3^+$ lymphocytes in cancer cell nests had significant impact on patient's disease free survival (dfs). Stage seemed to be the only factor with a significant impact on overall survival (os).

However, further analyzed by stage, $CD3^+$ infiltration (for dfs and os) and $CD8^+$ infiltration (for dfs only) along the invasive margin had a significant impact on survival in stage II CRC only.

Further statistical analysis needs to be done to investigate whether lymphocyte infiltration is an independent prognostic factor.

At present, we conclude that lymphocyte infiltration may play a role on survival of CRC patients, especially in stage II tumors.

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THE BIOMARKER POTENTIAL OF PROMOTER HYPERMETHYLATION IN HNSCC TREATED BY RADIOTHERAPY

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Background : During the carcinogenetic process of head and neck squamous cell carcinoma (HNSCC), multiple genetic as well as epigenetic alterations occur. Promoter hypermethylation, the most important epigenetic change, is a topic of intense research. In HNSCC, aberrant methylation status of several genes involved in cell cycle (p16INK4a), DNA repair (MGMT), cell adherence (ECAD) and apoptosis (DAPK) has been associated both with clinicopathological features and with outcome. However, data on the prognostic and predictive value of promoter hypermethylation in the radiotherapy setting are limited. More specifically, it is unclear whether epigenetic silencing of genes involved in the normal molecular response to ionizing radiation could influence radiosensitivity.
Material and methods : Genomic DNA was extracted from archival diagnostic paraffin-embedded tumor biopsies of 50 patients with HNSCC treated with hyperfractionated radiotherapy only. In addition, a control group was selected, consisting of smokers of which an archival vocal cord biopsy taken at the time of diagnosis of Reinke's oedema was available. After bisulfite treatment of the extracted DNA, methylation status of several genes will be assessed both with conventional and array methylation-specific PCR (MSP). Promoter hypermethylation will be investigated for genes already known to be potentially methylated in HNSCC, and new candidate genes. The latter were selected by an in silico algorithm, and included only genes involved in the normal molecular response to ionizing irradiation. The obtained methylation profiles will be related with clinicopathological parameters and with patient's outcome.
Results : Up till now, the conventional MSP's and the epi-arrays have been optimized on DNA extracted from HNSCC cell lines. The DNA extractions of the patient group have been performed, and the methylation analyses are ongoing.
Conclusions : This project aims to assess whether and to what extent aberrant methylation of genes involved in HNSCC carcinogenesis, and genes involved in the response of these tumors to ionizing radiation, influences radiosensitivity. The analyses are ongoing and the results will be presented at the time of the congress.

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CONTRIBUTION OF MEMBRANE-TYPE MATRIX METALLOPROTEASES TO TUMOR PROGRESSION: A GENOMIC APPROACH

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Abstract: The matrix metalloproteases or MMPs constitute a family of proteolytic enzymes which are involved in tumor and metastatic progression. MMPs can be divided into two groups: secreted MMPs and MMPs linked to the cell membrane (MT-MMPs). Among MT-MMPs, MT1-MMP is the most often overexpressed MMP in human tumours. Moreover, the expression of MT1-MMP by the cancerous cells promotes tumoral growth, metastatic dissemination as well as the acquisition of an angiogenic phenotype. These observations show that MT1-MMP is a potential target for the development of new anti-tumoral therapies. However, the exact mechanisms by which MT1-MMP affects tumor progression remain poorly defined. To better characterize the involvement of MT1-MMP in the tumor progression, our laboratory has compared, by using DNA microarrays, the transcriptomes of human mammary adenocarcinoma cells (MCF7), expressing or not the human MT1-MMP. This analysis revealed modulations of expression of genes implicated in different cellular processes including cell cohesivity (CD44, GJA1, CELSR2, MUC1, PKP1). The modulation of PKP1 expression was validated by RT-PCR and Western blotting. Moreover, MCF7 overexpressing MT1-MMP show a more cohesive morphology which is dependent of its catalytic activity. This cohesive morphology is characterized by a reduced number of migratory cells and an organisation into more compact colonies. These results suggest a direct or indirect modulation of cell cohesivity by MT1-MMP. Finally, the expression of MT1-MMP by MCF7 cells caused an increased mortality of mice after intraveinous injection.

Keywords: MT1-MMP, breast cancer, plakophilin 1

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DEVELOPMENT AND EVALUATION OF A NON INVASIVE METHOD TO ESTIMATE THE OXYGEN CONSUMPTION BY TISSUES

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Introduction : Oxygen is a key environmental factor in the development and growth of tumors, and their response to treatment. Tumor oxygenation depends on a balance between oxygen supply and consumption, and both should considered in developing strategies to reduce hypoxia.
The oxygen consumption rate of tumor cells can be measured by EPR using in vitro or ex vivo EPR measurement. The method is based on the variation of the linewidth of a paramagnetic material in the presence of consuming cells.
Purpose : Here, we developed a new EPR method to measure non invasively the tissue oxygen consumption. The protocol used was based on the measurement of pO_2 during a carbogen challenge protocol. The following sequence was used: 1) basal value during air breathing; 2) saturation of tissue with oxygen by carbogen breathing; 3) switch back to air breathing. The assumption was that the kinetics of return to the basal value after oxygen saturation will be mainly governed by the tissue oxygen consumption. This challenge was applied in hyperthyroid mice compared to control mice. This status is known to dramatically affect consumption rate of tumor and muscle cells.
Results : First we checked ex-vivo, that the treatment by L-thyroxine was able to change the oxygen consumption by tumors. We observed an oxygen consumption rate significantly enhanced for hyperthyroid mice compared to control mice. During the breathing challenge, we obtain typical evolutions of pO_2. The return kinetics to the basal values was faster in hyperthyroid mice than in control mice in tumor and muscle. The quantitative estimation of the return kinetics was carried out using a monoexponential curve. Kinetics constants (k expressed as min^{-1}) from hyperthyroid mice were higher than in tumors and muscles control mice.
Discussion : Our work was an attempt to provide a new method that may highlight differences in oxygen consumption by different tissues. The use of normal and hyperthyroid mice provided ideal models with tissues presenting differences in oxygen consumption rates. This method has the unique advantage of being non invasive and adapted to in vivo studies.

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TRANSPORT AND DELIVERY OF L-PROLYL-M-L-SARCOLYSYL-L-P-FLUORO-PHENYLALANINE-ETHYLESTER (PSF) TO TUMOR CELLS

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Many approaches are being explored to improve drug targeting and delivery of anticancer drugs. We investigated the mechanism of action of an alkylating tripeptide, PSF and found a particular targeting and delivery process based on the quick binding of PSF to blood cells (BC) and recognition by tumor-associated proteolytic enzymes subsequently generating its active metabolites. PSF was found stable in human plasma, it disappeared very quickly in whole blood along with the generation of a metabolite, m-sarcolysin while in the presence of melanoma cells (MC), 3 additional metabolites were generated and identified by mass spectrometry. Similarly, PSF bound to natural or artificial membranes but the generation of the active metabolites was only obtained by adding MC or proteolytic enzymes. Interestingly, tumor-shed proteases as well as enzymes mimicking ECM-degrading proteases were also able to generate the same metabolites with similar kinetics. 2D-gelelectrophoresis on membrane protein extracts of melanoma cells are performed in order to identify the families of enzymes involved in the proteolytic cleavage of membrane-bound PSF. Different enzyme inhibitor combinations indicated both the families of proteases involved, including metalloproteases, and the possible sequential generation of metabolites. Our data suggest that PSF can be transported by RBC and subsequently cleaved to release 4 active metabolites in the presence of protease rich environments such as tumor sites.

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ADAMTS-12 AS ANTITARGET IN CANCER

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ADAMTS (a disintegrin and metalloproteinase domain with ThromboSpondin motif) constitute a family of endopeptidase related to matrix metalloproteases (MMPs). These proteases are largely implicated in extracellular matrix degradation and tissue remodelling associated to different pathologic processes such as arthritis, tumor growth and angiogenesis.
Here, we have established the profile of ADAMT-12 expression in different cancer cell lines such as pulmonary and breast cancer cells. In human samples, a RT-PCR study revealed that ADAMTS-12 is overexpressed in breast cancer compared to normal breast tissues. In these specimens, ADAMTS-12 transcript level is positively correlated to vascular endothelial growth factor (VEGF-A) expression suggesting it a role in tumor growth and angiogenesis.
Then, we have stably transfected ADAMTS-12 cDNA in MCF-7 human breast cancer cells. These cells have been characterized in 2D-adhesion and -proliferation in vitro assays. ADAMTS-12 expression did not affect cell adhesion in different matrices tested. Similarly, cell proliferation rate in 2D culture conditions was not modulated by ADAMTS-12 production. In sharp contrast, proliferation of cells overexpressing ADAMTS-12 was drastically decreased in 3D soft agar assay. Interestingly, rat aortic rings cultured in the presence of media conditioned by ADAMTS-12 transfectants displayed a decrease of angiogenesis in comparison with rings cultured with conditioned media of control clones. Altogether, these results suggest that ADAMTS-12 could regulate negatively cancer progression by reducing anchorage independent growth and by regulating tumoral angiogenesis.

