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Current Status Review

Targetting VEGF in anti-angiogenic and anti-tumour therapy: Where are we now?

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Summary. Since the recognition of the importance of the vascular bed for growth and metastasis of solid tumours, many researchers have investigated the approach of attacking the tumour vascular bed instead of the tumour cells themselves in anti-cancer therapy. Such approaches have become possible with the increasing knowledge of the angiogenic process and the factors that regulate it. Especially the potent angiogenic factor VEGF has been the subject of extensive study in this regard. A number of studies showed that inactivation of this factor or its receptors led to a profound negative effect on the development of experimental tumours. However, despite the encouraging results obtained in animal studies, it remains to be established whether human tumours, which might be in a state of relative quiescence, are as sensitive to anti-VEGF treatment as the fast-growing tumours that are generally used in animal studies. If so, anti-VEGF treatment might certainly represent a powerful tool in anti-cancer therapy, either or not in combination with other blockers of angiogenesis.

Keywords: antiangiogenesis, antagonist, cancer therapy, VEGF

During the last two decades, starting with the dogma, postulated by Dr Judah Folkman in the early seventies that the formation of new blood vessels, a process called angiogenesis, is indispensable for the growth and metastasis of solid tumours, researchers have increasingly recognized that targetting the tumour vascular bed instead of the tumour cells themselves might represent a very effective and advantageous anti-cancer therapy (Folkman 1990; Barinaga 1997; Harris 1997). Given sufficient knowledge about the angiogenic process and the factors that regulate it, disruption of the tumour vascular bed should be achievable such that damaging surgery, chemotherapy or radiotherapy is avoided while uninvolved, distant sites in the body are not affected. Also, targetting the tumour vascular bed cirumvents the problem of chemotherapy-induced resistance of tumour cells, since the blood vessels in a tumour are composed of normal cels that are not as prone to mutations as the tumour cells. In this review, we aim to give a concise overview of the approaches that can be followed to inhibit angiogenesis, with particular emphasis on the most important angiogenic factor, namely vascular endothelial growth factor (VEGF).

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Inhibiting the angiogenic process

Angiogenesis is a very complex process involving production of proteases by the vessel endothelium and subsequent degradation of the blood vessel wall, deposition of new matrix proteins, proliferation of the endothelium,

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migration, extravasation and adherence of endothelial cells to the newly formed extracellular matrix and formation of a new lumen (Varner *et al.* 1995; Ellis & Fidler 1996; Bischoff 1997). Angiogenesis is required in a number of physiological processes like embryogenesis, wound healing and the female cycle. Under normal conditions, the vasculature in the healthy adult is in a state of quiescence, which is dependent upon an equilibrium between the activities of a large number of angiogenesis inhibitors and activators (Hanahan & Folkman 1996). When this balance is shifted in favour of the inhibitors, blood vessels may go into regression, while angiogenesis occurs when activators prevail. The latter situation is seen in a number of diseases associated with angiogenesis, like rheumatoid arthritis, diabetic retinopathy, psoriasis and cancer. The list of endogenous angiogenesis inhibitors includes platelet-factor 4, thrombospondin, angiostatin, endostatin, angiopoietin II and the 16 kD N-terminal fragment of prolactin (D'Angelo *et al.* 1995; Gengrinovitch *et al.* 1995; Kim Lee Sim *et al.* 1997; O'Reilly *et al.* 1997), while activators include acidic and basic fibroblast growth factors, hepatocyte growth factor/scatter factor, transforming growth factors α and β, angiopoietin I and, most importantly, vascular endothelial growth factor/vascular permeability factor (VEGF/VPF, further referred to as VEGF) (Klagsbrun 1991; van Belle *et al.* 1998).

All steps in the angiogenic process are potential targets for anti-angiogenic and anti-tumour therapy. Inhibition of metalloproteases by tissue inhibitor of metalloprotease (TIMP-4) has been shown to have a profound negative effect on tumour angiogenesis and growth in animal models (Wang *et al.* 1997). Angiostatin, with endostatin one of the most potent and promising angiogenesis inhibitors, possibly also works by blocking protease function. On another level, antibodies against integrin $\alpha_v\beta_3$, an endothelial surface protein that is important for the adherence of the endothelium to the basal membrane and numerous other endothelial functions, are able to stop angiogenesis in tumours (Varner *et al.* 1995). A lot of effort has also been invested in blocking the action of VEGF, as this factor plays a central role in tumour angiogenesis.

