VEGF Receptor Signaling in Tumor Angiogenesis

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ABSTRACT

The growth of human tumors and development of metastases depend on de novo formation of blood vessels. The formation of new blood vessels is tightly regulated by specific growth factors that target receptor tyrosine kinases (RTKs). Vascular endothelial growth factor (VEGF) and the Flk-1/KDR RTK have been implicated as the key endothelial cell-specific factor signaling pathway required for pathological angiogenesis, including tumor neovascularization. Inhibition of the VEGF tyrosine kinase signaling pathway blocks new blood vessel formation in growing tumors, leading to stasis or regression of tumor growth. Advances in understanding the biology of angiogenesis have led to the development of several therapeutic modalities for the inhibition of the VEGF tyrosine kinase signaling pathway. A number of these modalities are under investigation in clinical studies to evaluate their potential to treat human cancers.

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INTRODUCTION

Angiogenesis, the process by which capillaries sprout from preexisting blood vessels, is tightly regulated by a large number of proangiogenic and antiangiogenic factors. Tumor cells have an absolute requirement for a persistent supply of new blood vessels to nourish their growth and to facilitate metastasis. Thus, tumor vascularization is a vital process for the progression of a neoplasm from a small localized tumor to an enlarging tumor with the ability to metastasize [1, 2]. The angiogenic cascade leading to tumor vascularization can be divided into two general phases, the prevascular phase (referred to as the “angiogenic switch”) and the vascular phase [3, 4]. Once tumor cells undergo the transformation to an angiogenic phenotype, these malignant cells are capable of inducing phenotypic changes in endothelial cells as well as in other cell types [5, 6]. At that point, avascular tumors can acquire their own blood supply, which permits a rapid rate of growth. While tumors lacking adequate vasculature become necrotic [7] or apoptotic [8], tumors that have undergone neovascularization may not only enter a phase of rapid growth but may also have increased metastatic potential.

ANGIOGENIC FACTORS

A large number of proangiogenic factors and their cognate receptors have been identified, including basic fibroblast growth factor [9], platelet-derived growth factor (PDGF) [10], platelet-derived endothelial cell growth factor [11], fibroblast growth factor [12], angiopoietin-1 [13], transforming growth factor beta-1 (TGF-β1) [14], transforming growth factor alpha (TGF-α), and epidermal growth factor (EGF) [15]. Perhaps the best characterized of the proangiogenic factors is vascular endothelial growth factor (VEGF) also known as vascular permeability factor), which is relatively unique among growth factors in terms of its specificity for the vascular endothelium [16-18]. The VEGF family currently includes six known members: VEGF, placenta growth factor, VEGF-B, VEGF-C, VEGF-D, and VEGF-E [19-21]. These are secreted as dimeric glycoproteins, all of which contain the characteristic regularly spaced eight-cysteine residues referred to as the “cysteine knot” motif [20, 22, 23]. These glycoproteins belong to a structural superfamily of growth factors that also includes PDGF-BB and TGF-β2 [24].

VEGF, the most potent direct-acting angiogenic protein known [25, 26], is a diffusible endothelial cell-specific mitogen and angiogenic factor that also increases vascular permeability. It elicits a pronounced angiogenic response in a variety of in vivo models [27-31]. Endothelial cell survival in newly formed vessels is VEGF-dependent [32]. VEGF overproduction has been identified as a major factor underlying pathological angiogenesis in vivo in conditions such as psoriasis, macular degeneration, and tumor proliferation.

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Malignant transformation of cultured cells often results in an induction of VEGF expression. For example, Kieser et al. [34] reported that mutant murine p53 tumor-suppressor gene induced expression of VEGF mRNA in transient transfection assays. Oncogenic forms of the tumor-suppressor genes, Ras and Raf, have also been shown to upregulate VEGF expression [35]. Recently, constitutive expression of mRNA and proteins for VEGF and its cognate receptors was observed in most primary and metastatic melanoma cell lines and in SV40T-transformed melanocytes [36]. Neonatal melanocytes did not express VEGF or VEGF receptors, and VEGF expression could not be induced by exogenous growth factors [36].