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ESTABLISHMENT OF A PROGNOSTIC SIGNATURE IN PATIENTS WITH ER- MAMMARY TUMORS BASED ON GENETIC EXPRESSION PROFILES OF TUMOR INFILTRATING CD4+CELLS

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To further gain insight into the functional defects characterizing breast tumors infiltrating T cells, we performed a whole genome gene expression profiling study using Affymetrix Human GeneChip© Arrays on CD4+ T cells purified from primary invasive ductal breast carcinomas (N=10 pts).
Intriguingly, an unsupervised cluster analysis of purified CD4+ cells isolated from estrogen receptor negative (ER-) and ER+ primary tumors revealed important differences in gene expression profiles according to the ER status. A supervised analysis between CD4+ cells isolated from ER- and ER+ tumors revealed the presence of statistically significant differentially expressed genes involved in several immune response pathways. These results highlight the importance of tumor microenvironment on the global gene expression profiles of tumor infiltrating CD4+ cells in breast cancer.
A tumor infiltrating CD4+ signature index (TICD4SI) was computed based on 108 selected genes form the above analysis and its association with clinical outcome was assessed in several external publicly available datasets including more than 2500 breast cancer patients. Similar analysis was performed according to the already identified different molecular subtypes of breast carcinomas, namely ER-/Erbb2- ("basal-like"), Erbb2+ and ER+/Erbb2- ("luminal") subtypes. Interestingly, TICD4SI levels were higher in the ER-/Erbb2- ("basal-like") and Erbb2+ groups compared to the ER+ (luminal) subgroup. With respect to clinical outcome, TICD4SI showed a statistically significant association with survival only in ER-/Erbb2- (HR= 0,706; CI:{0,586-0,840}; p=0,000) and in Erbb2+ subtypes (HR= 0,750; CI:{0,585-0,963}; p=0,024) but not in the ER+ ("luminal") subgroup. Of interest, the TICD4S was the only signature among several others (Wound[1], IGS[2], Oncotype[3], GGI[4], Gene 70[5] and Gene 76[6]) that showed a statistically significant prognostic value in the already high risk ER-/Erbb2- ("basal-like") subgroup (high TICD4SI levels were associated with better clinical outcome).
This study is the first to demonstrate differences in gene expression profiles of tumor infiltrating CD4+ cells according to different molecular subtypes of breast carcinomas. Furthermore, it is also shows that higher expression TICD4SI levels provide better clinical outcome particularly in the high risk ER-/basal subtype.

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MARKERS OF CHEMOTHERAPY RESISTANCE OF BREAST CANCER CELLS UNDER HYPOXIC CONDITIONS

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Chemotherapy is an integral component of standard care for solid tumors. However, recurrence may occur, with a poor clinical outcome. Some of the primary factors leading to chemoresistance become to be understood. Overexpression of members of the ABC transporter family (MDR being the most well known), mutations as well as the development of therapeutic sanctuaries are well characterized to be responsible for drug resistance. Recently, the influence of hypoxia has also been recognized. Hypoxic conditions elicit cellular responses designed to improve cell survival through an adaptive process. Regulation of gene expression through HIF-1 (hypoxia-inducible factor-1) but also via other transcription factors plays an important role in this process. The aim of this work was to establish an in vitro model to study hypoxia-induced resistance to chemotherapy in breast cancer cells and to identify genes that may be responsible for the protective effect of hypoxia. MDA-MB-231 and SK-BR-3 cells were incubated in the presence of taxol or epirubicin: cell death, as measured by an increase in LDH release, was induced in a concentration-dependent manner by both drugs. Cell death was at least in part due to apoptosis since an increase in caspase 3 activity, in DNA fragmentation and in PARP cleavage was observed. Hypoxia per se had no effect on cell viability but it markedly protected MDA-MB-231 cells from taxol-induced cell death and apoptosis while it increased taxol-induced apoptosis in SK-BR-3 cells. We also observed that hypoxia had no effect on epirubicin toxicity in both cell types.
In order to identify the mechanism(s) responsible for hypoxia-induced protection, Affymetrix whole transcriptome studies were performed, in triplicates, for control cells, cells incubated in the presence of taxol and cells incubated in the presence of epirubicin, both under normoxia and hypoxia conditions. Unsupervised cluster analysis using centered correlation and average linkage showed that control cells are well separated from drug-treated cells. Moreover, for control cells and cells incubated in the presence of taxol, hypoxia was separated from normoxia while it was not the case for epirubicin-treated cells. These results are very well in accordance with the protection or the worsening of the drug-induced apoptosis brought by hypoxia for taxol but not for epirubicin. Amongst the genes which expression is modulated by hypoxia in these conditions, are several genes encoding chemokines or chemokine receptors. The role of these genes in regulating cell viability will thus be investigated. These results are a clear demonstration that hypoxia can have a direct protective effect on apoptotic cell death. Moreover, molecular profiling pointed to putative pathways responsible for tumour growth in challenging environmental conditions and cancer cell resistance to chemotherapeutic agents.

Acknowledgments : C. Michiels is a Researcher Director of FNRS. This work is part of the KeyMarker, a Biowin project financed by the Region Wallonne.

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DYNAMIC CONTRAST-ENHANCED MAGNETIC RESONANCE IMAGING (DCE-MRI) FOR THE DETECTION OF PROSTATE CANCER: A PILOT STUDY

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Background : Several randomized studies show that a higher radiation dose to the entire prostate improves the disease-free survival of patients with prostate cancer. However, further dose-escalation is limited by normal tissue toxicity. An alternative is dose-escalation to subvolumes of tumor cells within the prostate. Therefore, an accurate assessment of the intra-prostatic tumor distribution is necessary.
Aim : To determine the use of dynamic contrast-enhanced MRI (DCE-MRI) for the detection of prostate cancer.
Materials and Methods : Twenty patients with histology-proven prostate cancer were examined by dynamic contrast-enhanced T1-weighted MR imaging at 1.5T. Cancer regions and normal prostatic tissue were identified and delineated based on the histopathology of whole mount sections after radical prostatectomy. The time to peak (TTP), the maximal contrast enhancement (Cpeak), the speed of contrast uptake (WashIn) and the clearance of the contrast agent (WashOut) were measured in both the tumor and the normal prostatic tissue.
Results: Within individual patients, a consistently higher Cpeak (found in 17/20 patients) and WashIn (in 18/20 patients) was present in the tumor tissue compared to the normal prostatic tissue. The TTP and the WashOut were shorter in tumor tissue than in normal prostatic tissue in 90% (18/20) and 70% (14/20) of the patients, respectively. However, there seems to be an overlap between the parameter values of tumor and normal prostatic tissue, when all patients are considered together.
Conclusion : DCE-MRI seems a promising tool for the detection of prostate cancer. The use of these different parameters could enable a better discrimination between tumor tissue and normal prostatic tissue in individual patients. However, in order to compare patients, a normalization of perfusion parameters may be warranted.

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TH1-TYPE IMMUNOSTIMULATION OF MURINE SPLENOCYTES RESULTS IN PARACRINE SECRETION OF IFN-Γ AND HYPOXIC TUMOR CELL RADIOSENSITIZATION

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Purpose : In this study, we examined whether Th1-type immunostimulators such as OM-174 and IL-12/IL-18, may indirectly enhance tumor cell radiosensitivity through activation of immune cells and secretion of IFN-γThe mechanism of radiosensitization has been examined with regard to the production of nitric oxide (NO) through the inducible isoform of nitric oxide synthase (iNOS).
Methods : Splenocytes from Balb/c mice were exposed to OM-174 (activation through antigen-presenting cells) or IL-12/IL-18 (direct activation of T cells) for 24 h, and analyzed for the secretion of IFN-γ by ELISA. Next, EMT-6 mammary carcinoma cells were exposed to conditioned medium from activated splenocytes and their hypoxic cell radiosensitivity was estimated by clonogenic assay. The transcriptional activation of iNOS was examined by RT-PCR, the protein level by Western blotting and the enzymatic activity by Griess assay.
Results : Non-stimulated splenocytes secreted low levels of IFN-γ which did not affect iNOS expression nor the hypoxic radiosensitivity of EMT-6 tumor cells. Upon stimulation in 21% oxygen, the maximal rates of IFN-γ secretion induced by OM-174 and IL-12/18 were 70 and 110 ng/ml respectively. This caused significant activation of iNOS-mediated NO production in tumor cells and enhanced their hypoxic radioresponse by 2-times. The efficient activation of iNOS and radiosensitization of EMT-6 tumor cells could be mimicked by combining the immunostimulators with purified IFN-γ. Radiosensitization was abrogated by neutralizing anti-IFN-γ antibodies and by the metabolic iNOS inhibitor aminoguanidine. Hypoxia displayed a dual effect on the immune-tumor cell interaction, by downregulating the expression of the IFN-γ-driver gene while upregulating the iNOS gene.
Conclusions : This study demonstrates that IFN-γ-driven Th1-type immunostimulation may enhance the radioresponse of hypoxic tumor cells through paracrine secretion of IFN-γ

