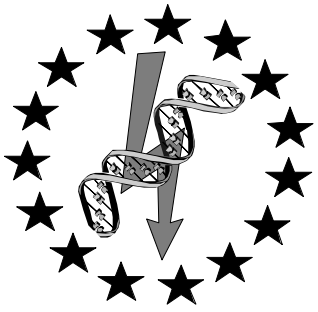
J Mol Med (2002) 80:B1–B8



Euroconference 2002

The stromal reaction in neoplastic and non-neoplastic disease – basic mechanism and therapeutic applications

Bonn, Königswinter/Petersberg, Germany
26 – 29 September 2002

Supported by the European Commission, DGXII, Human Potential Program, High-Level Scientific Conferences, HPCF-2002-00474

Short papers

Growth Factor Regulation of Angiogenesis and Lymphangiogenesis

Kari Alitalo and collaborators Molecular/Cancer Biology Laboratory and Ludwig Institute for Cancer Research, Haartman Institute and Biomedicum Helsinki, P.O.B. 63 (Haartmaninkatu 8), 00014 University of Helsinki, FINLAND

Angiogenesis and permeability of blood vessels are regulated by vascular endothelial growth factor (VEGF) via its two receptors VEGFR-1 and VEGFR-2. The VEGFR-3 receptor does not bind VEGF and its expression becomes restricted mainly to lymphatic endothelia during development. We have found that homozygous VEGFR-3 targeted mice die around midgestation due to failure of cardiovascular development. We have also purified and cloned the VEGFR-3 ligand, VEGF-C. Transgenic mice expressing VEGF-C show evidence of lymphangiogenesis. VEGF-D is closely related to VEGF-C, similarly processed and binds to the same receptors. Thus, VEGF-C and VEGF-D appear to be both angiogenic and lymphangiogenic growth factors. VEGF-C overexpression also led to lymphangiogenesis, intralymphatic tumor growth and lymph node metastasis in an orthotopic model of human breast carcinoma in immunoincompetent mice. Furthermore, soluble VEGFR-3, which blocks embryonic lymphangiogenesis, blocked lymphatic metastasis in breast and lung cancer models.

Veikkola et al., Cancer Res. 60: 203-212, 2000, Karkkainen et al., Nature Genetics 25: 153-159, 2000, Iljin et al., FASEB J. 2001 15:1028-1036, Enholm et al., Circ Res. 88:623-9, 2001, Karpanen et al., Cancer Res. 61:1786-90, 2001, Veikkola et al., EMBO J. 20:1223-31, 2001, Mandriota et al., EMBO J. 20:672-82, 2001, Makinen et al., Nat Med. 7:199-205, 2001, EMBO J. 20: 4762-4773, 2001, He et al., JNCI in press, Kubo et al., Proc. Natl Acad. Sci., in press, Petrova et al., EMBO J., in press.

In silico Protein Protein interactions

A. Fritz[1], M Fellenberg[1, 2], K. Albermann[1], A. Zollner[1], H.W.Mewes[2] and J. Hani[1]
1.Biomax Informatics AG, Lochhamer Str. 11, D-82152 Martinsried
2.MIPS, GSF - Forschungszentrum für Umwelt und Gesundheit, GmbH Ingolstädter Landstraße 1, D-85764 Neuherberg

Biomax has developed a method for the integrative analysis of protein interaction data. It comprises clustering, visualization and data integration components. The method is generally applicable for all sequenced organisms. We describe in detail the combination of protein interaction data in the yeast Saccharomyces cerevisiae with the functional classification of all yeast proteins. We evaluate the utility of the method by comparison with experimental data and deduce hypotheses about the functional role of so far uncharacterized proteins. Further applications of the integrative analysis method are discussed. The method presented here is powerful and flexible. We show that it is capable of mining large-scale data sets.

Inhibition of tumor vascularization

Yihai Cao
Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77 Stockholm, Sweden. Tel: (+46)-8-728 7596, Fax: (+46)-8-31 94 70, E-mail: yihai.cao@mtc.ki.se