The role of VEGF in tumour biology

The importance of VEGF in tumour biology is evident: in a number of human tumours there is a positive correlation between VEGF expression, vascular density and degree of malignancy (Takahashi *et al.* 1995; Viglietto *et al.* 1995). Furthermore, in animal models tumour growth is greatly affected by VEGF. In our laboratory we demonstrated that, upon injection in immunodeficient mice, transformed C127I mouse epithelial cells expressing VEGF develop into highly vascularized tumours within two to three weeks, while cells expressing an inactive VEGF mutant give rise to tumours with low vascular density only two months after injection (unpublished observation). Increased levels of angiogenesis and metastasis were also seen in melanoma cell lines, stably transfected with VEGF expression constructs (Claffey *et al.* 1996; Pötgens *et al.* 1996).

Inversely, inhibiting VEGF action by administering neutralizing antibodies or soluble VEGF receptors, resulted in decreased tumour angiogenesis and growth as well as in reduced metastatic spread in animal models. Also, inactivation of one of the VEGF receptors by a dominant-negative strategy was sufficient to completely abolish growth of glioma xenografts (Jin Kim *et al.* 1993; Kondo *et al.* 1993; Millauer *et al.* 1994; Asano *et al.* 1995).

Structure of VEGF

VEGF shares with platelet derived growth factor (PDGF) a characteristic cystine knot motif and is therefore regarded as a member of the superfamily of growth factors, which also includes Placenta Growth Factor (PlGF), VEGF-B, VEGF-C and VEGF-D (Orlandini *et al.* 1996; Park *et al.* 1994; Joukov *et al.* 1996; Olofsson *et al.* 1996). The cystine knot motif consists of eight cysteine residues which are involved in intra- and intermolecular disulphide bonding, leading to a dimer of twisted antiparallel β-sheets (Pötgens *et al.* 1994; Muller *et al.* 1997). The VEGF transcript is alternatively spliced to yield five mRNAs encoding proteins of 121, 145, 165, 189 and 205 amino acids which are secreted as glycosylated dimers. These VEGF isoforms share an N-terminal part of 110 amino acids that contains the complete cystine knot motif and is biologically fully active. The positively charged C-terminus, present in the larger isoforms, is responsible for retention of these variants to the heparan sulphates in the extracellular matrix (ECM) as inactive molecules. Cleavage of these ECM-bound proteins by proteases like plasmin or urokinase releases the N-terminal VEGF fragment. These data suggest that bio-availability of VEGF can be partly regulated by proteases (Houck *et al.* 1992; Keyt *et al.* 1996a; Plouet *et al.* 1997).

VEGF receptors

Two class III tyrosine kinase receptors with high affinity for VEGF have been well characterized, namely fms-like

tyrosine kinase 1 (Flt-1) and fetal liver kinase (Flk, the mouse homologue of the human kinase domain containing receptor KDR). Both are largely confined to proliferating endothelium, lining growing vessels (Hatva *et al.* 1995) and are comprised of an ectodomain of seven Ig-like loops, a transmembrane region and an intracellular split kinase domain. Recently, a third receptor, specific for the $VEGF_{165}$ isoform, has been identified (Soker *et al.* 1996).

Upon binding of a VEGF dimer to the KDR/Flk receptor, dimerization and trans-phosphorylation of the kinase domains occur. The resulting receptor phosphotyrosines act as docking sites that recruit signal transduction intermediates (Kroll & Waltenberger 1997; Mukhopadhyay *et al.* 1998). Subsequent signal transduction pathways ultimately lead to effects in the nucleus on the transcriptional level, leading to biological responses.

The biological significance of the Flt-1 receptor in the adult is not clear. During embryonic development, this receptor is indispensable as Flt-1 knockout mice die at E8-E9 due to blood vessel malformations (Fong *et al.* 1995). Although Flk-1 null mice also die at this stage of embryogenesis, they show a defect in endothelial cell differentiation (Shalaby *et al.* 1995) pointing at distinct roles for each receptor. In adult endothelial cells Flt-1 is not or only very weakly phosphorylated upon VEGF activation. Nevertheless, monocytes which express Flt-1 but not KDR, can be stimulated by VEGF or PlGF to express tissue factor. Furthermore, PlGF, which is a ligand for Flt-1 but not for KDR, stimulates tissue factor expression in human umbilical vein endothelial cells (HUVECs) definitely pointing to a biological role for the Flt-1 receptor in the adult cell (Clauss *et al.* 1996). However, since tumour angiogenesis and growth can be inhibited by blocking solely the KDR/Flk receptor, this receptor is thought to be responsible for mediating VEGF effects in the tumour angiogenic process (Millauer *et al.* 1994, 1996).