**ROLE OF VEGF IN ANGIOGENESIS**

Hypoxia appears to be an important stimulus for VEGF production in both malignant and normal cells [37, 38]. The induction of VEGF gene expression by hypoxia in tumor cells involves both an increase in the rate of gene transcription, mediated by the transcription factor hypoxia-inducible factor-1 [39], and an enhancement of the stability of VEGF mRNA [40]. This mechanism is discussed in more detail in a subsequent chapter on the role of VEGF in von Hippel-Lindau Syndrome by Adrian Harris. In addition to its effect in tumors, hypoxia-induced VEGF is capable of stimulating angiogenesis in a number of other sites, including endothelial cells [41], retinal pericytes [42], and the myocardium [43-46].

Transcription of VEGF mRNA is also induced by a variety of growth factors and cytokines, including PDGF, EGF, tumor necrosis factor alpha, TGF-β1, and interleukin 1-beta [19, 25]. In addition to its role in the paracrine stimulation of angiogenesis, VEGF may also have an autocrine stimulatory effect on tumor cells [47]. These autocrine and paracrine effects are summarized in Figure 1. The initial event of hypoxia-mediated transcription and factor secretion by the growing tumor and the stromal tissue leads to an upregulation and activation of growth factor receptors. This results in endothelial sprouting, increased vascular permeability, the expression of tissue matrix metalloproteinases (MMPs), and eventually the digestion of matrix, which is required for the endothelial cell to move. The increased endothelial cell mitogenesis and spread and activation of other factors lead to the formation and movement of endothelial cells, including other supporting cells like pericytes, and eventually lead to vessel extension, increased capillary integrity, differentiation of microvessel support cells, and formation of the vascular network. VEGF plays a role in the earliest events in this process. Recent evidence suggests that VEGF may not only play a role in inducing angiogenesis but also is important in promoting the survival of new vessels formed in tumors. Benjamin et al. [48] demonstrated that downregulating VEGF transgene expression using a tetracycline-regulated expression system results in the selective obliteration of immature blood vessels that have not yet recruited periendothelial cells in a human glioma xenograft model. Similar results were observed when the constitutive production of VEGF by the glandular epithelium was suppressed as a consequence of androgen-ablation therapy in human prostate cancer. These results underscore the pivotal role of VEGF in the stimulation and maintenance of newly formed vessels in tumors.

**VEGF AND FLK-1/KDR SIGNALING PATHWAY**

Three high-affinity cognate endothelial receptors for VEGF have been identified: VEGFR-1/Flt-1, VEGFR-2/Flk-1/KDR, and VEGFR-3/Flt-4. These receptors function as signaling molecules during vascular development [49]. VEGFR-1 and VEGFR-2 are cell surface receptor tyrosine kinases (RTKs), which are localized on endothelial cells during embryonic development. The coordinated patterns of expression of the genes for VEGF and its receptors suggest that these proteins participate in vascular development during embryogenesis [50, 51]. As shown in Figure 2, VEGF RTKs are single-pass transmembrane receptors that possess intrinsic cytoplasmic enzymatic activity, catalyzing the transfer of the gamma-phosphate of ATP to tyrosine residues in protein substrates.
VEGF RTKs, members of a large family of RTKs, are essential components of signal transduction pathways that affect cell proliferation, differentiation, migration, and metabolism. Activation of VEGF RTKs occurs through ligand binding, which facilitates receptor dimerization and autophosphorylation of tyrosine residues in the cytoplasmic portion. The phosphotyrosine residues either enhance receptor catalytic activity or provide docking sites for downstream signaling proteins [52, 53]. VEGFR-2 is exclusively expressed in endothelial cells and appears to play a pivotal role in endothelial cell differentiation and vasculogenesis [54, 55]. Many studies using molecular techniques have provided evidence for the role of VEGFR-2 in tumor vascularization, growth, and metastasis. For example, the manipulation of the cloned receptor to create a “dominant negative” mutation is one experimental technique that helps establish the relevance of Flk-1 to tumor angiogenesis. Millauer et al. [56] used a retrovirus encoding a dominant-negative mutant of the VEGF-2 receptor to prevent the growth of a transplanted glioblastoma tumor, demonstrating the biological relevance of the VEGF-2/Flk-1/KDR receptor/ligand system for tumor-associated angiogenesis in vivo. Recently, Bernatchez [57] used antisense oligomers directed against Flk-1 and Flt-1 to show that VEGF stimulation of endothelial cell proliferation, migration, and platelet-activating factor synthesis is Flk-1 dependent, whereas inhibition of Flt-1 expression failed to affect VEGF ability to modulate these activities. These studies have validated targeting of the VEGFR-2 signaling pathway for the development of antiangiogenic agents.

