Receptor tyrosine kinases as targets for inhibition of angiogenesis

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Anti-angiogenic agents potentially have broad applications in the clinic. Although most agents now in development are intended ultimately for use as anti-cancer drugs, patients with a range of disorders may benefit in the longer term. The signal recognition and transduction processes involved in controlling angiogenesis are complex and are likely to be dependent on the status of the target endothelial cell in a specific organ or tissue. In this review, the authors focus on signaling interactions that affect microvascular endothelium and the role of growth factors and their receptor tyrosine kinases in the regulation of microvessel physiology as they relate to the angiogenic process.

Angiogenesis, the sprouting of capillaries from pre-existing blood vessels, is a complex process involving many biological and cellular functions. The process begins when endothelial cells become activated and cause dissolution of the basement membrane, leading to migration of the endothelial cells. New capillary lumina are formed by realignment and vacuolization of the migrating endothelial cells. Capillary loops are then formed, followed by the deposition of new basement membranes around the vessels (for review, see Ref. 1). These events are illustrated in Figure 1. All of these processes depend on the tight regulation of factors that 'promote' or 'inhibit' these biological events. Signal recognition and transduction are complex processes that are likely to be dependent on the status of the target endothelial cell in a specific organ or tissue. This review will focus on signaling interactions that affect microvascular endothelium and will not address large vessel endothelium, which is important for controlling vasoconstriction, vasodilation, blood pressure and other physiological parameters that affect blood supply. In addition, the review will primarily address the role of growth factors and their receptor tyrosine kinases (RTKs) in the regulation of microvessel physiology as they relate to the angiogenic process.

During development, the angiogenic process is active to ensure the formation of the capillary network associated with developing organ systems. However, the turnover of endothelial cells in the normal human adult is very low, in the order of years, except during formation of the corpus luteum, pregnancy, wound healing following tissue injury, or when oxygen supply is compromised. Pathologic angiogenesis occurs under many conditions and is thought to be induced by local ischemia. Diseases in which angiogenesis is thought to play a critical role in the underlying pathology include: ocular diseases such as diabetic retinopathy, retinopathy of prematurity and age-related macular degeneration; vascular diseases such as ischemic heart disease and atherosclerosis; chronic inflammatory disorders such as psoriasis and rheumatoid arthritis; and solid tumor growth.

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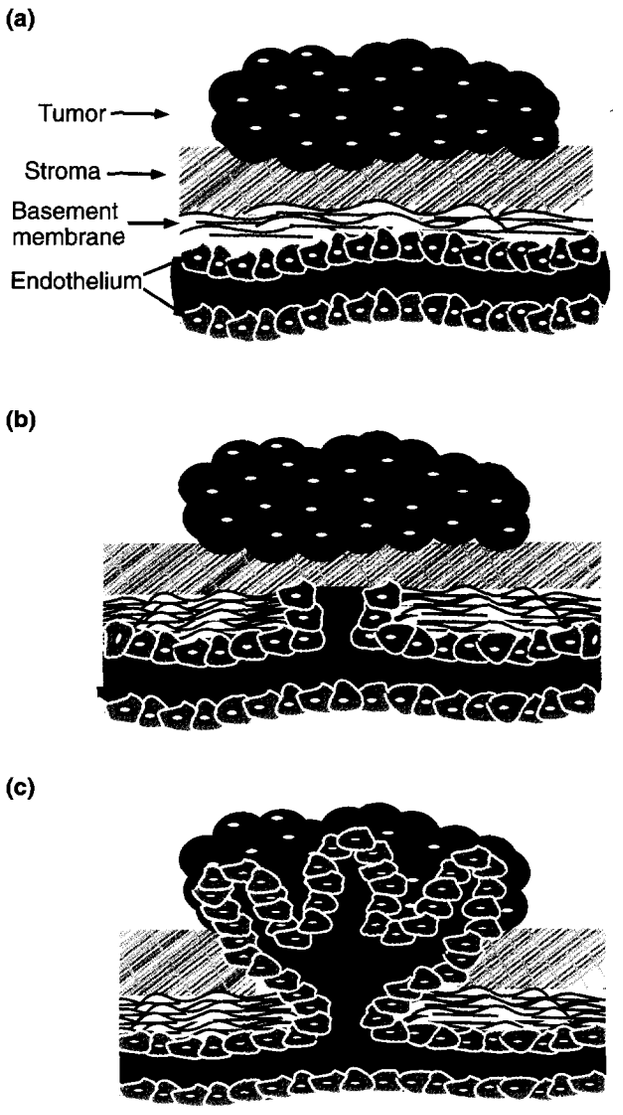

Figure 1. *Formation of new blood vessels by angiogenesis. Angiogenesis, the sprouting of new blood vessels from pre-existing ones, is a complex mechanism involving several biological processes as outlined in the text. (a) Small nonangiogenic tumor; (b) invasion and migration of endothelial cells through the basement membrane; (c) extension of vessels into the tumor.*

While RTKs are thought to be important in angiogenesis associated with these pathologic conditions, this review will primarily focus on the role of RTKs in tumor angiogenesis.

Angiogenesis in solid tumor growth

Results of research originating in the 1970s and continuing today have led to the conclusion that new blood vessel growth is required for the growth and metastasis of solid tumors. Immunohistochemical analysis of sections from growing tumors shows a preponderance of blood vessels, irrespective of tumor type. This is illustrated in Figure 2. These new blood vessels are required for tumors to expand beyond a minimum volume. Before tumors acquire the angiogenic phenotype, new blood vessel growth is kept in check by a balance of angiogenic and anti-angiogenic factors. Some of these factors are listed in Box 1.

It has been proposed recently by Hanahan and Folkman[2] that there is a 'switch' that perturbs the balance between these factors. The 'angiogenic switch' has been suggested to be a component of the tumor phenotype that is often activated during the preneoplastic stage in tumor development. With the balance disturbed, unchecked angiogenic factors released from hypoxic tumor cells migrate to nearby blood vessel endothelia, which signals the activation of biochemical events leading to the cellular changes associated with the angiogenic process. This is illustrated in Figure 3.

While tumors that lack adequate vasculature become necrotic[3] and/or apoptotic[4,5], tumors that have undergone neovascularization may not only enter a phase of rapid growth but may also have increased metastatic potential. The significance of angiogenesis in human tumors has been highlighted by recent studies that relate the angiogenic phenotype to patient survival. These studies found that the number of microvessels in a primary tumor has prognostic significance in breast carcinoma[6,7], bladder carcinomas[8], colon carcinomas[9] and tumors of the oral cavity[10].

