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