process involves ECM-degrading proteases and particularly matrix metalloproteinases (MMPs) that have been shown to be highly expressed and activated by serine proteases in tumoral microenvironment. By inhibiting plasmin, Tissue Factor Pathway Inhibitor-2 (TFPI-2) could modulate indirectly the activation of MMPs thus regulating ECM degradation and tumoral cell invasion. In the present study, we used a RNA interference method to stably knock down the expression of TFPI-2 in NCI-H460 non-small cell lung cancer cell line.

Methods: Micro interfering RNAs (miRNAs) were used to trigger sequence-specific TFPI-2 RNA degradation and then gene silencing. Two miRNA targeted TFPI-2 mRNA were designed and a non-silencing miRNA, showing no known homology to mammalian genes, was used as negative control. TFPI-2 mRNA level was measured by real-time RT-PCR and protein inhibition evaluated by western blot. Cell proliferation was measured by MTT assay. Cell migration was studied using a model based on Boyden chamber and using transwell inserts. To study the cell invasion through basement membrane components, cell culture inserts were coated with a thin layer of Matrigelâ.

Results: A specific inhibition of both TFPI-2 mRNA (between 76 and 85%) and protein was observed in NCI-H460 clones expressing miRNA. Cell proliferation was not modified by TFPI-2 RNA degradation. However, we showed that the downregulation of TFPI-2 expression was associated with a strong increase of cell invasion through basement membrane components while migration was less affected. Adhesion assays showed a slight effect of TFPI-2 inhibition on cell adherence to laminin and collagen IV matrix. Furthermore, TFPI-2 downregulation is associated with an increased expression of MMP-1 transcripts.

Conclusion: This study demonstrated that downregulation of TFPI-2 RNA by miRNA might favor the invasive behaviour of tumoral cell. To investigate these results, metalloprotease expression by tumoral cells will be now evaluated particularly when cocultured with stromal fibroblasts.

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Tumor-stromal cell interactions modulate metalloproteinase and kallicrein expression in direct and indirect co-culture cell models

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Background: The crosstalk between tumor cells and surrounding stromal fibroblasts is now considered crucial for cancer progression, particularly in invasive tumors such as lung carcinomas. Tumor-stromal cell interactions might provide signal for regulating protease and protease inhibitor secretion in the tumoral microenvironment and modulate extracellular matrix (ECM) proteolysis and then tumor invasion. The aim of this study was to develop co-culture models with cancer cells, derived from a non-small cell lung carcinoma (NSCLC), and fibroblast cells. Expression of several MMPs, kallicrein 6 and 8 (KLK) and Tissue Factor Pathway inhibitor 2 (TFPI-2) was then measured in these models.

Material and Methods: Two in vitro co-culture models were developed to evaluate the effects of direct or indirect contact between NSCLC NCI-H460 cells and CCD19-Lu fibroblast cells. In direct co-culture, both cells (ratio 1 :1) were cultured for 24 h in serum free medium. In indirect co-culture, conditioned media were collected from either confluent tumoral cells or fibroblasts grown in serum free medium during 24 h. Transcript levels of MMP-1, -2, -3, -9, -13 and –14, EMMPRIN (Extracellular Matrix MetalloPRoteinase Inducer), KLK6, KLK8 and TFPI-2 were measured using specific quantitative real-time RT-PCR. Protein expressions were evaluated by Western Blotting and immunofluorescence staining.

Results: We found a 3-fold and 8-fold increase of MMP-3 and MMP-9 expression respectively in the direct co-culture compared to cells grown alone. Although the level was lower, KLK6 mRNA was also enhanced in direct co-culture. In indirect co-culture with CCD19Lu cultured with NCI-H460 conditioned medium, we observed an increase in MMP-1, -3, -9 and TFPI-2 transcripts. Except for MMP3 and KLK6, no difference in transcripts level were observed in the other indirect co-culture model, i.e NCI-H460 grown in CCD19Lu conditioned medium.

Conclusion: Our results indicate that direct or indirect contacts between tumors and surrounding fibroblasts modulate the expression of various proteinases. This effect might be mediated by soluble or/and cell surface factors. Further investigations will be required to identify them.