Receptor tyrosine kinases (RTKs)

RTKs (also known as growth factor receptors) play an important role in many cellular processes. They make up a large class of receptors represented by at least 19 distinct subfamilies (for review, see Ref. 11). All of these molecules have an extracellular ligand-binding domain, a transmembrane domain and a tyrosine kinase domain. Upon ligand binding, receptors dimerize, the tyrosine kinase is activated and the receptors become autophosphorylated (for review, see Ref. 12). Receptor-specific phosphotyrosines serve as binding sites for other substrates. Some of these molecules are in turn phosphorylated, resulting in their activation and the ability to affect additional downstream molecules. Others do not have intrinsic enzymatic activity but serve as docking proteins for enzymes that are activated only when brought into association with the appropriate cellular compartment. The cascade triggered by RTK activation

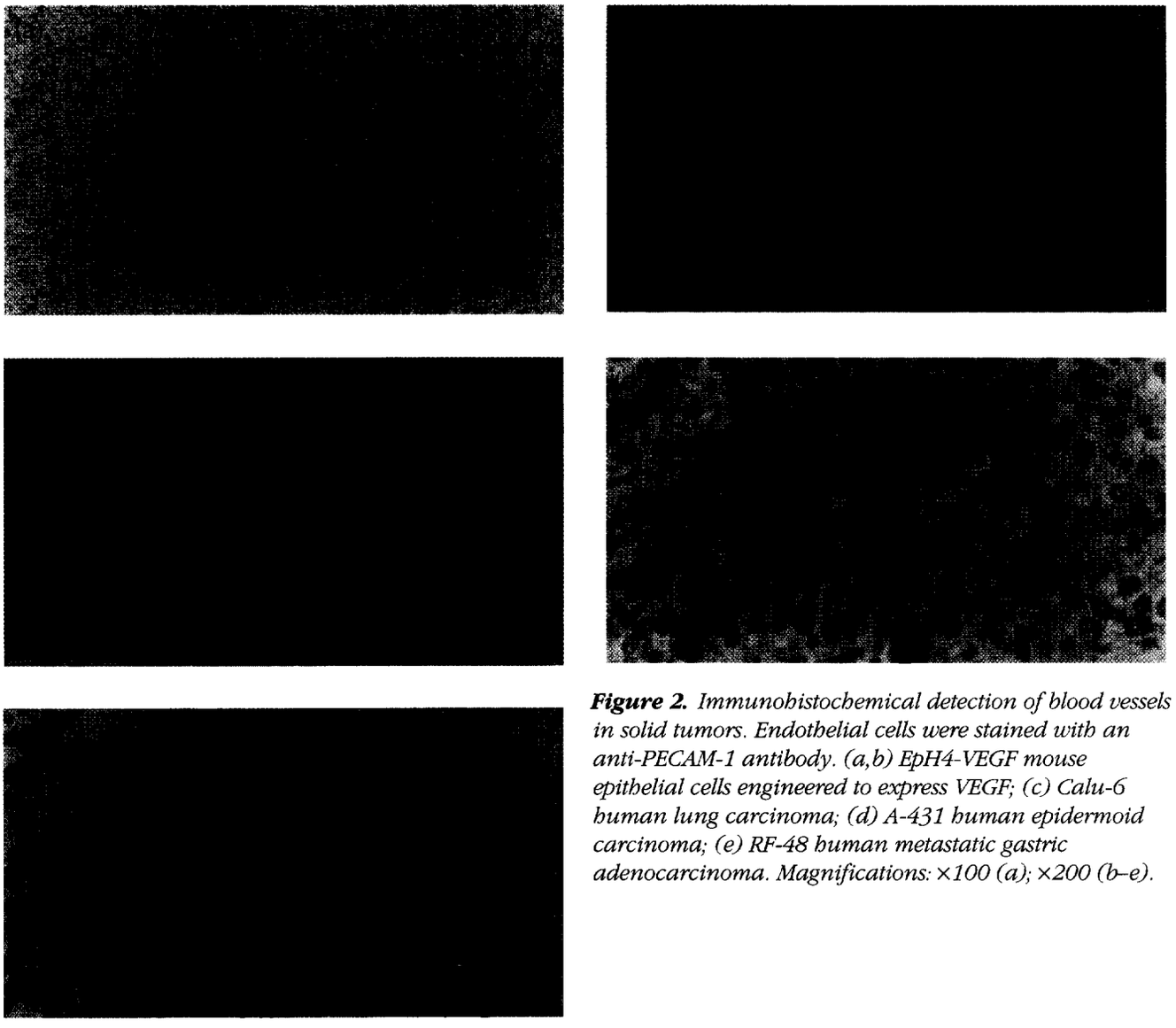

Figure 2. *Immunohistochemical detection of blood vessels in solid tumors. Endothelial cells were stained with an anti-PECAM-1 antibody. (a,b) EpH4-VEGF mouse epithelial cells engineered to express VEGF; (c) Calu-6 human lung carcinoma; (d) A-431 human epidermoid carcinoma; (e) RF-48 human metastatic gastric adenocarcinoma. Magnifications: ×100 (a); ×200 (b–e).*

modulates cellular events, determining proliferation, differentiation and morphogenesis in a positive or negative fashion.

Disturbances in the expression of growth factors, their cognate RTKs, or constituents of downstream signaling pathways are commonly associated with many types of cancer. Gene mutations giving rise to altered protein products have been shown to alter the regulatory mechanisms influencing cellular proliferation, resulting in tumor initiation and progression.

Uncontrolled growth responses are manifested via both autocrine and paracrine pathways. As shown in Figure 3, the paracrine pathway is thought to be predominant for angiogenesis. Growth factors released by tumor cells begin the signaling cascade that regulates gene transcription (Figure 3a) for those proteins involved in new blood vessel formation (Figure 3b).

RTKs in angiogenesis

There are several strategies for demonstrating the role of RTKs in angiogenesis. Early work usually focuses on defining a temporal and spatial correlation of ligand and receptor expression with biological events in model systems for angiogenesis, including wound healing, tumor growth and induced corneal angiogenesis. The role of RTKs in the angiogenic process can be better understood, however, by

examining the cellular phenotype following interference with receptor signaling. This can be achieved through several techniques, described below.

One strategy for interfering with receptor signaling is to inhibit ligand binding. This can be accomplished with specific receptor-binding antagonists such as ligand fragments, or with nonspecific antagonists such as suramin, with neutralizing antibodies to either the ligand or receptor, or with an excess of soluble receptor or ligand-binding protein, which will sequester the ligand.

