Control of VEGF expression via hypoxia and MAP kinase signaling

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Vascular endothelial growth factor (VEGF), a potent agonist secreted by virtually all cells, controls migration, division and survival of vascular endothelial cells. Disruption of one VEGF allele in mice has revealed a dramatic lethal effect in early embryogenesis, suggesting a key role in vasculogenesis and a very tight level of regulation.

To identify the signalling features controlling VEGF expression (i) we analyzed the regulation of VEGF mRNA in normal and transformed CCL39 fibroblasts, (ii) we dissected the VEGF promoter to identify the signalling pathway(s) controlling its activation in response to growth factors, oncogenes and hypoxic stress, and (iii) we explored the signalling elements controlling mRNA stability. Activation of p42/p44 MAPKs was found to be sufficient to turn on VEGF mRNA. We demonstrated that the p42/p44 MAPK (Erk) signalling cascade controls VEGF expression at least at two levels. In normoxic conditions, MAPKs activate the VEGF promoter at the proximal (−88/−66) GC-rich region where Sp1/AP-2 transcriptional factors bind. This action is mediated via MAPK-dependent phosphorylation of Sp1 at two sites, threonines 453 and 739. These phosphorylations, which occur in vivo when the p42/p44 MAPK signalling cascade is activated, have a physiological significance. Tetracyclin-dependent induction of a double Sp1 mutant (T453A, T739A), not phosphorylatable, suppresses MAPK-dependent VEGF expression. This result clearly identifies two MAPK phosphorylation sites controlling the functionality of Sp1.

Low O₂ tension, through a mechanism that is not fully understood promotes the induction of several genes, including VEGF. This pleiotypic response is under the control of the ‘master’ transcriptional factor HIF-1α, itself regulated by the posttranscriptional control of the subunit HIF-1α. Recently it was established that it is the product of the von Hippel Lindau tumour suppressor gene (pVHL), which targets HIF-1α to the proteasome, an action suppressed by hypoxia. Concomitant to this hypoxic stress, HIF-1α translocates and accumulates in the nucleus where VEGF and several other genes are ‘turned’ on. To address whether nuclear translocation is involved in HIF-1α stabilization, we specifically trapped HIF-1α either in the cytoplasm or nucleus. We established that the oxygen-driven proteasomal degradation of HIF-1α takes place equally well irrespectively of the sub-cellular localization. This result sharply contrasts with the mechanism of degradation of p53, which only occurs in the cytoplasm, and requires the nuclear shuttling activity of the E3-ubiquitin ligase, MDM2. Although we clearly established that p42/p44 MAPKs do not participate in the stabilization of HIF-1α, MAPKs are, however, capable of phosphorylating HIF-1α in vivo. We showed that in vivo phosphorylation of HIF-1α by p42/p44 MAPK strongly enhanced VEGF gene transcription. Finally we demonstrated that the stress-activated protein kinases (JNK and p38/HOG) are essential for increasing the stability of VEGF mRNA.

The VEGF secreted by the tumour cells then acts on VEGF-R1 and R2 to activate several signalling cascades, including PI3 kinase and p42/p44 MAPKs. We showed that the persistent activation of p42/p44 MAPK is critical for controlling cell survival and growth of vascular endothelial cells when grown at confluence.

In conclusion, our results point to at least three major targets of angiogenesis where p42/p44 MAPKs exert a determinant action. A positive action at the level of VEGF expression by targeting two transcription factors: Sp1 and HIF-1α and at the level of endothelial cells by promoting growth and survival. Therefore, inhibitors targeting specifically the Ras >> MAPK signalling cascade will have a profound impact on tumour development because they will suppress the amplification loop leading to cell survival and cell proliferation, at least at two levels: tumour cells and vascular endothelial cells.
References

1. Milanini J, Vinals F, Pouyssegur J, Pagès G. p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. J Biol Chem 1998; 273: 18,165–72.
2. Vinals F, Pouyssegur J. Confluency of vascular endothelial cells induces cell cycle exit by inhibiting p42/p44 MAPK activity. Mol Cell Biol 1999; 19: 2763–72.
3. Vinals F, Chambard J-C, Pouyssegur J. p70 S6 kinase-mediated protein synthesis is a critical step for vascular endothelial cell proliferation. J Biol Chem 1999; 274: 26,776–82.
4. Richard D, Berra E, Gothié E, Roux D, Pouyssegur J. p42/p44 MAP kinases phosphorylate hypoxia inducible factor 1 alpha (HIF-1α') and enhance the transcriptional activity of HIF-1. J Biol Chem 1999; 274: 32,631–7.
5. Gothié E, Richard D, Berra E, Pages G, Pouyssegur J. Identification of alternative spliced variants of human hypoxia-inducible-factor-1 alpha (HIF-1α'). J Biol Chem 2000; 275: 6922–37.
6. Pages G, Berra E, Milanini J, Levy AP, Pouyssegur J. Stress-activated protein kinases (JNK and p38/HOG) are essential for vascular endothelial growth factor mRNA stability. J Biol Chem 2000; 275: 26,484–91.
7. Richard D, Berra E, Pouyssegur J. Non-hypoxic pathway mediates the induction of hypoxia inducible factor 1 alpha (HIF-1α) in vascular smooth muscle cells. J Biol Chem 2000; 275: 26,765–71.
8. Berra E, Pagès G, Pouyssegur J. MAP kinases and hypoxia in the control of VEGF expression. Cancer Metastasis Rev 2000; 19: 139–45.
9. Berra E, Darren E, Richard E, Gothie E, Pouyssegur J. HIF-1 α-dependent transcriptional activity is required for oxygen-mediated HIF-1α degradation. Febs Lett (in press).
Angiogenesis and permeability of blood vessels are regulated by vascular endothelial growth factor (VEGF) via its two known receptors VEGFR-1 and VEGFR-2. Recently, additional VEGF genes have been cloned and new insight has been obtained into the molecular mechanisms regulating the function of the endothelial cells in lymphatic vessels. VEGF-C and VEGF-D have been shown to stimulate lymphangiogenesis, and their receptor VEGFR-3 has been linked to human hereditary lymphoedema, although there is evidence that other genes are also involved. Such molecules may make it possible to regulate angiogenesis and lymphangiogenesis, as well as the tissue edema involved in many diseases. The VEGFR-3 receptor tyrosine kinase is related to the VEGF receptors, but 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 the tenth day of embryonic development due to failure of cardiovascular development and that heterozygous missense mutations of VEGFR-3 that deactivate the tyrosine kinase activity are associated with human hereditary lymphedema. Two mouse models for lymphedema reproduce the essential features of this disease. We have also purified and cloned the VEGFR-3 ligand, VEGF-C, which is made as a precursor protein having an extended N-terminus and a C-terminal half containing extra cysteine-rich motifs characteristic of a protein component of silk. Transgenic mice expressing VEGF-C developed a hyperplastic lymphatic vessel network and show evidence of lymphangiogenesis. However, proteolytically processed VEGF-C was also capable of stimulating VEGFR-2 and was weakly angiogenic. VEGF-C induced vascular permeability, but its mutant point, which retained lymphangiogenic properties and activated only VEGFR-3, did not. 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. When overexpressed as a transgene in the RIP-Tag model of pancreatic β-cell tumors, VEGF-C induced the growth of peritumoral lymphatic vessels and was associated with lymphatic metastasis. VEGF-C overexpression also led to lymphangiogenesis and intralymphatic tumor growth in an orthotopic model of human breast carcinoma in SCID mice. Furthermore, soluble VEGFR-3 blocked these changes. However, VEGF-C is also induced in the blood vessels of various types of human cancer. Ongoing experiments address the role of the VEGFR-3 signaling pathway in embryonic and tumor angiogenesis and the mechanisms of lymphatic metastasis.

References

1. Korpelainen E, Alitalo K. Signaling angiogenesis and lymphangiogenesis. Curr Opin Cell Biol 1998; 10: 159–64.
2. Ferrara N, Alitalo K. Clinical applications of angiogenic growth factors and their inhibitors. Nature Med 1999; 5: 1359–64.
3. Veikkola T, Karkkainen M, Claesson-Welsh L, Alitalo, K. Regulation of angiogenesis via vascular endothelial growth factor receptors. Cancer Res 2000; 60: 203–212.
Autocrine and paracrine interactions of FGF2 with endothelial cells

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Autocrine and paracrine roles of FGF2 in angiogenesis

Tumor cells of different origin, macrophages, and T lymphocytes express fibroblast growth factor-2 (FGF2) in vitro and in vivo. FGF2 lacks a classic signal peptide for secretion. However, cell damage may cause the release of FGF2 from producing cells. Also, an alternative mechanism of exocytosis of FGF2, independent of the endoplasmic reticulum/Golgi pathway, has been proposed. Accordingly, FGF2 has been found to be associated with the extracellular matrix (ECM) of cell cultures and located in the basement membranes of blood vessels. On this basis, FGF2 is thought to exert its effects on endothelial cells via a paracrine mode consequent to its release by other cells and/or mobilization from ECM.

Besides experimental evidence for a paracrine mode of action for FGF2, some observations raise the hypothesis that FGF2 may also play an autocrine role in endothelial cells. Indeed, endothelial cells produce FGF2 that modulates cell proliferation and migration, as well as the production of proteinases and their receptors. In vivo, FGF2 expression occurs in the endothelium adjacent to neoplastic cells in several human tumor types. These neoplasms include neuroblastoma, astrocytoma, glioblastoma, meningioma, pheochromocytoma, melanoma, carcinomas of the stomach and colon, and adenocarcinomas of the larynx, endometrium, and cervix. Thus, FGF2 expression is a common feature of vascular endothelium during tumor angiogenesis.

These observations strongly support the hypothesis that neovascularization may be triggered by one or more molecules released by tumor cells and/or infiltrating inflammatory cells that induce FGF2 upregulation in the quiescent endothelium. In keeping with this hypothesis is the observation that tumor cells of different origin release one or more molecules able to interact with endothelium and to upregulate the expression of FGF2 that, in turn, stimulates the fibrinolytic potential of the endothelial cell in an autocrine manner. In addition, FGF2 itself, thrombin, nitric oxide, and interleukin-2 stimulate FGF2 production in endothelial cells.

FGF2 overexpression in endothelial cells

To investigate the biological consequences of endothelial cell activation by endogenous FGF2, immortalized Balb/c mouse aortic endothelial cells (MAE cells) were transfected with a retroviral expression vector harboring a human FGF2 cDNA. FGF2 transfectants express all FGF2 isoforms and are characterized by a transformed morphology and an increased saturation density. FGF2 transfectants show invasive and morphogenetic behavior in three-dimensional gel that is prevented by anti-FGF2 antibodies, revealing the autocrine modality of the process. The biological consequences of this autocrine activation were investigated in vivo. FGF2-transfected MAE cells induce the growth of highly vascularized tumors. In agreement with these observations, FGF2-transfected MAE cells induce an angiogenic response when implanted in the avascular rabbit cornea. Also, they cause an increase in vascular density and formation of hemangiomas in the chorioallantoic membrane (CAM) when injected into the allantoic sac of the chick embryo. Thus, the data demonstrate that pZipbFGF2-MAE cells induce highly vascularized spindle-cell hemangioendotheliomas in immunodeficient mice that are sustained by recruitment of host elements, including endothelial cells.

Cidofovir has been approved for the treatment of cytomegalovirus (CMV) retinitis in AIDS patients and possesses potent inhibitory activity against various papillomavirus (HPV)-induced tumors in animal models and patients. In addition, cidofovir inhibits the development of murine polyomavirus (PyV)-induced hemangiomas in rats by an as yet uncharacterized antiviral-independent mechanism. We investigated the effect of cidofovir on virus-independent vascular tumors originated by FGF2-T-MAE cells, a subclone of pZipbFGF2-MAE cells. In vitro, cidofovir was cytostatic for FGF2-T-MAE cells. Cidofovir did not affect FGF2-T-MAE cell sprouting in 3D fibrin gel and morphogenesis on Matrigel at non-cytotoxic concentrations. In vivo, cidofovir completely suppressed hemangioma formation on CAM induced by intra-allantoic injection of FGF2-T-MAE cells, without affecting the normal CAM vessels. Intratumoral or systemic administration of cidofovir caused a significant inhibition of the growth of subcutaneous, intraperitoneal, or intracerebral FGF2-T-MAE-xenografts in nude and SCID mice. Drug-induced apoptosis was observed in FGF2-T-MAE tumors as soon as 2 days after the beginning of treatment. Thus, cidofovir appears to inhibit the growth of endothelial-derived tumors via induction of
apoptosis without exerting a direct anti-angiogenic activity. Cidofovir may be explored for the treatment of tumors that are not associated with an oncogenic virus.

**FGF2 overexpression in tumor cells**

Various tumor cell lines express FGF2 \textit{in vitro}. \textit{In situ} hybridization and immunolocalization experiments have shown the presence of FGF2 mRNA and/or protein in neoplastic cells, endothelial cells, and infiltrating cells within human tumors of different origin. Antisense-FGF2 and FGF receptor-1 cDNAs inhibit neovascularization and growth of human melanomas in nude mice. Also, a significant correlation between the presence of FGF2 in cancer cells and advanced tumor stage has been reported. Recent observations have shown that a secreted FGF-binding protein can serve as an angiogenic switch for different tumor cell lines, including squamous cell carcinoma and colon cancer cells. Interestingly, targeting of FGF-binding protein with specific ribozymes reduces significantly the growth and vascularization of xenografted tumors in mice, despite the high levels of VEGF produced by these cells. These data suggest that modulation of FGF2 expression, release, and mobilization may allow a fine-tuning of the angiogenesis process, even in the presence of significant levels of VEGF. This hypothesis is supported by the capacity of the two factors to act synergistically in stimulating angiogenesis \textit{in vitro} and \textit{in vivo}.