Regulation of VEGF bio-activity

In therapeutic terms, how can we modulate tumour cells to decrease their VEGF production to levels that can no longer generate a vascular bed, or, on another level, how can the activity of tumour derived VEGF be inhibited to such an extent that the same effect is established? To approach this question, we have to take a closer look at the mechanisms involved in controlling VEGF activity.

Since quiescence in the vasculature is dependent on an equilibrium between angiogenic activators and inhibitors, the activities of both have to be controlled tightly. Several mechanisms are involved in regulating VEGF activity, both on the transcriptional and on the protein level. Obviously, a major regulatory step, of particular importance in tumour cell biology, is the production of VEGF itself. Tumours induce the establishment of their own vasculature at least in part by upregulating the synthesis of VEGF. This upregulation can occur as a result of activation of proto-oncogenes like c-Src or ras, or loss of tumour suppressor proteins like p53 and Von Hippel-Lindau (VHL) protein (Mukhopadhyay *et al.* 1995; 1997; Bouck 1996; Arbiser *et al.* 1997; Okada *et al.* 1998). The most important condition for upregulation of VEGF expression in tumour biology, however, is a state of hypoxia which eventually will occur in the centre of an expanding tumour nodule. Triggering of a hypoxia-sensing mechanism leads to VEGF-upregulation on the transcriptional and post-transcriptional level. The transcription factor Hypoxia Inducible Factor-1 (HIF-1) binds to an enhancer element in the VEGF-promoter, leading to direct transcriptional activation (Forsythe *et al.* 1996; Maxwell *et al.* 1997). At the post-transcriptional level, hypoxia-induced proteins bind to the 3′-untranslated region of the messenger RNA, thereby stabilizing the messenger (Ikeda *et al.* 1995; Levy *et al.* 1996).

Several mechanisms seem to be involved in regulating VEGF-activity on the protein level. First of all, as already mentioned, cells can activate ECM-bound VEGF by secreting proteases that release an active VEGF digest (Houck *et al.* 1992; Keyt *et al.* 1996a). Secondly, heterodimers of VEGF and PlGF are found to be produced by some tumour types (DiSalvo *et al.* 1995; Cao *et al.* 1996). Since PlGF has no affinity for the KDR/Flk receptor, these heterodimers can lead to homodimerization of Flt-1, but probably also to heterodimeric KDR/Flt-1 receptors (Kendall *et al.* 1996). Heterodimeric PlGF/VEGF molecules stimulate proliferation in HUVECs, but with a much lower activity than VEGF homodimers (Cao *et al.* 1996). Thus, production of PlGF represents a way for a cell to regulate biological activity of VEGF. A third method of controlling VEGF activity is the secretion by endothelial cells of soluble Flt-1 splice variants (sFlt-1) (Kendall & Thomas 1993). These block VEGF activity on two levels: they bind tightly to VEGF, thereby rendering the ligand unable to bind to the cognate cell surface receptors and they heterodimerize with KDR/flk. The resulting heterodimeric receptor is not able to transduce signals to the cell nucleus because it lacks one intracellular domain. Thus, sFlt-1 acts as a dominant negative variant (Kendall *et al.* 1996). Finally, the existence of the recently discovered $VEGF_{165}$-specific receptor (Soker *et al.* 1996) suggests differential activities for $VEGF_{121}$ and the larger isoforms, thereby

including differential splicing as a means of regulating VEGF activity.

Since VEGF upregulation on the transcriptional level can be caused by a number of different conditions as outlined above, the search for specific VEGF inhibitors should not focus on blocking VEGF expression on the transcriptional level but on levels of VEGF action beyond this stage. Potential approaches to block VEGF action include:

- inhibition of secretion of endogenous tumour VEGF;
- neutralization of VEGF in the (micro)circulation;
- prevention of VEGF receptor binding and subsequent signal transduction.

Inhibition of secretion of endogenous tumour VEGF

Tumours in which endogenous VEGF production is switched off are not able to establish or maintain a vascular bed. Benjamin & Keshet (1997) showed in an elegant way that shutting off recombinant VEGF expression in xenografts of C6 glioma cells in nude mice led to detachment of tumour endothelium and regression of blood vessels. This points to a crucial role for VEGF as a survival factor for the tumour vascular bed. The major question to be asked at this point is: is the vascular bed in a human tumour, that might be present in the body for many years before it becomes detectable, as vulnerable to VEGF withdrawal as the fast growing tumours that are commonly used in animal tumour models? As yet, the answer to this question is still open.

