1060 POSTER

The anaplastic lymphoma kinase receptor inhibits the apoptotic effect of the dependence receptor unc5

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Background: The Anaplastic Lymphoma Kinase (ALK) receptor is highly over-expressed and activated in several tumors protecting the cells from apoptotic cell death and promoting cell proliferation. The UNC5 receptors promote cell migration and axonal outgrowth when bound to their ligand Netrin-1 but induce apoptosis without ligand, therefore acting as dependence receptors. Recently it was shown that they also play an important role in tumorigenesis. Our study is aimed at determining the possible interaction of ALK, UNC5 and Netrin-1 and its effect on apoptosis and downstream signaling.
Methods: 293T cells over-expressing ALK/UNC5/Netrin-1 were analyzed for protein interaction and downstream signaling by Immunoprecipitation, Western Blotting or Dual Luciferase Assay, and for apoptosis by Annexin V staining.
Results: Interaction of the transmembrane receptors ALK and UNC5 depends on their extracellular domains and is blocked by a single chain anti-ALK antibody but not by Netrin-1. Apoptosis caused by UNC5 can be inhibited by ALK, similar to the protective effect of Netrin-1 on UNC5. Interaction of ALK and UNC5 led to decreased phosphorylation of Mitogen Activated Protein Kinase (MAPK) compared to ALK alone. Stimulation by additional Netrin-1 further decreased MAPK phosphorylation.
Conclusion: Our data provide evidence for an anti-apoptotic effect of the interaction of ALK and UNC5 accompanied by a decrease in MAPK phosphorylation. This makes the ALK receptor an ideal target for cancer treatment, by inhibiting its anti-apoptotic effect and simultaneously utilizing the dependence receptor UNC5 to induce apoptosis in tumor cells.

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The neuropilins: critical survival factors for non-small cell lung cancer cells

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Background: In this study, we examined the role of VEGF in non-small cell lung cancer (NSCLC) cells and the epigenetic mechanisms regulating expression of its receptors, in particular the Neuropilin receptors.
Methods: A549 and SKMES1 NSCLC cells were screened for VEGF receptors. The effect of VEGF on cell survival was examined using the BrdU assay. Cell cycle analysis was carried out following neutralisation of VEGF. Phosphorylation of Akt/PKB and Erk1/2 by VEGF was examined by confocal microscopy. VEGF receptor expression in response to the histone deacetylase inhibitor, TSA (Trichostatin A), was assessed, in addition to its effect on proliferation and apoptosis. VEGF levels were measured by ELISA in conditioned media of cells treated with TSA. The association between histone proteins and DNA was studied by ChIP analysis. The effect of TSA on acetylation of H3 and H4 histones was examined by Western blotting while the cell survival effects of VEGF following NP1 and NP2 gene silencing (siRNA) were determined using the MTT assay.
Results: VEGF increased proliferation of NP1- and NP2-expressing NSCLC cells. Neutralisation of VEGF induced growth arrest in the G0/G1 phase of the cell cycle. VEGF induced phosphorylation of Akt and Erk1/2. TSA upregulated the expression of VEGFR1 and VEGFR2 and downregulated NP1 and NP2. VEGF was unable to rescue cells from TSA-induced cell death. VEGF secretion by NSCLC cells was decreased in response to TSA. *De novo* protein synthesis was required for downregulation of the Neuropilin receptors by TSA but not for VEGFR1 and VEGFR2. TSA increased acetylation of H3 and H4 histones in both cell lines. Silencing of NP1 and NP2 receptors reduced survival of A549 and SKMES1 cells. VEGF was unable to rescue siNP1- and siNP2-treated NSCLC cells.
Conclusions: This study confirms that VEGF is a growth factor for NSCLC acting, at least in part, through NP1 and NP2, and implicates the Neuropilin receptors as critical survival factors for NSCLC cells.

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Analysis of Nanog gene in human gastrointestinal cancer

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Homeobox protein Nanog plays key roles in self-renewal and pluripotency in Embryonic stem (ES) cells. While Nanog expression was thought to be absent in somatic cells, recent reports suggested Nanog overexpression in several human tumor cells. We also found overexpression of Nanog gene in side population in human gastrointestinal (GI) tumor cell lines. However, expression of Nanog in human GI tumor tissue and functional role of Nanog in these cells still remains unknown. In order to clarify functional expression of Nanog, we investigated human GI cancer tissues and detected significant expression on Nanog by RT-PCR, western blot analysis and immunohistochemical staining. Nucleotide sequencing revealed that GI cancer cells we examined expressed Nanog pseudogene 8 (Nanog P8 gene). Overexpression and knockdown analysis of Nanog P8 in gastric cancer cell line, AZ521, and colon cancer cell line, SW480, exhibited cell proliferative activity of Nanog P8 in vitro. These data suggested that Nanog P8 might have a functional role in proliferation of human GI cancer cells. We are currently investigating tumorigenesis of Nanog P8-overexpressed cells in mice tumor xenograft model.