A second strategy for interfering with receptor signaling is to block signal transduction by overexpression of a dominant-negative receptor. Because receptor kinases typically dimerize to induce signal transduction through transphosphorylation (for review, see Ref. 13), prevention of receptor dimerization due to overexpression of kinase-deficient receptors will attenuate activation of signaling. Receptors can be made kinase-deficient by introduction of a point mutation in amino acids critical for kinase function, or deletion of the kinase or entire cytoplasmic domain. Dominant-negative receptors attenuate signaling by forming heterodimers with endogenous receptors, which may allow partial signaling, and by diluting the number of effective growth factor binding sites with inactive homodimers of the dominant-negative receptor.

The third strategy for understanding receptor function involves depleting the receptor protein. This can be accomplished by the introduction of exogenous agents such as antisense oligonucleotides, antisense RNA, or ribozymes, all of which lead to degradation of the receptor mRNA and gradual depletion of the protein in the cell. Alternatively, embryos, and perhaps animals, can be made that lack the receptor or ligand of interest by homologous recombination in embryonic stem cells and inactivation of the target locus in offspring following implantation. Because mice with such targeted gene deletions ('knockouts') are often not viable, it is most common to compare embryos at various stages of development with normal embryos for altered development of vascular systems.

Box 1. Angiogenic and anti-angiogenic factors involved in the regulation of new blood vessel growth in tumors

Angiogenic factors	Anti-angiogenic factors
Basic fibroblast growth factor (bFGF)	Platelet factor 4
Acidic fibroblast growth factor (aFGF)	Thrombospondin-1
Transforming growth factor-a (TGF-a)	Transforming growth factor-b
Transforming growth factor-b (TGF-b)	Interferon-a
Platelet-derived growth factor (PDGF)	Prolactin fragment
Insulin-like growth factor (IGF)	Angiostatin (fragment of plasminogen)
Vascular endothelial growth factor (VEGF)	Tissue inhibitors of metalloproteinases
Platelet-derived endothelial cell growth factor	TIMP-1
Granulocyte colony-stimulating factor	TIMP-2
Hepatocyte growth factor (HGF)	TIMP-3
B61	bFGF soluble receptor
Tie-2 ligand	Placental proliferin-related protein
Angiogenin	
Tumor necrosis factor-a (TNF-a)	
Growth hormone	
Placental growth factor	
Interleukin 8	
Proliferin	
Prostaglandins E_1 and E_2	

Using these strategies, a number of RTKs have been shown to be involved in angiogenesis, either directly or indirectly (Figure 4). Of particular interest are Flt-1 and Flk-1, the receptors for vascular endothelial growth factor (VEGF), as well as Tie-1 and Tie-2. Flt-1 and Flk-1 are also known as VEGFR1 and VEGFR2, respectively; the human homolog of Flk-1 is KDR. For the purposes of this review, the VEGF receptors will be referred to as Flt-1 and Flk-1. These receptors, as well as Tie-1 and Tie-2, which are also referred to as TIE (tyrosine kinase with immunoglobin and EGF homology domains) and TEK (tunica interna endothelial cell kinase), are expressed primarily on endothelial cells and play a direct role in angiogenesis. A summary of these endothelial-specific RTKs and their ligands is shown in Figure 5.

Other RTKs of potential interest in angiogenesis include the epidermal growth factor (EGF) receptor, the platelet-derived growth factor (PDGF) receptor, the fibroblast growth factor (FGF) receptor family, c-met proto-oncogene product (MET) and epithelial cell kinase (ECK). They have also been implicated in angiogenesis, but they have broader expression patterns that encompass other cell types as well as endothelial cells. The following is an overview of the

Figure 3. *Stimulation of tumor angiogenesis. (a) Angiogenic growth factors (green) are synthesized and released by tumor cells, particularly under hypoxic conditions. The growth factors are bound by their receptors (blue) on endothelial cells, initializing a signaling cascade leading to activation of genes in the nucleus (orange). (b) The process of mitogenesis and differentiation of endothelial cells leads to the formation of new blood vessels within the tumor (red), thus allowing appropriate nutrients and growth factors to support continued tumor-cell proliferation. Artwork by Doug Struthers.*

RTKs postulated to play a role in angiogenesis and a summary of the evident data.

VEGF receptors

mRNA for the receptor tyrosine kinases Flk-1 and Flt-1, and their ligand, VEGF (also known as VPF), is expressed in endothelial cell precursors and later in the endothelial cells of vessels throughout mouse and rat embryos[14–17]. Furthermore, protein expression of VEGF receptors was confirmed along the lumina of vessels in rat embryos by binding of ^{125}I-labeled VEGF (Ref. 18). Thus, the temporal and spatial patterns of the expression of VEGF and its receptors support their involvement in angiogenesis during development.

VEGF, Flt-1 and Flk-1 have also been implicated in the angiogenesis that occurs in many solid tumors, including gliomas[19,20], breast cancer[21], bladder cancer[22], colon carcinoma[9,23] and other cancers of the gastrointestinal tract[24]. A correlation has been observed between VEGF expression and vessel density in human breast tumors[7,25],