To investigate the impact of the modulation of FGF2 expression on the neovascularization at different stages of tumor growth, we generated stable transfectants (Tet-FGF2) from the human endometrial adenocarcinoma HEC-1-B cell line, in which FGF2 expression is under the control of the tetracycline-responsive promoter (Tet-off system). After transfection, independent clones were obtained in which FGF2 mRNA and protein were upregulated compared to parental cells. Also, the conditioned medium of Tet-FGF2 transfectants caused proliferation, urokinase-type plasminogen activator upregulation, migration, and sprouting of cultured endothelial cells. A 3-day treatment of Tet-FGF2 cell cultures with tetracycline abolished FGF2 overexpression and the biological activity of the conditioned medium without affecting their proliferative capacity. Tet-FGF2 cells formed tumors when injected s.c. in nude mice. Administration of 2.0 mg/ml tetracycline in the drinking water prior to cell transplantaion and continously throughout the whole experiment inhibited FGF2 expression in Tet-FGF2 tumor lesions. This was paralleled by a significant decrease in the rate of tumor growth and vascularization to values similar to those observed in lesions generated by parental HEC-1-B cells. Tetracycline administration 20 days after tumor cell implant, although equally effective in reducing FGF2 expression and inhibiting tumor vascularity, only minimally impaired the growth of established Tet-FGF2 tumors.

The results indicate that FGF2 expression deeply affects the initial tumor growth and neovascularization of HEC-1-B human endometrial adenocarcinoma in nude mice. In contrast, the growth of established tumors appears to be independent of the inhibition of FGF2 expression and decreased vascular density. The possibility that a significant reduction of angiogenesis may not affect the progression of large tumors points to the use of anti-angiogenic therapy in the early tumor stage.

**Concluding remarks**

FGF2 exerts angiogenic activity \textit{in vivo} and induces a pro-angiogenic phenotype in cultured endothelial cells. \textit{In vivo}, FGF2 exerts paracrine effects on endothelial cells when released by tumor and/or inflammatory cells. FGF2 may also play an autocrine role in endothelial cells \textit{in vitro} and \textit{in vivo}. FGF2 may therefore represent a target for anti-angiogenic therapies. In order to assess the angiostatic potential of different classes of compounds, novel experimental models can be developed based on the autocrine and/or the paracrine capacity of FGF2.

**Acknowledgements**

The work in our laboratory was supported by grants from Associazione Italiana per la Ricerca sul Cancro, Istituto Superiore di Sanità (AIDS Project), C.N.R. (Target Project Biotechnology), and M.U.R.S.T (Cofin 1999).
Endothelial cell growth and death in angiogenesis

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The survival of endothelial cells is critical for angiogenesis and the maintenance of blood vessel integrity. Recent studies have shown that the Akt protein kinase functions downstream of angiogenic growth factor and cell-matrix attachment signaling pathways to control endothelial cell survival [1]. Related studies have shown that Akt signaling also controls endothelial NO synthesis [2, 3] and endothelial cell migration to VEGF [4]; cellular responses that contribute to new blood vessel growth and stabilization of the vascular network. I will provide evidence showing that Akt signaling in endothelial cells is essential and sufficient for the differentiation of these cells into vascular structures in vitro and that enhanced Akt signaling in vascular endothelium is sufficient to promote blood vessel growth in rabbit model of hindlimb ischemia [5]. Therefore, the Akt signaling pathway in endothelial cells represents a logical target for the identification of drugs to treat vascular insufficiency.

References

1. Fujio Y, Walsh K. Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. J Biol Chem 1999; 274: 16349–54.
2. Fulton D, Gratton J-P, McCabe TJ, et al. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 1999; 399: 597–601.
3. Luo Z, Fujio Y, Kureishi Y, et al. Acute modulation of endothelial Akt/PKB activity alters nitric oxide-dependent vasomotor activity in vivo. J Clin Invest 2000; 106: 493–9.
4. Morales-Ruiz M, Fulton G, Sowa G, et al. Vascular endothelial growth factor-stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt. Circ Res 2000; 86: 892–6.
5. Kureishi Y, Luo Z, Shiojima I, et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nature Med 2000; 6: 1004–10.
Sprouting of new capillaries from pre-existing vessels, or angiogenesis, occurs in several physiological or pathological conditions, such as tumor progression, diabetic retinopathy or rheumatoid arthritis. This local hypervascularization is thought to result from release by tissues of growth factors interacting with their receptors on endothelial cells, which in turn migrate, proliferate and differentiate into new capillaries. Both fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) exert a potent angiogenic activity as determined by the usual corneal pocket assay, but the actual role of FGF2 in pathological angiogenesis remains questionable.

The possibility that inhibitors of angiogenesis might be useful as antitumor therapeutic agents was first suggested almost 30 years ago by J. Folkman. Several products that can inhibit tumor angiogenesis have recently entered clinical trials. The widespread expectation is that these antiangiogenic compounds have the potential to eradicate tumors by killing genetically stable target cells without inducing drug tolerance. However, most of them might not be as specific for tumor endothelial cells as predicted by the pre-clinical data. Therefore another field of investigation focused on the identification of agents targeting only angiogenic vessels but not established vessels. Such a goal requires the identification of ports of entry (receptors, antigens) which either are not expressed or are not activable by their cognate ligand in the normal adult vasculature.

**Theoretical issues**

There is no rationale for discovering new antiangiogenic agents. The demonstration that a given growth factor exerts a pharmacological angiogenic activity does not preclude that its inhibition might be beneficial in reducing pathological angiogenesis. For instance, basic peptides encoded by exon 6 of VEGF 189 aa can chase FGF2 from its storage sites in the corneal stroma and elicit a strong dose and sequence dependent angiogenic response, although they do not bind to VEGF receptors. The straightforward demonstration that heparin binding growth factors such as VEGF or FGF2 act by activating their receptors and not by releasing other growth factors from the extracellular matrix raises several methodologic points. Systemic delivery of growth factors is inefficient because they are sequestered onto heparan sulfates in the extracellular matrix of the vascular wall and therefore do not reach their target receptors.

The large number of heparin binding growth factor receptors makes the deciphering of their functions a difficult task. The measurement of the remaining functions exerted by mutant growth factors of which the binding domain is reduced to only one receptor provides an interesting clue, but the problem of the bioavailability is not solved. The pre-clinical and clinical data can demonstrate unambiguously that a growth factor or a growth factor receptor is up-regulated in pathological vessels and thus would represent a potential therapeutic target. This strategy has led to the discovery of many antiangiogenic agents. However long lasting treatments might affect normal endothelial cells. For instance the inhibition of the kinase activity of VEGFR2 induces pulmonary emphysema. Thus the elucidation of the functions mediated by a single factor requires the preparation of circulating agonists in order to inhibit only those which are not functional in the normal vasculature.

**Mechanisms of action involved in the VTA targeting of known receptors**

We had to construct circulating agonists mimicking the distinct domains of VEGF and FGF2 interactions with their receptors, and therefore we relied on the antidiotopic strategy. We raised several antidiotopic antibodies by priming lymphocytes in the lymph nodes with neutralizing IgG which inhibits production of VEGF or FGF2. The antidiotopic IgG inhibitor of VEGF (Ald-V) or FGF2 (Ald-F) were further purified by affinity chromatography, as the antiVEGF or antiFGF2 compound IgG previously conjugated to CN–Br sepharose. Their specificity for VEGF-R2 or FGF-R1 was ascertained by an radio receptor assay using heparan sulfates deficient CHO cells transfected with the corresponding cDNA sequences. Ald-V and Ald-F2 induced a similar mitogenic effect on microvascular endothelial cells, which were only inhibited by their corresponding immunogen, thus demonstrating the antidiotopic property of Ald-F2 and Ald-V.
Since both antiidiotypic antibodies stimulated endothelial cell proliferation, we determined whether systemic activation of endothelial cell proliferation could modulate tumor angiogenesis. MCF-7 cells grafted in ovariectomized nude mice were challenged with PI-IgG, Ald-F2, Ald-V or VEGF. We found that VEGF could not increase tumor growth whereas Ald-V or Ald-F2 treatments resulted in a similar increase in tumor volume (doubling time reduced to ca. 6 days). Tumor sections immunostained with antiCD31 antibodies demonstrated an increase in vascularization over Ald-V but not Ald-F2 treatments. Image analysis of low magnification photomicrographs demonstrated striking differences in the distribution of endothelial cells in tumor sections. In contrast, no difference in apoptotic cell counts was observed. Upon estrogen addition, xenografts of MCF-7 caused tumor formation, which was increased through distinct mechanisms involving Ald-V (angiogenesis) and Ald-F2 (stroma reaction).

The most striking result was that none of these angiogenic-factor receptors could be activated unless a converting factor had previously switched the phenotype of the endothelial cells.

Organ specificity of VTA

Although the VEGF promoter does not contain consensus steroid-responsive sequences, estrogen can up-regulate VEGF expression at a level comparable to that obtained upon FGF2 or Ald-F2 in MCF-7. Estrogen exposure of endothelial cells that do not express VEGF induced a dose-dependent increase of VEGF expression and a mitogenic effect, which was abolished by a neutralizing antiVEGF antibody. In contrast, any antiFGF2 neutralizing antibody was inefficient in E2-stimulated endothelial cell proliferation. We examined if an increase in VEGF bioavailability might be sufficient to allow the tumor take. When ovariectomized mice did not receive an estrogen pellet, they did not develop tumors whether they had been challenged with antiidiotypic antibodies or not. Similarly VEGF165-transfected MCF-7 cells did not develop tumors in the absence of estrogen whereas when estrogen was present their doubling time was even lower (5 days) than those growing in Ald-V or Ald-F2 challenged mice. Thus, although in vitro experiments have clearly shown that VEGF or estrogen stimulates the expression of VEGFR2, neither constitutive expression of VEGF in MCF-7 cells nor systemic activation of VEGFR2 could circumvent estrogen ablation. This indicates that VEGFR2 and FGFR1 are not functional in skin vessels in which the tumor vascularization occurs. Taken together, these data suggest that the permissive effect of estrogen on tumor angiogenesis resulted from the release by MCF-7 cells of soluble factors which would induce a phenotypic switch of dermal endothelial cells into tumor endothelial cells.

We previously showed that Ald-V failed to activate VEGF receptors in normal vessels, and did not induce vasodilatation leading to hypotension or vascular permeability [1]. However Ald-V induced corneal angiogenesis when inserted in the cornea. More surprisingly the surgical traumatism made during the insertion of the pellet in the corneal stroma is sufficient to induce the angiogenic switch of endothelial cells in the limbal vessels [2]. As expected VEGF and FGF2 induced a similar corneal angiogenesis in normal or castrated animals. Thus, the ‘converting factors’ in inflammatory and estrogen-dependent tumor takes are different.

VTA targeted on VEGFR2

Several lines of evidences have demonstrated that the most potent signaling pathway in the VEGF system is represented by the activation of VEGFR2. Selective inhibition of the binding of VEGF to VEGFR2 through antibodies [3] or peptides [4] is sufficient to reduce tumor growth. Knowing that VEGFR2, although it is expressed but not functional in the normal vasculature, does constitute a target for agonistic antibodies, we conjugated Ald-V to a toxin.

In our laboratory, we separated retina-derived capillaries clones of non angiogenic cells which do not differentiate in 3D cultures in the presence of VEGF from angiogenic cells which do make this differentiation. Although both strains make the differentiation upon FGF2 addition, VEGF induces an increase of bcl-2 expression and prevents TNF-α dependent apoptosis only in angiogenic cells. The immunoconjugate kills only angiogenic endothelial cells but not the non-angiogenic ones.

When injected in nude mice bearing PC3 (human prostate cancer cells) tumors the compound inhibited tumor progression by more than 80%. Conversely, a similar regimen of immunotoxin did not affect the normal vasculature nor the immunity of wild-type mice.

Until recently it has been taken for granted that VTA should bind only to antigens or receptors specifically expressed in angiogenic vessels. The proof of this concept has been mainly demonstrated by the work of P. Thorpe, who inserted foreign genes in tumor endothelial cells and therefore induced tumor infarction by having bispecific antibodies recognize the tissue factor [5]. Promising candidate molecules are represented by endoglin, endosialin, fibronectin isoform. The strategy of phage display [6] or gene subtraction has also provided some
evidence that the number of genes selectively expressed in tumor endothelial cells must have been underestimated because the endothelial compartment represents a very low amount of the tumor and thus the specific genes may have escaped to screening tumor libraries. However, the concept of specific expression should be revisited to achieve a safe Trojan horse strategy.

**References**

1. Malavaud B, Tack I, Jonca F et al. Activation of Flk-1/KDR mediates angiogenesis but not hypertension. Direct therapeutic implication in myocardial ischemia. Cardiovasc Res 1997; 36: 276–81.
2. Ortega N, Jonca F, Vincent S et al. Systemic activation of the vascular endothelial growth factor receptor flk-1 selectively triggers angiogenic endothelial cells. Am J Pathol 1997; 151: 1215–24.
3. Brekken RA, Overholser JP, Stastry VA et al. Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. Cancer Res 2000; 60: 5117–24.
4. Benetruy-Tournaire 2000
5. Huang X, Molema G, King S et al. Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. Science 1997; 275: 547–50.
6. Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 1998; 279: 377–80.
Molecular anatomy of blood vessels

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We, among others, developed a method that allows the identification of peptides with homing capability to different vascular beds after in vivo administration of a phage display random peptide library. The selection is based on the use of peptides that are expressed on the surface of the bacteriophage. Extensive previous work has established that peptide libraries can be used to probe tissue-specific and angiogenesis-related vascular homing. Taken together, this work has uncovered a novel vascular address system. We have isolated peptides that can home to normal blood vessels or sites of angiogenesis through the circulation via this vascular address system. Every normal or diseased organ appears to display a unique signature on its blood vessels that selected peptides can use as a target. We have also developed complementary methods of assessing the distribution of peptides guided to the vasculature, their tissue-specificity, and their target cells.