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Functional role of S100A4 in tumour stroma interaction

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Communication between tumor cells and host components, such as immune cells, fibroblasts and endothelial cells, contribute to the progression of cancer from its initial growth at a primary site in the body to its metastasis to distant organs. S100A4, one of many genes involved in stimulation of metastatic spread of tumor cells exerts its function as a stroma-cell derived factor. The exact mechanism of its metastasis-stimulating function remains poorly understood.
In the present study we investigated the effect of S100A4 genetic depletion in Polyoma Middle T oncoprotein (PyMT)- induced metastatic mammary tumors. Massive leukocyte infiltration is closely associated with the malignant transition in the PyMT tumors (adenoma/MIN). This is associated with increase in the concentration of extracellular S100A4 detected in the tumor microenvironment. In contrast, in PyMT S100A4(−/−) tumors, we observed substantial suppression in leukocyte infiltration in the transition period. S100A4 deficiency lead to significant decrease in particular in numbers of T-lymphocytes that invade developing tumor. A chemotaxis assay revealed that purified T lymphocytes migrate in response to S100A4. Invasion of T lymphocytes into the S100A4 positive fibroblast monolayer is greatly enhanced compared to the S100A4(−/−) ones. Both processes are blocked by anti-S100A4 antibodies. Moreover, co-injection of tumor cells with S100A4(+/+) but not the S100A4(−/−) fibroblasts in S100A4(−/−) mice, attracts significantly more T-lymphocytes to the site of growing tumor. This is also accompanied by increase in the amount of S100A4 released into the tumor microenvironment.
Engagement of high concentration of S100A4 in tumor microenvironment may cause T-cells to migrate to tumor sites and probably take part in stimulation of tumor vascularization and metastasis.

1064 POSTER

Invasive breast tumour cells induce up regulation of tumour endothelial marker 8 (TEM8) in monocytes

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Background: Tumor cell migration and metastasis share many similarity with leukocyte trafficking which is critically regulated by chemokines/chemokine receptors interactions in a co-ordinate fashion with cell-surface adhesion molecules. We previously reported that Tumour Endothelial Marker-8 (TEM-8), a putative adhesion molecule though to be involved in extra cellular matrix-remodeling and migration processes, is selectively over -expressed in highly invasive vs. non-invasive breast cancer cell lines (e.g. MDA-MB231 vs. ZR-75 respectively). Here we investigated the effect of soluble factors released fom cycling cancer cells on TEM8 expression in immature myeloid monocytes.

Methods: Human CD14$^+$ monocytes (MOs) were isolated from peripheral blood mononuclear cells, and resuspended in RPMI medium supplemented with 10% foetal calf serum (Reference MOs). MOs were cultured (time course covered 12, 24 and 48 hrs) with tumor cell supernatants (TCSs) obtained either from growing or starving MD-MBA-231 cells. TCSs – conditioned MOs were then scrutinized for TEM8 expression levels and for the production of invasive/pro-angiogenic factors [i.e. urokinase system (uPAR/uPA), metalloproteinase 9 (MMP9), and chemokines (CXCL8/IL8, CXCL5)] by using well-established methods (i.e. Real-time RT-PCR, flow cytometry, western blotting, ELISA, and zymography).
Results: MOs' phenotype and functions were not substantially modified by the addition of serum in culture medium. A kinetic profile of TEM8 expression revealed that in response to TCS from growing MDA-MB231 cells, by 48 hrs, MOs expressed maximal levels of TEM8 mRNA (approx. 70 fold increase over reference MOs). Around this time point, conditioned-MOs showed also the highest production of membrane-bound uPR, and secretion of MMP9 and CXCL8/IL8, CXCL5 chemokine. Of note, these modifications were absent in MOs incubated with TCS obtained from starving MDA-MB231 cells.
Conclusions: We speculate that TEM8 is invlved in cellular mechanisms that foster both leukocytes-dependent inflammatory angiogenesis and tumor cell migration/invasion processes.

1065 POSTER

Metastasis-promoting S100A4 protein affects the EGFR signalling pathway

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Expression of S100A4, a member of the S100-family of calcium binding proteins, has been associated with tumor invasion and metastasis. Recently we described the suppression of tumor development and metastasis formation in S100A4 deficient mice. Immunohistochemical staining of tumors in these mice indicated an abnormal recruitment and distribution of immune cells. Since S100A4 is expressed and secreted from different cell types from the tumor environment, including macrophages and leukocytes, extracellular S100A4 could influence cell motility and affect the recruitment of immune cells as well as their function at the tumor site.
Our recent finding showing that extracellular S100A4 attracts mouse T-lymphocytes isolated from spleen in a transwell migratory assay, is supporting this hypothesis. However, the cell surface receptor recognizing S100A4 and the signal transducing pathways triggered by S100A4 are not known. To identify proteins binding extracellular S100A4, we screened a phage display peptide library using multimeric S100A4 as bait and identified a peptide motif that mimics the KCCY/F sequence present in the EGF domain of EGF receptor ligands. Binding studies confirmed selective binding between S100A4 and a number of EGF receptor ligands, with the strongest interaction to Amphiregulin. Furthermore, extracellular S100A4 enhanced EGFR/ErbB2 signaling and Amphiregulin-dependent proliferation of S100A4$^{(-/-)}$/fibroblasts. The S100A4 had no effect on ligand shedding, a process known to convert the transmembrane EGF-family pro-ligand to the active soluble form. Alternative mechanisms which could explain the observed S100A4 effects are currently under investigation. We speculate that extracellular S100A4 can affect tumor progression by interacting with the EGFR/ligand complex leading to an enhancement of EGFR signaling, increasing cell motility and proliferation.