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Antitumor activity and mechanism of action of ultra-low dose endothelial-monocyte activating polypeptide-II combined with a tumor targeting derivative of TNF

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Endothelial-monocyte activating polypeptide-II (EMAP-II) is a tumor-derived antiangiogenic cytokine that can sensitize tumors to the damaging activity of high-dose tumor necrosis factor-a (TNF). However, high-dose combination of these cytokines cannot be used for systemic treatment of patients because of prohibitive toxicity. In order to overcome this limitation we investigated the combination of EMAP-II with NGR-TNF, a tumor vasculature-targeted TNF derivative currently tested in Phase II studies. We observed that ultra-low doses of EMAP-II and NGR-TNF can exert synergistic anti-tumor effects and can inhibit the tumor growth in murine RMA-lymphoma and B16-melanoma models, even in the absence of chemotherapy, with no evidence of toxicity. The dose-response curve was bell-shaped, maximal synergism being achieved when 0.1 ng of EMAP-II was given to mice 0.5 hours before 0.1 ng of NGR-TNF. Surprisingly higher doses of EMAP-II in the nanogram/microgram range could progressively inhibit the synergism.Studies on the mechanism of action underlying this synergistic antitumor activity showed that while RMA lymphoma and B16 melanoma tumor cells were resistant in vitro to a wide range of concentrations of EMAP-II and NGR-TNF, low-dose combination of these cytokines induced endothelial cell apoptosis in vivo within 8 hours from administration and, at later time points, caused reduction of vessel density and, in turn, massive apoptosis of tumor cells. Since EMAP-II / NGR-TNF combination could not induce direct cytotoxic effects on cultured HMEC-1 and HUVEC endothelial cells, it is likely that other factors present in the tumor microenvironment are critical for the observed in vivo activity. Vascular targeting of TNF was critical, as the combination of non-targeted TNF with EMAP-II was inactive in these murine models, supporting the hypothesis of vascular damage as the mechanism of action of EMAP-II / NGR-TNF combination. The observation that human as well as murine NGR-TNF can induce marked apoptosis of tumor cells in combination with EMAP-II suggests that TNF-R1 is primarily involved in the pro-apoptotic mechanism, as human TNF binds only TNF-R1.Furthermore, we observed that doses of EMAP-II higher than 1 ng were able to induce the release of soluble TNF-R1, a strong counter-regulatory inhibitor of TNF, accounting for the observed inhibition of the antitumor activity.The combination of NGR-TNF with ultra-low dose EMAP-II could be a new strategy for cancer therapy.

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The Vav3 proto-oncogene is a transcriptional target of the dioxin receptor that contributes to fibroblast shape and adhesion

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Recent studies are uncovering unanticipated roles of the dioxin receptor (AhR) in cell plasticity and migration. Nevertheless, the molecular pathways and the signaling involved remain largely unknown. Here, we report a mechanism that integrates AhR into these cytoskeleton-related functions. Immortalized (FGM) and primary mouse embryonic fibroblasts (MEF) lacking AhR (AhR-/-) had increased cell area and expanded cytoplasms that reverted to wild type spindle-like morphology upon AhR re-expression. The AhR-null phenotype was characterized by an increase in F-actin stress fibers, depolarized focal adhesions, enhanced spreading and attachment and reduced migration. We present evidences that the cytoskeletal alterations observed in AhR-deficient cells are due to diminished expression of Vav3, a GDP/GTP exchange factor for Rho/Rac GTPases and a new transcriptional target of AhR. Dioxin receptor was recruited to the vav3 promoter and maintained its constitutive mRNA expression in a ligand-independent manner. Consistently with these observations, AhR-/- fibroblasts had reduced Rac1 activity and increased activation of the RhoA/Rock pathway. Pharmacological inhibition of Rac1 shifted AhR+/+ fibroblasts to the null phenotype while a Rock inhibitor revert AhR-/- cells to the wild type morphology. Importantly, knockdown of vav3 transcripts by small interfering RNAs in fibroblasts induced cytoskeletal defects and changes in adhesion and spreading that closely mimicked those observed in AhR-null cells. By modulating cell phenotype through this Vav3-dependent pathway, AhR could regulate the shape, adhesion and migration of normal cells and, perhaps, contribute to the abnormal function of these pathways under pathological conditions.

