

VEGF and Tumor Angiogenesis

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Abstract

Based on successful preclinical data, over twenty anti-angiogenic agents, alone or in combination with conventional therapies, are now in clinical trial. In the year 2000, close to 1,000 manuscripts describing the basic molecular and cellular mechanisms that allow for an angiogenic response have been published. The goal of this review is to summarize the reasons that angiogenesis research has attracted the attention of both clinicians and basic researchers recently. In addition, the biology of vascular endothelial growth factor (VEGF), a promising target for anti-angiogenesis drug development, is reviewed.

Tumor Angiogenesis

Angiogenesis, the formation of new blood capillaries from existing vessels, is an important mechanism for supplying nutrients to cells that are distant from existing blood vessels (Folkman and Shing, 1992). Angiogenesis is critically important during embryonic development (Breier, 2000) and certain physiological circumstances in the adult, including wound healing (Iruela-Arispe and Dvorak, 1997).

Angiogenesis is a complex process that is mediated by the endothelial cells that line blood vessels (Daniel and Abrahamson, 2000). Unlike quiescent endothelial cells that rarely divide, angiogenic endothelial cells undergo a complex sequence of events that includes the secretion of metalloproteases and other matrix-degrading enzymes, cell migration into the newly created space, endothelial cell division and proliferation, and vessel formation. These are well regulated processes involving a number of stimulators such as fibroblast growth factor (FGF) (Nugent and Iozzo, 2000), vascular endothelial growth factor (VEGF) (Petrova et al., 1999), angiopoietins (Davis et al., 1996), activators of integrins (Eliceiri and Cheresh, 1999), and inhibitors such as thrombospondin (Roberts, 1996), angiostatin (O'Reilly et al., 1994), and endostatin (O'Reilly et al., 1997).

In addition to its important role in normal physiological processes, angiogenesis contributes to the pathology of a number of diseases (Patz, 1980; McLaren et al., 1996; Fava et al., 1994), including tumor progression (Carmeliet and Jain, 2000). This is because angiogenesis provides nutrients that maintain the viability of diseased tissue. Tumor-associated angiogenesis allows the

tumor to maintain its growth advantage and also facilitates metastatic spreading by establishing connections to the existing vasculature. A correlation has been observed between the density of microvessels in primary breast carcinoma and nodal metastases with respect to survival (Weidner et al., 1991; Horak et al., 1992). Similarly, a correlation has been reported between vascularity and invasive behavior in a number of other tumors (Wakui et al., 1992; Macciarini et al., 1992). These findings indicate that the number of vessels in tumor sections may be a prognostic factor for cancer patients (Bigler et al., 1993).

There are two major differences between pathological and normal angiogenesis. First, in diseased tissue, the regulatory mechanisms which "turn off" neovascularization in healthy tissue do not function normally; there is a shift in the balance of positive and negative angiogenesis regulators towards the positive molecules. The second major difference between pathological and normal angiogenesis is that the vessels formed in diseased tissue are highly disorganized, and their walls have numerous openings. This is because tumor vessels are not able to mature through the recruitment of smooth muscle cells and pericytes, leading to the formation of leaky vessels in the tumor. This phenomenon was critical for the discovery of VEGF (Senger et al., 1983).

Targeting the tumor vasculature may be a more effective therapeutic strategy than targeting the tumor itself for a number of reasons:

- 1) Cancer is considered to be a large group of diseases classified by the tissue of origin and the degree of tumor progression. With the advent of new technologies that can reveal a tumor's genetic profile such as microarray chips and chromosome dissection, cancer is being further divided into hundreds of identifiable gene-driven diseases (Brazma and Vilo, 2000). It is expected that a single chemotherapeutic agent will be effective in treating only a subset of these diseases. In contrast, a pharmaceutical that can effectively inhibit angiogenesis is likely to be effective against most of them.