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RAPID MONITORING OF OXYGENATION BY ^{19}F MAGNETIC RESONANCE IMAGING : SIMULTANEOUS COMPARISON WITH FLUORESCENCE QUENCHING

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Introduction: Methods to determine tumor oxygenation are of crucial importance for the prediction of therapeutic outcome (1). Many current methods are either highly invasive, non quantitative, or lack spatial resolution. Mason et al. have been successfully developing FREDOM MRI (fluorocarbon relaxometry using echo planar imaging for dynamic oxygen mapping) following direct intratumoral injection of the oxygen reporter molecule hexafluorobenzene (HFB) (2). The aim of this study was to develop an MRI fluorocarbon oximetry technique using snapshot inversion recovery (SNAP-IR) and compare it with fluorescence quenching fiber-optic probe oximetry (OxyLite™) performed simultaneously in experimental mouse tumors.
Materials and Methods: HFB was injected directly into the tumors of TLT bearing mice, along with the insertion of the fluorescent quenching OxyLite™ probe. Tumor pO_2 was modified using carbogen or lethal doses of the anesthetic gas. MRI pO2 maps were generated in 1.5 minutes with an in-plane spatial resolution of 1.88 mm using a SNAP-IR pulse sequence (Bruker, 4.7T with a tunable 1H/19F surface coil). The pulse sequence consisted of a non-selective hyperbolic secant inversion pulse (10 ms length), followed by acquisition of a series of 512 rapid gradient echo images (TR =10.9 ms, TE=4.2 ms, flip angle = 1°, matrix = 32x16, FOV = 60x30 mm, BW = 12.5 kHz, single thick slice [projection]). Calibration of HFB was performed by measuring R_1' in sealed tubes containing HFB respectively bubbled with N_2 (0% O_2), air (21% O_2) and carbogen (95% O_2) for 20 min in a 37° water bath before measurement.
Results: In vitro data: The respective R1' measurements for HFB in 0, 21, and 95% O2 were 0.105 ± 0.006 s-1, 0.407 ± 0.048 s-1, and 1.56 ± 0.08 s-1 providing a calibration curve of R1' = 0.1048 (± 0.0060) + [0.002000 (± 0.000105)] pO_2 (R1' in s-1 and pO_2 in mmHg). The relation pO_2 = (R_1' – 0.1048)/0.002 was used voxel-by-voxel for the remainder of our experiments.
In vivo data: Both MRI and OxyLite™ showed consistent increases in tumor pO_2 during carbogen breathing. However, there was a lack of correlation in the magnitude of response between the two techniques, despite similar baseline and post-mortem values. Color maps show that each region of the tumor responds differently to the respiratory challenge. From the histograms, we observe a clear shift to the right of the median pO_2 value under carbogen breathing conditions.
Discussion: The SNAP-IR pulse sequence allowed us to sample tumor oxygenation with an effective in-plane spatial resolution (1.88 mm) similar to that of FREDOM (1.25 mm) and with an acquisition time of 1.5 min, which is shorter than that of FREDOM (6.5 min). As the present sequence is more rapid than FREDOM, it could be particularly suitable to monitor acute changes of pO2 in tumors. The quantitative discrepancy might be due to the difference in sampling volumes of the techniques as well as to a 'reservoir' effect of HFB.

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SERUM DICKKOPF-1 (DKK-1) IN BREAST CANCER PATIENTS WITH BONE METASTASES

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The skeleton is the most common site for metastasis of breast cancer. Metastatic cells may lead to the development of osteolytic, osteoblastic or mixed lesions, depending on tumor-derived factors. Skeletal lesions of malignancies are induced by a multifactorial process notably implicating the osteoinductive factors (BMPs, Wnts) modulated by antagonists (noggin and dickkopf-1). Our group recently demonstrated that dickkopf-1 (Dkk-1) is expressed and secreted by breast cancer cell lines which induced osteolytic metastases in nude mice, and that increased levels of serum Dkk-1 in cancer patients were associated with bone metastases (Voorzanger-Rousselot, Br J Cancer, 2007). In the current study, we examined the expression of Dkk-1 in 33 bone metastasis biopsy specimens and in 2 breast cancer cell lines by immunohistochemistry and RT-PCR, respectively. Moreover, we confronted serum Dkk-1 (measured by two-site ELISA, Voorzanger-Rousselot, Br J Cancer, 2007) to classical serum bone turnover markers (CTx, Nordic; TRAP, Medac; BAP, Hybritech). Serum marker levels were determined in 22 breast cancer patients in complete remission, 24 patients with bone metastases before bisphosphonate (BP) therapy, and 25 patients after at least 3 months of BP treatment.
We previously described that circulating Dkk-1 levels were significantly higher in patients with breast cancer and bone metastases than in patients in complete remission, suggesting that metastastic cancer cells contribute to the elevation of serum Dkk-1 (Voorzanger-Rousselot, Br J Cancer, 2007). This suggestion is consistent with our observations that Dkk-1 was highly expressed in bone metastasis biopsies of breast cancer patients (median score = 6). Along the same line, Dkk-1 mRNA was expressed in breast cancer cell lines (MFC-7, MDA-MB-231), and was upregulated in osteoblast-like cells (MG-63) cocultured with MCF-7 cells. Thus, tumor-associated osteoblasts might also contribute to the increase in serum Dkk-1 levels. In addition, circulating Dkk-1 levels in metastatic patients under BP treatment were not significantly different from those determined in untreated patients. As expected, serum levels of CTx and TRAP were significantly lower in the patients under BP therapy. Thus, our observations support the hypothesis that elevated serum Dkk-1 levels in cancer patients are at least partly derived from cancer cells and tumor-associated osteoblasts, both cell types that are weakly affected by BPs in vivo. This hypothesis is further supported by the lack of correlation between Dkk-1 and bone turnover markers in breast cancer patients with bone metastases before and under BP therapy, suggesting that Dkk-1 derived from cancer cells and tumor-associated osteoblasts may mask possible correlations between normal osteoblast-derived Dkk-1 and classical bone turnover markers. Interestingly, negative correlations were observed in patients in complete remission.
In conclusion, our current data suggest that serum Dkk-1 is partly derived from metastatic cells and tumor-associated osteoblasts. Moreover, we found that BP treatment does not decrease circulating Dkk-1 levels and that Dkk-1 concentrations do not correlate with bone turnover markers in patients suffering with bone metastases. Thus, these observations indicate that Dkk-1 is not a bone remodelling marker in advanced breast cancer patients with skeletal complications and has a dual origin.

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ASSOCIATION BETWEEN FARNESOID X RECEPTOR EXPRESSION AND CELL PROLIFERATION IN ESTROGEN RECEPTOR-POSITIVE, LUMINAL-LIKE BREAST CANCER FROM POSTMENOPAUSAL PATIENTS

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The farnesoid X receptor (FXR, NR1H4), a member of the nuclear receptor superfamily of ligand-dependent transcription factors, is normally produced in the liver and the gastrointestinal tract, where it acts as a bile acid sensor. Surprisingly, we have recently detected FXR in breast cancer cell lines and tissue specimens (Journe, Breast Cancer Res Treat, in press). In the present study, the expression of FXR in breast cancer was evaluated and correlated with clinicopathological characteristics. FXR expression was scored (0 to 8) by immunohistochemistry on 204 breast cancer samples and correlated with classical biomarkers. Moreover, the effect of the FXR activator chenodeoxycholic acid (CDCA) was determined on breast cancer cells to complement clinicopathological analyses.
FXR was detected in 82.4% of samples with a high median expression score of 5. Its expression was significantly higher in the ER-positive subgroup than in the ER-negative one. FXR expression significantly correlated with ER expression and luminal-like markers (cytokeratins 8/18 and mucin 1). Moreover, in ER-positive tumors, FXR expression was significantly correlated with the proliferative markers Ki-67, topoisomerase II alpha, cyclin D, c-myc, p27, but only so in postmenopausal women, suggesting that lack of estrogens may disclose the association between FXR and cell proliferation.
In vitro experiments confirmed clinicopathological data since CDCA stimulated cell proliferation in ER-positive cancer cells (MCF-7) cultured in steroid-free medium (SFM), while it exerted no effect in ER-negative MDA-MB-231 cell growth. In addition, CDCA increased the expression of the proliferative markers cyclin D and p27 in MCF-7 cells cultured in SFM. FXR silencing by siRNA significantly reduced the mitogenic effect of CDCA in MCF-7 cells, and antiestrogens completely suppressed the stimulation of cell growth, suggesting that both FXR and ER contribute to the mitogenicity of CDCA. In addition, treatment of MCF-7 cells with CDCA for 24 hours induced ER downregulation and stimulated ER-mediated gene transactivation. Finally, coimmunoprecipitation using FXR antibody disclosed physical interactions between both receptors upon exposure to CDCA.
Altogether, our in vitro data indicate a positive crosstalk between FXR and ER, accounting for FXR-mediated ER activation and mitogenic effects of FXR agonists. In breast tumors, we show that FXR is expressed at a higher level in ER-positive, luminal-like malignancies, where it correlates with tumor cell proliferation in post-menopausal women. Thus, FXR could be a new marker of poor prognosis in ER-positive breast cancers.