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Combined inhibition of vascular endothelial growth factor and overexpression of Angiopoietin-2 enforces glioma regression

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Background: Angiogenesis inhibition appears to be promising therapies for glioblastoma, a highly vascularized brain tumor. Sunitinib (SU) is an oral

multitarget tyrosine kinase receptor inhibitor including those important for angiogenesis (vascular endothelial growth factor receptors) with both antiangiogenic and antitumor properties. However, if targeting the function of VEGF leads primarily to significant tumor regression it could also be followed by tumor relapses. One possible strategy to overcome this therapeutic obstacle might be to overexpress anti-vascular factors to destroy tumor vasculature, rather than inhibiting the formation of new vessels. Recently, the angiopoietin (Ang) family has been identified to cooperate with VEGF to control angiogenesis. Among Angs, Angiopoietin-2 (Ang2) is involved in vessel remodelling leading to angiogenesis or vessel regression depending on VEGF activity. Accordingly, it is of interest to investigate whether an Ang2 overexpression may impair tumor growth, in particular in the brain. The aim of this study was to evaluate and compare the effect of an anti-angiogenic strategy (by the use of SU treatment), an anti-vascular strategy (by a tumoral overexpression of Ang2) or the combination of both Ang2/SU, on glioma vasculature, angiogenesis and growth.

Materials and methods: 9L glioma cells were implanted in the striatum of Fischer 344 rats. Ang2 was overexpressed by 9L cells from a stable transfection. SU treatment was administered daily 3 days after 9L cell implantation. Four groups of animals were studied: 9L (n=10); 9L-SU (n=7); 9L-Ang2 (n=13); 9L-Ang2/SU (n=11). Tumor volume was analysed by MRI (7T, Bruker). Tumor vascularization was assessed by immunohistochemistry using antibodies against RECA-1.

Results: MRI results showed that, at day 18, each treatment leads to a significant reduction of the tumor volume in comparison with non-treated animals, i.e 72% (9L-SU), 86% (9L-Ang-2), 95% (9L-Ang2/SU) suggesting that combined inhibition of VEGF and overexpression of Ang2 enforces glioma regression. Immunohistochemistry study showed a significant decrease in tumor vessel density and length in the 9L-SU group in comparison with the other groups. Interestingly, this anti-angiogenic effect of SU treatment totally disappeared in presence of an Ang2 overexpression.

Conclusions: Collectively, our data reinforce the interest to consider anti-vascular strategies for glioma treatment, in parallel to anti-angiogenic strategies.

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Role of E2F1 in sporadic Burkitt lymphoma formation

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Treatment of Burkitt Lymphoma(BL),an aggressive non-Hodgkin´s lymphoma,requires intensive combination chemotherapy that is associated with systemic toxicity.A better knowledge of the molecular basis of BL lymphomagenesis might help to develop less toxic treatments.The molecular mechanism underlying sporadic BL(sBL)formation still remains largely unknown.No single genetic lesion,besides c-myc translocation has been associated to the pathogenesis of most sBL cases to date.Patients would benefit from novel therapies aiming at specific molecular markers that have yet to be identified.To gain insight into the molecular basis of sBL tumor formation we looked for genes whose expression was deregulated in most sBL cases and may therefore have a role in lymphomagenesis. Because c-myc induces the expression of activators E2F family members and they can behave like oncogenes in mouse models,we compared their expression in BL and lymphoblastoid B-cell lines(LCLs)and found that both mRNA and protein E2F1 levels,but not those of E2F2 and E2F3,were higher in BL than in LCL cell lines.Using qPCR and immunohistochemistry we determined that the relative expression of E2F1 mRNA and protein was also much higher in tumor samples derived from sBL patients than in control tissues or in samples from other B cell lymphomas. The elevated E2F1 expression is not a consequence of the high proliferation rate of these cells,because other lymphoma samples with a similar proliferation index showed no increased E2F1 expression. To asses whether elevated E2F1 is required for tumorigenicity of sBL we reduced E2F1 expression by specific shRNAs in DG75 sBL cell line.Reduction of E2F1 expression greatly decreases their ability to form colonies in soft agar and their tumor formation capacity when inoculated in immunodeficient SCID mice,reduces their proliferation capacity,causes their accumulation in the G2/M phase of the cell cycle and leads to hyperploidy. These findings indicate that E2F1 is involved in sBL formation and suggest that it might collaborate with c-myc in sBL lymphomagenesis by eliciting progression through G2/M. Since E2F1 is not required for proliferation of most normal cells and,in fact, E2F1-/- mice are viable until old age,our results suggest that E2F1 is a promising target for developing novel and less toxic treatments of sBL. Moreover,our data show for the first time that over-expression of any E2F family member might play a positive role in the formation of a human tumor.