- 2) Even if chemotherapeutics are developed that can effectively inhibit the gene products aberrantly expressed at a particular stage of a specific cancer, there is no guarantee that the drug will prevent disease progression. This is because cancer cell genomes are so unstable (Stoler et al., 1999). Genomic aberrations continually accrue and alter the character of both the primary tumor and its metastases. These changes can provide alternative cellular mechanisms that compensate for the loss of function of the gene product targeted by the chemotherapy. Drug resistance may not be an issue

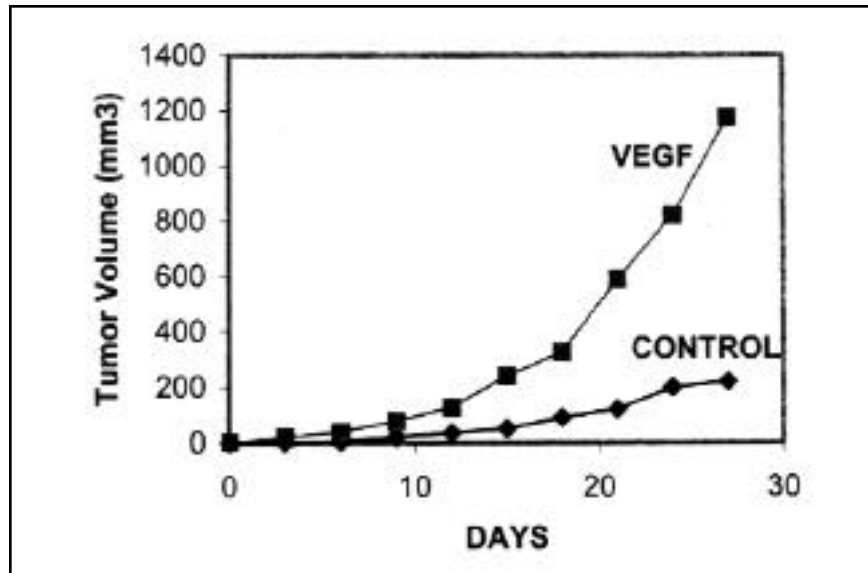


Figure 1: VEGF expression allows for more aggressive tumor growth. PANC1 cells were transfected with VEGF cDNA cloned into an expression vector. Subcutaneous injection of cells transfected with either VEGF-containing vector or empty vector was done using athymic mice. Tumor dimensions were measured at various times using a caliper, and tumor volumes were determined. The average tumor volume for five mice was calculated for each data point.

for pharmaceuticals that target endothelial cells, because they are genetically stable.

3) There are cell surface proteins that are expressed on angiogenic endothelial cells, but not on quiescent endothelial or other cells. These proteins include the receptors for VEGF (Vaisman et al., 1990), angiopoietins (Folkman and D'Amore, 1996), and ephrins (Yancopoulos et al., 1998), as well as certain adhesion molecules (Telo et al., 1998). Since these molecular targets are unique to activated endothelial cells, it is expected that anti-angiogenic drugs will be less likely to have adverse side effects such as bone marrow suppression, gastrointestinal symptoms, or hair loss that are characteristic of standard chemotherapy treatment.

4) Angiogenesis is related to metastasis (Yano et al., 2000) in that tumors with higher densities of blood vessels are more likely to spread, which means a poorer clinical outcome for affected patients. Also, the primary tumor may not begin to shed a large number of tumor cells until after it has a network of blood vessels. Angiogenesis inhibitors have the potential to block both tumor growth and the spread of a tumor to different parts of the body.

5) Anti-angiogenesis therapy may be more effective in combination with therapy directly aimed at killing tumor cells, because each therapy is targeted at a different cell type in the tumor.

Based upon successful preclinical data, several anti-angiogenic agents, alone or in combination with conventional therapies, are now in clinical trials (Felman and

Libutti, 2000) (Table 1). Given the complexity of the angiogenic process, the pharmaceuticals under clinical evaluation are directed against several therapeutic targets. The strategies that are being utilized are: 1) interference with angiogenic ligands, their receptors, or downstream signaling; 2) upregulating or delivering endogenous inhibitors; and 3) directly targeting the tumor vasculature through inhibition of endothelial cell proliferation or activation of endothelial cell apoptosis.