Angiogenesis is essential not only for primary tumor growth but also for metastasis. Overproduction of angiogenic factors and down-regulation of certain endogenous angiogenesis inhibitors in a tumor are both necessary and sufficient for switching on an angiogenic phenotype. Among known angiogenic factors, vascular endothelial growth factor (VEGF) is probably the most frequently used angiogenic stimulator by tumors. Thus, inhibition of VEGF-induced angiogenesis has become an attractive approach for potential cancer therapy. We have found that placenta growth factor-1 (PlGF-1), an alternatively spliced isoform of the PlGF gene, antagonizes VEGF-induced angiogenesis when both factors are coexpressed in murine fibrosarcoma cells. Overexpression of PlGF-1 in VEGF-producing tumor cells results in the formation of PlGF-1/VEGF heterodimers and depletion of the majority of mouse VEGF homodimers. The heterodimeric form of PlGF-1/VEGF lacks the ability to induce angiogenesis in vitro and in vivo. Similarly, PlGF-1/VEGF fails to activate the VEGFR-2-mediated signaling pathways. Further, PlGF-1 inhibits the growth of a murine fibrosarcoma by approximately 90% when PlGF-1-expressing tumor cells are implanted in syngeneic mice. In contrast, overexpression of human VEGF in murine tumor cells causes accelerated and exponential growth of primary fibrosarcomas and early hepatic metastases. Our data demonstrate that PlGF-1, a member of the VEGF family, acts as a natural antagonist of VEGF when both factors are synthesized in the same population of cells. The underlying mechanism is due to the formation of functionally inactive heterodimers. As cancer masses consist of heterogeneous populations of tumor cells that constantly undergo genetic mutations, they may switch their angiogenic profiles during cancer progression. If a tumor produces other angiogenic factors such as FGF-2, the tumor may become resistant to anti-VEGF treatment. In contrast, antagonists for single angiogenic factors, general angiogenesis inhibitors that block common pathways of tumor angiogenesis could bypass drug resistance and thus prove therapeutically effective against all cancer types. Angiostatin seems to be such a general inhibitor which blocks a common angiogenic pathway triggered by several angiogenic factors. In mice, angiostatin has been shown to be sufficiently potent in suppression of several types of tumors. These preclinical findings have led to clinical trials of kringle 1-3 in human cancer therapy. For clinical trials, several major obstacles related to dosages and protein activity of angiostatin should be overcome before a large number of cancer patients are tested. To overcome these obstacles, we are developing alternative approaches. These include isolation of more potent inhibitors, prolongation of half-lives, angiostatin gene therapy, targeting angiostatin into the tumor vasculature, and combination therapy with other angiogenesis inhibitors. In addition to improvement of these therapeutic strategies, it is inevitable to understand the molecular mechanisms of angiostatin on suppression of angiogenesis and tumor growth. We have previously shown that kringle 1-5 displays more potent antiangiogenic anti-tumor activity than angiostatin. We have now found that K1-5-induced endothelial cell apoptosis as an essential mechanism for its antiangiogenic and antitumor activity. K1-5-induced endothelial apoptosis involves activation of caspase pathways. These findings provide a mechanistic insight to the angiostatic action of K1-5 and angiostatin.

Design, development and biological investigation of inhibitors of angiogenesis and lymphangiogenesis

Prof. Dr. Athanassios Giannis, Institute for Organic Chemistry, University of Leipzig, Johannisallee 29, D-04301 Leipzig, Germany, Fax: (int)-341-97 36 599; e-mail: giannis@chemie.uni-leipzig.de

The formation of new blood capillaries from pre-existing ones - angiogenesis or
neovascularisation - is of fundamental importance for tumor growth and metastasis. For this reason inhibition of angiogenesis became an attractive target for the treatment of cancer. In the last 10 years research has been focussed on the identification of the protagonists of angiogenesis. Among others vascular endothelial growth factors (VEGF), angiopoietins and their receptors (VEGFR1-3,Tie2) were discovered and their implication in angiogenesis has been clarified. Furthermore the role of additional signalling systems like avb3-Integrin (vitronectin receptor) and Methionin Aminopeptidase Type 2 has been elucidated. All these systems represent interesting targets for the development of anti-angiogenic drugs.
In my lecture the I will discuss the synthesis of
a) ATP-analogues as inhibitors of Receptor Tyrosin Kinases involved in angiogenesis
b) RGD-Peptidomimetics as vitronectin antagonists, and
c) Fumagillin analogues as inhibitors of Methionin Aminopeptidase Type 2.
Biochemical and biological investigations will be discussed.

Literature:
V. Kirkin, R. Mazitschek, J. Krishnan, A. Steffen, S. J. Mandriotta, J. Waltenberg, M. S. Pepper, A. Giannis, J. P. Sleeman, Eur. J. Biochem 2001, *268*, 5530–5540: Characterisation of ATP analogues which specifically inhibit VEGF-C and VEGF-D-induced activation of VEGFR-3 but not VEGFR-2.
P. Stahl, R. Mazitschek, A. Giannis, H. Waldmann, Angew. Chem. 2002, *114*, 1222-1226; Angew. Chem. Int. Ed. 2002, *41,* 1174-1178: Natural Product Derived Receptor Tyrosin Kinase Inhibitors: Identification of IGF1R-, Tie-2 and VEGFR3 Inhibitors Inhibitors.
N. Wernert, A. Stanjek, S. Kiriakidis, A. Hügel, H. C. Jha, R. Mazitschek, A. Giannis, Angew. Chem. 1999, *111*, 3432-3435; Angew. Chem. Int. Ed. Engl. 1999, *38*, 3228-3231:
Inhibition of Angiogenesis In Vivo by ets-1 Antisense Oligonucleotides - Inhibition of Ets-1 Transcriptionsfactor Expression by the Antibiotic Fumagillin.

In vivo cellular and molecular multicolor imaging with gfp and rfp

M. Yang, P. Jiang, N. Yamamoto, R.M. Hoffman. AntiCancer, Inc., 7917 Ostrow St., San Diego, CA 92111, USA.

Strong fluorescent labeling with green fluorescent protein (GFP) along with inexpensive video detectors, positioned external to the mouse, allows the monitoring of details of tumor growth, angiogenesis, and metastatic spread in mouse models. Opening a reversible skin-flap in the light path increases detection sensitivity to the single-cell level on internal organs. Single tumor cells, expressing GFP, seeded on the brain can be imaged through a scalp skin-flap. Lung tumor micro-foci representing a few cells are viewed through a skin-flap over the chest wall while contralateral micrometastases were imaged through the corresponding skin-flap. Pancreatic tumors and their angiogenic micro-vessels were imaged via a peritoneal wall skin-flap. A skin-flap over the liver allowed imaging of physiologically relevant micro-metastases originating in an orthotopically implanted tumor. Single tumor cells on the liver arising from intraportal injection were also detectable (Yang, M., et al. Proc. Natl. Acad. Sci. USA **99**, 3824-3829, 2002). Using the anti-apoptotic gene *bcl-2*, dominant-negative *caspase 9* (C9DN), p53 and $p16^{INK4a}$, we showed by whole-body GFP imaging that disruption of the apoptosis and senescence programs confer tumor agressiveness and drug resistance of in lymphomas which arose in *Eμ-myc* transgenic mice (Schmitt, C.A., et al. Cancer Cell **1**, 289-298, 2002; Schmitt, C.A., et al. Cell **109**, 335–346, 2002). HT-1080 human fibrosarcoma labeled with GFP or RFP were injected i.v. into SCID mice. This resulted in individual green or red clones growing on the lung visualized by dual color imaging in living mice. These results indicate the possibility of color-coding cells of different genotypes *in vivo* for molecular imaging studies such as those described above.