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EXTRACELLULAR CALCIUM INCREASES THE GROWTH INHIBITORY EFFECTS OF IBANDRONATE IN BREAST CANCER CELLS

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Bisphosphonates are now standard therapy for the treatment of bone metastases. They inhibit the activity of osteoclasts and induce their apoptosis. In addition to their effects of bone cells, in vitro and animal studies show that bisphosphonates also directly inhibit the proliferation and induce apoptosis of breast cancer cells. In the current study, we examined the effects of an increase in extracellular Ca^{++} level on the antitumor activity of ibandronate in MDA-MB-231 and MCF-7 breast cancer cell lines.

In presence of 0.6 mM Ca^{++}, 30 µM ibandronate had no effect on MDA-MB-231 cells growth (crystal violet staining determination), and only a borderline effect on MCF-7 cells growth (13.6±6.6% inhibition) (mean±SD). By contrast, in presence of 2 mM Ca^{++}, ibandronate markedly inhibited cancer cells growth by 55.5±7.8% (MDA-MB-231 cells) and 76.1±4.6% (MCF-7 cells) ($p<0.01$). Dose-response curves showed that increasing Ca^{++} concentrations from 0.6 to 1.6 mM decreased ibandronate IC50 values from 100 to 30 µM in MDA-MB-231 cells and from 60 to 10 µM in MCF-7 cells. In addition, whereas 30 µM ibandronate barely induced cell apoptosis in presence of 0.6 mM Ca^{++}, it significantly increased the percentage of apoptotic cells (annexin V-PE-positive) in presence of 1.6 mM Ca^{++} (11.4 and 32.9% in MDA-MB-231 and MCF-7 cells, respectively). The enhancement of ibandronate activity was specifically noted with Ca^{++}, inasmuch as it was not observed with the other divalent cation Mg^{++} in the same range of concentrations. Of note, Ca^{++} chelation by EGTA at a concentration which did not affect cell growth (0.5 mM) significantly reduced the growth inhibitory effect of 30 µM ibandronate in culture medium containing 1.6 mM Ca^{++}. To address the effects of Ca^{++} on cell drug accumulation, cells were cultured in medium containing 0.6 or 1.6 mM Ca^{++} and were exposed to [^{14}C]-ibandronate for 4 hours. In presence of 1.6 mM Ca^{++}, cells accumulated much more [^{14}C]-ibandronate than cells cultured in presence of 0.6 mM Ca^{++} (4.6 and 11.4-fold increases in MDA-MB-231 and MCF-7 cells, respectively). Finally, when evaluating the inhibition of protein prenylation, we found that a high Ca^{++} level increased the intracellular activity of ibandronate. Thus, in both cell lines, 10 µM ibandronate was sufficient to produce a detectable inhibition of Rap1A prenylation (Western blotting, antibody from Santa Cruz Biotechnology, CA, USA) in presence of 2 mM Ca^{++}, while 100 µM ibandronate was required to achieve a similar effect in presence of 0.6 mM Ca^{++}. In conclusion, our data indicate that extracellular Ca^{++}, at physiologically relevant concentrations, increases the cytotoxicity of bisphosphonates by facilitating its accumulation by breast cancer cells. Therefore, Ca^{++} released during the process of bone resorption could enhance the antitumor effects of bisphosphonates.

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CHARACTERIZATION OF BONE MARROW-DERIVED CELLS RECRUITED IN DIFFERENT TUMOR TYPES IN MICE

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During tumor evolution, the invasion of cancer cells into surrounding tissue is associated with considerable tissue remodeling called "cancer induced stromal reaction" or "desmoplastic reaction". The main constituents of cancer stroma are inflammatory cells, small blood and lymphatic vessels, fibroblastic and myofibroblastic cells, and extracellular matrix components. Although roles of inflammatory cells and endothelial cells have been reported to be involved in tumor immunity and neoangiogenesis, those of fibroblasts which participate in cancer progression have not been fully elucidated yet.

Fibroblasts, which are widely distributed and play a key role in tissue fibrosis, represent a main source of interstitial collagens. This cell type is heterogeneous with respect to a number of phenotypic and functional features. This heterogeneity may arise not only from the cellular activation and differentiation processes but also from their different cellular origins. Fibroblasts and myofibroblasts, i.e. activated fibroblasts expressing α-smooth muscle actin, which produce collagen and extracellular matrix proteins constitute the "desmoplastic reaction" and have been suggested to represent an important player in tumor invasion.

In the present study, we investigated in vivo the putative contribution of bone marrow-derived cells into two different cancer types: malignant murine keratinocytes (PDVA and BDVII). Mice were engrafted with bone marrow isolated from transgenic mice expressing green fluorescent protein (GFP), and the different types of cancer cells were subcutaneous injected. Bone marrow-derived cells positive for GFP were detected in each type of tumor xenografts. This was confirmed by immunohistochemistry stained against GFP. Interestingly, in the two types of malignant murine keratinocytes (PDVA and BDVII), bone-marrow derived cells were mostly localized in connective tissue bundles. These cells were fusiforms with a fibroblast-like morphology and expressed CD45, Thy1.1, and α-SMA. Moreover, these cells were specifically associated with collagen deposition.

Our results suggested that bone marrow-derived cells (GFP^+) are efficiently recruited into tumor, expressed several fibroblastic markers in vivo, and actively take part in the desmoplastic reaction.

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POTENT ROLE OF PAI-1 AS REGULATOR OF PRO- AND ANTI-TUMORAL FACTORS.

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Proteases and their inhibitors are key regulators during angiogenesis and tumor invasion. PAI-1, an inhibitor of plasminogen activators, has been demonstrated as pro-angiogenic and pro-tumoral factor in different murin models of cancer. Using the tumor transplantation model, we have previously demonstrated that the PAI-1 production by host cells is very important and essential for tumor invasion. Indeed, the production of PAI-1 by carcinoma cells doesn't compensate the lack in host tissue in PAI-1 deficient mice. This model allows observing host and cancer cell behaviour through the collagen interface. The comparison of gene expression in PAI-$1^{-/-}$ versus wild-type mice is a great interest to explain the deficient migration of endothelial cells and myofibroblastes in PAI-$1^{-/-}$ tumor transplants.

A gene array (Superarray Inc., Frederick, MD) has been used to compare 96 pathway genes in mouse angiogenesis, expressed in tumor transplants. Relative mRNA expression levels of genes were compared one week after the tumoral transplantation in PAI-1-/- and WT mice. At this time point, collagen gel was only invaded by host cells, while tumor cell invasion did not yet occur. Upon 96 genes spotted, we first selected those which were upregulated (minimum two fold) in PAI-1-/- mice and their modulation was verified by semi-quantitative RT-PCR. We evidenced an upregulation of Maspin/SerpinB5 ($p<0,0001$), CXCL-1/Growth Related Oncogene-α (GRO-α; $p<0,001$), CXCL-4/Platelet Factor 4 (PF4; $p<0,05$) and Collagen-18α1 ($p<0,05$) in tumor transplants engrafted in PAI-1-/- mice. Maspin being a serine protease inhibitor like PAI-1, its up-regulation in PAI-1 deficient mice may compensate for the lack of this inhibitor. Maspin and CXCL-4 have been both identified as anti-angiogenic factor in different models of angiogenesis. Collagen 18α1 is a precursor of endostatin, an anti-angiogenic and anti-tumoral protein. Altogether these factors, known as anti-angiogenic factor, could therefore explain the reduction of neo-vascularisation observed in PAI-1-/- mice.

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IDENTIFICATION OF DISCRETE TUMOR-INDUCED MYELOID-DERIVED SUPPRESSOR CELL SUBPOPULATIONS WITH DISTINCT T-CELL SUPPRESSIVE ACTIVITY

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The induction of $CD11b^+Gr\text{-}1^+$ myeloid-derived suppressor cells (MDSCs) is an important immune-evading mechanism employed by tumors. However, the exact nature and function of MDSCs remain elusive, especially because they constitute a heterogeneous population which has not yet been clearly defined. Here, we identified two distinct MDSC subfractions with clear morphologic, molecular and functional differences. These fractions consisted of either mononuclear cells (MO-MDSCs), resembling inflammatory monocytes, or low-density polymorphonuclear cells (PMN-MDSCs), akin to immature neutrophils. Interestingly, both MO-MDSCs and PMN-MDSCs suppressed antigen-specific T-cell responses, albeit by employing distinct effector molecules and signaling pathways. Blocking IFN-γ or disrupting STAT1 partially impaired suppression by MO-MDSCs, for which nitric oxide (NO) was one of the mediators. In contrast, while IFN-γ was strictly required for the suppressor function of PMN-MDSCs, this did not rely on STAT1 signaling nor NO production. Finally, MO-MDSCs were shown to be potential precursors of highly anti-proliferative NO-producing mature macrophages. However, distinct tumors, irrespective of host genetic background, differentially regulated this inherent MO-MDSC differentiation program, indicating that this was a tumor-driven phenomenon. Overall, our data refine the tumor-induced MDSC concept by uncovering mechanistically distinct MDSC subfractions, potentially relevant for MDSC-targeted therapies.