**Figure 2.** Representative structure of vascular endothelial growth factor (VEGF) tyrosine kinase receptors. The VEGF receptor family is represented by seven immunoglobulin-like loops in the extracellular domain, which binds VEGF. Two VEGF receptors form a dimer to activate autophosphorylation of tyrosine residues on the cytoplasmic domain. Ig = immunoglobulin; VEGF = vascular endothelial growth factor; Y = phosphorylated tyrosine residues.

**Table 1.** Therapeutic agents that have been developed to specifically target the VEGF pathway

<table>
<thead>
<tr>
<th>Agent</th>
<th>Target</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiozyme™ (Ribozyme Pharmaceuticals; Boulder, CO)</td>
<td>VEGFR</td>
<td>[64]</td>
</tr>
<tr>
<td>rhumAb-anti-VEGF Antibody (Genentech; South San Francisco, CA)</td>
<td>VEGF</td>
<td>[65-67]</td>
</tr>
<tr>
<td>Soluble Flt-1 (Genentech)</td>
<td>VEGF</td>
<td>[68]</td>
</tr>
<tr>
<td>Anti-Flk Antibody (Imclone; Somerville, NJ)</td>
<td>VEGFR-2</td>
<td>[69]</td>
</tr>
<tr>
<td>SU5416 (SUGEN; South San Francisco, CA)</td>
<td>VEGFR</td>
<td>[59, 70, 71]</td>
</tr>
<tr>
<td>ZD4190 (AstraZeneca; Macclesfield, UK)</td>
<td>VEGFR</td>
<td>[72-74]</td>
</tr>
<tr>
<td>PTK787/ZK22584/CGP 41251 (Novartis AG; Basel, Switzerland)</td>
<td>VEGFR</td>
<td>[75]</td>
</tr>
</tbody>
</table>

**Therapeutic Strategies for Inhibiting VEGF Pathway**

VEGF and its receptors have been implicated in the angiogenesis that occurs in many solid tumors including breast cancer [58], colon cancer [59], hepatoma [60], bladder cancer [61], gastric cancer [62], and prostate cancer [63]. Since formation of solid tumors is angiogenesis dependent, several strategies have been developed for targeting the VEGF pathway as part of anticancer therapy (Table 1) [59, 64-75]. Potential approaches for blocking VEGF action include inhibiting secretion of endogenous tumor VEGF, neutralizing VEGF in the microcirculation, and preventing VEGF binding and subsequent signal transduction. A number of these strategies for inhibiting tumor angiogenesis by selectively targeting the VEGF signaling pathway are currently being tested in early phase I/II clinical trials.