Inhibition of VEGF in human macrophages and rheumatoid synovial cells by blockade of the NFκB pathway using adenoviral gene transfer

Serafim Kiriakidis, Evangelos Andreakos, Claudia Monaco, Brian Foxwell, Marc Feldmann & Ewa Paleolog
Kennedy Institute of Rheumatology Division, Imperial College of Science, Technology and Medicine London, United Kingdom

Vascular endothelial growth factor (VEGF) is a multifunctional cytokine, which regulates angiogenesis. VEGF is expressed in rheumatoid arthritis (RA) synovium and is elevated in the serum of RA patients. The aim of our study was to investigate whether the transcription factor NFκB, which is a key molecule in the expression of many pro-inflammatory cytokines in RA, is also involved in the production of VEGF.
Human monocyte-derived macrophages and cells from RA synovial tissue (SMC) were infected with adenoviral vectors encoding IκBα (Adv IκBα) the endogenous inhibitor of NFκB nuclear translocation, or control adenovirus, encoding for β-galactosidase (Adv β-gal). A multiplicity of infection of 100:1, at which infection efficiency was > 95%, was used. After infection cells were stimulated with LPS or CD40 ligand (CD40L) for 24 hours. SMC culture media were collected 48 hours after adenoviral gene transfer. VEGF release was measured by ELISA.
We observed that LPS-induced production of VEGF in human macrophages was completely inhibited (>90%) following adenoviral transfer of IκBα. We also observed strong inhibition (70%) of the CD40L-induced VEGF production in macrophages. In contrast, control adenovirus was without effect. Spontaneous VEGF release by SMC was significantly ($p<0.001$) inhibited following infection with Adv IκBá.
Our results show for the first time that NFκB is required for VEGF production by macrophages and SMC, thus joining the list of other NFκB-dependent genes involved in the pathogenesis of RA. These data add to the understanding of signaling pathways involved in the induction of VEGF expression, which is the most important factor regulating angiogenesis.

Angiogenesis: how does it really look like? Studies by 3-D corrosion casts.

Moritz A. Konerding, Department of Anatomy, Johannes-Gutenberg-University Mainz
Phone: +49-6131-3922549 Fax: +49-6131-3924710 E-mail: konerdin@mail.uni-mainz.de

The vascular system plays a role of key importance during tumor growth and metastasis formation. In addition, the effectiveness of almost all therapeutic modalities, including drug therapy and radiotherapy, is influenced by the micro-architecture and the gradients of essential nutrients around each vessel. The knowledge that tumor vasculature is abnormal has led to concepts such as angiogenic attack and vascular targeting. However, even though our knowledge of the mechanisms underlying angiogenesis has increased dramatically in the past 25 years, few quantitative data are available on the vascular network architecture and pattern formation in tumors.
Analyses of microvascular networks in normal tissues as well as in secondary angiogenesis have been based on light microscopic and/or angiographic 2D methods, which enable a morphometric assessment of the gross network, but cannot adequately describe the three dimensional microvascular architecture. This can best be done with 3D reconstructions of corrosion casts. Reproducibility tests and tests for the possible errors indicate that a maximum result deviation of 2.5% may be expected. All parameters describing the microvascular unit such as inter-vessel and inter-branch distances, vessel diameters and branching angles can be assessed after injection of a pre-polymerized resin into the vascularity and subsequent corrosion of the surrounding tissue.
The results obtained with 3D morphometry of corrosion casts include the proof that the tumor inherent vasculature is tumor type specific. Different experimental tumors display characteristic microvascular units. This was also proven in human colorectal tumors. The tumor-type specific vasculature is primarily primed by the tumor cells themselves; in the first attempt to define the influence of FGF-2 as a single, defined growth factor on the 3D tumor vascular pattern it could be proven that FGF-2 production and release confer to tumor cells the ability to stimulate the formation of new blood vessels without altering the basic vascular architecture. Systematic studies of precursor lesions have revealed that angiogenesis and the establishment of a new vascular architecture sets in long before the change into the malignant phenotype.
Comparisons of the results of corrosion casting studies of tumor vascular architecture with those in chronic inflammation reveal some similarities but also fundamental differences. Changes of the vascular architecture in inflammation include also focal structural dilatations of precapillary and capillary vessels needed for flow and shear stress reduction facilitating adhesion and transendothelial migration of lymphocytes.

