

# Vascular Endothelial Growth Factor and Angiogenesis

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**Abstract**—Angiogenesis is a hallmark of wound healing, the menstrual cycle, cancer, and various ischemic and inflammatory diseases. A rich variety of pro- and antiangiogenic molecules have already been discovered. Vascular endothelial growth factor (VEGF) is an interesting inducer of angiogenesis and lymphangiogenesis, because it is a highly specific mitogen for endothelial cells. Signal transduction involves

binding to tyrosine kinase receptors and results in endothelial cell proliferation, migration, and new vessel formation. In this article, the role of VEGF in physiological and pathological processes is reviewed. We also discuss how modulation of VEGF expression creates new therapeutic possibilities and describe recent developments in this field.

## I. Introduction

Humans are complex multicellular organisms, and all cells require a dependable, finely controlled supply of oxygen. The diffusion of oxygen through tissues is limited to 100 to 200  $\mu\text{m}$ ; therefore, a highly developed vascular system has evolved to ensure that all cells are within this distance of a supply of oxygen. The system needs to be maintained through angiogenesis, the process of new blood vessel development from pre-existing vasculature. This involves endothelial cell division, selective degradation of the basement membrane and the surrounding extracellular matrix, endothelial cell migration, and the formation of a tubular structure. Once blood vessels have been established, the endothelial cells undergo tissue-specific changes to generate functionally distinct vessels. During embryogenesis, blood vessels form by the differentiation of endothelial cell precursors (angioblasts), which associate to form primitive vessels. This process is called vasculogenesis.

Angiogenesis is subject to a complex control system with proangiogenic and antiangiogenic factors. In adults, angiogenesis is tightly controlled by this "angiogenic balance", i.e., a physiological balance between the stimulatory and inhibitory signals for blood vessel growth. In normal circumstances, the formation of new blood vessels occurs during wound healing, organ regeneration, and in the female reproductive system during ovulation, menstruation, and the formation of the placenta. It is also an important factor in several pathological processes such as tumor growth, rheumatoid arthritis, diabetic retinopathy, and psoriasis. A switch to the angiogenic phenotype depends on a local change in the balance between angiogenic stimulators and inhibitors.

One of the most important proangiogenic factors is vascular endothelial growth factor (VEGF<sup>1</sup>). VEGF also

potentiates microvascular hyperpermeability, which can both precede and accompany angiogenesis. In this review, we summarize the properties and functions of VEGF and its place in the process of angiogenesis in malignancy and other conditions.

## II. Vascular Endothelial Growth Factor

### A. The Locations and Structures of Vascular Endothelial Growth Factor

The VEGF family currently comprises seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and PlGF. All members have a common VEGF homology domain. This core region is composed of a cystine knot motif, with eight invariant cysteine residues involved in inter- and intramolecular disulfide bonds at one end of a conserved central four-stranded  $\beta$ -sheet within each monomer, which dimerize in an antiparallel, side-by-side orientation (Neufeld et al., 1999; Ortega et al., 1999). Figure 1 represents the three-dimensional structure of VEGF.

VEGF-A is a 34- to 42-kDa, dimeric, disulfide-bound glycoprotein. In normal tissues, the highest levels of VEGF-A mRNA are found in adult lung, kidney, heart, and adrenal gland. Lower, but still readily detectable, quantities of VEGF-A transcript levels occur in liver, spleen, and gastric mucosa. VEGF-A exists in at least seven homodimeric isoforms. The monomers consist of 121, 145, 148, 165, 183, 189, or 206 amino acids (Fig. 2). The primary VEGF-A transcript derives from a single VEGF-A gene, coding for eight exons (Poltorak et al., 1997; Neufeld et al., 1999). The amino acids encoded by exons 1 to 5 and 8 are conserved in all isoforms except VEGF-A<sub>148</sub>, whereas variable alternative splicing occurs in exons 6 and 7, which encode two distinct heparin-binding domains. The presence or absence of these domains influences solubility and receptor binding. The heparin-binding domain encoded by exon 6 determines