Angiogenic vasculature as a therapeutic target

It is well established that angiogenesis, the recruitment of new blood vessels, is an important rate-limiting step in solid tumor growth. New antitumor therapies based on the premise that inhibiting angiogenesis suppresses tumor growth are currently being tested in clinical trials. Angiogenesis is a multi-stage process that involves the release and activation of angiogenic factors, endothelial cell migration and proliferation, and differentiation into newly formed capillaries. The neovascularization of diseases with an angiogenic component differentially expresses many cell surface receptors in the endothelium. Identification of novel molecules characteristic of angiogenic vasculature by techniques such as in vivo phage display will improve our understanding of the plasticity of the activated endothelial phenotype and suggest new therapeutic strategies. Thus far, identification and isolation of such molecules has been slow, in large part because endothelium-derived cells undergo marked phenotypical changes when grown in culture. Angiogenic vasculature is an appealing target for cancer therapy since it is composed of non-malignant cells; such endothelium-derived cells are genetically stable and presumably less prone to acquire drug resistance. Targeting angiogenic blood vessels has other advantages such as an improved accessibility to the drug, and an intrinsic amplification mechanism. The idea of therapy directed at angiogenic vasculature has been proposed for more than a decade but its impact has not yet been fully realized. However, an intense effort is currently underway for the development of antiangiogenic strategies because of the fact that solid tumors cannot progress without new blood vessels.

In vivo selection of peptides from phage display libraries enables homing probes to be isolated through a functional screening: the ability to home selectively to blood vessels of an organ, tumor, or defined site. The strategy has allowed the identification of peptides that selectively target the vasculature of normal tissues and that of tumors. These results indicate that the vascular endothelium is modified by the tissue microenvironment in ways that allow differential targeting with circulating peptide ligands.

The system can also permit the identification endothelial cell surface receptors expressed in vivo. This can be accomplished by the discovery of the receptors corresponding to selected ligands. We have assembled a panel of peptide motifs that target the blood vessels of tumor xenografts. These motifs include the sequences ACDCRGDCFG (termed RGD-4C), NGR, and GSL. The RGD-4C peptide binds selectively to v integrins and it has been shown to home to angiogenic vasculature. We identified an aminopeptidase (CD13) as a vascular receptor for the NGR motif. CD13 is strongly expressed in angiogenic blood vessels. Peptides that home to tumor vasculature have been used as carriers to deliver cytotoxic drugs, proapoptotic peptides, cytokines, and gene therapy. Generally, the coupling of homing peptides can yield targeted agents that are more effective and less toxic than the parental agents. Taken together, these results indicate that it is possible to isolate ligand-receptor pairs for targeted therapy by in vivo phage display.

Each of these peptides binds to different receptors that are selectively expressed on the vasculature of the target tissues. It is interesting to note that many of these tumor vascular markers are proteases. This finding is not surprising, given the invasive features of malignant tumors; in fact, some of the selective vascular markers are vascular proteases that not only serve as receptors for circulating ligands but also modulate angiogenesis. Also, it is intriguing that some of the markers also serve as viral receptors: v integrins are receptors for adenoviruses, CD13 are receptors for coronaviruses, and MMP-2/MMP9 are receptors for echoviruses. It is
tempting to speculate that bacteriophage – prokaryotic viruses – may use the same cellular receptors of eukaryotic viruses. In fact, the structure of the phage capsid protein provides good evidence bacteriophage share ancestry with animal viruses. More than an evolutionary biology footnote, these findings do suggest that the receptors isolated will have internalization capability, a key feature if one wishes to utilize peptide motifs as therapy carriers targeted to certain cell populations.

Exploring the heterogeneity of the blood vessels may further our understanding of tumor endothelium specificity and may define the role of endothelial cell markers play in angiogenesis and metastasis.

References

Arap W, Pasqualini R, Ruoslahti E. Current Opinion in Oncology 1998; 10: 560.
Arap W, Pasqualini R, Ruoslahti E. Science 1998; 279: 377.
Brooks PC, Clark RA, Cherech DA. Science 1994; 264: 569.
Burg MA, Pasqualini R, Arap W et al. Cancer Research 1999; 59: 2869.
Carmeliet P, Jain RK. Nature 2000; 407: 249.
Carmeliet P, Valtanen H et al. Nature Biotechnology 2000; 18: 1185.
Ellerby HM, Arap W, Ellerby LM et al. Nature Medicine 1999; 5: 1032.
Folkman J. Nature Medicine 1995; 1: 27.
Folkman J. Nature Biotechnology 1997; 15: 510.
Folkman J, in DeVita Jr VT, Hellman S, Rosenberg SA (eds) Cancer: Principles and Practice. Lippincott-Raven, Philadelphia/New York 1997; 3075.
Folkman J. Molecular Medicine 1995; 1: 120.
Fidler IJ, Ellis LM. Nature Medicine 2000; 6: 500.
Iruela-Arispe ML, Dvorak HF. Thrombosis & Haemostasis 1997; 78: 672.
Jain RK. Microcirculation 1997; 4: 1.
Koivunen E, Arap W, Rajotte D et al. Journal of Nuclear Medicine 1999; 40: 883.
Koivunen E, Arap W, Valtanen H et al. Nature Biotechnology 1999; 17: 768.
McLean JW, Fox EA, Baluk P et al. American Journal of Physiology 1997; 273: H387.
Mustonen T, Alitalo K. Journal of Cell Biology 1995; 129: 895.
O’Reilly MS, Boehm T, Shing Y et al. Cell 1997; 88: 277.
Pasqualini R. Quarterly Journal of Nuclear Medicine 1999; 43: 159.
Pasqualini R, Ruoslahti E. Nature 1996; 380: 364.
Pasqualini R, Koivunen E, Ruoslahti E. Nature Biotechnology 1997; 15: 542.
Pasqualini R, Koivunen E, Kain R et al. Cancer Research 2000; 60: 722.
Rak JW, St Croix BD, Kerbel RS. Anti-Cancer Drugs 1995; 6: 3.
Rajotte D, Arap W, Hagedorn M et al. Journal of Clinical Investigation 1998; 102: 430.
Rajotte D, Ruoslahti E. Journal of Biological Chemistry 1999; 274: 11593.
Scott JK, Smith GP. Science 1990; 249: 386.
Smith GP, Scott JK. Methods in Enzymology 1993; 217: 228.
Thurston G, McLean JW, Rizen M et al. Journal of Clinical Investigation 1998; 101: 1401.
Trepel M, Grifman M, Weitzman MD, Pasqualini R. Human Gene Therapy 2000; 11: 1971.
Watson CA, Camera-Benson L, Palmer-Crocker R, Pober JS. Science 1995; 268: 447.
Wickham TJ, Haskard D, Segal D, Kovacs I. Cancer Immunology, Immunotherapy 1997; 45: 149.
Zetter BR. Annual Review of Medicine 1998; 49: 407.
In silico cloning of novel endothelial specific genes: Their role in angiogenesis

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The method

Sequence data analysis in the postgenomic era presents a unique challenge to biologists. Efforts to ascribe biological meaning to genomic data, whether by identification of function, structure or expression pattern are lagging behind sequencing efforts [1]. Nevertheless, the sequence databases house a wealth of information waiting to be extracted.

The endothelium plays a pivotal role in many physiological and pathological processes, including angiogenesis. Endothelium is known to be an exceptionally active transcriptional site. To advance our understanding of endothelial cell biology and to elucidate potential pharmaceutical targets we have developed a new database screening approach to permit the identification of novel endothelial specific genes. The technique combines two independent methods of differential sequence data analysis.

The first strategy involves an expressed sequence tag (EST) cluster expression analysis of the human UniGene gene index [2]. Recurrent gapped BLAST searches [3] were performed at very high stringency against ESTs grouped into two pools. These two pools comprised endothelial cell and non-endothelial cell libraries derived from dbEST [4]. The second strategy employed another datamining tool, the on-line SAGEmap xProfiler. xProfiler that is a part of the National Center for Biotechnology Information's (NCBI) Cancer Genome Anatomy Project (CGAP) [5, 6].

When used alone, the two approaches gave a discouraging number of false positives, however, when combined predictions proved exceptionally accurate. Endothelial specificity of expression was confirmed by a combination of RTPCR and in situ hybridisation.

The genes

Four novel highly endothelial specific genes are being characterised. These have been named endothelial cell-specific molecules 1 (ECSM1 – UniGene entry Hs. 13957), 2 (ECSM2 – UniGene entry Hs. 30089), 3 (ECSM3 – UniGene entry Hs. 8135), and 4 (ECSM4 or magic roundabout (MR) – (UniGene entry Hs. 111518).

ECSM1 has no protein or nucleotide homologues. It codes for a small protein of 24 aa (the longest and most up-stream open reading frame identified in the contig sequence). The size of the mRNA from 5' RACE supports this conclusion.

ECSM2 corresponds to the cDNA from the patent 'cDNAs encoding novel polypeptides from human umbilical vein endothelial cells' [7], EMBL acc. E10591. A 205 aa polypeptide coded by this cDNA is a transmembrane protein with a suggested role in cell adhesion in that it is serine and proline rich, though no exact function has yet been identified.

ECSM3 is a novel endothelial specific zinc containing matrix metalloprotease that is probably involved in matrix remodelling during angiogenic sprouting.

ECSM4 – the fourth gene is the most exciting find in that it shows homology to a family of transmembrane neuronal specific receptors involved in axon guidance, notably the roundabout gene. In view of this homology we have called this gene MR. Roundabout is an axon repulsion mediator whose cognate ligand is slit [8, 9]. A homologue called frazzled acts as an axon attractor in response to its ligand netrin. The receptors are completely neuronal specific and modular, that is the extracellular domain recognises the ligand but the intracellular domain determines the neuronal response and they are interchangeable. The true protein product of MR is likely to be larger than the 417 aa coded in the AK000805 clone since the open reading frame has no apparent up-stream limit, and size comparison to human roundabout 1 (1651 aa) suggests a much larger protein. This has recently been confirmed by 5' RACE analysis. In situ hybridisation has given very interesting results. The analysis has confirmed that expression of MR is completely restricted to the endothelium in man, however, expression is tightly restricted to endothelium within particular vascular beds e.g. the large vessels of the placenta.

Recently intriguing associations between neuronal differentiation genes and endothelial cells have been discovered. For example, a neuronal receptor for vascular endothelial growth factor (VEGF) neuropilin 1 [10] was
identified. VEGF was traditionally regarded as an exclusively endothelial growth factor. Processes similar to neuronal axon guidance are now being implicated in guiding migration of endothelial cells during angiogenic capillary sprouting. Thus EphrinB ligands and EphB receptors are involved in demarcation of arterial and venous domains [11]. It is possible that MR may be an endothelial specific homologue of human roundabout 1 involved in endothelial cell repulsive guidance, presumably with a different ligand since similarity is contained within the cytoplasmic i.e. effector region and guidance receptors are known to have a highly modular architecture [12].

Expression of endothelial specific genes is rarely 100% restricted to the endothelial cell. KDR and FLT1 are both expressed in the male and female reproductive tract: on spermatogenic cells [13], trophoblasts, and in decidua [14]. KDR has been shown to define haematopoietic stem cells [15]. FLT1 is also present on monocytes. In addition to endothelial cells VWF is strongly expressed in megakaryocytes [16, 17], and in consequence present in platelets. Similarly, multimerin is present both in endothelial cells [18] and platelets [19]. Endothelial and haematopoietic cells are descended from the same embryonic precursors and many cellular markers are shared between the two cell lineages (for a review see [20]). RTPCR analysis showed that the genes identified here (ECSM1–3 and MR) have a greater endothelial specificity than does even the classic endothelial marker von Willebrand factor. This is an observation predicted by the in silico analysis.

Our combined datamining approach together with experimental verification is a powerful functional genomics tool. This type of analysis can be applied to many cell types not just endothelial cells. The challenge of identifying the function of discovered genes remains, but bioinformatics tools such as structural genomics, or homology and motif searches can offer insights that can then be verified experimentally.

References

1. Boguski MS. Biosequence exegesis. Science 1999; 286: 453–5.
2. Schuler GD. Pieces of the puzzle: Expressed sequence tags and the catalog of human genes. J Mol Med 1997; 75(10): 694–8.
3. Altschul SF, Madden TL, Schäffer AA et al. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 1997; 25: 3389–402.
4. Boguski MS, Schuler GD. Establishing a human transcript map. Nat Genet 1995; 10: 369–71.
5. Strausberg RL, Dahl CA, Klausner RD. New opportunities for uncovering the molecular basis of cancer. Nat Genet 1997; 15: 415–6.
6. Cole KA, Kriman DB, Emmert-Buck MR. The genetics of cancer – A 3D model. Nat Genet 1999; 21(1): 38–41.
7. Shibayama S, Hirano J, Ono H. cDNA encoding novel polypeptide from human umbilical vein endothelial cell. European Patent Office. Publication number: 0 682 113 A2.
8. Kidd T, Brose K, Mitchell KJ et al. Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell 1998; 92(2): 205–15.
9. Brose K, Bland KS, Wang KH et al. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell 96(6): 795–806.
10. Soker S, Takashima S, Miao HQ et al. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 1998; 92(6): 735–45.
11. Adams RH, Wilkinson GA, Weiss C et al. Roles of ephrinB ligands and EphB receptors in cardiovascular development: Demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev 1999; 13(3): 295–306.
12. Bashaw GJ, Goodman CS. Chimeric axon guidance receptors: The cytoplasmic domains of slit and netrin receptors specify attraction versus repulsion. Cell 1999; 97(7): 917–26.
13. Obermair A, Obraca A, Poht M et al. Vascular endothelial growth factor and its receptors in male fertility. Fert Ster 1999; 72(2): 269–75.
14. Clark DE, Smith SK, Sharkey AM, Charnock-Jones DS. Localisation of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy. Human Reproduction 11(5): 1080–8.
15. Ziegler BL, Valtieri M, Porada GA et al. KDR receptor: A key marker defining hematopoietic stem cells. Science 1999; 285: 1553–8.
16. Sporn LA, Chavin SI, Marder VJ, Wagner DD. Biosynthesis of von Willebrand protein by human megakaryocytes. J Clin Invest 1985; 76(3): 1102–6.
17. Nichols WL, Gastineau DA, Solberg LA, Mann KG Jr. Identification of human megakaryocyte coagulation factor V. Blood 1985; 65(6): 1396–406.
18. Hayward CP, Bainton DF, Smith JW et al. Multimerin is found in the α-granules of resting platelets and is synthesized by a megakaryocytic cell line. J Clin Invest 1993; 91(6): 2639–9.
19. Hayward CP, Cramer EM, Song Z et al. Studies of multimerin in human endothelial cells. Blood 1998; 91(4): 1304–17.
20. Suda T, Takakura N, Oike Y. Hematopoiesis and angiogenesis. Int J Hematol 2000; 71(2): 99–107.
Human methionine aminopeptidase – a novel target associated with inhibition of angiogenesis

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In the last decade, the recognition of angiogenesis as a promising new anti-cancer target has proceeded simultaneously with the discovery and characterization of a novel anti-angiogenic anti-cancer drug class (fumagillin) and its target enzyme (methionine aminopeptidase 2, MetAp2). The parallel progress made in initial studies of fumagillin/angiogenesis and MetAp2 is reviewed below, followed by results of more recent studies conducted after the establishment of their enzyme–substrate relationship. Finally, some current ideas concerning cellular mechanisms and consequences of MetAp2 inhibition by fumagillin and related compounds are discussed.