**Oligonucleotides**

Secretion of VEGF can be inhibited by antisense oligonucleotides or expression constructs specific for VEGF, which have been successfully used in models of thyroid carcinoma [76], glioma [77], and melanoma [78]. Ribozymes are RNA molecules that possess the dual properties of RNA sequence-specific recognition and site-specific cleavage of other RNA molecules. Ribozymes that target the VEGF receptor mRNAs...
were developed, and their biological activities in cell culture and in an animal model were assessed [64]. Ribozymes targeting Flt-1 or KDR mRNA sites reduced VEGF-induced proliferation of cultured human vascular endothelial cells and specifically lowered the level of Flt-1 or KDR mRNA present in the cells. Anti-Flt-1 and KDR ribozymes also exhibited antiangiogenic activity in a rat corneal pocket assay of VEGF-induced angiogenesis [64].

**Anti-VEGF Monoclonal Antibodies**

Several anti-VEGF monoclonal antibodies have been developed and have demonstrated efficacy in a wide variety of human tumors in xenograft models [25, 65-67, 79-81]. Initial studies by Kim et al. [65] demonstrated treatment with a murine monoclonal antibody specific for VEGF (mumAb VEGF A.4.6.1) potently suppressed angiogenesis and growth in a variety of human tumor cell lines transplanted in nude mice. Subsequently, a recombinant humanized mAb VEGF version of this antibody, rhumAb VEGF, was developed for clinical evaluation in the treatment of solid tumors and other disorders [82]. A phase I clinical trial of rhumAb VEGF in 25 cancer patients showed that multiple doses of anti-VEGF were safe and well tolerated [83]. In a phase II trial of rhumAb VEGF in 15 patients with hormone refractory prostate cancer, administration of 10 mg/kg of rhumAb VEGF intravenously every two weeks resulted in three possible mixed responses [84].

Similarly, Prewett et al. [69] demonstrated that treatment with an anti-Flk-1 mAb significantly suppressed the growth of primary murine Lewis lung, 4T1 mammary, and B16 melanoma tumors and the growth of Lewis lung metastases. This antibody also completely inhibited the growth of established epidermoid, glioblastoma, pancreatic, and renal human tumor xenografts. Histological examination of anti-Flk-1 mAb-treated tumors showed evidence of decreased microvessel density, tumor cell apoptosis, decreased tumor cell proliferation, and extensive tumor necrosis. These findings support the conclusion that anti-Flk-1 mAb treatment inhibits tumor growth by suppressing tumor-induced neovascularization and demonstrate the potential for therapeutic application of anti-VEGF receptor antibody in the treatment of angiogenesis-dependent tumors [69]. Based on these results, the anti-Flk-1 antibody has been introduced into the clinic.

**Soluble VEGF Receptor**

An alternative approach would be to use a soluble recombinant Flt-1 receptor to inhibit the VEGF signaling pathway. Using this approach, a soluble full-length recombinant Flt-1 could be used to bind to circulating VEGF [68]. Another approach would be to produce tumor cells transfected with cDNA encoding the native soluble Flt-1 (sFlt-1) truncated VEGF receptor, which can function by sequestering VEGF or by forming inactive heterodimers with membrane-spanning VEGF receptors in a dominant negative fashion [85]. Both of these techniques have demonstrated preclinical efficacy. Although VEGF has been successfully neutralized using these strategies in preclinical models, their potential application may be limited by the significant amount of protein that would be needed to treat humans. Therefore, preventing RTK signal transduction may be a more appropriate means of tumor control in humans.

**Small Molecule Inhibitors**

The 3-substituted indolinone compound, SU5416, is a specific and potent catalytic inhibitor of VEGFR protein kinases [86] (Fig. 3). It inactivates Flk-1/KDR by binding in the adenine-binding pocket (Fig. 4). It is a specific VEGFR inhibitor that has virtually no inhibitory activity against serine threonine protein kinases and tyrosine kinases, such as Src, FGF receptor, Met, and Abl and has little activity against PDGF receptor [70]. At present, SU5416 is the most clinically advanced VEGF RTK-selective tyrosine kinase inhibitor being developed for antiangiogenic treatment of cancer [87]. As would be expected by its mechanism of action, SU5416 inhibits tumor growth in vivo in a dose-dependent manner, whereas it has no effect on tumor cells in vitro [70]. SU5416 has shown activity in a large number of tumor xenografts in nude mice including melanoma, glioma, fibrosarcoma, and lung, epidermoid, mammary, and prostate carcinomas [70] as well as in neurogenic sarcoma xenografts [71]. Recently, Shaheen et al. [59] evaluated the effect of SU5416 on tumor angiogenesis and metastasis in a human colon cancer xenograft model. In this study, SU5416 inhibited tumor metastases, microvessel formation, and cell