So how can endogenous VEGF expression be eliminated? Several approaches have proven to be successful in doing so, each resulting in decreased tumour angiogenesis – and, consequently, growth. Based on the dependence of hypoxia-mediated VEGF expression on c-Src (Mukhopadhyay *et al.* 1995), Ellis *et al.* (1998) examined the effect of inactivating c-Src function by introducing antisense c-Src expression constructs in HT29 colon tumour cells. Indeed, VEGF expression was abrogated with significant effects on tumour angiogenesis. However, approaches that aim at disrupting signalling pathways are not desirable in anti-VEGF therapy since their interference with VEGF expression is not likely to be specific. Far more obvious is the use of antisense oligonucleotides or expression constructs, specific for VEGF itself. Indeed, when transfected in C6 rat glioma cells, antisense VEGF expression constructs abrogated VEGF expression *in vitro* under hypoxic conditions, and upon xenografting in mice the resulting tumours exhibited a greatly reduced vascular density, reduced growth rate and a large degree of necrosis (Saleh *et al.* 1996). In a similar study using human glioblastoma cells, essentially identical results were obtained (Cheng *et al.* 1996).

Interference with endogenous VEGF expression can also take place on the post-transcriptional level. In our laboratory we have constructed a series of mutants of VEGF, of which some were observed to be secreted at very low levels in different expression systems. This inefficient secretion was probably not due to improper folding since secreted proteins had a dimeric conformation. In cotransfection experiments, these mutants inhibited wild-type VEGF expression *in vitro*. Furthermore, production of these proteins in an *in vivo* tumour model resulted in significantly inhibited tumour growth (manuscript in preparation). A similar dominant-negative mutant (VEGF-Cys101Ser) that inhibited wild-type VEGF secretion in COS-1 cells was found by Claffey *et al.* (1995). The mechanism of inhibition of VEGF secretion by this mutant has not been elucidated. Possibly, intracellular dimers are targetted for degradation instead of secretion. Such mutants might prove to be of high value in anti-angiogenic gene therapeutic approaches.

Neutralization of VEGF in the (micro)circulation

Neutralization of circulating VEGF has proven to be a very promising method of inhibiting angiogenesis in mouse tumour models. Neutralizing anti-VEGF antibodies are very effective in inhibiting growth and metastasis of a variety of human and rat tumours in nude mice (Jin Kim *et al.* 1993; Kondo *et al.* 1993; Asano *et al.* 1995; Borgström *et al.* 1996). The discovery that endothelial cells and some tumour types can secrete a soluble splice variant of the Flt-1 receptor *in vivo* that still has high affinity for VEGF and probably interacts with full length Flt-1 or KDR/Flk in a dominant-negative manner (Kendall & Thomas 1993; Kendall *et al.* 1996) led reseachers to investigate the potential use of recombinant soluble Flt-1 in anti-angiogenic therapy. Indeed, tumours grown as xenografts from cells engineered to produce recombinant sFlt-1, are significantly growth-inhibited (Dr K. A. Thomas personal communication).

Prevention of VEGF receptor binding

Although neutralization of VEGF using the strategies outlined above is successful in treating experimental tumours in mice, their potential application in anti-cancer therapy in humans is most probably limited due to the enormous amounts of protein that would be needed to treat human subjects, since the entire circulation should

be saturated with VEGF-neutralizing agents. Because the VEGF receptor KDR/Flk seems to be confined mainly to proliferating (tumour) endothelium and since antagonizing KDR/Flk-1 is sufficient to abolish tumour angiogenesis in a number of tumour types in nude mice (Millauer *et al.* 1994, 1996), targetting this receptor instead of its ligand would be advantageous. Therefore, VEGF receptor antagonists could represent a more appropriate means of anti-tumour therapy in humans.