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IMPLICATION OF VEGF RECEPTOR SOLUBLE FORMS, SVEGFR1 AND SVEGFR2, IN ANGIOGENESIS

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Angiogenesis is essential for successful placentation and for tumour growth. Among the various angiogenic factors, we are particularly interested in VEGF family and their receptors. They are key players for normal endothelial function by controlling vascular tonicity, for reproductive and tumour initiation of angiogenesis as well. Indeed, trophoblasts and cancer cells instigate neovascular progression by inducing VEGF production. VEGF is implicated in endothelial cell proliferation, migration and survival, it also increases vascular permeability. The two main VEGF receptors implicated in angiogenesis are VEGFR1 and VEGFR2. A variant transcript of VEGFR1 lead to a soluble form: sVEGFR1. It is detected in plasma of pre-eclamptic women, during ischemia and in some cancer cases. A soluble form of VEGFR2, named sVEGFR2, was recently detected in plasma of healthy people, in leukaemia and in systemic erythematosus lupus cases. However, this form remains uncharacterized and its physiological or pathological role is still unknown.
The aim of this study was to understand and to characterize the role of sVEGFR2 in angiogenesis and in endothelial function. For this, the effect of sVEGFR2 was tested and compared to sVEGFR1 one, in aortic ring assay, in a 3D model of tube-like structure formation assay, in endothelial cell proliferation, apoptosis and migration assay as well. The impact of these receptor soluble forms was tested on VEGF-induced signalling pathways implicated in proliferation and survival. The ability of sVEGFR1 and sVEGFR2 to bind endothelial cells was evaluated as well.
In aortic ring assay, an ex vivo model of angiogenesis, we observed that sVEGFR1 and sVEGFR2 were able to abolish VEGF-induced angiogenesis. In tube-like structure formation assay, similar results were obtained with sVEGFR1 and sVEGFR2: inhibition of VEGF-induced tube-like structure formation. VEGF-induced HUVEC proliferation and migration were also abolished by sVEGFR1 and sVEGFR2 and VEGF-induced phosphorylation of ERK1/2 and Akt was decreased by sVEGFR1 and by sVEGFR2 as well. Additionally, we demonstrated that sVEGFR1 and sVEGFR2 were able to bind endothelial cells in presence of VEGF.
Our results evidence that sVEGFR1 and sVEGFR2 inhibit VEGF-induced angiogenesis in a similar way. In a mechanistic point of view, we demonstrate that sVEGFR1 and sVEGFR2 prevent VEGF-induced angiogenesis by blocking endothelial cell proliferation and migration and signalling pathway leading to these processes as well. Our results suggest that these soluble receptors act, not only as a circulating VEGF trap, but also by direct cellular interactions. These results contribute to identify factors by which it could be possible to regulate the balance between pro-angiogenic and anti-angiogenic factors.

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IDENTIFICATION OF NEW PROTEINS INVOLVED IN CELL ADAPTATION TO HYPOXIA IN A HIF-1 INDEPENDENT WAY

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Our work takes place in the global effort that aims to have a better knowledge of the mechanisms by which tumor cells adapt to low oxygen tension during cancer development. It has been shown that a hypoxic area rapidly develops in the center of a tumor because the preexisting blood vessels are pushed away to the periphery of the growing tumor mass. Hypoxic conditions are responsible for biochemical modifications within tumor cells that allow them to adapt not only to hypoxia, but also make them more resistant to chimio or radiotherapy treatments. The master regulator of this adaptative response is HIF-1 (Hypoxia Inducible Factor-1) However, if HIF-1 is important for adaptation to hypoxia, it does not explain all the complexity of this response and the other factors involved still wait to be identified.
Our aim is to discover new hypoxia mediators that are HIF-1 independent in order to point up some new targets for cancer therapy. For this purpose, we utilized a well-established hypoxia model using HepG2 cells and identified some putative candidates through 2D-DIGE analyses in tandem with mass spectrometry.
HepG2 cells were incubated under normoxia (N), true hypoxia (H) or chemical hypoxia (C) i.e. incubation in the presence of Cobalt Chloride. $CoCl_2$ inhibits HIF-prolylhydroxylases and thus prevents HIF-1α degradation under normoxia. We compared 2D-DIGE gel profiles from N vs H and N vs C experiments. Results from N vs H pointed up the hypoxia-responsive proteins that are either HIF-1 dependent or independent while N vs C revealed the proteins that are only under the control of HIF-1. In that way, by excluding proteins common to both experiments, we were able to identify some hypoxia-responsive HIF-1 independent proteins from the N vs H experiment. More than 60 proteins have been identified by mass spectrometry and clustered as follows: cytoskeleton proteins (cortactin, dynein, coronin, CAPZA2, tubulin,...), translation regulating proteins (elongation factor 2, HNRPK, HNRPC, HNRPAB, aconitase,...), stress proteins (HSP90, trap-1, PDI, PAK2,...), kinases (BMK1, MAPKK2, UMP-CMP kinase,...) and other proteins. As a starting point, we focused on the cytoskeleton proteins as they may play a role in the cancer cell migration that could be enhanced under hypoxia. We have confirmed the up-regulation under hypoxia of dynein by Western blot, but no correlation was observed with the mRNA level measured by real-time P.C.R. Further investigations will be performed for the other proteins.
Our results identified several new HIF-1-independent proteins that are up/down regulated or post-translationally modified in response to hypoxia. These proteins may represent new attractive targets for cancer therapy.

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PROTEOMIC ANALYSIS DISCLOSES CERULOPLASMIN AS A NEW CANDIDATE BIOCHEMICAL MARKER OF BREAST CANCER BONE METASTASES

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Breast cancer is a heterogeneous disease with high range of metastatic potential The skeleton is the most common site of breast cancer colonization. Several markers have been studied to detect and monitor bone metastasis in breast cancer patients. However, they appear to provide unequivocal predictive information and much work has to be done to find more specific biomarkers of bone metastasis. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) has emerged as a powerful tool for new biochemical markers discovery. This technique allows the resolution of a complex protein mixture. Biological fluids are often preferred for developing such markers since their use allows a non invasive and more comfortable analyses for the patient, in comparison with the use of material from biopsies. Serum is a common source of proteins for 2D-PAGE because of high protein concentration. The major drawback of serum samples is the high proportion of albumin and immunoglobulins. Thus, albumin and immunoglobulins shoulde be removed from the serum sample in order to detect minor proteins.
We compared 2D gels of native and depleted serum from healthy premenopausal women. After the depletion process, 92 spots were at least two fold up-leveled. Moreover, comparing 2D gels from healthy postmenopausal and premenopausal woman depleted serum, we found that 109 spots were at least two fold up- or down-leveled in the postmenopausal group. The highest up-leveled proteins in postmenopausal woman serum were identified as markers of postmenopausal status and were undetectable without Alb/IgG depletion, confirming the efficiency of the depletion process.
Then, we applied 2D-PAGE to depleted serum and compared 2D gels from premenopausal women, women with breast cancer and women with bone metastasis from breast cancer in order to find a protein profile associated with cancer-related bone diseases. Moreover, a recent study reveals that ceruloplasmin mRNA is upregulated in PC-3 prostate cancer cells exposed to TGFβ, an abundant growth factor in bone. We are currently examining the effect of TGFβ on several breast cancer cell lines to bring mechanical basis of the increase of ceruloplasmin in serum of breast cancer bone metastasis patients.
In conclusion, we found that ceruloplasmin levels were not significantly different between premenopausal women and women with breast cancer but were increased at least two fold in women with bone metastases from breast cancer as confirmed by quantitative Western blot analyses. These preliminary data suggest that ceruloplasmin could be a new candidate biochemical marker to detect and monitor bone metastases.

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INTERACTION BETWEEN VIRUS LIKE PARTICLES (VLP) AND NATURAL KILLER (NK) CELLS.

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Human papillomavirus (HPV) is the etiological agent of uterine cervical cancer. This virus cannot be easily produced in vitro, but L1, the major virus capsid protein, can self-assemble into virus-like particles (VLP), which have the same morphology than the viral capsid. HPV-VLP are able to enter in keratinocytes, which are the targets of HPV infection, but also in immune cells such as dendritic cells (DC), which are activated in the presence of HPV-VLP. Depending on the HPV type, the virus used different endocytosis pathways to enter cells, clathrin-mediated endocytosis for HPV16 and caveola-mediated endocytosis in HPV31 entry. Since we observed an increased proportion of NK cells in cultures derived from HPV-associated cervical lesions biopsies, we tried to determine if HPV16-VLP could interact with NK cells.
Using fluorescent HPV16-VLP, we have shown that VLP penetrate into the NK cell line, NK92MI. Primary NK cells isolated from blood of healthy donors also internalised VLP, but the kinetics seemed to be different than those for NK92MI or keratinocyte cell line, Caski, used as positive control. We also carried out cultures of primary NK cells in the absence or presence of VLP and we observed that a concentration of 1 to 5 µg of VLP induced NK cell proliferation. Cytotoxic activity of the NK cells against K562 did not seem to be affected by the presence of VLP. Since the interaction between NK cells and DC is important for the induction of adaptive immune response, we study the effect of DC cultivated in presence of VLP on the activation of NK cells. Preliminary data showed an increase of CD69 cell surface expression and IFNγ production by NK cells in the presence of DC activated by VLP.
The results suggest that NK cells recognize HPV16-VLP. We will confirm these data by studying the mechanism of internalisation with inhibitors known to be involved in VLP entry and using HPV31-VLP, which used another endocytosis pathway. We will also more carefully analyse the interaction between DC and NK cells in presence of both type of HPV-VLP.