Literature

Konerding MA, Fait E, Gaumann A: 3D microvascular architecture of precancerous lesions and invasive carcinomas of the colon. Br. J. Cancer, 84 (10), 1354-1362 (2001).
West CA, He CH, Su M, Secomb T, Konerding MA, Young AJ, Mentzer SJ: Focal topographic changes in the inflammatory microcirculation associated with lymphocyte slowing and transmigration. Am J Physiol Heart Circ Physiol. 2001, 281(4):H1742-50.
Konerding MA, Fait E, Dimitropoulou C, Malkusch W, Ferri C, Giavazzi R, Coltrini D, Presta M (1998) Impact of fibroblast growth factor-2 on tumor microvascular architecture. A tridimensional morphometric study. *Am J Pathol* 152: 1607-1616.
Konerding MA, Malkusch W, Klapthor B, van Ackern C, Fait E, Hill SA, Parkins C, Chaplin DJ, Presta M, Denekamp J (1999) Evidence for characteristic vascular patterns in solid tumours: quantitative studies using corrosion casts. *Br J Cancer* 80: 724-732.
Konerding MA, Miodonski AJ, Lametschwandtner A (1995) Microvascular corrosion casting in the study of tumor vascularity: a review. *Scanning Microsc* 9: 1233-1243.

Proteome analysis in lymphangiogenesis

Rene C. Krieg and Cloud P. Paweletz
NCI-FDA Clinical Proteomics Initative, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD, 20892, USA

With the sequencing of the human genome completed the functional elucidation for all expressed genes has become the predominant biological task. While DNA is the information archive, the spatio-temporal expression of proteins defines all biological processes. Lymphangiogenesis has recently become a hotspot in metastasis research, as initial dissemination via lymphatic vessels is believed to be the main reason for tumor spreading. However, most results in lymphangiogenesis research were obtained by immunohistochemistry using a small selection of antibodies. Yet, the challenges and opportunity within proteomics is much more than merely developing a list of expressed proteins, but rather observed protein changes need to be analyzed within a pathological frameset. To that end we employ laser microdissection for proteomic analysis using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), surface enhanced laser desorption ionzation (SELDI) mass spectrometry, and protein microarrays for the proteomic analysis of microdissected subpopulations of cells. More specifically, our program is focused on the understanding of mechanisms of carcinogenesis. These studies employ:
a) Differential protein profiling and discovery technologies, such as (2D-PAGE) coupled with mass spectrometry for new target and biomarker discovery.
b) High-throuphput proteomic pattern profiling using surface enhanced laser desorption and ionization (SELDI) mass spectrometry to identify disease-related proteins and protein patterns.
c) Multiplexed phospho-specific antibody arrays and general antibody & lysate arrays for signal transduction pathway profiling.

Tumor cell and stromal cell interactions during breast cancer progression

A. NOEL and J-M. FOIDART
Laboratory of Tumor & Development Biology, University of Liège, Tour de Pathologie (B23),B-4000 Liège, Belgium

Cancer cells are not simply isolated islands of cells residing in a specific organ; they are surrounded by a modified extracellular matrix and by stromal cells of the host tissue, both of which influence tumor progression. Breast neoplastic stroma contains a heterogenous cell population composed of fibroblasts, myofibroblasts (expressing smooth muscle actin), endothelial cells and inflammatory cells. Those cells produce a variety of cytokines, growth factors and proteases which influence neoplastic cell properties. We have previously demonstrated that fibroblasts promote the tumorigenicity of human breast adenocarcinoma MCF7 cells. This is related at least to their capacity to produce proteases such as matrix metalloproteinases (MMPs). MMPs form a large family of structurally related zinc endopeptidases that are collectively able to degrade a wide variety of extracellular matrix proteins. Their activity is controlled by physiological inhibitors or TIMPs.
Membrane type 1 metalloprotease (MT1-MMP) is a transmembrane metalloprotease that plays a major role in the extracellular matrix remodeling, directly by degrading several of its components and indirectly by activating pro-MMP2. By immmunohistochemistry and in situ hybridization, we provide

evidence that MT1-MMP is exclusively produced by myofibroblastic cells, and not by breast neoplastic cells. To gain new insight into the role of MT1-MMP during early steps of cancer progression MCF7 cells were transfected with MT1-MMP and/or MMP2 cDNAs. All clones overexpressing MT1-MMP 1) are able to activate endogenous or exogenous pro-MMP2, 2) display an enhanced in vitro invasiveness, 3) induce the rapid development of highly vascularized tumors when injected subcutaneously in nude mice, and 4) promote blood vessels sprouting in the rat aorting ring assay. The angiogenic phenotype of MT1-MMP producing cells is associated with an up-regulation of Vascular Endothelial growth Factor (VEGF) expression.
Altogether our data emphasize the key role played by stromal MMPs such as MT1-MMP during cancer progression. They also provide a new mechanism of MT1-MMP action, the upregulation of VEGF expression. These information change our view of MMPs which are not simple regulators of matrix remodeling, but are also regulators of gene expression.
Reference : Sounni N.E. et al. (2002) FASEB J. 16: 555-564.

Molecular mechanisms of tumoral angiogenesis

Karl H. Plate
Institute of Neurology, Johann Wolfgang Goethe-University, Frankfurt am Main, Germany