![Figure 3. Structural formula of SU5416. The chemical name of the agent is 3-{(2,4-dimethylpyrrol-5-yl) methylidene}-indoline-2-one.](attachment:image_url)
proliferation [59]. These findings indicate that targeting the VEGF receptor/ligand system with SU5416 decreases tumor vascularity and vessel density and increases tumor cell apoptosis and is a rational approach to inhibiting tumor growth. In a phase I clinical trial with SU5416 after enrollment of 69 patients, the drug was well tolerated at dose levels of 4.4-145 mg/m²/day, and stable disease was seen in patients with Kaposi’s sarcoma and in patients with non-small cell lung, colorectal, and basal cell cancers [88, 89].

Another substituted indolin-2-one inhibitor of RTKs, SU6668, inhibits the signaling of the VEGF receptor (Flk-1/KDR) and also targets the PDGF and fibroblast growth factor receptors [86, 90, 91]. This novel drug is believed to inhibit tumor growth by preventing angiogenesis and by its direct effects on the tumor cells and the surrounding stromal cells, which support tumor cell growth. SU6668 has recently entered phase I human trials for the treatment of solid tumors.

Several other VEGF RTK inhibitors have shown preclinical efficacy. AstraZeneca Pharmaceuticals (Macclesfield, UK) have identified a series of substituted 4-anilinoquinazolines that are potent inhibitors of VEGFR-1 and VEGFR-2. Of these, ZD4190 is an orally active inhibitor of Flt-1 and Flk-1/KDR that prevented VEGF-mediated proliferation of endothelial cells in vitro [72]. Following chronic oral administration, this compound significantly inhibited the growth of various human tumor xenografts in vivo including breast, colon, lung, ovarian, and prostate carcinomas [73]. ZD4190 has also been shown to reduce significantly vascular endothelial permeability in experimental models [74]. Two additional oral inhibitors of VEGF RTK activity, ZK222584 and CGP 41251, are under development by Novartis Pharma (Basel, Switzerland). Originally identified as an inhibitor of protein kinase C, CGP 41251 has been shown to inhibit the ligand-induced autophosphorylation of VEGF-R2/Flk-1/KDR without affecting the activity of other RTKs such as VEGF-1/Flt-1 and FGF [92]. CGP 41251 has been shown to have a broad antiproliferative effect in vitro and inhibits the angiogenic response to VEGF in vivo [92]. Similarly, ZK222584 has been shown to inhibit angiogenesis and growth of human ovarian carcinomas in vivo in a dose-dependent manner [75]. In mice, this compound was associated with increased survival time, decreased tumor weight, and decreased ascites volume when administered orally [75].

CONCLUSION

The future of RTK inhibitors as therapeutic agents will be clearer over the next several years as results of the current clinical trials become available and as the factors regulating angiogenesis and the interactions between these factors are more fully elucidated. Drug resistance is a major problem with chemotherapy agents because many tumors are genetically unstable. Since cytotoxic and antiangiogenic therapies are aimed at different cellular targets, clinical strategies to combine antiangiogenic agents with cytotoxic therapy are being devised. It is hoped that combining these agents will be more effective in controlling tumor growth; cytotoxic agents will reduce the tumor burden of already vascularized tumors, while antiangiogenic agents will prevent neovascularization and growth of small and occult metastatic foci as well as the formation of new metastatic lesions.

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