With the recent unravelling of the VEGF structure by X-ray diffraction analysis, the domains involved in the receptor–ligand interactions have been resolved (Keyt *et al.* 1996b; Muller *et al.* 1997; Walsh & Grant 1997). The three dimensional structure of VEGF resembles that of PDGF as predicted from the conserved cystine knot motif, present in both factors (Keck *et al.* 1997). VEGF is secreted from the cell as an antiparallel, symmetrical dimer with at each pole a binding site for one KDR/Flk receptor subunit. All information for receptor binding is located in the N-terminal 110 amino acids of the molecule, the fragment that is released from the ECM-bound larger VEGF splice variants after cleavage by plasmin (Houck *et al.* 1992; Keyt *et al.* 1996a; Keck *et al.* 1997). Alanine scanning experiments in which charged amino acids are systematically mutated to neutral alanine residues have confined the KDR/Flk binding domains to two positively charged regions (positions 36–47 and 84–90), while Flt-1 binding is mediated by a negatively charged loop, located between positions 63 and 67 (Keyt *et al.* 1996b; Muller *et al.* 1997). These results have very recently been confirmed by swapping these domains with the corresponding domains of PDGF (Siemeister *et al.* 1998). Using this knowledge, VEGF heterodimeric variants have been created with an intact receptor binding site at one pole and a mutated one at the other pole. Such molecules can only occupy receptor monomers and in this way prevent receptor dimerization and subsequent signal transduction. Indeed these molecules display antagonism in proliferation and receptor phosphorylation assays (Fuh *et al.* 1998; Siemeister *et al.* 1998) and might be promising therapeutic agents. However, at this stage it is important to note that the latter studies demonstrated antagonism on endothelial proliferation and KDR/Flk binding only. Since induction of endothelial proliferation is an activity that is not unique to VEGF, it is clearly important to investigate the effect of such heterodimers on the unique activities of VEGF such as the induction of vascular permeability. Also, the concept of VEGF binding to only two receptors is clearly too simple, since a $VEGF_{165}$-isoform specific receptor has recently been characterized (Soker *et al.* 1996). Surprisingly, inhibition of this receptor by the $VEGF_{165}$-specific exon-7 amino acid sequence also leads to inhibition of KDR/Flk-mediated endothelial cell proliferation (Soker *et al.* 1997). Clearly, the whole array of VEGF receptors and their functions and interplay is not yet fully understood and extensive *in vivo* studies will be needed to evaluate the use of VEGF antagonists.

Conclusions and future perspectives

Although numerous studies have shown that inhibiting VEGF action on different levels (transcription, secretion, receptor binding or receptor activation) represents a powerful approach for anti-tumour therapy, application of this principle to cancer treatment in humans could encounter some major problems. First, it is not yet known whether an established vascular bed in human tumours, which might be resident and in a state of relative quiescence, is as responsive to anti-VEGF treatment as the fast growing tumours that are studied in animal experiments. Furthermore, antagonizing VEGF action by neutralizing antibodies or soluble receptors would require almost unachievably high amounts of protein. Therefore, targetting the tumour vascular bed by attacking the KDR/Flk receptor would be advantageous. Knowledge about the structure of VEGF, together with the identification of the domains in VEGF that are responsible for KDR/Flk binding, have already led to the design of molecules that are able to antagonize KDR/Flk function *in vitro*. If these VEGF antagonists prove to be effective *in vivo*, how can they be delivered to the tumour? Repeated systemic or intratumoral administration of protein is the most simple method, but it is difficult to predict its efficiency since the biological half-life of these antagonists has to be taken into account. Gene therapy, in theory, is far more promising. The ideal scenario would be that tumour cells produce recombinant VEGF variants that, upon heterodimerization with endogenous, hypoxia-induced VEGF, not only inactivate this VEGF, but also lead to local secretion of receptor antagonists. With the current knowledge about the VEGF structure and its receptor binding determinants it should be possible to design such recombinant molecules. Currently we are investigating the activities of such mutants *in vivo*. Also, dominant-negative VEGF mutants that merely inhibit wild-type VEGF secretion might be very valuable in a gene therapeutic approach. However, a technical problem that still exists is how to introduce mutant VEGF expression constructs efficiently into tumour cells. Especially when aiming at inhibiting wild type VEGF expression, it is important that the majority of tumour cells express dominant-negative mutants, as VEGF produced by nonrecombinant

tumour cells will be able to stimulate angiogenesis at distant sites in the tumour. With the currently available methods to introduce DNA *in vivo* into cells, the goal of highly efficient DNA transfer probably cannot be achieved since these methods are either inefficient or suffer from safety problems or immunological problems (reviewed by Finkel & Epstein 1995). However, progress is being made on the field of gene transfer by using adeno-associated viral vectors that seem to lack the extensive immune response that is evoked by adenoviruses (reviewed by Flotte & Carter 1995).

Clinical trials using VEGF for therapeutic angiogenesis have already started (Isner *et al.* 1996) and it is to be expected that efficient, clinically applicable, therapies blocking VEGF action will become available in the next few years. We suspect that, although other anti-angiogenic compounds like angiostatin and endostatin have been proven to be very effective in blocking angiogenesis in experimental tumours, therapies that block VEGF might prove to be a very valuable additional tool to be added to the list of anti-cancer treatments.

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