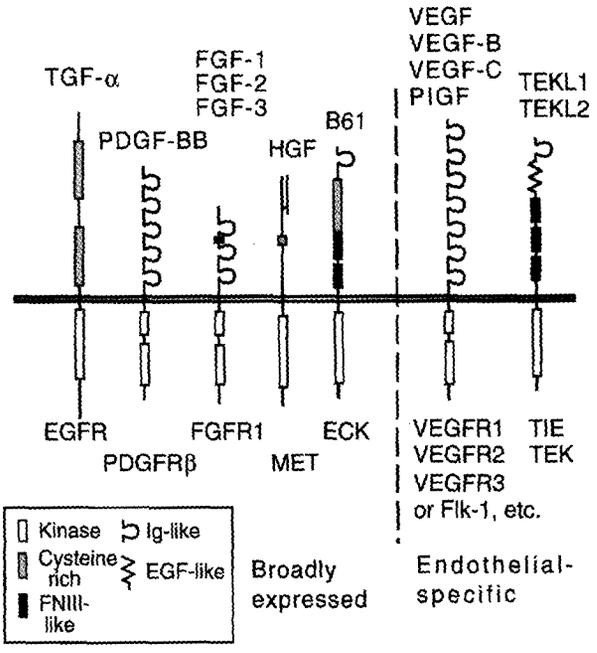

Figure 4. *Receptor tyrosine kinases (RTKs) in angiogenesis. Multiple sequence alignments of tyrosine kinase catalytic domains allow RTKs to be classified into distinct families. RTKs implicated in angiogenesis are shown. The horizontal line represents the plasma membrane with the extracellular domain above and the cytoplasmic domain below. TGF-α, transforming growth factor-α; EGFR, epidermal growth factor receptor. PDGF-BB, platelet-derived growth factor; PDGFRβ, PDGF β-receptor. FGF-1, -2, -3, fibroblast growth factor; FGFR1, FGF receptor type 1. HGF, hepatocyte growth factor; MET, HGF receptor. B61, ligand for ECK; ECK, epithelial cell kinase. VEGF, -B, -C, vascular endothelial growth factor; PlGF, placental growth factor; VEGFR1, -2, -3 (Flk-1, etc.), VEGF receptors. TEKL1, -2, Tie-2 ligands; TIE, tyrosine kinase with immunoglobin and EGF homology domains; TEK, tunica interna endothelial cell kinase. FNIII, fibronectin type III repeat.*

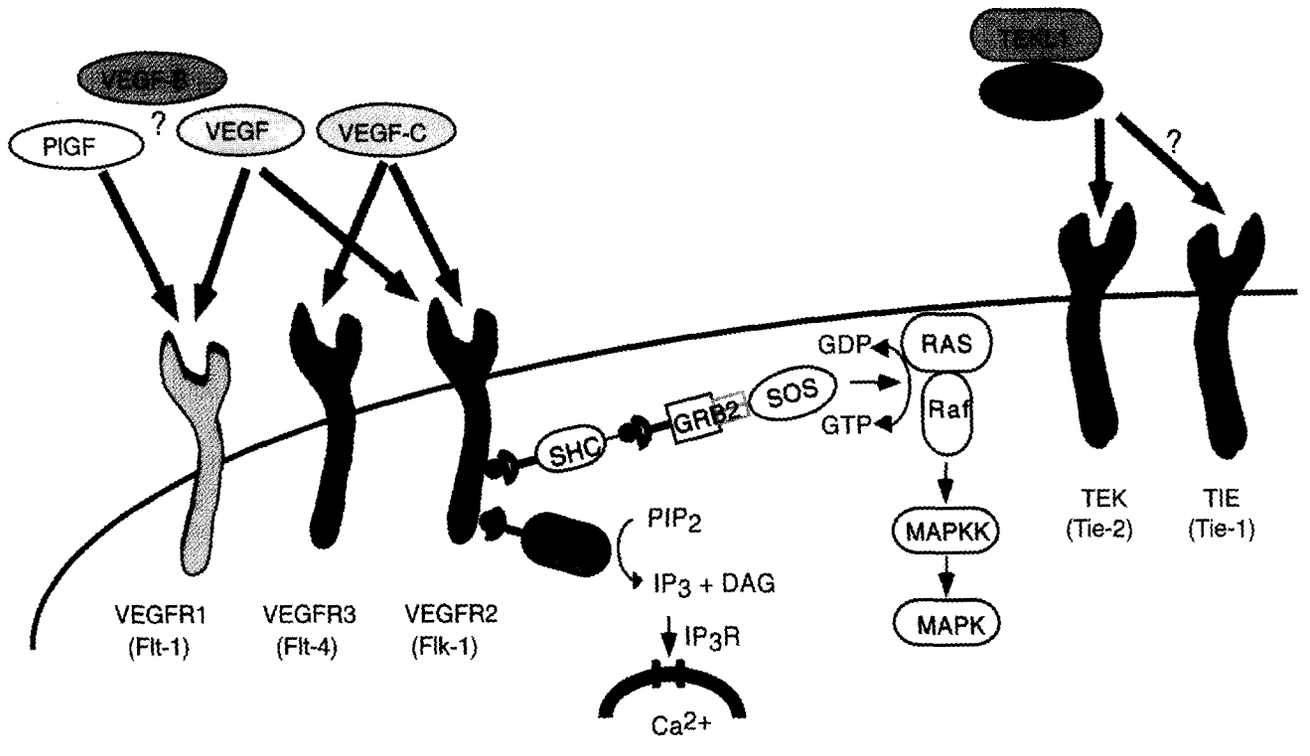

Figure 5. *Signaling through Flk-1 and Tie-2 family receptors. The three known vascular endothelial growth factor receptors (VEGFR1, -2, -3) have differential binding affinities for the various members of the VEGF family. Two Tie-2 ligands have recently been discovered (TEKL1, TEKL2). Ligand binding leads to receptor dimerization and activation, and the resulting phosphotyrosines on the receptors serve as binding sites for other proteins. Typical signal transduction pathways are illustrated.*

renal cell carcinoma[26] and colon cancer[9]. In highly vascularized glioblastoma, transcripts for all three proteins were identified by *in situ* hybridization. Flk-1 was found to be dramatically upregulated, localized to the necrotic areas, when compared with low-grade gliomas. The receptors were detected in the endothelial cells of the vessels, whereas the VEGF was found in the tumor cells. None of the mRNAs were expressed in normal brain tissue in which angiogenesis does not occur[19,20].

VEGF is mitogenic for endothelial cells *in vitro*. In such a system, a neutralizing antibody against Flk-1 inhibited mitogenesis[27], as did a truncated soluble form of Flt-1, which competed for binding of VEGF to its receptors[28]. Similarly, ribozymes that cleave *flk-1* or *flt-1* mRNAs reduced the growth of human microvasculature endothelial cells, presumably by decreasing the amount of receptors on the cells[29]. However, mutant forms of VEGF that preferentially bind to Flt-1 do not stimulate endothelial cell proliferation, which raises questions regarding the role of Flt-1 in angiogenesis[30].

The genes for VEGF and its receptors have been disrupted through targeted mutagenesis in mice. Embryos homozygous for mutant *flk-1*, *flt-1* or VEGF were resorbed by days 10–12 of development. In the case of *flk-1* disruption, no endothelial cells or vessels were observed in the embryo or yolk[31]. This indicates that Flk-1 is required for development of mature endothelial cells. In contrast, embryos lacking Flt-1 had mature endothelial cells, but the vessels were large and disorganized[32]. In two studies with disruption of the VEGF gene, heterozygous as well as homozygous embryos were resorbed[33,34]. Mature endothelial cells were detected before resorption, but the vessels in the embryos and yolks were abnormal. Possibly, the newly identified members of the VEGF family, VEGF-B (Ref. 35) or VEGF-C/VRP (Refs 36,37) (Figure 5), can substitute for VEGF and allow maturation of endothelial cells, but cannot substitute for VEGF in later steps of angiogenesis.