<sup>1</sup> Abbreviations: VEGF, vascular endothelial growth factor; PlGF, placental growth factor; tPA, tissue type plasminogen activator; uPA, urokinase type plasminogen activator; bFGF, basic fibroblast growth factor; Flt, fms-like tyrosine kinase; VEGFR, vascular endothelial growth factor receptor; KDR, kinase domain region; Flk, fetal liver kinase; PAF, platelet activating factor; aFGF, acid fibroblast growth factor; PAI-1, plasminogen activator inhibitor 1; PLC $\gamma$ 1, phospholipase C  $\gamma$ 1; Erk, extracellular regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3'-kinase; Bcl-2, B-cell lymphoma 2; EPO, erythropoietin; HIF-1, hypoxia inducible factor-1; IGF, insulin-like growth factor; ROS, reactive oxygen species; TNF, tumor necrosis factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IL, interleukin;

HUVEC, human umbilical vein endothelial cells; Stat, signal transducer and activator of transcription; COX, cyclooxygenase; NSAID, nonsteroidal inflammatory drug; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; AP-1, activator protein 1; MMP, matrix metalloproteinase; EPC, endothelial progenitor cell; HLH, helix-loop-helix; LH, luteinizing hormone; hCG, human chorionic gonadotrophin; DC, dendritic cell; MVD, microvessel density; VEGF-A<sub>s</sub>, serum concentration of vascular endothelial growth factor; SU5416, semaxanib; NP-1, neuropilin-1; AIDD, apoptosis-induced drug delivery; RA, rheumatoid arthritis; OA, osteoarthritis; OHSS, ovarian hyperstimulation syndrome.

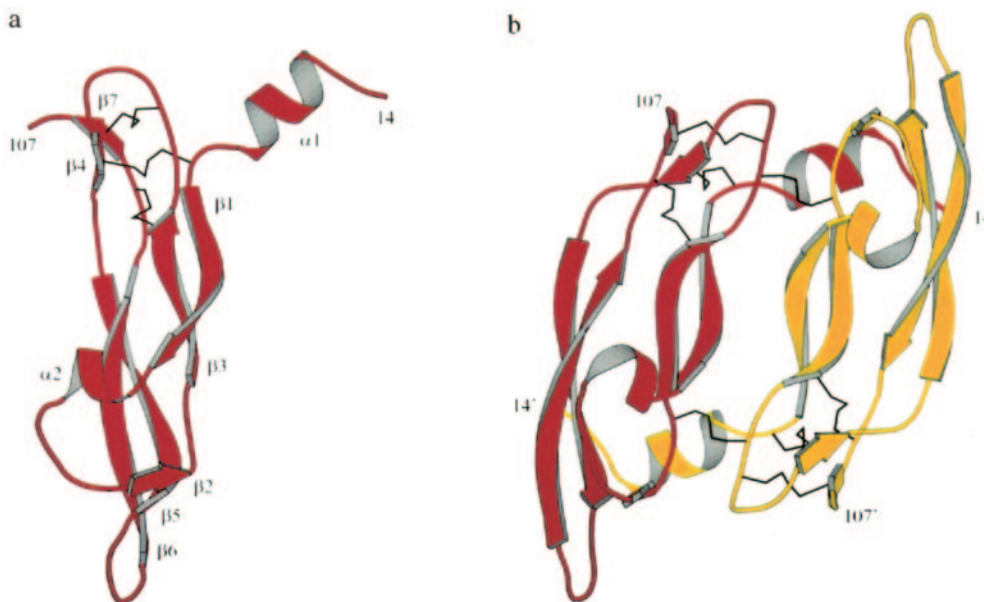


FIG. 1. Ribbon representation of the receptor-binding domain of VEGF showing a monomer in a and a dimer in b. The two disulfide bonds are shown as black lines. Helix  $\alpha 1$  consists of residues 16 to 24, and the central four-stranded b-sheet is formed by b1 (residues 27–34), b3 (51–58), b5 (73–83), and b6 (89–99), with the characteristic cysteine knot motif at one end [strands b4 (67–69) and b7 (103–105)], and a short three-stranded b-sheet [strands b2 (46–49), b5, and b6] at the other end. VEGF monomers dimerize in an antiparallel way.