MetAp2

Eukaryotic methionine aminopeptidases were cloned and characterized in the mid-1990s. Their reaction mechanisms have been extensively described and reviewed. These enzymes catalyse the co-translational removal of initiator methionines at N-termini of a subset of eukaryotic proteins. To be a substrate, a nascent protein must, at a minimum, contain a small, uncharged amino acid next after methionine at the N-terminus. This substrate specificity is highly conserved; the identity of actual cellular substrates remains under investigation.

In eukaryotes, two types (1 and 2) of methionine aminopeptidase have been defined, based upon sequence alignments. They are more similar to their prokaryotic counterparts than they are to each other, although their catalytic domains are homologous. In SDS-polyacrylamide gels, purified MetAp1 migrates as a polypeptide of molecular mass somewhat smaller than that of MetAp2 (ca. 40 vs. ca. 60 kDa). Purified MetAp2 is dependent on cobalt, as well as on zinc, manganese, and nickel; which of these metals are critical physiologically has not been established unambiguously.

In yeast, neither type 1 nor type 2 methionine aminopeptidase is essential, but the double null construct is lethal, suggesting that the two classes have overlapping roles. In the absence of evidence from knockout animals or highly selective inhibitors, it is not known whether similar functional redundancy exists in higher eukaryotes, including mammalian cells.

Fumagillin and its analogues

Fumagillin is a natural product identified originally from a fungal (Aspergillus fumigatus) contamination of an endothelial cell culture, producing local rounding up of the endothelial cells. Fumagillin and certain structurally similar molecules, notably the semisynthetic analogue TNP-470, have become important tool compounds in the study of angiogenesis. They inhibit the growth of cultured endothelial cells at nanomolar or greater potency, but are inactive against most other cell lines at concentrations less than several micromolar. Selectivity for endothelial lines is not absolute, however, as recently a few non-endothelial cell lines have been found to be inhibited by nanomolar concentrations of fumagillin. Thus, although the cellular mechanism of fumagillin is not completely understood, it is characterised by inhibition of DNA synthesis (late G1 block) and by expression or activation of various cyclins and cyclin-dependent kinases. Cell strain-selective transcriptional effects are considered to be the ultimate step in the cell biology of fumagillin-like compounds. TNP-470 inhibits angiogenesis in vivo, and has demonstrated anti-tumour activity in several animal models. It is currently in Phase II/III clinical trial as a single agent as well as an agent in combination with conventional cytotoxics such as paclitaxel.

Fumagillin and MetAp2

In the late 1990s, the cellular target of fumagillin was established to be MetAp2. Mammalian cell extracts subjected to conventional affinity chromatography yielded MetAp2 as the fumagillin binding protein. A second part of the study reported that among various methionine aminopeptidase-engineered yeast strains evaluated for sensitivity to fumagillin, only a deletion strain lacking the type 1 enzyme (ΔmetAp1) was inhibited. This finding is
consistent with the inability of fumagillin to inhibit MetAp1 and with lethality of the double mutant (redundancy of function of type 1 and type 2 enzymes). Taken together, these biochemical and genetic data strongly indicate that fumagillin inhibits MetAp2 in yeast, leading to growth inhibition and ultimately to the death of the organism. Coincidentally, fumagillin was shown to be an irreversible inhibitor of MetAp2, but not MetAp1. Crystal structure studies of enzyme and enzyme–inhibitor complexes have provided elegant substantiation of this finding.

It is tempting to make the extrapolation that in vivo and clinical activity of TNP-470 is due to inhibition of MetAp2, and that later generation inhibitors of this enzyme will also be clinically active anti-tumour agents by virtue of their anti-angiogenic activity. While it is clear that MetAp2 is a target of fumagillin in higher eukaryotes, a compelling genetic component of the argument is lacking. Biochemical and biological data have established two important correlations, however, in the case of mammalian cells. First, limited structure-activity data based on TNP-470 show a correlation between MetAp2 inhibition and anti-proliferative activity. Second, TNP-470 inhibits endothelial cell proliferation and fumagillin-MetAp2 binding with the same dose dependence. Moreover, antisense oligonucleotides to human MetAp2 block endothelial cell proliferation. All of this evidence suggests that MetAp2 is a critical target for the anti-angiogenic, and thus anti-tumour activity of fumagillin and related compounds.

It remains, however, to establish how inhibition of MetAp2 by fumagillin results in the observed biological and pharmacological activity in mammalian cells. No correlation exists between cell strain sensitivity to fumagillin and related compounds and cellular content of MetAp1 and MetAp2, as both fumagillin sensitive and fumagillin insensitive strains contain similar amounts of type 1 and type 2 enzyme. Two attempts to explore the relationship between MetAp2 inhibition and biological effect have recently been described. Proteomics methodology has been employed to determine critical substrates of MetAp2 in cells by subtractive analysis (cells ± fumagillin) of proteins subjected to two-dimensional gel electrophoresis. Several proteins were identified which appeared to be altered as anticipated under conditions of MetAp2 inhibition, but none had any obvious relevance to the DNA/cell cycle effects of fumagillin. A second approach, based on an early observation that TNP-470 inhibits activation of cyclin dependent kinases, led to the finding that this fumagillin analogue induces p53 activation and expression of p21$^{CIP/WAF}$, a natural inhibitor of cyclin-dependent kinases. These inductions are reportedly specific for endothelial cells, providing a possible explanation for selective anti-angiogenic activity of fumagillin and related compounds. A clear relationship between this effect and MetAp2 inhibition is not evident from these data, however, and it is necessary to postulate that an event downstream of MetAp2 action is cell-type dependent and favors p53 activation and cell cycle blockade in endothelial cells. Additional studies of this sort should lead to a clearer understanding of the operation of fumagillin-like compounds on their target MetAp2 in a cellular setting.

**Conclusion**

Based on current knowledge and understanding, it appears very likely that in vivo and clinical activity of fumagillin analogues such as TNP-470 arises from inhibition of MetAp2 in cells, and that later stage inhibitors of this enzyme will also be clinically active anti-tumour agents by virtue of their anti-angiogenic activity. Current efforts are aimed at identifying such inhibitors and exploring the cellular consequences of MetAp2 inhibition to find the molecular basis of its link with angiogenesis.

**References**

1. Bradshaw RA, Brickey WW, Walker, KW. N-terminal processing: the methionine aminopeptidase and N$^{\text{acetyl}}$ transferase families. TIBS 1998; 23: 263–7.
2. Ingber D, Fujita T, Kishimoto S et al. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. Nature 1990; 348: 555–7.
3. Liu S, Widom J, Kemp CW et al. Structure of human methionine aminopeptidase-2 complexed with fumagillin. Science 1998; 282: 1324–7.
4. Sin N, Mheng L, Wang MQW et al. The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAp2. PNAS(US) 1997; 94: 6099–103.
5. Yeh J-R, Mohan R, Crews CM. The anti-angiogenic agent TNP-470 requires p53 and p21$^{CIP/WAF}$ for endothelial cell growth arrest. PNAS(US) 2000; 97: 12782–7.
Modulation of angiogenesis by ginsenosides

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Ginseng has been reported both to suppress the growth of new blood vessels in tumours, and conversely to promote angiogenesis during wound healing. To resolve this apparent contradiction, we have been studying pure ginsenosides extracted from Panax ginseng for their modulatory effects on angiogenesis. In an in vitro wounding assay, two ginsenosides induced a concentration-dependent proliferation of serum-starved human umbilical vein endothelial cells. In a mouse sponge-granuloma model, one of these ginsenosides produced a marked angiogenic response. Further studies will focus on the angiogenic or antiangiogenic activities of pure ginsenosides and their molecular mechanisms of action, with a view to develop novel ginsenoside derivatives for the appropriate treatments of angiogenic diseases.

Acknowledgement

This work has been supported by Research Grants Committee of Hong Kong.
VEGFs, angiopoietins and ephrins: Emerging views on the individual and collaborative actions of these angiogenic factors

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The recently discovered Angiopoietins join the vascular endothelial growth factor (VEGF) family as the only known growth factor families largely specific for vascular endothelial cells. A single member of the very large Ephrin family of growth factors, EphrinB2, also appears to have selective actions on blood vessels.

Emerging data indicates that VEGF and the Angiopoietins work in complementary and coordinated fashion during normal vascular development and re-modeling. While VEGF is critical for the initiation of vessel formation, Angiopoietin-1 seems to serve an important later role during vessel maturation and stabilization, by optimizing formation of the vessel wall. While the hypervascularity formed in the presence of excess VEGF is leaky and fragile (and associated with tissue edema and hemmorhage), vessels made in the presence of excess Angiopoietin-1 are actually resistant to vascular leak induced by VEGF or inflammatory mediators. Correcting an imbalance towards excess VEGF seen in many pathologic states, by either blocking the excess VEGF or administering additional Angiopoietin-1, could decrease plasma leakage and the resulting edema and thus have important clinical benefit in numerous disease settings, including diabetic retinopathy, tumor-associated ascites, brain edema associated with tumors or ischemic stroke, as well as in arthritis and other inflammatory conditions.

Recent gene knockout studies suggest that Angiopoietin-2 also plays a key role within the vessel wall, in regulating vessel de-stabilization and vessel regressions, and in regulating development of lymphatics. Interestingly, recent work with EphrinB2 also suggest a key role in vessel wall formation, particularly for arterial vessels.

Re-examination of tumor angiogenesis, in the context of considering the roles of these various factors, has led to a new view of how tumors interact with the vasculature. In contrast to prevailing dogma suggesting that tumors arise as avascular masses that require new angiogenesis for their initial vasculaturization and further growth, we suggest that tumors can instead grow by coopting existing vessels. Development of a potential therapeutic we term our ‘VEGF Trap’, which is perhaps the most potent VEGF antagonist described and can completely block tumor angiogenesis and endothelial proliferation, has allowed us to conclusively demonstrate that tumors can indeed grow invasively by vessel cooption in the absence of angiogenesis or endothelial proliferation; tumor growth is however limited by using the VEGF Trap to block new angiogenesis. In addition to regulating vessel formation and survival, the balance between VEGF and Angiopoietins seems to regulate the function and quality of tumor vessels, e.g., tumor vessels are often subject to excess VEGF and are thus leaky and fragile (and associated with tissue edema and hemmorhage), due to relative lack of the vessel stabilization, maturation and anti-permeability functions provided by Angiopoietin-1. In addition, Angiopoietin-2 and EphrinB2 are dramatically induced in tumor vessels, providing perhaps the best early markers of coopted or angiogenic tumor vessels, and thus targets of anti-angiogenesis approaches.

The discovery of multiple new angiogenesis regulatory factors, together with novel characterization approaches made possible by the use of knockout and transgenic technologies, is clearly leading to a new understanding of the molecular basis of blood vessel development that seems likely to have important therapeutic implications.

References

1. Suri C, Jones PF, Patan S et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 1996; 67: 1171–80.
2. Maisonpierre PC, Jones PF, Wiegand SJ et al. Angiopoietin-2: A natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997; 277: 55–60.
3. Holash J, Maisonpierre PC, Compton D et al. Vessel cooption, regression, and growth tumors mediated by angiopoietins and VEGF. Science 1999; 284: 1994–98.
4. Thurston G, Rudge J, Ioffe E et al. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nature Medicine 2000; 6: 460–63.
5. Yancopoulos GD, Davis S, Gale NW et al. Vascular-specific growth factors and blood vessel formation. Nature 2000; 407: 242–8.
Inhibition of VEGF signal transduction in cancer

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There is a significant unmet need for cytostatic (i.e. non-cytotoxic) therapy to stabilise or slow the progression of solid tumour disease, particularly in non-hormone dependent cancers. Anti-angiogenics are one approach which might satisfy this need. Experience from the anti-hormonal cytostatics suggests that chronic dosing will be required and hence oral delivery of drug is preferable. Side effects will also need to be compatible with the clinical benefit.

There is evidence that VEGF contributes to tumour growth through the promotion of both angiogenesis and vascular permeability. Sequestration of VEGF with antibody has been shown to reduce tumour growth in animal models (e.g. [1]). There are several technical options for modulation of VEGF activity. We have sought to abrogate VEGF signalling by inhibiting VEGF receptor-associated tyrosine kinase (VTK) activity with low molecular weight compounds. The in vitro properties of such an agent would include potent inhibition of VTK enzyme(s) in isolation, and in vascular endothelial cells, at concentrations which do not show direct effects on the normal growth of endothelial or tumour cells. The anti-tumour effects in vivo should therefore be attributable to inhibition of VEGF signalling rather than a direct effect on tumour cell proliferation.