We have investigated the molecular mechanisms of angiogenesis in stroke, breast cancer, and two distinct types of brain tumors, namely glioblastoma and hemangioblastoma. In all pathologies examined, we observed upregulation of VEGF and of VEGF receptors –1 and –2 in a cell type scecific fashion. VEGF was predominantly expressed around necrotic areas, suggesting a hypoxia-dependent upregulation of the VEGF gene in vivo. Whereas Angiopoietin-1 and tie2 expression was not significantly altered, we observed a cell-type specific upregulation of Angiopoietin-2 in vascular cells, suggesting a modulation of tie2 function mainly via Ang-2 expression levels. The expression of VEGF, VEGFR-2 and Ang-2 witin the same tumor microenvironment (e.g. in perinecrotic regions) suggests a connection between VEGFR-2 and Tie-2 signaling pathways. The hypoxia-inducible transcription factors HIF-1a and HIF-2a were also up-regulated in stroke and tumor specimens, suggesting that hypoxic induction of several genes underlies the observed vascular phenotypes. Our results thus suggest a coordinate upregulation of genes (synexpression groups) mediated by hypoxia or by hypoxic mimicry (e.g. von Hippel Lindau Tumor Suppressor gene loss of function) which mediate the vascular and metabolic responses to hypoxic/ischemic and neoplastic brain injury.
Adaptation of cells to hypoxic stress and induction of angiogenesis by hypoxia represent important aspects in solid tumor growth. It has recently been shown that two transcription factors, HIF-1a and HIF-2a play a key role in regulating these responses by transactivating multiple genes, including VEGF (vascular endothelial growth factor). To investigate the role of HIF-1a and HIF-2a in brain tumor vascularization and growth we modulated HIF expression in a GS9L glioma model.
GS9L glioma cells were stably transfected with HIF-1a, HIF-2a or dominant negative mutant constructs. Several clones were isolated and expression confirmed by Western Blot and Northern Blot analysis. The effect of HIF modulation on the expression of hypoxia inducible genes such as VEGF, LDHa (lactate dehydrogenase) and Glut-1 (glucose transporter) under both normoxic and hypoxic conditions was investigated. HIF-2a overexpression lead to marked induction of VEGF, while in clones expressing dominant negative mutants VEGF expression was repressed under hypoxic conditions. Our data confirm that VEGF expression is regulated by HIF-1/2a and can be repressed by HIF dominant negative mutants.

Automation in microscope-based fluorescence cell screening

Andreas Plesch,
MetaSystems GmbH, Altlussheim, Germany, aplesch@metasystems.de

Conventional manual cell screening under the fluorescence microscope is tedious, particularly if several simultaneous markers are to be detected. Moreover, manual microscopy is limited to qualitative or at best semi-quantitative analyses.
We developed an automated slide scanning platform for high throughput fluorescence cell screening. The RCDetect operating mode allows rapid identification of rare cells e.g. isolated tumor cells in bone marrow or peripheral blood. The combination of different markers, labeled with distinguishable fluorochromes provides the high sensitivity and specificity that is crucial for reliable rare event detection at occurrence rates of down to 1 in a million and below.
In the MetaCyte operating mode the system works as a scanning image cytometer. During the scan numerous cell features can be measured in up to 6 individual color channels. Applications include fully automated interaphase FISH analysis to detect numerical chromosome aberrations as well as translocations. The system can be adapted to various preparation techniques such as cell suspensions, cytospin preparations, tissue sections, and tissue micro arrays.
The principles of the automated system as well as several application examples and results will be presented.

Normal and tumor angiogenesis are perturbated by an Ets-1 dominant negative mutant in a mouse ear angiogenesis model.

Albin POURTIER-MANZANEDO[1¤], Chantal VERCAMER[1], Eric VAN BELLE[2], Virginie MATTOT[3] and Bernard VANDENBUNDER[1].
[1]FRE 2353 CNRS/Institut Pasteur de Lille/Université Lille 1 and [3]UMR 8526 CNRS/Institut Pasteur de Lille/Université Lille 2, Biology Institute of Lille, 1 Rue Calmette, F-59021 Lille Cedex, France; [2] Cardiology B and Hemodynamics, Hôpital Cardiologique, Lille-2 University, Avenue Oscar Lambret, F-59037 Lille, France ; [¤] Development Biology Laboratory, UPRES EA 1033, Bâtiment SN3, Sciences and Technologies University of Lille-1, USTL, F-59655 Villeneuve d'Ascq Cedex, France. Albin.pourtier@univ-lille1.fr (00 (33) 3 20 33 62 22).

Ets1 is the founder of a family of transcription factors characterized by a conserved DNA binding domain [Laudet, 1999; Sharrocks, 1997]Ets1 is expressed in activated endothelial cells during blood vessel formation in the embryo [Lelievre, 2001]. Ets1 expression collapses in quiescent endothelial cells of large blood vessels or adult capillaries, but this expression is re-induced during healing [Ito, 1998; Tanaka, 1998; Wernert, 1992] and pathological processes such as tumor development [Wernert, 1994]. Ets1 transcripts also accumulate in the fibroblastic stroma surrounding invasive tumors [Wernert, 1994; Bolon, 1995; Bolon, 1996]. Altogether, Ets1 expression generally correlates with the occurrence of invasive processes during normal or pathological developments.
Ets1 expression is induced upon stimulation by angiogenic factors such as acidic and basic FGFs, VEGF and EGF of proliferating and migrating endothelial cells. This expression is switched off as cells reach confluence which can be compared to the loss of Ets1 expression in endothelial cells of adult tissues [Tanaka, 1998; Wernert, 1992].

Members of the Ets family of transcription factors were shown by us and others to be involved in morphogenic properties of endothelial cells in vitro. To investigate the role of these factors in the transcriptional regulation of angiogenesis in vivo, we set up a non-traumatic model that allows daily macroscopic examination of both growth factor- and tumor-induced angiogenesis in mouse ears. In the same animal, we were thus able to record variations in the patterns of neovessels induced and cell populations recruited by the angiogenic factors FGF-2 and VEGF. In this model, inhibition of FGF-2-induced angiogenesis by the pharmacological compound TNP-470, was readily observed, demonstrating that the mouse ear model is also useful in the evaluation of anti-angiogenic strategies.
Our functional analysis of Ets transcription factors activity utilized a competitor protein, Ets1-DB, a dominant negative Ets1 mutant lacking the transactivation domain. Retrovirus-mediated expression of Ets1-DB inhibited FGF-2-induced angiogenesis, while the expression of Ets1-DB in cancerous and stromal cells disturbed tumor-induced angiogenesis. These results illustrate the value of the ear model and highlight the role of Ets family members in the transcriptional regulation of tumor angiogenesis.