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SiRNA mediated downregulation of Bcl-2 in small cell lung cancer cell lines - impact on protein level and cell viability

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Background: The identification of activated proto-oncogenes has made possible to consider such genes as targets for gene silencing-based therapies. One of such targets is the BCL2 gene, known for its anti-apoptotic activity and overexpressed in most cases of small cell lung cancer (SCLC). The success of any gene silencing strategy depends on three main factors: the achievement of a suitable pharmacokinetic and biodistribution profile of the gene silencing drug; relevance of the target gene for tumor viability; and the potency and selectivity of the gene silencing drug. In this context, the objective of the present study was to evaluate the in vitro potency of different gene silencing molecules in terms of Bcl-2 downregulation and cytotoxicity against SCLC cells, as well as to evaluate the relevance of BCL2 as a molecular target in this type of tumor.

Materials and methods: An antisense oligonucleotide (ODN) and two small interfering RNA (siRNA) sequences targeting specifically the BCL2 mRNA at different positions, were transfected into SCLC cells that overexpress the Bcl-2 protein. The levels of Bcl-2 protein were determined through flow cytometry. Cell growth inhibition was evaluated by the resazurin reduction method, whereas cell viability was evaluated by flow cytometry with 7-aminoactinomycin D staining in direct correlation to Bcl-2 protein signal. Combined treatments of anti-BCL2 ODN or siRNA and the cytotoxic drug cisplatin were evaluated using both the Bliss Independence and Loewe criteria, and error propagation applied to infer about the uncertainty of the predictions of additivity.

Results: In experiments where tumor cells were submitted to 1 or 2 treatments with G3139 ODN or anti-BCL2 siRNAs, a significant sequence-specific downregulation of Bcl-2 protein levels was observed that, surprisingly, did not cause any sequence-specific inhibition of cell growth. Under treatment conditions resulting in the strongest Bcl-2 downregulation, anti-BCL2 siRNA-treated SW2 cells presented higher viability than cells treated with a non-targeted sequence. Combined treatments of anti-BCL2 ODN or siRNA and the cytotoxic drug cisplatin were merely additive on cell growth inhibition.

Conclusions: Overall, these results point out for the higher potency of siRNA, as compared to ODN, in silencing the BCL2 oncogene, but most importantly, question some previous established concepts on the role of Bcl-2 protein in SCLC cell lines.

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Towards combined treatments targeting Met and downstream survival signals: from cell biology to preclinical trials

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Studying the nature of Met oncogene signals and establishing therapeutic approaches to target Met-triggered neoplasia represent two major aims of our laboratory.

The Met oncogene plays a major role during tumorigenesis, by conferring to neoplasic cells growth advantage, invasive abilities and decreased sensitivity to therapeutic agents. It is hence not surprising that Met is a prognostic marker for a variety of tumours. More recently Met received further attention as it can confer resistance to EGFR inhibitors in lung, medulloblastoma multiforme and breast tumours, by substituting EGFR activity. We recently uncovered novel mechanisms involving well-known proto-oncogenes, which are activated by Met to promote hepatocyte survival in developing livers. First, Met acts through the PI3K-Akt pathway to induce the translation and nuclear translocation of Mdm2, which is an endogenous antagonist of p53 activity and cell death. Besides, Met triggers Mdm2 transcription through a novel molecular mechanism currently under investigation. Interestingly, we found that these pathways also contribute to the survival and the anchorage-independent growth of cancer cells with aberrant Met.

Based on these findings, we are developing new therapeutic approaches combining drugs targeting both Met and its survival signalling targets. With