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STUDY OF THE MECHANISMS INVOLVED IN THE MODULATION OF CANCER CELL APOPTOSIS UNDER HYPOXIA

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Cancer cells located in the tumour core are often deprived of oxygen. Moderate tumour hypoxia induces cell resistance to radio- and chemo-therapies. Although this resistance has been observed for a long time, the mechanisms underlying it are not yet well understood.
The aim of this work was to highlight the cellular responses to hypoxia leading or not to cancer cell resistance according to the cell line, the chemotherapeutic agent used and the presence or not of a wild-type p53 transcription factor. For this purpose, we studied the behaviour of 8 cell lines originating from various organs and harbouring or not a wild-type p53 factor when submitted to 5 chemotherapeutic agents targeting different cellular pathways. The level of apoptosis under normoxia or hypoxia in the presence or not of the chemotherapeutic agents was monitored by the measure of caspase 3 activity and the detection of PARP cleavage. The actual cell death was assessed by measuring the LDH release. Although the effect of the different agents was more or less the same on a particular cell line, the responses to these agents varied according to the cell line: hypoxia protected some cell lines while it had no effect or aggravated the mortality of others.
In order to delineate pathways involved in these various cell responses to hypoxia, p53 protein level was studied. Indeed, the absence of a functional p53 transcription factor often leads to cell resistance. The abundance of the p53 protein as well as of post-translationally modified forms of this factor was thus investigated. The results obtained showed variations according to the cell line and the pro-apoptotic agent. For example, in HepG2 cells, p53 protein level was increased when cells were incubated in the presence of all the chemotherapeutic agents but Ser-15 was phosphorylated only when etoposide was used. In these cells, p53 abundance was decreased under hypoxia compared to normoxia, correlating with the apoptotic profile. p53 could therefore be a factor mediating the effect of hypoxia on apoptosis but other pathways are certainly also involved. These results show that hypoxia has different effects on the apoptosis induced by chemotherapeutic agents according to the cell line. Although in the majority of the cases, hypoxia protected cells against apoptosis as observed in vivo, hypoxia had no effect or even aggravated apoptosis induced in other cell types. The mechanisms responsible for the differential effects observed in this work are under investigation but could involve p53.

Acknowledgements : A. Sermeus and J.-P. Cosse are recipients of a FRIA fellowship (Fonds pour la Recherche dans l'Industrie et l'Agriculture, Belgium) and Carine Michiels is research director of FNRS (Fonds National de la Recherche Scientifique, Belgium).

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ANALYSIS OF MOLECULAR CELLULAR SIGNALLING PATHWAYS LEADING TO APOPTOSIS IN ER STRESSED HUMAN LYMPHOMA AND MYELOMA CELLS.

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Human myeloma and lymphoma are cancers of the lymphoid tissue. Treatment of these types of cancer is still difficult or even impossible depending on the type and stage of the disease and tumor localization.. Therefore, a search for new therapies is of interest. Since these tumour cells often contain a highly developed endoplasmic reticulum (ER), we decided to explore the cellular mechanisms involved in ER-stress-induced cell death, initially using different human myeloma and lymphoma cell lines. The specific primary response to ER-stress is a survival mechanism, known as the unfolded protein response (UPR) allowing the cell to overcome ER malfunction. UPR occurs via three mechanisms: reduced translation of misfolded proteins via PERK, enhanced production of ER chaperones and ER-associated degradation (ERAD). However, if and when the stress is continuous or without relief, the damaged cell undergo apoptosis. Using several different ER-stress inducing inhibitors we have studied typical pro-apoptotic events in several human myeloma and lymphoma cell lines. Our results suggest that caspase-2, -3, -8 and -9 are processed and may play a role in ER-stress induced apoptosis. Using BCL-2 overexpressing or caspase-8 or FADD deficient Jurkat lymphoma T cells, we demonstrated that the mitochondrion plays an important role in the process while caspase-8 and FADD are probably redundant. We are currently exploring in a similar way the role played by RIP1 and the eIF2-alpha kinases, PERK and PKR, in ER-stress induced apoptosis.

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INHIBITION OF TUMOR-ASSOCIATED LIPOGENESIS CHANGES MEMBRANE LIPID COMPOSITION AND AFFECTS MEMBRANE STRUCTURE AND FUNCTION

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One of the most common molecular changes in cancer is the overexpression of lipogenic enzymes, such as fatty acid synthase (FASN) and acetyl-CoA carboxylase (ACC). Here, to gain more insight into the role of ACC overexpression and activation in cancer cells, we have treated LNCaP prostate cancer cells with soraphen A, a potent inhibitor of ACC. Treatment with soraphen A caused an almost complete inhibition of fatty acid synthesis. Besides a drastic decrease in the cellular content of triglycerides, soraphen A markedly reduced the cellular phospholipid content particularly in detergent-resistant membrane microdomains (DRMs) and induced a shift of cholesterol from detergent-resistant to detergent-soluble fractions. Importantly, MS-analysis showed that soraphen A caused a decrease in saturated and an increase in poly-unsaturated fatty acyl chains. In addition to changes in the lipid content and composition of membranes and DRMs, soraphen A also caused a shift of several typical DRM-associated proteins to detergent-soluble fractions. Among these are the DRM-associated scaffolding proteins flotillin-1 and flotillin-2, Lyn tyrosine kinase and beta-catenin. Taken together, these findings reveal that in cancer cells lipogenesis affects the lipid composition of membranes and plays a key role in the formation and composition of membrane microdomains, which are essential for the proper functioning of cellular membranes and tumor cell behavior.

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EFFECTS OF INTERMITTENT HYPOXIA ON HIF-1, NRF2 AND NFKB ACTIVITY

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It was recently demonstrated that tumor blood flow is irregular leading to periods of intermittent hypoxia. Intermittent hypoxia affects endothelial cell functions and protects them against radiotherapy. However, the mechanisms responsible for this protection are still not well known. In order to better understand the anti-apoptotic effect of intermittent hypoxia on endothelial cells, we studied the activity of HIF-1, Nrf2 and NFkB, three transcription factors known to be activated by hypoxia and by reactive oxygen species (ROS) generated during reoxygenation.
Intermittent hypoxia is the repetition of hypoxia and reoxygenation phases. Hypoxia promotes the stabilization and the activation of HIF-1 which is responsible for cell adaptation to this condition by increasing the transcription of genes involved in glycolysis, angiogenesis, cell cycle and oxygen transport. During reoxygenation, low or moderate oxydative stresses can be generated by the production of ROS. Low oxidative stress is sufficient to induce Nrf2 transcription factor which increases the transcription of genes involved in detoxification and anti-oxidant defence processes. A higher level of ROS can promote the activation of NFkB which regulates inflammatory processes, cell proliferation and cell survival.
EAhy926 endothelial cells were exposed to four cycles of one hour hypoxia followed by 30 minute reoxygenation. Cell viability was evaluated under intermittent hypoxia (IH) and chronic hypoxia (CH) by Ethidium Bromide/Orange Acridine staining. A decrease in cell mortality was observed under IH and CH with respect to normoxia. In parallel, the production of ROS was measured in the course of hypoxia-reoxygenetion cycles using H2DCF-DA. A small increase in ROS production was evidenced during the first reoxygenation period.
The activity of HIF-1, Nrf2, and NFkB was then studied. First of all, we observed a stabilization of HIF-1α subunit after each hypoxia step and its degradation during each reoxygenation phase. Moreover, HIF-1α translocation into the nucleus was also observed under hypoxia by immunofluorescence whereas no translocation was evidenced for Nrf2 and NFkB p65 subunit neither under hypoxia nor during reoxygenation phases. These results were confirmed by western blot using cytoplasmic and nuclear extracts.
The transcriptional activity of these three transcription factors was also assessed. An increase in HIF-1 target gene transcripts (Aldolase and Glut-1) was observed under IH and CH measured by real-time RT-PCR. On the other hand, a decrease in Nrf2 (NQO1, HO-1 and GCS) and NFkB (IL8) target gene transcripts was observed with respect to normoxia. Results obtained using reporter systems for HIF-1, Nrf2 and NFkB confirmed these observations.
In conclusion, HIF-1α stabilization and HIF-1 activation were evidenced under intermittent hypoxia like under chronic hypoxia. However, no activation of Nrf2 and NFkB was observed under intermittent hypoxia despite the production of ROS measured in these conditions. These results suggest that the protection against apoptosis observed under intermittent hypoxia does not require Nrf2 and NFkB, but could be linked to HIF-1 activation.