Bolon, I., E. Brambilla, et al. (1996). Lab. Invest. **75**: 1-13.
Bolon, I., V. Gouyer, et al. (1995). Am J Pathol **147**(5): 1298-310.
Ito, M., T. Nakayama, et al. (1998 Biochem Biophys Res Commun **246**(1): 123-7.
Laudet, V., C. Hanni, et al. (1999). Oncogene **18**(6): 1351-9.
Lelievre, E., F. Lionneton, et al. (2001). Int J Biochem Cell Biol **33**(4): 391-407.
Sharrocks, A. D., A. L. Brown, et al. (1997). Int J Biochem Cell Biol **29**(12): 1371-87.
Tanaka, K., N. Oda, et al. (1998). J Cell Physiol **176**(2): 235-44.
Wernert, N., F. Gilles, et al. (1994). Cancer Res **54**(21): 5683-8.
Wernert, N., M. B. Raes, et al. (1992). Am J Pathol **140**(1): 119-27.

Computer-assisted interpretation of hybridized microarrays – Molecular profiling to predict survival in lymphoma

Andreas Rosenwald, M.D.
Metabolism Branch, National Cancer Institute (NCI), Bethesda, MD, USA

Initial gene expression profiling of diffuse large B-cell lymphoma (DLBCL) using Lymphochip cDNA microarrays revealed that this single diagnosis contains at least two distinct diseases that differ in the expression of hundreds of genes (1). One DLBCL type, termed germinal center B-like (GCB) DLBCL, strongly resembles normal germinal center B cells whereas the other type, termed activated B-like (ABC) DLBCL, instead resembles mitogenically activated blood B cells. These DLBCL types have distinct clinical outcomes following anthracyclin-based chemotherapy.
An international consortium, the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) has been formed to use gene expression profiling to provide a molecular diagnosis for all lymphoid malignancies. An expanded analysis of 274 DLBCL cases confirmed the existence of the GCB and ABC DLBCL subgroups but demonstrated that additional subgroups exist. Importantly, two recurrent oncogenic events in DLBCL, t(14;18) and c-rel locus amplification, were only observed in GCB DLBCL, demonstrating that the DLBCL gene expression subgroups represent pathogenetically distinct diseases.
As expected, GCB DLBCL patients had a relatively favorable prognosis, but the DLBCL subgroup distinction did not fully capture the variability in survival of these patients. Clinical data were therefore used to discover genes that influence survival. The genes that predicted survival were functionally classified into gene expression signatures of biological processes (2). Most of the predictor genes fell into gene expression signatures that reflected the cell of origin, proliferation rate, and the host immune response (stroma reaction). These gene expression signatures were combined to create a multivariate outcome predictor that was validated on an independent set of cases. The outcome predictor was used to stratify patients into quartiles that had strikingly distinct 5-year survival rates of 73%, 71%, 34% and 15%. The outcome predictor functioned independently of the International Prognostic Index (IPI). This gene expression-based outcome predictor incorporates defined biological features of DLBCL tumors and should therefore prove useful in identifying alternative therapies for DLBCL patients who are not likely to be cured by conventional multi-agent chemotherapy.

Alizadeh, A. et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 403, 503-511 (2000).
Shaffer, A.L. et al. Signatures of the immune response. Immunity 15(3), 375-385 (2001)
Rosenwald, A. et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large B-cell lymphoma. N Engl J Med 346(25), 1937-1947 (2002)

Regulation of *VE-cadherin* and *fli-1* genes by the Ets-1 transcription factor in endothelial cells.

E Lelièvre, F Lionneton, V Mattot, N Spruyt, D Stehelin and F Soncin*.
CNRS UMR8526 - Institut de Biologie de Lille - 1, rue Calmette - 59021 Lille Cedex - France [*fabrice.soncin@ibl.fr]

Ets-1 is the funding member of the ETS family of winged helix-turn-helix transcription factors defined by a conserved DNA binding domain that recognizes the core sequence GGAW, named the ETS Binding Site (EBS). ets-1 is expressed in endothelial precursors during vasculogenesis and in endothelial cells during angiogenesis. Functional EBS have been identified in numerous promoters of genes that are involved in angiogenesis, among which *tie-1*, *tie-2*, *uPA*, *flt-1* or *flk-1* are regulated by Ets-1. We have over-expressed Ets-1 in endothelial cells and in fibroblasts using a retroviral strategy. Over-expression of Ets-1 reduces endothelial cell density at confluence and increases the expression of the endothelial-specific *VE-cadherin* gene. Ets-1 binds to two EBS located in the proximal region of the *VE-cadherin* promoter and mutation of these sites abolishes transactivation of the promoter[1].
The transcripts levels of the ETS transcription factor *fli-1* were also specifically up-regulated in endothelial cells, and not in fibroblasts, which over-expressed Ets-1. Analysis of the mouse *fli-1* gene promoter reveals that the 1 kb region spanning the transcription starts and part of exon 1 is responsible for the response of the promoter to Ets-1. Two known Spi-1 responding EBS and a novel EBS are necessary for the activation of the -270/-41 promoter fragment by Ets-1. The rest of the promoter activity is located in the -986/-505 region where three active EBSs have been identified. Furthermore, endogenous Fli-1 was found to be bound to its own promoter and to be able to promote the transactivation of its gene. These results suggest that Ets-1 activates an auto-regulatory loop of expression of *fli-1* in endothelial cells[2].