In order to screen for VTK inhibitors, the cytoplasmic portion of the VEGF receptor Flt-1, which encodes the tyrosine kinase (TK) domain, was cloned and expressed. The extracted enzyme was intrinsically active. Screening of a panel of kinase inhibitors revealed a distinct selectivity profile for Flt-1 TK compared to another TK derived from the EGF receptor. Hints of a structure–activity relationship in an anilinoquinazoline series were confirmed by robotic synthesis. Further medicinal chemistry led to the identification of highly potent Flt-1 TK inhibitors which were also active against the TK activity of another VEGF receptor, KDR.

In order to satisfy the perceived clinical requirements of an anti-angiogenic (see above), several in vivo attributes were considered to be necessary. A successful VTK compound would need to have (a) pharmacokinetics compatible with chronic dosing, (b) anti-tumour at well-tolerated doses and (c) evidence to support the proposed mode of action in vivo. Unfortunately, neither the degree nor duration of VTK inhibition required to achieve anti-tumour activity in animal models (or man) was known. Furthermore, the best early VTK inhibitors identified had suboptimal bioavailability in mice.

Further medicinal chemistry led to a series of compounds with much improved bioavailability though this was accompanied by some loss of potency. Encouragingly, consistent with the proposed anti-angiogenic mechanism, a prototype compound was found to be active in all human tumour xenograft models tested (n = 9). Comparison of a series of compounds revealed that the best anti-tumour activity correlated with sustained blood levels of drug. This indicates that sustained inhibition of VEGF signalling is required – at least in a rapidly growing xenograft model.

ZD4190 [2] was selected from this group of compounds for clinical development. ZD4190 is a potent (IC50 0.05 μM) inhibitor of KDR TK activity and VEGF signalling in human umbilical vein endothelial cells but only has direct effects on endothelial and tumour cell proliferation at much higher concentrations (>10 μM). In xenograft models, ZD4190 was significantly active (50 mg/kg/day) against all tumours tested and was well tolerated during chronic dosing (28 days). In all cases, tumours were established (0.5 cm³) before dosing was commenced. In one particularly aggressive xenograft, PC3, sustained inhibition of growth has been demonstrated with daily dosing of ZD4190 for 10 weeks. In line with expectations, when therapy was withdrawn tumour growth resumed at the normal rate after a short delay. Acute effects of ZD4190 on tumour vascular permeability have also been demonstrated using gadolinium-enhanced magnetic resonance imaging [3].

Further rat pharmacology provides support for the proposed mode of action of ZD4190. We have previously described the use of the anaesthetised rat to show the acute hypertensive effects of large doses of VEGF or bFGF [4]. Orally dosed ZD4190 (25 mg/kg) inhibits the effects of VEGF, but not bFGF, in this model indicating that this compound can inhibit VEGF activity in vivo. During chronic dosing in the immature (growing) rat, ZD4190 produces epiphyseal hypertrophy at the femur growth plate. This is consistent with the inhibition of angiogenesis which is a critical step in the conversion of cartilage to bone during growth. Similar effects have also recently been described with VEGF antibody [5].

Another VTK inhibitor, ZD6474, with a similar in vitro and in vivo efficacy profile, has superseded ZD4190 in development. This compound has been used to explore further the anti-tumour profile of a VTK inhibitor. In order to examine the effect of tumour size on efficacy, treatment of PC-3 and Calu-6 xenografts with ZD6474 was delayed until tumours had grown to a volume of up to 1.4 cm³. In the Calu-6 xenograft, ZD6474 restrained the
growth of tumours irrespective of size at the start of treatment. In contrast, ZD6474 administration caused marked regression of PC-3 tumours with the most dramatic effects seen on the largest tumours. The observation of regression suggested that in some tumours, chronic treatment may not be mandatory for control of tumour growth. This was confirmed in further experiments with the PC-3 xenograft which demonstrated that intermittent treatment with ZD6474 was effective in controlling/reversing tumour growth [6].

In summary, we have identified ZD6474, a potent inhibitor of VTK activity in vitro with pharmacokinetic properties compatible with chronic oral administration. This compound significantly inhibits the growth of a range of histologically distinct, established tumour xenografts in vivo, and at doses which are unlikely to have any direct effect on tumour cells. This and other in vivo evidence supports the proposed mode of action of ZD6474. ZD6474 is in phase I clinical development for the treatment of cancer. Another inhibitor of VEGF signaling, AZD2171, is also in development.

References

1. Kim KJ, Li B, Winer J et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature (London 1993); 362: 841–4.
2. Wedge SR, Ogilvie DJ, Dukes M et al. ZD4190: An orally active inhibitor of VEGF signalling with broad-spectrum anti-tumour efficacy. Cancer Res 2000a; 60: 970–5.
3. Wedge SR, Waterton JC, Tessier JJ et al. Effect of the VEGF receptor tyrosine kinase inhibitor ZD4190 on vascular endothelial permeability. Proc Am Assoc Cancer Res 1999; 40: 2741.
4. Curwen JO, Ogilvie DJ. Production of the angiogenic factors VEGF and bFGF at tumour sites may also confer an acute haemodynamic advantage to the tumour. Br J Cancer 1997; 75: P89.
5. Ryan AM, Eppler DB, Hagler KE et al. Preclinical safety evaluation of rhuMAbVEGF, an antiangiogenic humanised monoclonal antibody. Tox Path 1999; 27: 78–85.
6. Wedge SR, Ogilvie DJ, Dukes M, et al. VEGF receptor tyrosine kinase inhibitors as potential anti-tumour agents. Proc Am Assoc Cancer Res 2000b 41: Abst. 3610.
Drug based approaches which selectively damage tumour vasculature

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Tumour vasculature represents an appealing target for the development of new cancer treatments. Most of the research effort has been focused on antiangiogenic therapy. Many agents are currently undergoing clinical evaluation and even more are at the preclinical development phase. These agents target one or more of the key processes involved in angiogenesis e.g. endothelial cell proliferation, migration, and basement membrane degradation. Such agents can prevent the growth of new blood vessels but will have little or no effect on the vasculature already preexisting in the tumour at the time treatment commences. As a result, recently there has been an increased interest in developing agents, which irreversibly damage the already formed neovasculature in tumours. One of the most effective group of agents identified to date are certain tubulin depolmerising agents the most studied compound being Combretastatin A4 phosphate (CA4P) which is in phase I trials.

CA4P has been demonstrated to induce rapid, selective and extensive blood flow reductions in a large number of experimental tumour systems. These effects have been observed in spontaneous as well as transplanted tumour models. More recently ongoing clinical studies have demonstrated that tumour blood flow reductions are also seen in humans following administration of CA4P. Although the mechanisms responsible for these effects have not been completely defined it has been shown that CA4P induces rapid shape changes in proliferating endothelial cells in culture. The fact that these changes occur over a similar time course to that seen with blood flow effects in vivo and that quiescent endothelial cells are much more resistant to such changes provides further evidence that this process is a key component in its action. These selective effects on cell shape reflect the critical role the tubulin cytoskeleton plays in maintenance of the elongated shape of endothelial cells when they are newly formed. Whilst a change in cell shape is not critical event for many cellular functions for an endothelial cell in a vessel such a change can result in rapid alterations in vessel function.

The ability of CA4P to induce blood flow changes in tumours at doses below that required to see antiproliferative/cytotoxic effects in normal tissues is in contrast to other tubulin depolmerising agents such as colchicine and the vinca alkaloids. In vitro the effect of CA4P on endothelial cell shape is reversed 4 h after drug exposure unlike colchicine and vinblastine where changes persist for at least 24 h following drug exposure, reflecting the rapid reversibility of the tubulin binding properties of CA4P. In addition CA4P has a more rapid distribution and terminal half life in the plasma. These two factors reduced drug exposure to critical tissues and reversibility of action will minimise the antiproliferative effects of CA4P. However, since the vascular changes once initiated in vivo may lead to irreversible vascular dysfunction in the individual vessels due to additional effects like coagulation the vascular effects of CA4P can be fully retained.

Although CA4P can induce significant and extensive vascular shutdown in tumours it at best can induce stable disease when administered in a daily schedule to tumour bearing mice. This lack of overt response reflects the fact that even in tumours where complete vascular shutdown is induced a viable rim of tumour cells feeding off the normal vasculature surrounding the tumour remains. These cells and the stimulus from the localised hypoxia induced as a result of the ischemia result in continued growth and revascularisation. The most effective strategy to eliminate these remaining cells is to combine CA4P with conventional radiation and cytotoxic treatments. Several studies including those from our own laboratories have shown significant benefit using this approach. Interestingly whilst little or no response to CA4P has been observed in experimental systems when used as a single agent, clinical responses have been reported.

CA4P has been shown to be an effective neovascular damaging agent in a large number of experimental tumour systems however it is clear that there is a heterogeneous response with some tumours being more sensitive than others. One protective factor, which appears to be a determinant of response to CA4P and other vascular damaging agents, is nitric oxide (NO). NO plays a key role in several functions including preventing neutrophil adhesion to the vessel wall, increasing vessel patency and increasing cell survival during ischemic insult. Several studies in experimental tumours have established that NO synthase inhibitors can significantly enhance the activity of CA4P. Another factor, which may be a determinant of vascular response, is vessel maturity as determined by the number of vessels staining for smooth muscle actin. Identification of these modulators of response should help identify new strategies to enhance the activity of tumour vascular damaging agents.

In addition to cancer there are several other disease pathologies where abnormal neovascularisation is an integral part of disease progression these include ocular disorders (macular degeneration and retinopathy), psoriasis and arthritis. Approaches which selectively damage neovasculature potentially offer a way of reversing...
the disease pathology by rendering nonfunctional the newly formed vasculature. The results obtained with CA4P in an experimental model of ocular neovascularisation indicate it can not only stop but reverse the process of neovascularisation. Further studies evaluating the potential of CA4P in pathologies outside of oncology are now ongoing.

In summary, the development of neovascular damaging agents is a separate approach to antiangiogenic therapies since the primary aim is not to stop new vessel formation but to seek out and destroy newly formed and immature vessels. CA4P is the lead compound in the emerging field of tubulin binding vascular damaging agents (TBVDAs). It has been established that the drug causes vascular dysfunction in a range of experimental tumours and also in tumours in humans. The mechanism of action centers around the critical role of tubulin in maintainance of cell shape in recently formed endothelial cells.
Promoting angiogenesis: Angiogenesis gene therapy for peripheral vascular disease

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Severe peripheral artery occlusive disease: Treating 'no option' patients with angiogenic growth factors

Patients with severe peripheral artery occlusive disease (PAOD) and multi-segment disease not amenable to surgical or endovascular therapy may benefit from the use of angiogenesis based therapy. A number of naturally occurring growth factors can induce and/or promote angiogenesis by stimulating endothelial cell growth and migration. Among growth factors directly acting as endothelial mitogens, two are particularly potent: fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). Recent preclinical studies have shown that both recombinant FGF and VEGF may successfully augment collateral development in models of hind-limb ischemia. The gene transfer by viral and non-viral DNA vectors that express these factors, have been shown to also induce angiogenesis in a number of animal models and, recently, in clinical trials as well.

Gene therapy with angiogenic growth factors

Because of their very short half-life, the use of recombinant angiogenic growth factors is difficult. High dosage or repeated protein administration may lead to hypotension, proteinuria or even atherosclerotic plaque instability. Gene transfer holds promise for the angiogenic treatment of ischemic diseases by providing a sustained but low synthesis of the growth factor restricted at the site of administration.

Clinical data with angiogenic gene therapy applied for the treatment of PAOD are limited. Both viral and non-viral vectors are used to transfer the angiogenic gene to the ischemic tissues. The possibility of using a modified catheter in an intra-vascular delivery of an adenovirus expressing a marker gene, Lac-Z, has been demonstrated in patients with peripheral artery disease [1]. Preliminary clinical results suggest that intra-muscular [2, 3] or intra-arterial [4] administration of a non-viral vector encoding VEGF may lead to gene transfer. Therapeutic benefit was also described in patients with severe peripheral artery disease [2] or Buerger’s disease [3].

Non-viral gene therapy with FGF-1

We selected non-viral approaches for the treatment of ischemic diseases using the FGF-1 gene as a therapeutic gene. An expression cassette containing a naturally occurring truncated form of FGF1 fused to a heterologous secretion signal, flanked by a strong viral promoter and polyadenylation sequences, was engineered. This expression cassette was inserted into a plasmid backbone with a conditioned origin of replication and the resulting plasmid named NV1FGF [5].

Efficacy following intra-muscular gene transfer of NV1FGF into the ischemic muscle was demonstrated in a rabbit model of chronic hind-limb ischemia obtained by complete excision of femoral artery. FGF1 gene transfer led to the formation of angiographically visible collateral blood vessels. Angiographic score showed a significant effect of FGF1 gene transfer dose, as compared to β-galactosidase (β-Gal) marker gene (B. Witzenbichler, A. Mahfoudi, D. Branellec and J. Isner, unpublished data).

NV1FGF phase I study in patients with severe PAOD: Preliminary results

Based on the available preclinical data, supporting the safety and biological activity of FGF1 plasmid in various models, a phase I, multi-center, open label trial was initiated in patients with severe PAOD. This study is ongoing but preliminary data will be presented.

Objectives and study design

The primary objective of this study is to evaluate the safety and tolerability of increasing single and repeated (2) doses of NV1FGF administered by intra-muscular injection. The secondary objectives of the study are to...
determine the biological activity of NV1FGF on collateral artery development as well as to evaluate change in hemodynamic (ankle brachial index (ABI), toe brachial index (TBI), transcutaneous oxygen pressure (TcO2)), clinical (rest pain, healing of ulcers) and biological parameters (serum levels of FGF-1, serum levels of anti-FGF-1 antibodies and plasmid biodistribution into plasma, urine and tissues).