A variety of techniques have been used to investigate the role of VEGF signaling in tumor angiogenesis. Dominant-negative Flk-1 lacking the kinase domain blocked the activation of the endogenous Flk-1 tyrosine kinase in cultured cells[38]. It also inhibited the growth of eight out of nine types of tumors implanted subcutaneously into nude mice, and significantly reduced vessel density in the small tumors that did form[38,39]. Furthermore, embryonic stem cells with disrupted VEGF grew very poorly as subcutaneous implants in nude mice compared with control embryonic stem cells[34]. Also, a reduction in VEGF expression following the introduction of antisense DNA constructs inhibited the growth of C6 rat glioma cells in nude mice as well as reducing vessel density within the tumor[40]. Human melanoma cells in nude/SCID mice were also inhibited using antisense constructs[41]. Likewise, reduction of VEGF levels with neutralizing antibodies inhibited the growth of human rhabdomyosarcoma, glioblastoma multiforme and leiomyosarcoma in Beige nude/xid mice[42], and of fibrosarcoma in BALB/c nude mice[43]. Thus, there is strong evidence that VEGF signaling through the Flk-1 tyrosine kinase is required for angiogenesis in solid tumor growth as well as in development.

Tie-1 and Tie-2

Tie-1 and Tie-2 (TEK) are receptor kinases whose expression is most prevalent in the vascular endothelium during embryonic development[44–46]. In adults, the Tie receptors are weakly expressed, but are induced during active angiogenesis. For example, Tie expression is upregulated in skin capillaries during wound healing[47] and in angiogenesis associated with metastatic melanomas[48]. Transgenic mouse embryos expressing a dominant-negative Tie-2 receptor were developmentally delayed, had compromised heart development and exhibited signs of hemorrhage[49], suggesting that Tie-2 kinase activity is important for vasculogenesis. Mouse embryos lacking Tie-1 (Refs 50, 51) or Tie-2 (Refs 49, 51) exhibited somewhat different phenotypes. Embryos of Tie-1 knockout mice died in mid- to late gestation or shortly after birth as a result of breathing problems. All embryos of mice lacking Tie-1 exhibited peripheral and abdominal hemorrhage and/or edema. Because their vasculature appeared to be properly developed, these observations suggest that Tie-1 is important for maintaining vascular integrity. In contrast, embryos lacking Tie-2 died earlier in gestation and had obviously retarded growth of the head and heart. The vasculature of Tie-2 knockout mice was abnormally developed, suggesting that Tie-2 is important for vasculogenesis. Thus, the expression of Tie receptors in embryos and adults, the effect of expression of dominant-negative receptors in transgenic mice and the effect of deletion of Tie receptors on development strongly implicate them as important RTKs in the angiogenesic process.

EGF receptor

EGF and transforming growth factor type-α (TGF-α) bind to EGF receptors with comparable affinity, but TGF-α appears to be a more potent mediator of angiogenesis in a hamster cheek pouch assay[52]. TGF-α is secreted from some tumor

cells[53] and is seen in psoriatic epidermis[54]. Thus, signaling through EGF receptors has been classified as angiogenic. In support of this classification, inhibitors of EGF or TGF-α binding have been used in experimental models to validate the pathway. For example, a fragment of EGF has been demonstrated to inhibit EGF-induced angiogenesis in a vitelline membrane assay[55]. Furthermore, neutralizing antibodies prevent TGF-α-induced tube formation by human omentum microvascular endothelial cells in collagen gels[56]. Although these observations appear to confirm that ligand-induced angiogenesis can be inhibited if the initial induction is prevented, they do not validate EGF receptors as critical signaling receptors for angiogenesis. Gross abnormalities of the vasculature have not been reported in mouse embryos lacking TGF-α (Refs 57,58) or EGF receptors[59–61], or with an EGF receptor mutation that attenuates signaling[62]. In addition, the observation that activation of EGF receptors can induce the expression of VEGF (Ref. 63) suggests that EGF and TGF-α are more likely to be indirect angiogenic factors.

FGF receptors

When a secreted form of FGF-1 (acidic FGF) was expressed in porcine arteries by *in vivo* gene transfer, it induced neointimal hyperplasia and angiogenesis within the neointima[64]. Neointimal hyperplasia was not observed in expanded polytetrafluoroethylene (ePTFE, Gore-Tex) vascular grafts coated with FGF-1 (Ref. 65), but significant enhancement of endothelialization was induced by the FGF-1 coating. In *in vitro* models of angiogenesis, blocking FGF-2 (bFGF) interaction with its receptor by antibodies to FGF-2 (Refs 66,67), platelet factor 4 (Ref. 67), or mutations of the heparin-binding site[68] resulted in inhibition of various steps involved in angiogenesis, such as induction of endothelial cell protease expression[66,68], cellular invasion[66] and formation of capillary-like tubes[67,68]. There are also correlative observations relating the expression of FGF-2 and FGFR1 to cardiac development[69,70] and endothelium re-establishment after vessel injury[71]. Dominant-negative FGF receptors have been targeted to the eye lens[72,73], epidermis[74] and lung[75], but not to endothelium. Mouse embryos homozygous for deletion of FGFR1 died early in development (before E10.5) and exhibited gross abnormalities in mesodermal patterning[76,77]. Neither group[76,77] specifically reported on the effects that FGFR1 deletion has on vasculogenesis. Thus, although FGFs and their receptors have been implicated in angiogenesis, validation of a specific role for one of the four known FGF receptors or ten known FGFs is still lacking.

HGF/SF receptor (MET)

Hepatocyte growth factor/scatter factor (HGF/SF) and its receptor, MET (HGFR), have many important roles in embryogenesis (for review, see Ref. 78). However, regulation of vasculogenesis does not appear to be one. Embryos homozygous for deletions of HGF/SF (Refs 79,80) or MET (Ref. 81) developed a normal vascular system, but died between E13.5 and E16.5 from abnormal development of the placenta and liver. In matrix and cornea models, HGF/SF induced angiogenesis which was inhibited with anti-HGF/SF antibodies[82,83]. Analysis by RT-PCR (reverse transcriptase polymerase chain reaction) of cells infiltrating a Matrigel plug containing HGF/SF revealed the expression of several angiogenic factors and chemokines, including VEGF (Ref. 84). However, anti-VEGF antibodies were effective at only partial attenuation of HGF/SF-induced angiogenesis[84]. Thus, it is not clear whether HGF/SF-induced angiogenesis occurs through a direct or an indirect mechanism.