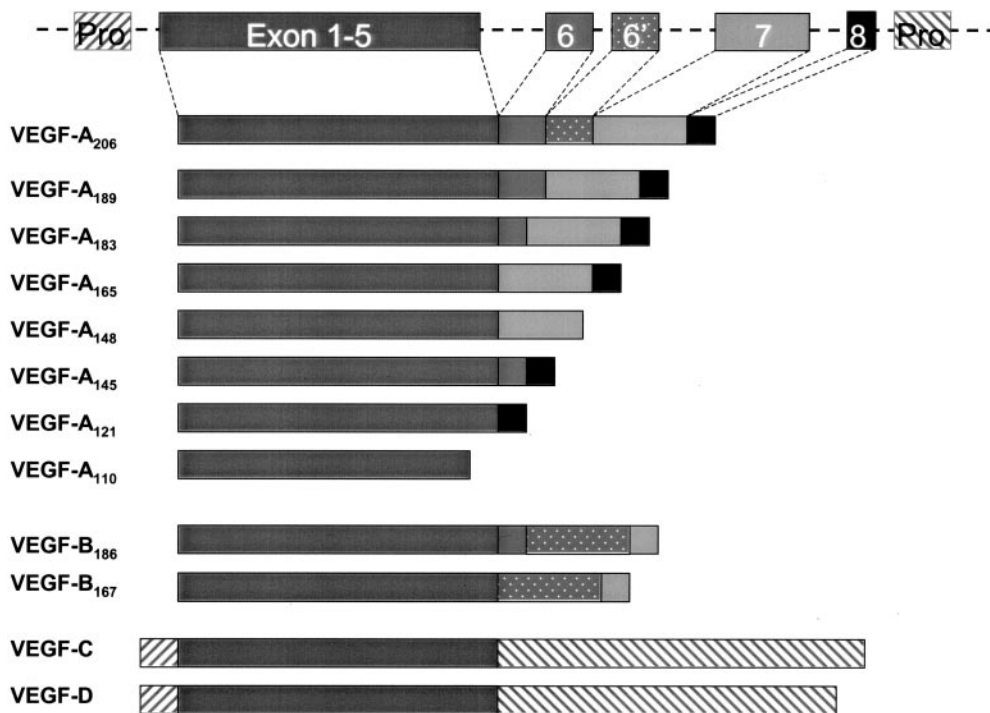


FIG. 2. Gene structure of VEGF-A, VEGF-B, VEGF-C, and VEGF-D. The VEGF-A gene consists of eight exons that give rise to seven isoforms of 121, 145, 148, 165, 183, 189, and 206 amino acids through differential splicing. An additional VEGF-A isoform of 110 amino acids results from proteolytic cleavage. VEGF-B exists as two isoforms of 167 and 186 amino acids. VEGF-C and VEGF-D are proteolytically released from their respective propeptides. All VEGF family members share a highly preserved VEGF homology domain, encoded by exons 1 to 5.

binding to the extracellular matrix, and therefore isoforms containing this domain (VEGF-A<sub>145</sub>, VEGF-A<sub>189</sub>, and VEGF-A<sub>206</sub>) are bound tightly to cell surface heparin-containing proteoglycans in the extracellular matrix, whereas those lacking the domain are diffusible. VEGF-A<sub>165</sub>, which contains only one heparin-binding region

encoded by exon 7, is moderately diffusible, and VEGF-A<sub>121</sub>, which lacks the domains encoded by both exons 6 and 7, is highly diffusible (Ortega et al., 1999).

Plasmin is involved in the degradation of the extracellular matrix, both directly through digestion of components of the basement membrane and indirectly by

activating collagenases from zymogens, and it liberates VEGF-A<sub>165</sub>, VEGF-A<sub>189</sub>, and VEGF-A<sub>206</sub>. Cleavage of VEGF-A<sub>165</sub> and VEGF-A<sub>189</sub> by plasmin results in fragments containing the 110-amino terminal amino acids (VEGF-A<sub>110</sub>) that are highly diffusible (Houck et al., 1992). Plasmin is generated from plasminogen by plasminogen activators, and it has been postulated that endothelial cells secrete tissue and urokinase type plasminogen activators (tPA and uPA) in response to VEGF and basic fibroblast growth factor (bFGF) (Houck et al., 1992; Cohen et al., 1995; Neufeld et al., 1999). VEGF-A<sub>189</sub> is also cleaved directly by uPA into 38-kDa homodimers, independently from the activation of plasmin, probably within the sequence encoded by exon 6 (Plouet et al., 1997). These homodimers have a weaker ability to circulate, in comparison to the 34-kDa homodimers generated by plasmin, but they have a higher mitogenic activity. It is postulated that VEGF-A<sub>206</sub> can undergo a similar maturation process following exposure to uPA. In contrast, VEGF-A<sub>165</sub> is not cleaved by uPA.