NV1FGF was given intra-muscularly into the diseased limb. Patients were followed regularly for evaluations up to 6 months after NV1FGF administration.

Preliminary safety results

Forty patients received NV1FGF in either single or repeated (2) doses. NV1FGF was well tolerated. Pain at the injection site was very rarely reported. There were no signs of systemic or local inflammatory reaction, nor reported complications or serious adverse events possibly or probably related to NV1FGF administration. There were no abnormalities in the laboratory assessments of hematology, blood chemistry or urinalysis parameters.

Blood samples were regularly collected for batch analysis of circulating levels of FGF-1 in serum, and biodistribution of the NV1FGF plasmid in urine and plasma. All the serum samples were negative for FGF-1. Biodistribution in plasma was limited and transient. No plasmid was detected in urine.

Preliminary clinical activity results

Signs of biological and clinical activity have been observed in most patients. An interim analysis conducted in the first 15 patients treated indicated that for all clinical parameters (ABI, TBI, TcO2, Pain, ulcer healing) the direction of NV1FGF effect was improvement.

Conclusions

Single and repeated doses (<2) of NV1FGF were well tolerated by the patients, confirming the safety of the gene therapy strategy for delivery of angiogenic growth factors. Preliminary biological and clinical data warrant the initiation of placebo-controlled phase II studies.

References

1. Laitinen M, Mäkinen K, Manninen H et al. Adenovirus-mediated gene transfer to lower limb artery of patients with critical limb ischemia. Human Gene Ther 1998; 9: 1481–6.
2. Baumgartner I, Pieczek A, Manor O et al. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation 1998; 97: 1114–23.
3. Isner J, Baumgartner I, Rauh G et al. Treatment of thromboangiitis obliterans (Buerger’s disease) by intramuscular gene transfer of vascular endothelial growth factor: preliminary clinical results. J Vasc Surg 1998; 28: 964–973.
4. Mäkinen K, Laitinen M, Manninen H et al. Catheter-mediated VEGF gene transfer to human lower limb arteries after PTA. Circulation 1999; 100: 1–770.
5. Soubrier F, Cameron B, Manse B et al. pCOR: A new design of plasmid vectors for nonviral gene therapy. Gene Ther 1999; 6: 1482–8.
There are currently more clinical trials being performed on anti-angiogenic agents as potential cancer treatments than of drugs that fall into any other category of activity. This shows a remarkable commitment to the belief that anti-angiogenesis may be of therapeutic benefit in the treatment of cancer.

Around fifty angiogenesis inhibitors are currently in phase I or phase II trials. A few have progressed to phase III trials, although none has yet received FDA approval. It appears that as with other drugs designed for use in oncology, there is going to be a high fallout rate of anti-angiogenics from the trials. These include some matrix metalloprotease inhibitors and VEGF tyrosine kinase inhibitors such as SU101. Despite this, a low toxicity anti-angiogenic with proven biological efficacy has the potential to be a pharmaceutical heavyweight.

Angiogenesis is a complex multistep process that presents several targets for intervention. Primary anti-angiogenic targets identified include (1) inhibition of matrix metalloproteases, (2) antagonism of VEGF induced angiogenesis, (3) abrogation of endothelial cell adhesion to the extracellular matrix and (4) inhibitors of endothelial proliferation that show specificity for the endothelial cell in their activity. Representative lead compounds with each of these activities will be described.

In recent years, there has been much excitement generated by the discovery of naturally occurring angiogenesis inhibitors that are proteolytical fragments of larger molecules that are devoid of such activity. Examples include, angiogiostatin that is a fragment of plasminogen and endostatin that is a fragment of collagen type VIII. The progress of such compounds in clinical trials will be described.

Another major consideration in the potential use of anti-angiogenic drugs in the treatment of cancer is what therapeutic approach should be adopted. Four possibilities are immediately apparent (1) the adjuvant situation, (2) as a single agent therapy, (3) combination therapy together with, for example, conventional cytotoxics or radiotherapy and (4) in prevention of what has been termed ‘incipient angiogenesis’. Combination therapy is most likely to be the first application of anti-angiogenic drugs. Examples of these approaches will be discussed and the case put forward for ‘optimal therapy’ involving use of an anti-angiogenic in combination with low dose chemotherapy. While use of anti-angiogenics in oncology clearly leads the field and is likely to do so in the foreseeable future, the use of anti angiogenics in other pathologies will be considered. This will aim to highlight the advantages as well as the pitfalls of such an approach.

References

1. Fox SB, Gasparini G, Harris AL. Angiogenesis: Pathological, prognostic and growth factor pathways and their link to trial design and use of anti-angiogenic drugs. Lancet Oncol 2001 (in press).
2. Hegedorn M, Bikfalvi A. Target molecules for anti-angiogenic therapy: From basic research to clinical trials. Crit Rev Oncol Hematol 2000; 34: 89–110.
3. Talks K and Harris AL. Current status of antiangiogenic factors. B J Haematol 2000; 109: 477–89.
Methionine aminopeptidase-2: A biologically relevant target of the anti-angiogenic compound fumagillin

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Methionine aminopeptidase-2 (MAP2) is inhibited by fumagillin, a natural product that inhibits growth of endothelial cells. Fumagillin has anti-angiogenic activity in vivo, presumably owing to selective MAP2 inhibition. TNP-470, a fumagillin derivative, is in phase II clinical trial. Evidence of cellular action of fumagillin on MAP2 was found in both yeast and cultured mammalian cells. Deletion of the related enzyme methionine aminopeptidase-1 (MAP1) sensitized yeast to fumagillin, while episomal overexpression of yeast wt MAP2 in a chromosomal Δmap1 strain reversed sensitization. Expression of human MAP1 afforded full resistance, indicating complementation of yeast MAP1 function. To link MAP2 to fumagillin action in mammalian cells, murine melanoma (B16F10) cells, which like endothelial cells are sensitive to pM-nM concentrations of fumagillin, were transfected with a plasmid carrying a CMV promoter driving human MAP2 and a gene for G418 resistance. Colonies selected in 500–800 nM G418 were isolated and evaluated for fumagillin sensitivity after 72 h. Growth inhibition curves of most G418-resistant clones were rightward-shifted. The amount of shift varied depending on the clone and is currently being correlated with MAP2 content by western blotting. These results suggest that MAP2 is a functionally relevant target of fumagillin in mammalian cells and that overexpression of the protein renders yeast and mammalian cells resistant to fumagillin.

Gastric injury activates sequential expression of VEGF, bFGF, Ang2 and Ang1 genes in mucosa bordering necrosis: Key factors for initiation of angiogenesis and wound healing?

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Angiogenesis, an essential component of wound healing is regulated by angiogenic growth factors. Our aims were to: (a) determine in normal and wounded gastric mucosa expression of VEGF, bFGF, Ang1, Ang2 and their temporal and spatial relationship; and (b) to characterize ultrastructural features of angiogenesis in wounded gastric mucosa in vivo. Methods: Ninety rats received intragastrically 8 ml/kg of 50% ethanol or saline (placebo) and gastric samples were obtained 3, 6, 24, 48 and 72 h after injury. Studies: (1) quantification of angiogenesis with videoimage analysis system; (2) VEGF, bFGF, Ang1 and Ang2, mRNAs by quantitative RT/PCR and proteins by Western blotting and immunohistochemistry; and (3) transmission EM. Results: Twenty-four hours after injury, 81% of microvessels in the mucosa bordering necrosis demonstrated endothelial sprouting, formation of microvascular tubes and ultimately microvessels reconstruction by 48–72 h. Mucosal injury triggered a significant overexpression of VEGF, bFGF, Ang2 and Ang1 mRNAs and proteins in the mucosa bordering necrosis (VEGF 385%–630% with peak at 3 h; bFGF 220%–310% with peaks at 6–24 h; Ang2 and Ang1 220%–390% with peaks at 6–24 h; all P < 0.001. Increased signal for bFGF and Ang2 co-localized with VEGF in sprouting microvascular tubes. Conclusions: (1) Gastric injury triggers angiogenesis in mucosa bordering necrosis. (2) Angiogenesis is preceded by overexpression of VEGF mRNA and protein, bFGF, Ang2 and Ang1 mRNAs and proteins, respectively. (3) Co-localization of VEGF, Ang1, Ang2 and bFGF suggests their local interactions in a temporally and spatially ordered manner.
NSAIDs inhibit endothelial proliferation by interference with cell cycle regulatory proteins: Cyclin D, p21 and pRb. A key to anti-angiogenic action?

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Angiogenesis involves endothelial cell migration, proliferation and tube formation. Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit angiogenesis in vivo and in vitro, but the molecular mechanisms are not fully explained. This study was aimed to determine in rat aortic endothelial and in HUVEC cells whether a nonselective NSAID, indomethacin (INDO) affects bFGF-induced angiogenesis, endothelial cell proliferation and expression of the cell cycle regulatory proteins. Methods: Rat aortic endothelial cells and HUVEC were cultured with and without INDO (0.1–0.5 mM) for 16 h. Then, vehicle or bFGF 1–10 ng/ml were added for 3–48 h. Studies: (1) in vitro angiogenesis; (2) cell proliferation with 3H-thymidine uptake; (3) expression of: (a) cyclins D1 and E; (b) cyclin kinase inhibitors (CKIs) p21 and p27; and (c) pRb phosphorylation. Results: INDO inhibited angiogenesis in vitro by 9-folds (P < 0.001); reduced endothelial cell proliferation (both at baseline, and bFGF-stimulated) by 7–15-folds (P < 0.001) at each study time (3–48 h), significantly inhibited bFGF-activated cyclin D1 protein expression (>163% inhibition; P < 0.001), increased baseline and bFGF-activated CKI p21 (but not p27) expression by >98% (P < 0.001), and abolished bFGF-induced pRb phosphorylation. Conclusions: (1) indomethacin dramatically inhibits in vitro angiogenesis and endothelial cell proliferation; (2) the latter action is mediated by INDO-induced interference with cyclin D1, pRb phosphorylation and by activation of CKI p21.

Single local injection of VEGF and angiopoietin-1 DNAs dramatically accelerates gastric ulcer healing and improves quality of scar

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Ulcer healing is critically dependent on angiogenesis. We have previously shown that gastric wounding triggers activation of VEGF and angiopoietin 1 (Ang1) genes. We studied whether single injection of naked DNA encoding for (a) VEGF, (b) Ang1 or (c) a + b affects healing of gastric ulcers. Methods: Gastric ulcers were induced in rats and 1 h later 100 g of plasmid DNA encoding for cDNA of either rhVEGF165, rhAng1 (both myc and 6xHis epitope tagged), combination of both, or control plasmid were injected into the submucosa around the ulcer. In some groups VEGF neutralizing antibody was injected i.v. 2 and 4 days after ulcer induction. Gastric specimens were obtained at 2, 7 and 14 days. Studies: (1) Ulcer size. (2) Quantitative histology. (3) Immunostaining for VEGF, Ang1, Factor VIII RA. (4) Expression of tagged VEGF mRNA. (5) Western blotting. Results: At 14 days in groups receiving plasmids encoding rhVEGF165 or rhAng1, ulcer healing was accelerated by >400%, and >190% vs. control plasmid (P < 0.001). Healing was preceded by a significant increase in angiogenesis in granulation tissue at 7 days, by 72% and 280%, respectively (P < 0.005); correlation between enhanced angiogenesis and ulcer healing was: r = 0.99. In group injected with rhVEGF plasmid, VEGF165 neutralizing antibody blocked angiogenesis and reversed acceleration of ulcer healing (by 26 ± 18%). Co-injection of rhVEGF + rhAng1 plasmids improved quality of ulcer healing and microvessel maturation. Conclusions: (1) Single injection of naked DNA encoding for VEGF and/or Ang1 significantly accelerates gastric ulcer healing. (2) This action is reversed by VEGF neutralizing antibody, indicating essential role of VEGF. (3) This study demonstrates feasibility of gene therapy for ulcer treatment.
Mutations interfere with VEGFR-3 function in primary lymphedema

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Vascular endothelial growth factor (VEGF) is the major regulator of vasculogenesis and angiogenesis through VEGF receptors 1 and 2 [1]. VEGF receptor-3 (VEGFR-3) binds VEGF-C and VEGF-D and it is required for normal cardiovascular development during embryogenesis. In adult tissues, VEGFR-3 expression becomes restricted mainly to the lymphatic endothelium. We have cloned and analyzed the human VEGFR-3 gene and characterized missense mutations affecting the receptor signaling function in patients with hereditary lymphedema [2–4]. All disease linked mutant VEGFR-3 alleles analyzed encoded a tyrosine kinase negative protein. According to our studies, the mutant receptor interferes with the wild-type receptor signaling, and the turnover time of the mutant receptor is longer than that of the wild-type receptor, leading to accumulation of the mutant receptor on the cell surface. These phenomena lead to reduced VEGFR-3 signaling and hypoplasia of the subcutaneous lymphatic vessels in lymphedema patients. Our results establish the importance of VEGFR-3 in normal lymphatic development and mutations in VEGFR-3 as a cause of primary lymphedema. We also have developed a mouse model for lymphedema, with one inactive VEGFR-3 allele. Like human patients, these mice show a reduced number of lymphatic vessels in the skin. This mouse model now allows us to study the possible treatments for lymphedema with VEGFR-3 as a target.

References
1. Karkkainen MJ, Petrova TV. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. Oncogene 2000; 19: 5598–605.
2. Iljin K, Karkkainen MJ, Lawrence EC et al. VEGFR-3 gene structure, regulatory region and sequence polymorphisms. FASEB J (in press).
3. Karkkainen MJ, Ferrell RE, Lawrence EC et al. Missense mutations interfere with vascular endothelial growth factor receptor-3 signaling in primary lymphedema. Nat Genet 2000; 25: 153–59.
4. Irrthum A, Karkkainen MJ, Devriendt K et al. Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR-3 tyrosine kinase. Am J Hum Genet 2000; 67: 295–301.