B61 receptor (ECK)

During embryogenesis, ECK has been implicated as having a role in pattern formation in gastrulation, hindbrain segmentation and limb development[85]. It is unknown whether ECK is expressed during development of the vascular system. In contrast, B61, a ligand for ECK, is expressed in endothelial cells of the developing vascular system and the endocardium of the developing heart, and has been postulated to have a role in vasculogenesis/angiogenesis[86]. The expression of ECK has been demonstrated in human umbilical vein endothelial cells, and B61 was shown to induce angiogenesis in a cornea model[87]. Because B61 can be induced by TNF-α (Ref. 88), which has been reported to be an angiogenic factor[89], Pandey and coworkers[87] investigated a putative role for B61 in TNF-α-mediated angiogenesis. Antibodies to B61 inhibited TNF-α-induced angiogenesis in a cornea model, indicating that B61–ECK interactions are responsible for this angiogenesis[87]. Although this clearly demonstrates that TNF-α induces angiogenesis via an indirect mechanism, it has yet to be determined if B61 stimulation of ECK represents a direct or an indirect mechanism.

PDGF receptors

Spatial and temporal expression of PDGF-BB and the PDGF β-receptor (PDGFRβ) suggests that they may play a role in angiogenesis. Both are expressed in vessels in human placenta[90], healing wounds, adenocarcinoma[91] and glioblastoma[92]. There is some discrepancy as to what cell types

express the receptor and whether the growth factor acts by an autocrine or a paracrine mechanism, although this may be tissue-dependent. There are also some contradictory studies addressing the expression patterns of the PDGF receptor in *in vitro* tube formation assays[93–95]. It is likely that PDGF is involved in angiogenesis, but, like EGF/TGF-α, it may play an indirect role by inducing VEGF (Refs 96,97). It may also exert growth-stimulatory effects on pericytes[91] and fibroblast-like cells[95,98] that surround the endothelial cells.

IGF-1 receptor

As with PDGF, insulin-like growth factor-1 (IGF-1) has been observed in angiogenic tissues, where it is known to be released by monocytes[99,100]. It also induces *in vitro* tube formation by endothelial cells[101] and stimulates the growth of fibroblast-like cells in vascular explants[98]. IGF-1 has been implicated in the angiogenesis that occurs in diabetic retinopathy because it is increased in the vitreous of patients and stimulates growth of human retinal endothelial cells[102]. Furthermore, IGF-1 induces angiogenesis in rabbit corneas[102,103]. Recently, IGF-1 was found to induce the expression of VEGF in several colon carcinoma cell lines[127]. Thus, IGF-1 may exert its angiogenic effects indirectly through VEGF.

Therapeutic strategies for inhibition of RTKs

Inhibition of angiogenesis as a possible mode of therapeutic intervention was first proposed by Folkman[104]. Advances in the understanding of the biology of angiogenesis have led to several potential modes for intervention, as illustrated in Figure 6. These include inhibition of angiogenic factors from surrounding cells, neutralization or inhibition of angiogenic factor binding to endothelial cells, signal transduction inhibition, prevention of basement membrane breakdown and inhibition of cell–cell interactions. Some of the molecules that inhibit these processes and which are in development as anti-angiogenic agents are listed in Table 1.

The role of RTKs in the formation of new blood vasculature associated with human disease has provided a strong rationale to identify ways of inhibiting the function of these enzymes. The approaches to achieve this objective have been quite varied. The vast majority of efforts have focused on the inhibition of FGF and VEGF receptors. In addition, other RTKs have been implicated in angiogenesis, but therapeutic strategies are few or lacking. Nonetheless, these targets would be amenable to many of the approaches that have been taken using the FGF and VEGF receptor targets. The therapeutic modalities that have been studied for abrogation of FGF- and VEGF-dependent signaling include the use of nucleotides (gene therapy and antisense), proteins (antibodies, receptor and ligand decoys) and low-molecular-weight compounds.

Millauer and coworkers[38,39] have shown that expression of a dominant-negative form of Flk-1 in subcutaneous implants of tumor cells from a wide variety of sources can lead to tumor growth inhibition. These findings suggest that introduction of inactive forms of Flk-1 into tumor endothelia may repress formation of new blood vasculature at the site of tumor growth. This study provides the rationale to support the use of viral and nonviral DNA transfer methods to enable expression of receptor-specific proteins resulting in inhibition of receptor function in the presence of ligand. In addition, the use of antisense oligonucleotides *in vitro* has been shown to reduce VEGF production by endothelial cells grown under conditions of oxygen depletion, leading to a block in DNA synthesis associated with VEGF-dependent proliferation of endothelial cells[105]. Furthermore, an antisense oligomer corresponding to bFGF has been shown to block proliferation of bovine aortic endothelial cells[106]. Taken together, the expression of dominant-negative and inactivating receptors or the use of antisense strategies that target local expression of ligands such as VEGF or FGF may provide a means to modulate receptor function. The limitations of the use of such techniques are more related to the pharmacological requirements for such agents in a clinical setting – namely, delivery of genes or nucleotides to the appropriate cells at the site of angiogenesis followed by a sustained effect on the process.

Several lines of evidence support the use of proteins to inhibit the angiogenic component of human disease. For instance, it has been shown that the use of anti-VEGF antibodies can result in inhibition of the growth of human tumor cells in nude mice[23]. In this case, VEGF expression was shown to be a common feature of human colorectal neoplasms. Growth of these neoplasms as subcutaneous lesions or as hepatic metastases could be substantially reduced following intraperitoneal injection of anti-VEGF antibodies. In a similar fashion, anti-VEGF antibodies have been shown to block angiogenesis of human retinal pigment epithelial cells grown *in vitro*[107]. Anti-FGF antibodies have also been shown to block *in vitro* angiogenesis[108]. In addition to antibodies directed against ligands, anti-Flk-1 antibodies[27] and soluble Flk-1 receptor decoys[109] were utilized to inhibit VEGF-induced proliferation of human umbilical vein endothelial cells.