In conclusion, we have identified two novel Ets-1 target genes which expression is correlated with blood vessel development. We also show that several ETS transcription factors can together finely regulate gene expression by a different activation of the various EBS present in the promoters of these genes.

[1]Lelièvre E et al. Oncogene (2000) 19:2438-2446.
[2]Lelièvre E et al. J Biol Chem. (2002) 277: 25143-25151.

Development of novel diagnostic and therapeutic tools in Europe - economic aspects

Axel Wellmann, Institute of Pathology, University Bonn, Germany
Axel.Wellmann@ukb.uni-bonn.de

Laser based microdissection enables researchers of various different fields to deal with very minute quantities of sample that may be available for particular tissues or may be of special interest within a specific tissue. However, the possibilities of this technology is still in a phase of being shaped. In addition to serving as a techique for detection of precancerous cells it is helping to record pattern of gene expression in various cell types. It was applied already by the enormous Cancer Anatomy project (CGAP) to identify the Tumor Gene Index, which seeked in part to define patterns of gene expression in normal, precancerous and malignant cells. In the US FY 99 alone, this project forked over $63.5 M.

Furthermore it can be applied to collect cells for all sorts of proteomic studies, using an array of tools like 2D Gel, SELDI and others. Not only big pharmaceutical companies but also smaller, university laboratories are looking into all sorts of high-through put technologies and projects. Even though it takes time, current top of the line 2D gel platforms, completed with robots to cut apart the gels and computers to analyze the information, can study a couple of thousand proteins a day. And according to the journal Science, mass spectrometers now in the prototype phase have the potential to go orders of magnitude faster than the current variety, making it possible to analyze hundreds of thousands of proteins per day. GeneProt has raised US 122 million capital since it was founded in 2000 and Celera Inc. has set aside US$ 1 Billion for proteomic research.

However these technologies still all require a lot of starting material. For one gel alone one has to isolate about 50.000 cells. Depending of the type of tissue and cell the project requires this will take several man working hours just for one analysis.

Therefore, one has to develop new automated microdissectors to accelerate these processes in the not only financially huge market. First developmental steps for automated tools for high throughput isolation are already done; some seems to be promising.

Not surprisingly, these very complex and expensive technologies, which usually lead to incredible data volumes can only be conducted competitively in larger international/european research networks which also depend on generous public support.

Recommended Literature
Srinivas PR Proteomics in early detection of cancer. Clin Chem. 2001 10:1901-11.
Bichsel VE, Cancer proteomics: from biomarker discovery to signal pathway profiling. Cancer J. 2001 1:69-78.
Hanash SM, Integrating cancer genomics and proteomics in the post-genome era. Proteomics. 2002 1:69-75.
Craven RA, Laser capture microdissection and proteomics: possibilities and limitation. Proteomics. 2001 0:1200-4.
Simone NL, Laser capture microdissection: beyond functional genomics to proteomics. Mol Diagn. 2000 4:301-7.
Proteomics, Frost and Sullivan
http://www.cordis.lu/en/home.html
http://europa.eu.int/comm/research/press/2001/pr0208en.html

The stromal reaction under physiological and pathological conditions

N. Wernert, Institute of Pathology, University of Bonn

The extracellular matrix (ECM) appears during evolution with the onset of multicellular life and the formation of a mesoderm during gastrulation is an almost universal feature of development of higher animals. Mesodermal cells and the ECM constitute the stroma which affects an array of fundamental cell functions such as cell adhesion, proliferation and differentiation important for both physiological and pathological processes.

Angiogenesis and lymphangiogenesis are pivotal for development, wound healing, diabetic retinopathy, inflammation, tumor growth and metastasis. The stroma is likewise involved in diseases like arteriosclerosis and restenosis. Multiple roles can also be attributed to the tumor stroma: during tumor invasion, stromal fibroblasts participate in the degradation of the ECM by secreting matrix degrading proteases. Stroma derived factors such as scatter factor as well as interactions between neoplastic cells and the ECM play a role for both tumor cell migration and proliferation. Analyses of stromal reactions take into account more and more actors, the roles of which can be determined by transgenic approaches or laser-asssisted microdissection combined to large scale expression studies at the levels of the transcriptome and the proteome. Protein-protein interactions can be analysed *in-silico* or *in-vivo* by techniques like fluorescence resonance energy transfer (FRET) measurement. Transcription is an important level of regulation of stromal reactions and the Ets 1 transcription factor has been linked to the regulation of both angiogenesis and the expression of matrix-degrading proteases. Specific inhibitors of pathological stromal reactions directed against different receptors and downstream signalling components are currently developed for therapeutic purposes.