There are potential concerns relating to the possible efficacy of using anti-angiogenic approaches for treating cancer. First, as tumors grow, they produce a wider variety of angiogenic activators. Therefore, if only one activator (for example, VEGF) is blocked, tumors may utilize or upregulate another activator (for example, FGF). Second, there is microvascular heterogeneity in tumors, and not all activated endothelial cells express the same cell surface markers. Therefore, a pharmaceutical targeting a specific marker may not effectively inhibit tumor progression. Third, it remains uncertain whether anti-angiogenesis agents will shrink tumor size or simply prevent further tumor growth. If the latter is the case, then therapy will involve combining the angiogenesis inhibitor with more traditional chemotherapies.

Preclinical studies using animal models provide hope that these concerns may be unwarranted. For example, endostatin administration to mice harboring solid tumors derived from several different tissue sources leads to shrinkage of the tumor, presumably by inducing tumor cell apoptosis (Dixelius et al., 2000). A similar finding was found for the VEGF-receptor inhibitor, SU5416

DRUG	SPONSOR	TRIAL	MECHANISM
Drugs that block matrix breakdown			
Marimastat	British Biotech	Phase III	Synthetic MMP inhibitor
AG3340	Agouron	Phase II	Synthetic MMP inhibitor
COL-3	Collagenex	Phase I/II	Synthetic MMP inhibitor tetracycline derivative
Neovastat	Aeriea	Phase III	Naturally occurring MMP inhibitor
BMS-275291	Bristol-Myers	Phase I/III	Synthetic MMP inhibitor
Drugs that inhibit endothelial cells directly			
Thalidomide	Celgene	Phase I/II	Unknown
Squalamine	Magainin	Phase II	Dogfish shark liver extract; Inhibits Na-H exchanger
Endostatin	EntroMed	Phase I	Endothelial cell inhibitor
Drugs that block activators of angiogenesis			
SU5416	Sugen	Phase III	Synthetic VEGF receptor inhibitor
SU6668	Sugen	Phase I	Synthetic receptor kinase inhibitor
Interferon-alpha	Commercially available	Phase I/III	Inhibits VEGF and bFGF production
Anti-VEGF ab	Genetech	Phase II	Monoclonal antibody to VEGF
Drugs that inhibit endothelial-specific integrin/survival signaling			
EMD121974	Merck KCoA	Phase I/II	Synthetic blocker of endothelial cell integrin
Drugs with non-specific mechanisms of action			
CAI	NCI	Phase I	Inhibitor of calcium influx
Interleukin-12	Genetics Inst.	Phase I/II	Up-regulation of interferon- γ and IP-10
IM862	Cytran	Phase III	Unknown

Table 1: Angiogenesis Inhibitors in clinical trials for cancer. Taken from <http://cancertrials.nci.nih.gov/news/angio/table.html>