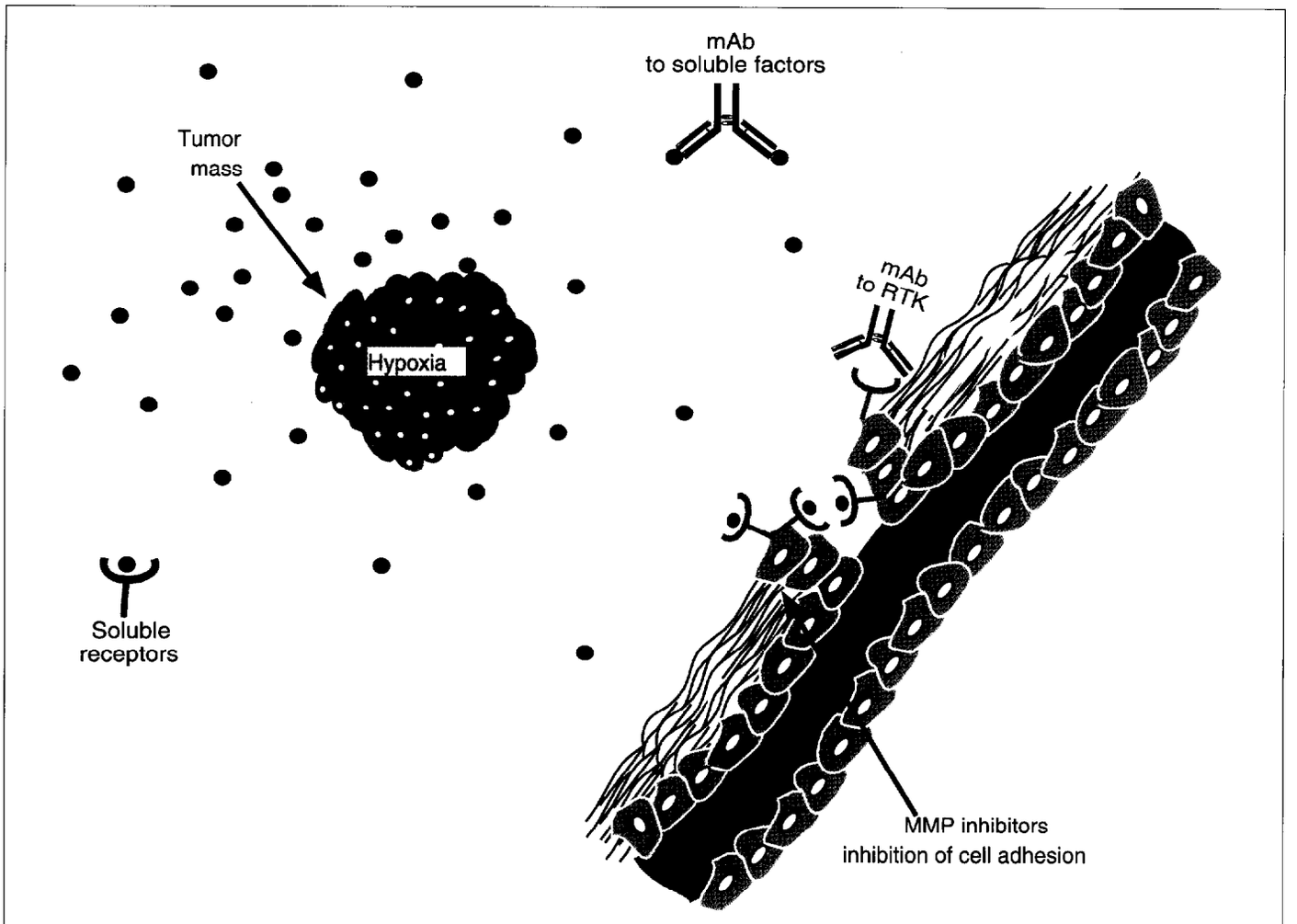

Figure 6. *Possible strategies for inhibition of angiogenesis. Because tumor angiogenesis involves several distinct processes, any one of several strategies might be successful in inhibiting the overall mechanism. These are summarized in the text; angiogenic inhibitors in development are listed in Table 1. mAb, monoclonal antibody; RTK, receptor tyrosine kinase; MMP, matrix metalloproteinase.*

The use of low-molecular-weight compounds to treat angiogenesis represents an ever-expanding area of research activities. Suramin, a nonspecific inhibitor of growth factor receptors, was shown to block bFGF-dependent proliferation of bovine aortic endothelial cells[106,110], angiogenesis in the chicken chorioallantoic membrane[106,110] and bFGF-dependent rat corneal angiogenesis[110]. Recently, inhibitors of the tyrosine kinase activities associated with Flk-1 following VEGF stimulation have been used to block VEGF-dependent proliferation of endothelial cells. For instance, genistein was shown to inhibit tyrosine phosphorylation events and cell proliferation in response to VEGF stimulation of bovine aortic endothelial cells[111]. In addition, lavendustin A blocked angiogenesis following subcutaneous implantation of VEGF-coated sponges[112]. More recently, Strawn and coworkers[113] were able to show that several distinct classes of compounds were effective at inhibiting Flk-1 tyrosine autophosphorylation in cells. In addition, some of these agents also abrogated cell signaling events that resulted in inhibition of endothelial cell proliferation following VEGF stimulation. Studies such as these provide a rationale to develop potent and selective inhibitors of Flk-1 for use as agents to inhibit angiogenic processes associated with human disease, including cancer.

Future directions

Most anti-angiogenic agents are in development as anti-cancer therapeutics. Based on the fact that angiogenesis

Table 1. Selected angiogenic inhibitors in development[a]