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ANTI-VEGF TREATMENTS IN EPITHELIAL OVARIAN CANCER : IS IT ONLY ANTI-ANGIOGENESIS ?

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Background : The Akt/mTOR/p70S6K1 (Amp) pathway is activated in 55-68% of epithelial ovarian cancers. pS6 and p4EBP1 are downstream targets and presumed to be representative for the activation of Amp. pS6 has been shown to be a reliable biomarker for mTOR inhibition while p4EBP1 has been shown to be associated with tumor grade and reduced survival in ovarian cancer. VEGF-A and angiogenesis are important targets because bevacizumab or VEGF-TRAP as a single agent induces encouraging responses in refractory patients. pVEGFR2 has been shown to be a biomarker for bevacizumab treatment in inflammatory breast cancer patients. Our primary objective was to evaluate the correlation between the Amp pathway and VEGF mediated mechanisms.
Material and methods : Epithelial ovarian cancer FFPE material from 1999-2004 was collected in a tissue micro array. Immunohistochemistry was performed using pS6, p4EBP1 and pVEGFR2. An H-score was used to quantify the staining.
Results : Patients (n=89) were FIGO stage I in 21%, stage II in 4%, stage III in 62% and 11% stage IV. 65 patients had available tissue material of both primary tumor as well as other (multiple) abdominal metastatic lesions. Considering both primary as well as metastatic lesions together, pVEGFR2 was weakly correlated with p4EBP1 (R=0.12; p=0.008). More profound correlation was found between pS6 and pVEGFR2. (R=0.33; p<0.0001). The correlation of pS6 and pVEGFR2 was present in tissue of primary tumors (R=0.28; p=0.007) but was more pronounced in tissue of metastatic lesions. (r=0.39; p<0.0001). A very plausible explanation for the found correlation is that VEGF-A is able to activate the VEGFR2 that on its turn is capable to (co-)activate the Amp signaling pathway in tumor cells similar to endothelial cells. In the group of patients who received uniform first line combination taxane-platinum based chemotherapy, a co-expression of pS6 and pVEGFR2 on tumor cells was associated with reduced overall survival. This means that a presumed activated VEGFR2/AKT/mTOR/p70S6K1 signaling pathway is potentially a biological important mechanism in ovarian cancer tumor cells and a possible target for anti-VEGF treatments besides angiogenesis.
Conclusions : Although bevacizumab seems to be very active, it is at present associated with severe treatment induced toxicities. Inhibiting the Amp pathway with mTOR inhibitors could potentially also influence VEGF-A mediated mechanisms in ovarian cancer with the potential of more manageable side effects. This study provides evidence that there is a relationship between the Amp pathway and pVEGFR2 in ovarian cancer. Since the correlation of activated VEGFR2 and pS6 was found on tumor cells, this suggests that VEGF-A might be a key-stimulating growth factor to the tumor cells itself by influencing downstream cell signaling proteins. The anti-tumoral activity of anti-VEGF treatments could be explained by reducing an important tumor cell growth factor besides anti-angiogenesis. Further research is necessary and ongoing.

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NEW INSIGHTS IN THE BIOLOGY OF THE FIBROTIC FOCUS: HISTOPATHOLOGY MEETS MOLECULAR BIOLOGY IN BREAST CANCER SURGICAL PATHOLOGY.

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Introduction: A fibrotic focus (FF) is a scar-like area in the centre of an invasive breast tumour and can be regarded as a focus of exaggerated reactive stroma formation. FF is a practical, easily assessable and reproducible integrative histological prognostic parameter in breast cancer. Furthermore, its presence is associated with an expansive growth pattern (GP), the presence of hypoxia, angiogenesis and lymphangiogenesis. Little is known about the molecular pathways involved in the biology of the FF.
Materials and methods: 65 patients were selected of whom micro-array data of the tumour and HE slides for histological analysis were available. The GP (expansive, infiltrative or mixed) and the presence and size of a FF (no FF, <1/3th tumour area, >1/3th tumour area) were assessed. Differences in biological pathways were identified with global testing. The correlations of GP and FF with common breast cancer signatures and with clinico-pathological variables and survival were investigated.
Results: Tumours with a large FF showed activation of Ras signalling and of the Hif-1α pathway. Furthermore, unsupervised hierarchical cluster analysis with hypoxia and (lymph)angiogenesis-related genes showed that Hif-1α, VEGFA and CA9 were overexpressed. The presence of a FF, especially a large FF, was associated with the basal-like subtype (p=0.009), an activated wound healing signature (p=0.06) and a poor prognosis 76-gene signature (p=0.004). The presence of a FF (p=0.02) and especially of a large FF (p=0.004) was also associated with early development of distant metastasis.
Conclusion: Our results sustain the hypothesis that hypoxia-driven angiogenesis is essential in the biology of a FF. Ras and Akt might play a role as downstream modulators of Hif-1α and VEGFA signaling. Our data furthermore suggest that VEGFA does not only drive angiogenesis, but also lymphangiogenesis in tumours with a FF. Our data also show an association between the presence of a FF and unfavourable molecular signatures and encourages further exploration of an integrated use in breast cancer surgical pathology.

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ABERRANT METHYLATION OF THE ADENOMATOUS POLYPOSIS COLI (APC) GENE PROMOTER IS ASSOCIATED WITH THE INFLAMMATORY BREAST CANCER PHENOTYPE

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Disruption of the adenomatous polyposis coli (APC)/β-catenin pathway may be involved in breast carcinogenesis and tumour progression. Aberrant methylation of CpG-rich sites in the promoter region is recognised as a more common mechanism of inactivation of this tumour suppressor gene in primary breast cancer than somatic gene mutation. To what extent epigenetic alterations of APC differ according to specific breast cancer phenotypes remains to be elucidated. Our aim was to explore the role of the APC/β-catenin pathway in the inflammatory breast cancer (IBC) phenotype, which is the most aggressive manifestation of primary epithelial breast cancer and is considered a separate entity, both from a clinical and biological perspective. Promoter hypermethylation changes in the APC gene were investigated in DNA from formalin fixed and paraffin embedded normal breast tissue (N=28), inflammatory breast tumours (N=21) and non-inflammatory breast tumours (N=30) using laser-assisted microdissection and the nested methylation-specific PCR (MSP) technology. APC gene promoter methylation was also analysed in frozen biopsies using the quantitative MethyLight technique. APC and phospho-β-catenin protein levels were analysed by immunohistochemistry. Using nested MSP, we demonstrate that the APC gene promoter is hypermethylated in 71% of IBC samples and 43% of non-IBC samples (P=0.047). This could be confirmed in another data set by using the MethyLight technique: 75% of IBC samples displayed APC gene promoter hypermethylation versus 44% of non-IBC samples. Higher frequencies of APC gene promoter hypermethylation were found in poorly differentiated and advanced-stage breast tumours. We observed no significant association between APC gene promoter hypermethylation and APC protein levels. The cytoplasmic/nuclear ratio of phospho-β-catenin was significantly higher in IBC than in non-IBC (P=0.014). In conclusion, the subcellular distribution of phospho-β-catenin differs according to specific breast cancer phenotypes. We are the first to report on the association of aberrant methylation of the APC gene promoter with the inflammatory breast cancer phenotype, which could be of clinical and biological importance. A study comparing APC gene promoter hypermethylation in serum samples from patients with IBC and non-IBC is ongoing.

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SET UP ACCURACY OF PATIENTS WITH HEAD AND NECK TUMORS USING THE MEGAVOLTAGE COMPUTED TOMOGRAPHY IMAGING CAPACITY OF THE HELICAL TOMOTHERAPY SYSTEM

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Purpose : Tomotherapy is a new modality of radiation treatment equipped with an on-board imaging device (MVCT) which allows for daily patient set-up verification and correction in the medial-lateral (m-l), cranial-caudal (c-c), anterior-posterior (a-p) and transversal angular (roll) direction. In this study, we measured the positioning accuracy and its effect on dose distribution in order to evaluate different MVCT protocols for patients with head and neck tumors.
Materials/Methods : The daily set-up errors of 75 patients immobilized with head and shoulder masks were detected by matching the MVCT with the treatment plan CT images. This step was accomplished automatically by the system's software (automatic deviations) and manually by the nurses (accepted deviations). Total displacement, systematic and random errors were then analyzed on a patient and population basis. Moreover, the following 3 MVCT protocols were evaluated :
- Daily protocol: *MVCTs are realized on a daily basis*
- 1st five days protocol: *MVCTs are realized during the first five fractions* - the systematic deviations are calculated and automatically corrected for during the rest of the treatment
- Alternate week protocol: *MVCTs are realized on alternate weeks* – during the "no-MVCT" days the systematic deviations calculated during the prior MVCT week are automatically corrected for.