Effect of prostaglandins and adenosine on angiogenic growth factors in skeletal muscle cells and in endothelial cells

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Although it is well known that regular contractile activity induces an increased capillarisation in skeletal muscle, little is known about the regulation of angiogenesis in this tissue. Several growth factors may be involved in the angiogenic process, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Furthermore prostaglandins and adenosine, two naturally secreted vasodilators in contracting skeletal muscle, have been shown to stimulate proliferation of endothelial cells. To investigate whether prostaglandins and adenosine regulate VEGF and bFGF gene expression in skeletal muscle tissue, we added 0.1 mg/ml indomethacin (a cyclooxygenase inhibitor) and 50 μM AOPCP (a 5’-nucleotidase inhibitor) to primary skeletal muscle cells and endothelial cells in culture. Northern blot analysis of the skeletal muscle cells and endothelial cells revealed the existence of VEGF and bFGF mRNA in both cell types. Densitometric mRNA/GAPDH levels for VEGF in skeletal muscle cells were increased 3-fold after 2 h and 6-fold after 26 h of indomethacin treatment compared to muscle cells with no inhibitor added (P < 0.05). Addition of indomethacin to endothelial cells increased VEGF mRNA levels 3.5-fold after 48 h (P < 0.05). Addition of indomethacin to skeletal muscle or endothelial cells did not have an effect on bFGF mRNA levels in either cell type. Inhibition of 5’-nucleotidase in skeletal muscle and endothelial cells did not alter VEGF or bFGF mRNA/GAPDH levels. These results indicate that prostaglandins reduce VEGF mRNA in skeletal muscle cells and endothelial cells but not bFGF mRNA levels, whereas adenosine does not appear to regulate VEGF or bFGF mRNA levels in these cell types. Supported by the Danish National Research Foundation (504-14).
Vascular endothelial growth factor (VEGF) plays a crucial role in the proliferation of endothelial cells

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In this stimulating study, we tested the mitogenic activity of VEGF on bovine retinal microvascular endothelial cells (BRMECs) by BRMECs with different VEGF concentrations (0–200 ng/ml). Our results demonstrate that VEGF stimulates the proliferation of BRMECs in a concentration-dependent manner. VEGF can promote BRMEC proliferation at 1–5 ng/ml VEGF and above 50 ng/ml, while proliferation was significantly less at the intermediate concentrations. Immunoblotting analysis revealed that the VEGF did not cause a dose-dependent change in KDR receptor expression. However, while exogenous VEGF induced tyrosine phosphorylation of KDR it was significantly reduced at 20 ng/ml VEGF. This was associated with an increase in SH-PTP1 (a membrane associated protein tyrosine phosphatase) and eNOS when BRMECs were exposed to 10 and 20 ng/ml VEGF. Immunoprecipitation and selective immunoblotting demonstrated that activation of KDR by VEGF enhances SH-PTP1 activity and eNOS expression, which in turn lead to two diverse events: one is that SH-PTP1 dephosphorylates KDR which weakens VEGF mitogenic activity, the other is that eNOS increases NO production, which in turn lowers SH-PTP1 activity via S-nitrosylation. Our results demonstrate a complex association between different membrane proteins which is highly dependent on VEGF concentration.

Fabrication of alginate scaffolds containing controlled delivery microspheres of VEGF and bFGF to enhance scaffold vascularization

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Tissue graft regeneration relies on the formation of new blood vessels (angiogenesis) for the supply of essential nutrients and removal of waste products. The angiogenesis process is regulated by a variety of growth factors, including the most potent vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). To enhance the vascularization of tissue-engineered implants following their implantation to replace a failing organ, our group has developed 3D alginate scaffolds containing controlled delivery microspheres of VEGF or bFGF. The composite scaffolds were characterized a highly porous structure, wherein the microspheres were evenly distributed throughout the scaffold. In vitro, the composite scaffolds released the growth factors over a month. In vivo, following implantation into rat peritoneum, the released VEGF or bFGF significantly enhanced the rate and the extent of blood vessel formation within the composites, as compared to control composites without growth factors. Histological cross-sections of the composite alginate scaffolds, 21 days after implantation, revealed that the VEGF or bFGF composites contained 70 capillaries per square millimetre, while the control composites contained only 20 capillaries per square millimetre. The enhanced vascularization should contribute to biograft viability and integration into the host.
Interaction of endostatin, an endogenous angiogenesis inhibitor, with heparin

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Endostatin (22 kDa) is a C-proteolytic fragment of collagen XVIII, localized in vascular basement membrane zones of various organs. It is an inhibitor of angiogenesis and tumor growth, and it binds heparin and heparan sulfate, which are complex sulfated polysaccharides. Endostatin might inhibit angiogenesis by binding to the heparan sulfate proteoglycans of the cell surface, which are involved in growth factor signaling. The aim of this work is to study the interaction between endostatin and heparin by surface plasmon resonance and to characterize the structural features of the heparin sequence involved in endostatin binding. Recombinant human endostatin produced by 293 EBNA cells bound immobilized heparin with an affinity constant in the micromolar range. The binding was strongly inhibited by heparin and dermatan sulfate (97% and 49% inhibition, respectively). Heparan sulfate was a very weak inhibitor (12% inhibition), and chondroitin sulfate did not inhibit the binding. Heparin oligosaccharides also inhibited the interaction. The inhibition increased with increased oligosaccharide length (10% for a hexasaccharide up to 80% for a hexadecasaccharide). The minimal size required for endostatin binding seems to be a dodecasaccharide (57% inhibition). Protection assays are in progress to determine the sequence of heparin involved in the binding, together with competition experiments to determine if endostatin binding to heparin prevents the binding of FGF-2 and thus interferes with the angiogenic activity of this growth factor.

Neoangiogenesis in the injured rat spinal cord: A key to axonal regrowth

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Mediators of extracellular matrix protein degradation, the matrix metalloproteinases (MMPs) involved in inflammation, angiogenesis and wound healing, are interesting targets for neuroprotection. Recent data reported an activation of MMP2 (gelatinase A) and MMP9 (gelatinase B) after cerebral ischemia and spinal cord injury (SCI). The present study uses in situ zymography to locate at cellular level the gelatinase activity in a rat spinal cord closed contusion model. The kinetic of gelatinase activation was monitored by in situ zymography on 20 μm cryostat sections. The spinal sections were incubated overnight with fluorescein-quenched DQ gelatin (25 μg/ml). The gelatin digestion yielded cleaved fluorescent peptides enabling the detection of gelatinolytic activity at cellular level. A strong gelatinase activity was detected 24 and 48 h after injury, at the lesion site in and around vascular structures, infiltrated cells and neurons. After a week and up to 2 months, the gelatinolytic activity was associated with a fibrous scar at the lesion epicentre and with longitudinally oriented blood vessels penetrating the lesion from both stumps and cells located in the necrotic area. The present data suggest that spontaneous tissue repair following spinal cord contusion includes a rapid neovascularisation of the necrotic zone by longitudinally oriented growing blood vessels, followed by the regrowth of nerve fibres, including supraspinal fibres, along the same axis. We conclude that revascularisation gives rise to a supportive matrix for nerve regeneration and permits the ingrowth of nerve fibres at the lesion site. The present findings indicate that a combination of substances can reduce MMP activity and stimulate angiogenesis to promote the neuritic extension may provide a new therapeutical approach for the treatment of SCI.
RNA splicing alterations associated with angiogenesis

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It is becoming increasingly clear that alternative mRNA splicing represents an important cellular control mechanism. A recent publication estimated that at least 35% of disease-associated genes are alternatively spliced [1]. The importance of alternative splicing in the field of angiogenesis is exemplified by a number of genes including the hypoxia-induced factor-1α (HIF-1α), fibroblast growth factor receptor-1 (FGFR1), vascular endothelial growth factor (VEGF) and VEGF-receptor 1 (VEGF-R1, FLT-1). Exonhit Therapeutics SA has developed an innovative approach to qualitative genome analysis that allows the identification of alterations in mRNA splicing, using its gene profiling technology DATAS (Differential Analysis of Transcripts with Alternative Splicing). DATAS allows the isolation of a library of alternatively spliced sequences associated with specific disease states. This is achieved through isolation of mRNA from two cellular populations (for example angiogenic and non-angiogenic tumour material), followed by reverse transcription to generate cDNA. Heteroduplexes between mRNA from one population and cDNA from the other population are allowed to form and are isolated. RNase H treatment is then used to release mRNA that does not hybridise to the heteroduplex. This RNA, which encodes the alternative splice event, can then be isolated, reverse transcribed into cDNA and cloned. Thus, a library of alternatively spliced mRNA sequences for a given condition can be isolated. DATAS has been applied to in vivo and in vitro hypoxic and angiogenic model systems in order to obtain a library of alternatively sliced clones associated with angiogenesis. This angiogenic library contains genes involved in early signal transduction events, cell survival and metabolic adaptation. Validation of the library has been performed and demonstrated that both alternative spliced and differentially expressed angiogenic sequences are present. This library, therefore, provides a powerful tool for the characterisation of alternative splicing during angiogenesis and offers a route for the identification of new anti-angiogenic drug targets.

Reference

1. Hanke J et al. TIGS 1999; 15(10): 389–90.

Expression of vascular endothelial growth factor and study of papillary blood vessels pattern in primary superficial bladder carcinoma

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Background: Angiogenesis is crucial during the development and progression of solid tumours. This study was performed to understand the clinical significance of vascular endothelial growth factor (VEGF) expression and study the pattern of papillary blood vessels in primary superficial bladder cancer. Materials and methods: A cohort of 43 cases of 13 pTa (30.2%) and 30 pT1 (69.8%) papillary transitional cell bladder carcinomas were studied using immunohistochemistry to detect VEGF. The immunoexpression of CD31 but PAS, reticulin and orcein stain were examined. Expression of VEGF was correlated with clinical and pathological indicators of bladder cancer in order to determine their prognostic value. Results: High expression of VEGF was detected in 43 cases (100%). In 10 cases (23.3%) the VEGF immunoreactivity was heterogeneous. There was no positive association of VEGF expression with histological grading, multifocality and stage. The pattern of VEGF immunoreactivity was not related with risk of tumour recurrence (P > 0.1). All papillary blood vessels were surrounded by a PAS and reticulin positive extracellular matrix; however, elastin (orcein stain) was never observed. These vessels were also positive for CD31 but none of the blood vessels observed were only positive for CD31. In our series, we did not observe any occurrence of neoangiogenesis or necrosis. Conclusions: According to our results, papillary blood vessels network in superficial urothelial bladder cancer is formed by capillaries and venules and represent an adequate vascular system. Expression of VEGF is one of the characteristics of these tumours and may play a role in the development and maintenance of papillary vascular network. In this study, VEGF expression does not provide any prognostic information, but may be a potential target for anti-VEGF strategies in the treatment of, or prophylaxis against, recurrent superficial bladder cancer.
In vitro and in vivo characterization of RGD based protein carriers for tumor vasculature targeting

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Introduction: RGD-based protein conjugates to target αvβ3 on the tumor vasculature were developed. By covalently attaching RGD peptides to a protein backbone, macromolecular conjugates were produced that are expected to exert a long circulation time in the body and enable the attachment of multiple effector molecules. The aim of this study was to analyze the biodistribution of these conjugates in tumor-bearing mice.

Methods: A nαvβ3 directed RGD peptide was chemically coupled to a protein backbone at peptide:protein ratios 2:1, 5:1, 10:1 and 25:1. Binding of the resulting conjugates to endothelial cells was analyzed by flow cytometry and by using an αvβ3-mediated endothelial cell adhesion assay. Furthermore, the biodistribution of the RGD conjugates was studied in tumor-bearing mice using radiolabeled conjugates and immunohistochemistry. For this latter purpose, we developed a polyclonal rabbit antibody specific for the RGD-peptide conjugated to the protein backbone.

Results: Binding of the RGD conjugates to endothelial cells was dose-dependent and dependent on the number of peptide molecules per protein backbone. The conjugates were furthermore still able to inhibit the adhesion of endothelial cells to αvβ3 ligand vitronectin. In vivo studies revealed that all RGD conjugates localized in the tumor. Moreover, this localization appeared to be endothelium specific. Furthermore, the RGD conjugates were also present in liver, kidney and spleen in various cell types. Discussion: Coupling of RGD peptide to a protein backbone resulted in conjugates with specificity for interaction with the αvβ3 integrin on endothelial cells. The conjugates appeared to localize in the tumor vasculature. However, the localization in other organs needs further investigation into the cellular distribution patterns in these organs as well as the quantitative comparison of tumor vasculature vs. non-tumor distribution. The effects of attachment of effector molecules to the RGD-protein conjugates will furthermore be studied in relation to anti-tumor activity and toxicity elsewhere in the body.