Wernert N, Raes MB, Lassalle PH, Dehouck MP, Gosselin B, Vandenbunder B, Stéhelin D (1992) C-ets 1 proto-oncogene is a transcription factor expressed in endothelial cells during tumor vascularisation and other forms of angiogenesis in humans. Am J Pathol, 140: 119-127.
Wernert N, Gilles F, Fafeur V, Bouali F, Raes MB, Pyke C, Dupressoir TH, Seitz G, Vandenbunder B, Stehelin D (1994) Stromal expression of c-ets 1 transcription factor correlates with tumor invasion. Cancer Res, 54: 5683-5688.
Wernert N (1997) The multiple roles of tumor stroma. A review. Virchows Arch, 430: 433-443.
Valter M M, Hügel A, Su Huang H J, Cavenee W K, Wiestler O D, Pietsch T, Wernert N (1999) Expression of Ets-1 transcription factor is related to neoangiogenesis and VEGF/VEGFR-1 expression in human gliomas. Cancer Res, 59: 5608-5614.
Wernert N, Stanjek A, Kiriakidis S, Hügel A, Jha H C, Mazitschek R, Giannis A (1999)Inhibition of in vivo angiogenesis by *ets-1* antisense oligonucleotides - Inhibition of Ets-1 transcription factor expression by the antibiotic fumagillin. Angew Chem Int Edit, 38: 3228-3231.
Behrens P, Rothe M, Wellmann A, Krischler J, Wernert N (2001) The Ets 1 transcription factor is upregulated together with MMP 1 and MMP 9 in the stroma of preinvasive breast cancer. J Pathol, 194: 43-50.
Wernert N, Löcherbach C, Behrens P, Wellmann A, Hügel A (2001) Presence of genetic alterations in microdissected stroma of human colon and breast cancers. Anticancer Res, 21: 2259-2264.
Wellmann A, Wollscheid V, Lu H, Ma ZL, Albers P, Schütze K, Rohde V, Behrens P, Dreschers S, Ko Y, Wernert N (2002) Analysis of Microdissected Prostate tissue with ProteinChip Arrays - a way to new insights into carcinogenesis and to diagnostic tools. Int J Mol Med, 9: 341-347

Combined image analysis with laser microdissection and laser pressure catapulting (LMPC) for functional genomics and proteomics

Karin Schütze[1], Andreas Plesch[2], Renate Burgemeister[1], Stefan Thalhammer[1] and Bernd Sägmüller[1]

[1] P.A.L.M. Microlaser Technologies AG Am Neuland 9 und 12, 82347 Bernried
[2] MetaSystems GmbH, Robert-Bosch-Str. 6, 68804 Altussheim

The increasing interest in sampling high numbers of morphologically defined or fluorescently labeled cell areas, single cells or chromosomes for subsequent genetic or proteomic analysis asked for the development of an automated laser microdissection and catapulting device. The RoboSoftware of the PALM microdissection system (PALM® MicroBeam) allows to outline multiple selected specimen throughout the object slide using computer graphic tools and sort them applying different colors. The system is able to collect specimen marked with a certain color and automatically catapults them into a collection device. This process is fast and completely without any mechanical contact. Furthermore, as the selected areas may be separated from the neighboring tissue by microdissection prior to catapulting, there is no danger of contamination with unwanted specimen.

Here we demonstrate for the first time the ”Metafer P” image analysis system which combines LMPC with the MetaSystems.software

”Metafer P” allows the automated detection of rare cells (Metafer4-RCDetect) or the automated finding of chromosome metaphases (Metafer4-Msearch). The rare events are collected and displayed in a software gallery from where their coordinates are transferred to the PALM® RoboSoftware for microdissection or catapulting.
The catapulted samples are collected into a corresponding buffer and forwarded to the desired downstream application.

In proteomic studies the samples are either catapulted into a protein lysis buffer solution and subsequently transferred to the SELDI wafer (Westphal et al., Methods in Enzymology) to analyze protein mass spectra. We also tested the possibility of protein lysis on the SELDI chip after catapulting a cell area directly onto the wafer.

The same attempts we performed using a MALDI wafer. Selected samples of human renal carcinoma cells were analyzed by Microprobe MALDI MS showing inhomogeneous distribution of different types of carcinoma cells and inhomogeneous molecular distribution within cell compartments.

We could demonstrate, that the combination of Image analysis with - laser microdissection and laser pressure catapulting (LMPC) together with SELDI or MALDI are promising tools for functional genomic-proteomic studies.

References:
Westphal et al. *Non-Contact Laser Catapulting: A Basic Procedure for Functional Genomics and Proteomics* (Methods in Enzymology; in press)

Clinical Molecular Signature Discovery via Laser Capture Microdissection and Gene Expression Analysis

Birgit Anderegg

Arcturus GmbH 64546 Mörfelden-Walldorf

Laser Capture Microdissection (LCM) has been developed to provide a fast, robust method for capturing specific cells from tissue for subsequent molecular analysis of pure populations of proteins and nucleic acids. When the LCM technology is used in clinical molecular signature discovery, cells are first procured via LCM, followed by mRNA isolation and a million-fold linear amplification process. Amplified antisense RNA (aRNA) is then hybridized to a microarray, followed by bioinformatic analysis of the microarray data. Arcturus Applied Genomics (AAG), a division of Arcturus in Carlsbad, CA, uses the LCM technology and proprietary gene expression analysis techniques, to identify gene expression “signatures”/profiles and biomarkers. The signatures and biomarkers will lead to a new generation of therapeutic and diagnostic breakthroughs by providing new levels of sensitivity in the detection and stratification of disease, and by assisting in the selection of therapeutic regimens. AAG’s first products will allow for early detection of breast cancer, and will identify lesions that are likely to require more aggressive treatment. In collaboration with researchers at Harvard Medical School, LCM was used to isolate cells from surgical specimens that were identified histologically as atypical hyperplasia (ADH), ductal carcinoma in situ (DCIS), invasive ductal carcinma (IDC), or normal. This grading and sub-typing of breast cancer, with the corresponding molecular signatures, will lead to a microarray chip for diagnostic purposes.