Results : The systematic accepted deviations reached 0.4mm (SD=1.7mm), 1.1mm (SD=1.5mm), -1.2mm (SD=1.4mm) and 0.5° (SD= 0.6°) in the m-l, c-c, a-p and roll direction respectively. The corresponding random deviations were equal to 1.4mm, 1.5mm, 1.5mm and 0.6°.
A t-test detected a difference between the accepted and automatic deviations in m-l and a-p directions. Contrary to the first five days protocol, for the MVCTs realized on alternate weeks were sufficient to predict the systematic deviations that exist in all 3 directions during the "no-MVCT" weeks. In this protocol, 80% of the patients would have CTV-PTV margins inferior to 4mm. The effect of these observations on dose distribution is being evaluated.
Conclusion : The systematic and random accepted deviations are comparable to previous studies. There existed a statistical difference between the accepted and automatic deviations. Additionally, the systematic deviations calculated during the alternate week MVCT protocol were similar to the ones calculated existing during the weeks where no MVCTs were realized. However, the clinical relevance of these observations can be discussed and their effects on dose distributions are currently being studied.

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EXPANDED IFN-Γ$^+$ CD8$^+$ T CELLS REVEAL RADIOSENSITIZING EFFECTS IN TUMOR-RELEVANT HYPOXIA

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Activated T lymphocytes are known to exert cytolytic effects, while their ability to modulate tumor cell radiosensitivity has never been described. This study examined the radiosensitizing potential of activated CD8+ T cells, obtained by CD3/CD28-induced expansion. To model the hypoxic tumor microenvironment, all experiments were performed in 1% oxygen. Purified mouse CD8$^+$ T cells displayed an IFN-γ$^+$/granzyme B$^+$ phenotype, and drastically enhanced the radiosensitivity of EMT-6 mammary carcinoma cells up to 1.8-fold. A similar radiosensitization was caused by conditioned medium that was collected from activated CD8$^+$ T cells and was abrogated by anti-IFN-γ antibodies and by aminoguanidine, a metabolic inhibitor of the inducible isoform of nitric oxide synthase (iNOS). These results indicate that the observed radiosensitization does not require direct immune-tumor cell interactions, but is linked to the release of IFN-γ, a potent activator of iNOS in target tumor cells. While radiosensitization was clearly observed at effector to target ratios of 1:3 to 3:1, the cytotoxicity of CD8$^+$ T cells was significantly impaired by hypoxia even at a 30:1 ratio. In addition, hypoxia revealed a dual effect on IFN-γ and iNOS gene expression. RT-PCR, FACS and ELISA data in agreement detected down-regulation of IFN-γ in hypoxic immune cells. Contrasting, hypoxic tumor cells showed transcriptional iNOS up-regulation, which was essential to explain the radiosensitizing effects. In conclusion, this study demonstrates the radiosensitizing potential of activated CD8$^+$ T cells, and suggests a link between immunity and radioresponse.

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AN INFLAMMATORY BREAST CARCINOMA SIGNATURE IS ASSOCIATED WITH REDUCED RELAPSE FREE SURVIVAL IN PATIENTS WITH NON-INFLAMMATORY BREAST CANCER.

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Purpose : Inflammatory Breast Cancer (IBC) is an aggressive form of locally advanced breast cancer associated with poor patient survival. We hypothesize that a gene expression profile characteristic for IBC might be related to tumour aggressiveness and might be able to stratify nIBC patients with respect to prognosis.

Experimental Design. RNA was extracted from 19 IBC samples and 40 non-stage matched nIBC samples and hybridized onto Affymetrix chips. With the nearest shrunken centroid algorithm, a gene signature predictive of IBC was identified. Using a centroid mediated classification algorithm, this signature was applied onto publicly available gene expression data from 1157 nIBC samples (7 data sets) with survival data of 881 nIBC samples (4 data sets). Samples were classified as "IBC-like" or "nIBC-like". Relapse free survival (RFS) between these groups was compared by the Kaplan-Meier method for each dataset individually. In addition, we classified the same 1157 nIBC breast cancer samples according to several other signatures with prognostic relevance, including the Cell-of-Origin Subtypes, the Wound Healing Response (WHR) signature, the Genomic Grade Index (GGI), the Fibroblastic Neoplasm Signature (FNS) and the Invasiveness Gene Signature (IGS). We compared these classifications with the classification according to the IBC signature. Cox regression analysis was performed to identify the most predictive signature with respect to RFS.

Results : Kaplan-Meier analysis revealed a significant difference between nIBC breast tumours classified as "IBC-like" and "nIBC-like" with respect to RFS in all 4 data sets. Patients with an "IBC-like" phenotype demonstrate a shorter RFS interval. Classification according to the IBC signature is significantly associated with classification according to the cell-of-origin subtypes ($p<0.0001$), the WHR signature ($p<0.0001$), the IGS ($p<0.0001$), the FNS ($p=0.025$) and the GGI ($p<0.0001$). Breast tumours having an "IBC-like" phenotype generally belong to the Basal-like (32.8%), ErbB2-Overexpressing (22.6%) or Luminal B (29.6%) subtypes, have an activated WHR (71.6%), express the IGS (75.7%), are less frequently of the DTF phenotype (44.7%) and have a GGI of 3 (71.1%). Significant associations were found between the IBC signature and the Nottingham Prognostic Grade ($p<0.0001$) and Estrogen Receptor status ($p<0.0001$). Using a conditional backward Cox regression analysis on the entire data set of 881 nIBC samples, the IBC signature was identified as an independent predictor of RFS (Exp(B)=1.532, C.I.=1.100–2.133, $p=0.012$), together with the GGI and the WHR signature.

Conclusions : Our data demonstrate that nIBC breast tumours having an "IBC-like" phenotype have a significantly reduced RFS interval. This suggests that IBC and nIBC tumours demonstrate the same phenotypic traits with respect to aggressive tumour cell behavior. Despite the fact that the tumour/stroma ratio differs between IBC and nIBC and that IBC is generally of high grade, gene signatures only related to activated tumour stroma and tumour grade still add valuable information regarding patient survival. This suggests that the IBC signature represents a different aspect of aggressive tumour behavior, such as augmented tumour cell motility. Interestingly, the IBC signature maintains its prognostic capacity despite different treatment strategies used throughout the different data sets.

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THE IMPACT OF OXYGEN DEFICIENCY ON CELL CHARACTERISTICS AND ON THE CYTOTOXICITY OF GEMCITABINE: IN VITRO DATA.

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The efficacy of many cytotoxic drugs and irradiation is directly linked with adequate oxygen supply. As hypoxic regions are frequently found in solid tumours, this implies that tumours often contain viable cells that are more resistant to chemotherapy and radiotherapy. In this study, we investigated the impact of moderate hypoxia (1% O_2) and anoxia (0% O_2) on the characteristics of three tumour cell lines. In addition, the cytotoxicity of gemcitabine was studied under normoxic versus hypoxic or anoxic conditions.

The human tumour cell lines investigated are A549 (lung carcinoma cell line, wt p53), A549-E6 (transfected cell line with abrogated p53) and A549-LXSN (vector transfected control). During exposure to low oxygen conditions, cells were kept in an anaerobic chamber, continuously flushed with a premixed gas containing 1% or 0% oxygen. At different time points (1h, 24h and 48h hypoxia; 24h and 96h reoxygenation), cells were sampled. Growth curve studies determined the influence on cell proliferation. The effect of hypoxia on cell cycle and apoptosis was flow cytometrically examined after Vindelov staining and Annexin V - PI labelling. To validate the hypoxic conditions, expression of VEGF was measured by real time qPCR. For the cytotoxicity experiments, cells were treated with gemcitabine (0 – 100 nM) for 24h. Exposure times to low oxygen conditions were varied, ranging from 4 till 24 hours. The chemosensitivity was assessed by the colorimetric sulforhodamine B assay.

Hypoxic or anoxic incubation for 24h or 48h inhibited cell proliferation in all cell lines examined, but only significantly at some time points. After four days of reoxygenation, proliferation was restored. We observed a small but non-significant increase in G_1 phase and a small decrease in the number of cells in S phase after 48h hypoxia in A549 and A549-LXSN. No significant induction of apoptosis was found in any cell line, at any time point. In all cell lines, the expression of VEGF increased after 24h hypoxia, which was even more pronounced after 48h hypoxia. Moreover, upregulation of VEGF after anoxic incubation was much higher than after hypoxic incubation.

Preliminary results suggest that oxygen deficiency could be involved in mediating the sensitivity to gemcitabine. A549 cells were treated with gemcitabine for 24 hours and subsequently placed under 1% O_2. After 24 hours, cells were reoxygenated. Hypoxic exposure induced a small decrease in chemosensitivity to gemcitabine. Using anoxic conditions, a gradual increase in chemoresistance was seen when cells were exposed to anoxia for a longer time prior to treatment with gemcitabine. However, these results are only preliminary and experiments have to be repeated and expanded before final conclusions can be drawn.

In conclusion, an in vitro model was established and could be used to examine the effects of cytotoxic agents under low oxygen conditions.

The research described in this abstract was supported by the Fund for Scientific Research Flanders (FWO-F).

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