Identification of novel modulators of angiogenesis using arrayed adenoviral expression libraries

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Biomedical research is entering the post-HUGO era. Many predicted ORFs/cDNAs can be extracted from the genome sequence, but definitely not every single ORF/cDNA, let alone splice variants. The search for novel proteins and their function is the next logical step in the quest for a complete understanding of the biology of the cell and the organism. At Galapagos, we have constructed an arrayed adenoviral expression library (PhenoSelect library) derived from placenta. By screening the PhenoSelect library in a number of high through-put assays, we want to assign novel functions to both known and previously unidentified genes. Recently we have set-up two such assays, which allow us to screen for novel modulators of angiogenesis. These assays study endothelial cell proliferation and capillary formation, two key processes during angiogenesis. To select for secreted proteins, we infect producer cells (HeLa cells) with the adenoviruses of our PhenoSelect library and then transfer the resulting conditioned medium to the assay plate containing the target cells (HUVEC). If an angiogenic growth factor is present in the conditioned medium, this factor will, depending on the assay, induce either proliferation or capillary formation. Currently we are screening the first 10,000 viruses from our PhenoSelect library in the HUVEC proliferation and capillary formation assay. Resulting hits will be evaluated in endothelial cell migration assays, expression studies and in animal models.
Acidosis prevents endothelial cell apoptosis by Axl activation

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Endothelial cells (EC) are exposed to an acidic environment in a variety of pathological and physiological conditions. We have previously shown [1] that hypercarbic acidosis (pH 7.0) inhibits EC proliferation and protects EC from serum deprivation-induced apoptosis. In order to characterize the mechanism(s) involved in this process, a cDNA array screening was performed on human umbilical vein endothelial cells (HUVEC) cultured in a medium with 2% fetal calf serum at pH 7.4 or 7.0. After 6 h at pH 7.0 tyrosine kinase receptor Axl expression was enhanced 3.3-fold as compared to pH 7.4. This modulation was further confirmed by RT-PCR (3 ± 0.9-fold; P < 0.03), Northern (3.6 ± 0.1-fold; P < 0.0003) and Western blot (10 ± 1.8-fold; P < 0.004). In a 48-h time course study the highest induction was achieved between 24–48 h. Northern blot analysis showed that the ligand of Axl, the survival factor growth arrest specific gene 6 (GAS-6), was also enhanced about 2-fold, after 48 h at pH 7.0. Functional analyses demonstrated that full length Axl cDNA overexpression reduced serum deprivation-induced apoptosis in HUVEC cultured at pH 7.4 by 69.4 ± 7% (P < 0.0001), compared to mock transfected cells. Further, either overexpression of soluble Axl or antisense GAS-6 mRNA partially reverted the effect of acidosis to protect EC from apoptosis. These data suggest that the ligand-activated Axl pathway may play a role in EC survival during acidosis.

Reference

1. D’Arcangelo D, Facchiano F, Barlucchi LM et al. Circ Res 2000; 86: 312–8.

Different effect of high and low shear stress on PGDF release by endothelial cells and smooth muscle cell migration

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It has been previously shown that, in the presence of an intact endothelium, low shear stress (SS) enhances and high SS inhibits artery wall thickening. The mechanism(s) responsible for this modulatory effect of SS are poorly characterized. In the present study we analysed the effects of conditioned media (CM) from bovine aortic endothelial cells (BAEC) exposed to laminar SS of 5 dyne/cm² (SS5) or 15 dyne/cm² (SS15), for 16 h, on SMC migration. In a Boyden chamber assay SMC migration in response to CM from BAEC exposed to SS5 (CMSS5) and SS15 (CMSS15) was 45 ± 5.5 and 30 ± 1.5 cells/field, respectively (P < 0.05). PDGFAA levels in CMSS5 and CMSS15 were 9.2 ± 7 and 18 ± 5 ng/ml, respectively (P < 0.05); PDGFBB levels in CMSS5 and CMSS15 were 38 ± 10 and 53 ± 11 ng/ml, respectively P < 0.05). CMSS15 induced phosphorylation of both PDGF receptor α (PDGFRα) and β (PDGFRβ) on SMC. In the presence of CMSS15, neutralizing antibody anti-PDGFAA enhanced SMC migration to a level comparable to that of CMSS5; in contrast antibodies anti-PDGFB abolished SMC migration. SMC transfection with dominant negative (dn) PDGFRα increased SMC migration in response to CMSS15 (P < 0.001), whereas overexpression of either dnPDGFRβ or inactive PDGFRβ mutants markedly reduced SMC migration in response to CMSS5, CMSS15 or recombinant PDGFB. Overexpression of wild type PDGFRα inhibited SMC migration in response to CMSS5, CMSS15 or recombinant PDGFB. In summary, lower SMC migration in response to CM from BAEC exposed to high vs. low SS is due, at least in part, to PDGFRα-mediated inhibition of PDGFRβ-dependent SMC migration. These results suggest that the ability of high SS to inhibit artery wall thickening in vivo may be related to activation of PDGFRα on SMC, by PDGF isoforms secreted by the endothelium.
Boron and wound healing

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During wound healing, angiogenesis and the remodelling of the extracellular matrix are stimulated through local production of several growth factors, and specific cytokines (such as VEGF) play an important role in the stimulation of wound healing. The effects of boron on wound healing have been studied, and it has been demonstrated clinically that boron greatly improves the healing of deep wounds with tissue loss. The aim of our study was to determine the mechanisms implicated in the wound healing under the effects of boron. By using different models, we have investigated the action of boron on the formation and turnover of extracellular matrix, and found that it modulates the formation of extracellular matrix by increasing the release of its components, such as proteoglycans and the protease activity (collagenase, cathepsin D). We also investigated the boric acid effect on the expression of angiogenic factor VEGF, and we show that more VEGF is synthesized in the assay (with boric acid) than in the control (without boric acid). By conclusion, the effects of boron in the wound healing could be explained, in part, by its action on the extracellular matrix and by the stimulation of VEGF release.

Antitumour effects of tumour necrosis factor are host mediated and vasculature targeted

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Tumour necrosis factor is a cytokine, which has, alone or in combination with IFN-γ and/or chemotherapeutic agents, a powerful anticancer effect in cancer patients as well as in animal models. Its potentially lethal shock-inducing effect, however, limits its application to loco-regional treatment. We could previously show, e.g. by selectively inhibiting the hemodynamic shock with methylene blue [1], that both effects are not inevitably linked. In order to selectively mimic its antitumour effects through downstream molecules and/or targets, we have studied its in vivo mechanisms of action. Using histological analysis, TNF and IFN-γ of different species and muteins thereof, transfected tumour cells and various knockout mice, we show that host-mediated effects are the major and necessary effects of the antitumour activity. The effectiveness of the treatment correlated with apoptosis of endothelial cells, which was observed in the tumour vasculature but not in the normal vasculature. Also the observed synergism between low dosed systemic TNF and StealthTM—liposome encapsulated doxorubicin is due to host-mediated effects, most probably on the tumour vasculature.

Reference

1. Cauwels et al. Immunity 2000; 13: 223–31.
The density of endoglin immunostained blood vessels is prognostic for survival in prostate cancer

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Vascular density is a promising prognostic marker in a variety of tumour types. Most often vascular density is determined by the use of a pan-endothelial antibody, such as von Willebrand factor. Pan-endothelial markers may however not be ideal for this purpose, as they do not stain all micro-vessels and particularly not the newly formed ones. Endoglin, a receptor for some members in the TGF-β family, is expressed on proliferating endothelial cells and may be a useful marker of ongoing angiogenesis. Immunohistochemical staining of endoglin and von Willebrand factor was examined in 72 cases of prostate cancer. Micro-vessels were counted in the most vascularized fields, on hot spots. Double staining for smooth muscle actin and endoglin or von Willebrand factor, respectively, was performed in order to evaluate vessel maturation. Endoglin stained tumour vessels were generally small, and only 14% were double stained with actin. The double staining for actin and von Willebrand factor was 38%. The endoglin and von Willebrand factor vascular counts were correlated and both were prognostic for clinical outcome. The median survival times were shorter for patients with tumours containing vascular counts above median than for patients with vascular counts below median (4 vs. 12 years, \( P = 0.0007 \), and 5 vs. 10 years, \( P = 0.018 \), respectively). The endoglin vascular count was correlated with Gleason grade, tumour stage, metastasis, tumour cell immunoreactivity for TGF-β1, and tumour cell proliferation index, while the von Willebrand factor vascular count was correlated with Gleason grade and TGF-β1 immunoreactivity. In conclusion, endoglin marks principally small probably newly formed tumour vessels in the prostate and seem to be a promising prognostic marker for prostate cancer patients.

Increased angiogenesis and tumour size in β3-integrin deficient mice is associated with increased VEGF-receptor levels

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\( αvβ3 \) Integrin expression is upregulated on endothelial cells during neovascularisation. Here we investigated the effect of β3-integrin deficiency on angiogenesis in vivo and in vitro. We report that in both retinopathy of prematurity and tumour assays angiogenesis is supported in β3-integrin and β3/5-double knockout mice. Analysis of B16F0 subcutaneous tumours revealed that the β3-deficient angiogenic vessels express various endothelial markers and that vessel density and tumour size were significantly enhanced in β3-null mice when compared to wild types. In ex vivo aortic ring cultures β3-deficient rings responded to exogenous VEGF with increased microvessel sprouting when compared to controls, implicating an increase in VEGF-receptor function. Since both \( αvβ3 \) and \( αvβ5 \)-integrins are vitronectin receptors and there is evidence that \( αvβ5 \) is a receptor for VEGF, it was necessary to examine a possible compensatory role of β5-integrin. Interestingly, endothelial cells isolated from knockout mice were deficient for β3-integrin, but had normal expression levels and function of all other integrins tested. In contrast, Western blot and FACS analysis of endothelial cells established a 2-fold increase of the VEGF receptor Flk-1 in β3-deficient cells. Furthermore, reintroduction of β3 into knockout cells reduced Flk-1 expression to wild-type levels. Taken together these data demonstrate that β3-integrin deficiency enhances angiogenesis and that β3-integrin is involved in Flk-1 downregulation.
Identification of specific targets for inhibition of endothelial cell proliferation and differentiation

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Endothelial cells represent an attractive source of diagnostic and therapeutic molecular targets during diverse diseases that depend on angiogenesis. Here we use gene expression analysis to find genes that are both relevant and unique to the angiogenic process as defined by an in vitro model. Analysis of the data identified 154 genes that are modulated during the process and 105 genes that enriched for expression in endothelial cells as compared to normal tissues. A subset of 39 genes was found to be common to both groups and of the 21 known genes found in this group, 16 genes have been associated with endothelial cell biology and/or angiogenesis. The identification of αvβ5 integrin in the genes enriched for endothelial cell expression allowed us to further validate our approach. Thus, this study identified a small number of genes fitting defined biological hypotheses that can be assessed as therapeutic targets.

Endostatin inhibits angiogenesis by stabilization of newly formed endothelial tubes

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Endostatin decreased VEGF-induced formation of endothelial tubes and microvessels sprouting from aortic rings and blocked their network. After cessation of treatment, the survival time of endostatin plus VEGF-treated tubes was approximately twice as long as that of tubes treated with VEGF alone. Endostatin antibody blocked VEGF-induced endothelial tube formation and disrupted existing tubes. Endostatin immunostaining was localized between endothelium and basement membrane, and in inter-endothelial junctions of new, but not of quiescent, blood vessels. In tumors grown in SCID mice, endostatin immunostaining was strongest when the blood vessels involved were undergoing maturation and was significantly prominent in vessels of the tumor marginal zone, where angiogenesis is highly active. These data indicate a new antiangiogenic action of endostatin in the stabilizing and maturing endothelial tubes of newly formed blood vessels. Thus, strategies accelerating vascular stabilization and maturation could be promising in tumor therapy.
Screening of new modulators of angiogenesis from marine sources

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Angiogenesis has been associated with many life-threatening pathologies, including tumor progression, diabetic retinopathies, haemangiomas, arthritis, arteriosclerosis, psoriasis, etc. The recognition of the involvement of angiogenesis in major disease states has been accompanied by an increased interest in research involved in identifying and developing angiogenesis inhibitors. One of the main objectives of our work is to carry out a high throughput screening for angiogenesis modulators. As a source of compounds, we use extracts and fermentation broths derived from marine organisms, and supplied by Instituto Biomar S.A. (León, Spain). Active compounds are selected by means of a number of in vitro and in vivo assays resembling angiogenesis processes. After purification and structure elucidation, the new active compounds are patented. The searching strategies and some results of our screening program will be presented.

Acknowledgement

This work is supported by funds from EC and Spanish MCT (Programa FEDER 1FD97-0693).

Monoclonal anti-idiotypic antibodies targeting VEGF receptors

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The demonstration of tumor progression angiogenesis has led to the development of new anti-angiogenic cancer therapies. Our laboratory has developed an anti-idiotypic strategy to construct circulating agonists of angiogenic growth-factor receptors. The specificity of these anti-idiotypic antibodies (Ald Abs) is very useful for analysing the intracellular signals and the differentiation effects mediated by each receptor. In a previous work, we demonstrated that polyclonal VEGF Ald Abs could behave as circulating agonists for VEGF-R2. These Ald Abs were able to stimulate endothelial cell proliferation in vitro and tumor angiogenesis in vivo. They also blocked tumor progression when coupled to a cytotoxic compound. We report the production of monoclonal Ald Abs for VEGF by fusing the myeloma and splenocytes from mice primed with anti-VEGF antibodies. Antibody specificity has been determined by ELISA and SPRIA on the extracellular domains of VEGFR1 and VEGFR2. Specific agonists of VEGFR2 induced endothelial cell proliferation but not migration in the nanomolar range. These antibodies also stimulate tumor progression in syngeneic mouse models. We are currently coupling these Ald Abs to a toxin in order to determine their potential anti-angiogenic activity. This work should result in the development of a new class of anti-angiogenic agents.
I-309 binds to and activates endothelial cell functions and acts as an angiogenic molecule in vivo

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Chemokines, among their several other functions, have been shown to induce angiogenesis or to act as angiostatic molecules modulating the activity of growth factors such as fibroblast growth factor 2 (FGF-2) and vascular endothelial growth factor (VEGF). We first detected the CC chemokine receptor (CCR) 8 message in human umbilical vein endothelial cells (HUVEC) by reverse transcription polymerase chain reaction (RT-PCR) and RNase Protection Assay (RPA). We then sought to investigate the potential role exerted by the CC chemokine I-309, a known ligand of such receptor, in both in vitro and in vivo angiogenesis assays. Our results show that I-309 binds to endothelial cells, stimulates chemotaxis and invasion of these cells, and enhances HUVEC differentiation into capillary-like structures in an in vitro Matrigel assay. Furthermore, I-309 is an inducer of angiogenesis in vivo in both the rabbit cornea and the chick chorioallantoic membrane assay.