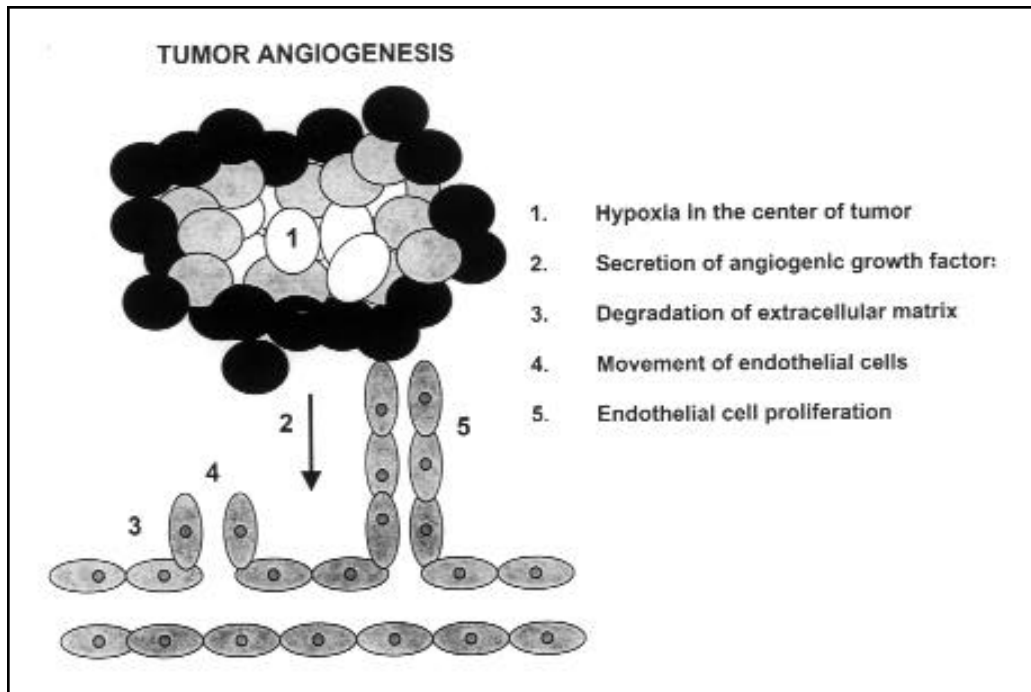


Figure 2: Tumor angiogenesis. Once a tumor grows to a certain size, the cells in the center are too far away from existing blood vessels to receive the necessary nutrients for cell survival. The lack of oxygen stimulates the production of VEGF, which is secreted from the starved cells. VEGF binds to receptors on endothelial cells of existing blood vessels, stimulating a series of events, including the secretion of matrix degrading enzymes, cellular movement into the newly created space, and cell proliferation. The endothelial cells then form tubes, and provide the necessary nutrients to the tumor.

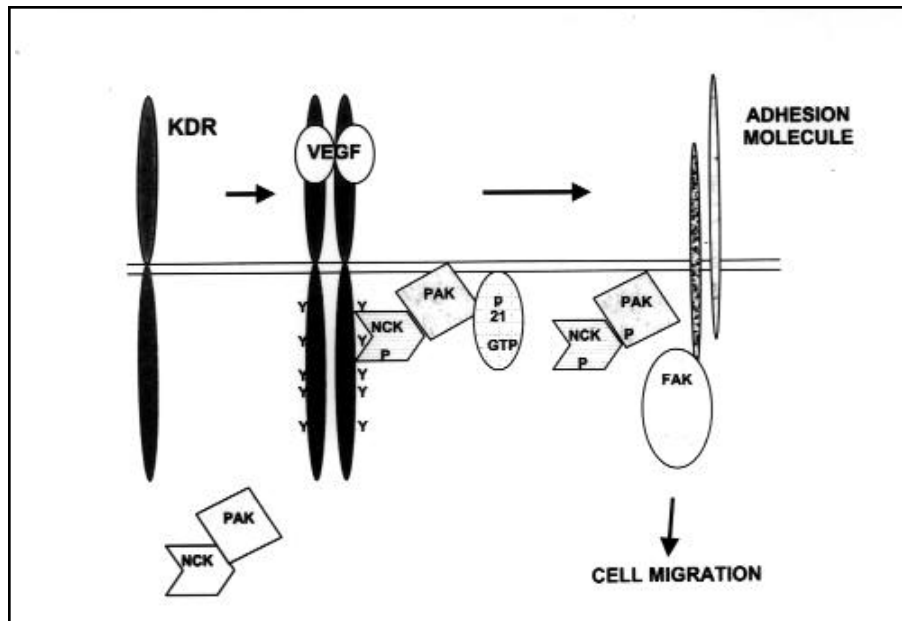


Figure 3: Signaling mechanism by which VEGF stimulates the assembly of focal adhesion.

(Shaheen et al., 1999). Furthermore, no drug-resistance was observed for at least one year of administration of endostatin (Boehm et al., 1997). It remains to be seen whether similar encouraging results will be seen in human patients as well.

VEGF as a target for tumor angiogenesis

VEGF has received attention as a target for therapeutic angiogenesis (Ferrara and Davis-Smyth, 1997). VEGF expression is up-regulated by hypoxia (Shweiki et al., 1992), and it serves as a major angiogenic factor in normal vascular development (Shalaby et al., 1995; Fong et al., 1995). The notion that VEGF's actions are predominantly specific to endothelial cells is supported by the findings that VEGF receptors are expressed on endothelial but few other cells. The expression of VEGF correlates both temporally and spatially with the onset of neovascularization (Ferrara and Davis-Smyth, 1997). Furthermore, an essential role for VEGF in tumor angiogenesis has been demonstrated in animal models by the findings that neutralizing VEGF antibodies and dominant-negative VEGF receptors inhibit both angiogenesis and the progression of the disease (Kim et al., 1993; Millauer et al., 1994).