1066 POSTER

A simple method to prepare tumour stem cells from the human breast cancer cell line MDA-MB 231

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Background: The commercially available cell line MDA-MB 231 is reported to contain considerable amounts of tumor initiating cells. As described in several papers these cells can be enriched by FACS or prepared via "mammo-spheres", three dimensional cellular aggregates. The procedures are all more or less time consuming and of limited efficiency. Here we present a very easy way to select a homogeneous cellular fraction of potential breast cancer stem cells.
Methods: The cells were cultured beyond confluency until viable cells escape from the monolayer into the culture medium. Then, the supernatant was transferred into a new culture flask and grown again beyond confluency.
Results: After 16 cycles, the suspension consisted of a homogenous cellular population presenting high CD44 and hardly detectable CD24 immunoreactivity, a generally accepted feature of breast cancer stem cells. The cells showed an intense staining of vimentin, a mesenchymal intermediate filament protein and no cytokeratin 18, the epithelial counterpart in breast epithelial cells. Time-lapse videography reveals that these cells are very motile but can be restrained by inhibition of the FAK system. Furthermore, a partial differentiation seems to be induced by FAK inhibition as indicated by the expression of cytokeratins.
Conclusion: Focusing on tumor stem cells in basic research and cancer therapy comes of age – here we present a convenient protocol to easily prepare the required cells for further experiments.

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Characterization of primary tumour stromal cells and their potential role in the breast cancer microenvironment

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Background: The importance of the primary tumour microenvironment in breast cancer development and progression has emerged in recent years. Tumour-derived stromal cells have been shown to promote epithelial tumour cell growth, migration and invasion. Although the tumour promoting effect of stromal-epithelial interactions is recognized, the precise mechanisms involved are poorly understood. The aim of this study was to isolate primary tumour stromal cells from breast cancer specimens and investigate their potential mode of action in the breast tumour microenvironment, based on expression of genes associated with cancer progression.
Methods: Following written informed consent, specimens of human breast cancer were harvested from patients undergoing surgery. Cells were isolated from tumour- and tumour-associated normal regions of breast tissue. Breast tissue obtained from reduction mammoplasty served as normal controls. Following tissue dissociation and digestion, stromal cells were isolated by differential centrifugation and characterised. Following culture of stromal cells, RNA was extracted, reverse transcribed and relative quantitative PCR performed using primers targeting Fibroblast Activation Protein (FAP), Transforming Growth Factor β (TGFβ), Transforming Growth Factor β Receptor II (TGFβRII), Matrix Metalloproteinase 3 (MMP3), and Vascular Endothelial Growth Factor A (VEGF A).
Results: Expression of TGFβ, which is known to induce epithelial to mesenchymal transition (EMT), was upregulated in tumour compared to normal stromal cells, while there was no difference in expression of its principle receptor, TGFβRII. This was supported by changes in epithelial cell cytoskeleton, with reduced cell-cell adhesion and E-cadherin expression observed in epithelial cells cultured in the presence of tumour stromal cells. The proangiogenic factor VEGFA, and the invasion associated gene MMP3, were also upregulated in the tumour stromal cell population. In contrast, the level of FAP detected in tumour stromal cells was lower than that detected in normal stromal cells.
Conclusion: Tumour stromal cells have the potential to stimulate angiogenesis and epithelial to mesenchymal transition through secretion of paracrine factors such as VEGF A and TGFβ. Further understanding pathways involved in stromal cell induced tumour progression is essential to inhibit initiation of the metastatic cascade.

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Increasing the efficiency of gene therapy by using protein transduction domains

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Background: Cancer is the main disease addressed by gene therapy. Despite the recent developments there are still some limitations. One important limitation of gene therapy strategies is that vectors are not efficient in vivo. It is necessary to deliver the gene products to every cell; otherwise, the remaining malignant cells will proliferate and disease will relapse. A promising approach to increase the efficiency of gene therapy is to increase the transmission of the gene product. The secretion of therapeutic agents from transduced tumor cells and the subsequent internalization by neighboring untransduced cells would increase the effect of gene therapy. This study aims to increase the transmission of therapeutic agents by using protein transduction domains (PTDs).
Methods: Cre recombinase – lox P system is selected as a reporter tool for examining the cargo delivery efficacies of several PTDs. Eukaryotic expression vectors were constructed to produce Cre fusion proteins with the protein transduction domain of HIV-1 TAT protein, and the herpes simplex virus (HSV) VP22. Reporter cells are transfected with Cre fusion protein vectors. To observe intercellular trafficking properties and subcellular distribution of the fusion proteins, transfected cells are examined by FACS and immunohistological staining.
Results: Cre mediated recombination in transfected cells indicated the functional protein production and successful transfer of the protein to the