Class of inhibitor	Chemical	*In vitro* and *in vivo* inhibitory effects	Stage of development	Company
Polypeptides				
Interferon α	Glycoprotein	EC migration; release of angiogenic factor	Clinical trials	Hoffmann-La Roche, Schering Plough
Platelet factor 4 (recombinant)	Tetrameric protein	EC proliferation; capillary formation; tumor angiogenesis	Clinical trials	Repligen
Angiostatin	Plasminogen fragment	EC proliferation; angiogenesis; metastatic growth; tumor growth	Preclinical studies	EntreMed
Bactericidal/permeability-increasing (BPI) protein derivative 23	Recombinant protein fragment	FGF-induced angiogenesis; B16 melanoma metastasis	Preclinical studies	Xoma
Humanized anti-$\alpha_v\beta_3$ antibody (LM609)	Monoclonal antibody	Cell binding to fibrinogen; tumor growth; TNF-α- and bFGF-induced angiogenesis; angiogenesis in CAM	Preclinical studies	Ixsys
Anti-VEGF monoclonal antibody	Monoclonal antibody	VEGF-induced angiogenesis *in vitro* and *in vivo*; tumor growth and metastasis	Preclinical studies	Genentech
Anti-Flk-1 monoclonal antibody (DC101)	Monoclonal antibody	VEGF binding to Flk-1; VEGF-induced phosphorylation of Flk-1; tumor growth	Preclinical studies	ImClone Systems
Soluble Flt-1 receptor	Receptor fragment	Receptor binding to VEGF and PlGF; VEGF-induced EC mitogenesis	Preclinical studies	Merck
Carbohydrates				
Tecogalan (DS4152)	Sulfated polysaccharide–peptidoglycan complex	bFGF binding to EC; EC proliferation; angiogenesis in CAM and tumors; tumor growth	Clinical trials	Daiichi
bFGF carbohydrate inhibitor (GM1474)	Sulfated carbohydrate	bFGF-induced EC proliferation; tumor growth; metastases in B16 model	Preclinical studies	Glycomed, Ligand
Glyceptor mimetic inhibitor of bFGF (GL14.2)	Carbohydrate	Binding of bFGF or VEGF to cell surface glycosaminoglycan; tumor growth	Preclinical studies	Glycan, ProsCure
Antibiotics				
AGM1470 (TNP 470)	Fumagillin analog	Expression of cyclins and activation of cyclin-dependent kinases; EC migration and proliferation; collagenases; tumor angiogenesis and growth	Clinical trials	Takeda, Abbott
Polycations and polyanions				
Suramin	Polyanionic compound	Binding of bFGF to receptor; tumor growth and angiogenesis	Clinical trials	Warner-Lambert/NIH
Small molecules				
Inhibitors of Flk-1	Small-molecule inhibitors	Flk-1 phosphorylation; VEGF-induced EC proliferation; angiogenesis in CAM; tumor growth, angiogenesis, and metastasis	Preclinical studies	SUGEN
Inhibitors of VEGF-Flt binding	Small molecule inhibitors	VEGF binding; VEGF-induced calcium changes and proliferation in ECs; VEGF-induced vascular permeability	Preclinical studies	Texas Biotechnology

Inhibitors of phosphatidic acid	Small-molecule inhibitors	FGF-, VEGF- and PDGF-induced SMC and EC mitogenesis; VEGF-induced EC migration in Matrigel	Preclinical studies	Cell Therapeutics
Thalidomide/analogs	Polycyclic teratogen	EC responses; TNF-α formation; bFGF-induced corneal angiogenesis	Clinical trials	EntreMed, Bristol-Myers Squibb
Batimastat/Marimastat	Small-molecule inhibitors	Matrix metalloproteinases; hemangioma growth; EC invasion; *in vivo* angiogenesis	Clinical trials	British Biotech
Urokinase receptor antagonists	Small-molecule inhibitors	Plasminogen activation; EC capillary tube formation; corneal and *in vivo* Matrigel angiogenesis; tumor growth and angiogenesis	Preclinical studies	Chiron
Oligonucleotides				
VEGF antisense oligonucleotide	Oligonucleotides	Expression of VEGF in tumor cells; ischemia-induced retinal neovascularization	Preclinical studies	Hybridon
Ribozymes targeting VEGF receptors	Nucleotides	VEGF-stimulated growth of human microvascular ECs and corneal angiogenesis	Preclinical studies	Ribozyme Pharmaceuticals

[a]bFGF, basic fibroblast growth factor; CAM, chorio-allantoic membrane; EC, endothelial cell; Flk-1, vascular endothelial cell factor receptor-2 (VEGFR2); Flt-1, vascular endothelial cell factor receptor-1 (VEGFR1); PDGF, platelet-derived growth factor; PlGF, placental growth factor; SMC, smooth muscle cell; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

inhibition not only blocks primary tumor growth but also reduces metastasis to distant sites[114], inhibitors may be useful in first-line therapies as well as in an adjunctive situation. Other diseases are also likely to benefit from this type of therapy. For example, abnormalities of microvessels in the papillary dermis are thought to be important in sustaining epidermal hyperproliferation in dermatological disorders such as psoriasis and scleroderma[115,116]. Biopsies of human tissues from psoriatic lesions and contact dermatitis have been shown to have increased levels of VEGF and Flk-1 (Refs 117,118). In addition, the receptors for VEGF, Flk-1 and Flt-1 were found to be overexpressed in papillary dermal microvascular endothelial cells[117].

Retinal neovascularization, the final common pathway leading to vision loss in diseases such as retinopathy of prematurity, age-related macular degeneration and diabetic retinopathy, is another potential therapeutic area for angiogenesis inhibitors. VEGF is present in ocular fluid of patients with diabetic retinopathy and other retinal disorders[119]. In animal models, elevated intraocular levels of VEGF are associated with active retinal neovascularization, and decreased levels parallel the regression in proliferative retinopathy[120,121]. The potential use of Flk-1 inhibitors as specific therapy for ischemic retinal disease is suggested by significant inhibition of retinal neovascularization observed with antisense VEGF and soluble VEGF receptor chimeric proteins[109].

Rheumatoid arthritis, although characterized by inflammation and immunoproliferation, is another disease in which angiogenesis has been implicated in the disease process. Growth of the pannus, which contributes to the destruction of joint cartilage, is thought to be dependent on local angiogenesis. Several investigators have shown the expression of VEGF in synovial fluid, subsynovial macrophages, fibroblasts surrounding microvessels in the pannus, vascular smooth muscle cells and synovial lining cells from patients with rheumatoid arthritis[122–124]. In atherosclerosis, the development of the atherosclerotic plaque is associated with neovascularization in the thickened intima and media of vascular walls. Although the mechanism and stimulus for neovascularization are unknown, the plaques have been shown to have angiogenic activity, as measured by the ability to induce growth of new vessels in a rabbit cornea model[125]. Also, conditioned medium from smooth muscle cells stimulated endothelial cell proliferation[126], and a neutralizing antibody to VEGF was able to attenuate the angiogenic activity.

It is apparent that anti-angiogenic agents will potentially have broad applications in the clinic. While there are several strategies for inhibiting angiogenesis, the data generated from many laboratories suggest that targeting receptors for angiogenic growth factors will lead to new treatments. As discussed previously, there are several approaches to disrupting the signaling of receptor tyrosine kinases, including

the use of antibodies or receptor decoys to block ligand binding, and reducing the function of the receptor using antisense or dominant-negative technology. Additionally, because RTKs represent proteins with enzymatic function, they lend themselves to pharmacological intervention with small-molecule compounds. Irrespective of the mechanism for inhibition, it is likely that these agents will be used for chronic therapy in most diseases. This will therefore necessitate the development of drugs with a good safety profile and a convenient route of administration.

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