VEGF elicits a strong angiogenic response in a variety of in vivo models. VEGF can also participate in the angiogenic response by increasing microvascular permeability (Dvorak et al., 1995). Also, VEGF stimulates several endothelial cell responses in cell culture including proliferation, migration, survival, and secretion of matrix-degrading enzymes.

In order to test the ability of VEGF to augment neovascularization and tumor growth in vivo, we have compared the rates of tumor growth in cells transfected with the VEGF gene versus control cells. Several cell lines derived from solid tumors express VEGF in cell culture, although to varying degrees. One of these cell lines, PANC1, expresses significantly less growth factor than the others. These human pancreatic ductal epithelial cells contain a number of genetic alterations, including the expression of oncogenic RAS and loss of p53 protein function. The molecular reasons that PANC1 cells express low levels of VEGF are not clear. Subcutaneous injection of PANC1 cells into immuno-compromised mice that have not been transfected with the VEGF expression vector result in a relatively slow rate of solid tumor growth. A significantly greater rate of tumor growth was observed for the VEGF-expressing cells (*Figure 1*).

These results are consistent with the hypothesis that angiogenesis is necessary for tumor growth, and that VEGF is a potent stimulator of the angiogenic response. The cartoon shown in *Figure 2* summarizes the current thinking on the physiological mechanism by which VEGF participates in tumor angiogenesis. Once a tumor grows to a certain size, the cells in the center become too far from existing blood vessels to receive necessary oxygen

and nutrients. Molecular sensors within these "starved" cells recognize the decrease in oxygen and initiate processes for producing angiogenic growth factors, most notably VEGF. VEGF is secreted from the tumor and binds to high affinity signaling receptors on the endothelial cells of existing blood vessels. This leads to the formation of new blood capillaries, which provide the necessary nutrients for tumor cell survival and tumor growth.

Elevations in VEGF levels have been detected in the serum of some cancer patients (Kondo et al., 1994), and a correlation has been observed between VEGF expression and microvascular density in primary breast cancer sections (Toi et al., 1994). A postoperative survey indicated that the relapse-free survival rate of patients with VEGF-rich tumors was significantly worse than that of VEGF-poor tumors, suggesting that VEGF expression is associated with stimulation of angiogenesis and with early relapse in primary breast cancer.

Two pharmaceuticals that inhibit VEGF actions are currently being evaluated in clinical trials. SU5416 is a synthetic inhibitor of VEGF receptor tyrosine kinase activity (Mendel et al., 2000). Sugen, the company developing this compound, reported in May, 2000, some encouraging results from a Phase I/II clinical trial involving 27 patients with colorectal cancer treated with SU5416 in combination with 5-fluorouracil/leucovorin (Via et al., 2000). Thirty-seven percent of patients had a complete or partial response to treatment, with their tumors reduced by greater than 50% of their original size. Forty-four percent of patients had stable disease, meaning that their tumors were unchanged, having neither increased nor decreased in size. Only seven percent of patients showed no response to the treatment. SU5416 is currently being evaluated in a number of Phase I and II clinical studies on patients with several different types of cancer.

Genentech reported in May, 2000, similar encouraging results from a Phase II clinical study evaluating their anti-VEGF antibody in combination with 5-fluorouracil/leucovorin in metastatic colorectal cancer. Forty percent of patients receiving anti-VEGF showed a positive response compared to 17% for patients receiving 5-fluorouracil/leucovorin alone. The time to disease progression was nine months for anti-VEGF-treated patients compared to 5.2 months in patients not receiving the antibody. The company is planning a Phase III trial in colorectal cancer to evaluate anti-VEGF as first-line therapy.

Cellular mechanisms of VEGF action

The VEGF gene encodes five alternatively spliced protein isoforms (Tischer et al., 1991). All but the 121-amino acid isoform contain a heparin-binding sequence. VEGF is expressed as a dimer, and the cysteine amino acids that are involved in both the inter- and intra-disulfide bonds are known. VEGF is one member of a family of four pro-

teins that includes placenta-derived growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D (Ferrara and Davis-Smyth, 1997). Most studies on these growth factors have utilized homodimers, although VEGF/PlGF heterodimers have been identified (Cao et al., 1996). The three dimensional structure of VEGF is very similar to platelet-derived growth factor, and both growth factors share conserved cysteine amino acids (Keck et al., 1989).

VEGF exhibits high affinity binding to two distinct endothelial cell receptor tyrosine kinases, the fms-like tyrosine kinase (FLT1) (Shibuya et al., 1990) and the kinase insert domain containing receptor (KDR) (Terman et al., 1991). Both receptors possess a single membrane-spanning domain, insert sequences within their catalytic domains, and seven immunoglobulin-like domains in the extracellular regions. Both receptors are related to the platelet-derived growth factor (PDGF) family of receptor tyrosine kinases. KDR and FLT1 are members of a family of receptor tyrosine kinases that includes FLT4 (Kaipainen et al., 1993), which is predominantly expressed in lymphatic vessels.

Although the expression of both VEGF receptor types occurs in adult endothelial cells, including human umbilical vein endothelial cells, recent findings suggest that KDR and not FLT 1 is able to mediate the mitogenic and chemotactic effects of VEGF (Keyt et al., 1996). KDR mediates other VEGF-induced cellular responses (Fujio and Walsh, 1999; Shen et al., 1999), including an enhancement in the expression of matrix-degrading enzymes, inhibition of apoptosis, and regulation of nitric-oxide synthase expression. A number of cell signaling proteins that mediate diverse biological functions of VEGF have been identified, including NCK, phospholipase C, mitogen activated protein kinase (MAPK), PI3-kinase, focal adhesion kinase (FAK), and paxillin (Abedi and Zachary, 1997; Guo et al., 1995).

The angiogenic response involves changes that occur in endothelial cell interactions with the extracellular matrix, as well as changes in cell-to-cell interactions. Endothelial cells are linked to each other by tight and adherens-type junctions and are linked to the extracellular matrix by a variety of integrin and other adhesion molecules (Carmeliet et al., 1999). VEGF activates endothelial cells, in part through stimulating signal transduction pathways that regulate the enzymatic components of adhesion complexes. VEGF-induced tyrosine phosphorylation of VE-cadherins (Esser et al., 1998), a component of adherens-type cell-to-cell junctions, has been implicated as a key step in endothelial cell migration. Experimental evidence supporting a role for VEGF in regulating cell-to-matrix interactions includes the findings that VEGF enhances the expression of eleven and twenty integrins (Senger et al., 1997), and neutralizing antibodies to v5 integrins block growth factor-induced neovascularization (Brooks et al., 1994).

Part of the significance of VEGF-induced changes in cell-to-cell and cell-to-matrix interactions in the angiogenic response is the importance of endothelial cell migration, an event that requires the disruption of these interactions. It is well established that cell migration is dependent upon the assembly of focal adhesions (FA) (Ilic et al., 1995), and recent work in our laboratory has clarified, in part, the signaling pathway that mediates VEGF-induced assembly of FA (Figure 3). VEGF binding to KDR leads to receptor autophosphorylation and the recruitment of NCK to the cell surface. NCK is an adaptor protein containing one src-homology 2 (SH2) and two src-homology 3 (SH3) domains. In quiescent cells, NCK interacts via its second SH3 domain to the p21-activated kinase (PAK). VEGF-induced recruitment of NCK to the cell surface allows for PAK recruitment as well. PAK kinase activity is activated, with subsequent association with the focal adhesion kinase, FAK, and the assembly of focal adhesion complexes.

There are several aspects of this signaling pathway that require further clarification. It is not clear whether NCK binds directly to activated KDR or interacts through a protein intermediate. The molecular mechanism that allows for PAK activation, and PAK's precise role in the assembly of focal adhesions are not known. Finally, the signaling events coupling focal adhesion assembly to cell migration are not clear.

Summary

The viability of tumor cells is dependent upon the nutrients provided by the vasculature. Tumor growth is dependent upon new blood vessel formation, and so, in theory, anti-angiogenesis inhibitors will starve tumor cells and thus block tumor growth. This conclusion holds for all solid tumors, irrespective of their tissue of origin, the specific oncogenes or tumor suppressor genes expressed, or the degree of metastasis. The next couple of years will prove very exciting as information about how this theory will translate into practice becomes available.

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