VEGF-B was discovered in 1995. It is abundantly expressed in the adult myocardium, skeletal muscle, and pancreas. In mouse embryonal tissues, high expression is seen in the developing heart, brown fat, muscle (including the smooth muscle layer in embryonic arteries), and in the spinal cord. The VEGF-B gene is composed of seven exons. Exons 3 and 4 encode for invariant cysteine residues that are responsible for a cysteine knot motif with two disulfide bridges. Alternative splicing of exon 6 generates two isoforms of VEGF-B, VEGF-B<sub>167</sub> (21 kDa) and VEGF-B<sub>186</sub> (32 kDa). The C-terminal domain of VEGF-B<sub>167</sub> is hydrophilic, structurally related to the heparin-binding domain of some VEGF-A isoforms, and interacts easily with a coreceptor neuropilin-1. Conversely, the C-terminal domain of VEGF-B<sub>186</sub> is hydrophobic, modified by *O*-linked glycosylation, and requires limited proteolysis to bind to neuropilin-1. VEGF-B<sub>167</sub> is bound to the surface of cells or pericellular heparan sulfate proteoglycans, whereas VEGF-B<sub>186</sub> is secreted freely. This difference in behavior is explained by the presence of a basic region at the C terminus of the VEGF-B<sub>167</sub> isoform, which is not present in the VEGF-B<sub>186</sub> isoform. Both VEGF-B<sub>167</sub> and VEGF-B<sub>186</sub> are produced as disulfide-linked homodimers and can generate disulfide-linked heterodimers with VEGF-A when coexpressed. In the absence of heparin, the VEGF-B<sub>167</sub>-VEGF-A<sub>165</sub> heterodimer remains associated with the cell. As the homodimers of VEGF-A<sub>165</sub> diffuse efficiently into the extracellular space, VEGF-B<sub>167</sub> appears to determine the release of the heterodimers from the cell surface and may therefore control the bioavailability of VEGF-A (Waltenberger et al., 1994; Olofsson et al., 1996, 1998; Neufeld et al., 1999; Ortega et al., 1999; Scrofani et al., 2000). The two VEGF-B isoforms are differentially expressed, with VEGF<sub>167</sub> being predominant, suggesting that splicing events are strictly con-

trolled (Olofsson et al., 1996, 1998, 1999; Joukov et al., 1997; Neufeld et al., 1999; Scrofani et al., 2000).

In adult tissues, VEGF-C is expressed most prominently in heart, placenta, ovary, small intestine, and the thyroid gland, whereas in embryonal tissue, expression occurs where lymphatic vessels undergo sprouting from embryonic veins, such as the perimetanepric, axillary, and jugular areas. The gene for VEGF-C spans more than 40 kb of DNA and consists of seven exons. The VEGF homology domain of VEGF-C is encoded by exons 3 and 4, and exons 5 and 7 encode cysteine-rich motifs. In comparison to other ligands of the VEGF family, there is a spacing of the cysteine residues at the C terminus, reminiscent of the BR3P sequence (C-terminal silk domain). Two 2.4-kb and 2.0-kb VEGF-C mRNAs can be detected by Northern blotting of RNA from many embryonal and adult tissues. Tumor cells almost exclusively express the 2.4-kb mRNA form. The identity of the 2.0-kb VEGF-C mRNA remains to be determined. Newly synthesized VEGF-C is a preproprotein, with a predicted molecular mass of 46.9 kD, consisting of an N-terminal signal sequence followed by an N-terminal propeptide, the VEGF homology domain, and a cysteine-rich C-terminal propeptide. The VEGF homology domain contains three putative glycosylation sites. VEGF-C is secreted as a disulfide-bonded homodimer that is proteolytically processed from the precursor polypeptide, and the secreted form contains the C-terminal silk domain. The fully processed VEGF-C is a noncovalent dimer (Joukov et al., 1996, 1997; Witzensbichler et al., 1998; Ortega et al., 1999; Li and Eriksson, 2001).

VEGF-D is found in adult tissues, particularly lung, heart, skeletal muscle, colon, and small intestine. In embryonal tissues, it is abundant in the developing lung (Yamada et al., 1997; Ortega et al., 1999; Li and Eriksson, 2001). The human gene of VEGF-D is located on chromosome Xp22.31. The human cDNA encodes a protein of 354 amino acids. Together with VEGF-C, VEGF-D defines a subfamily of the VEGFs, with close similarities in VEGF homology domains and long N- and C-terminal domains. VEGF-D is synthesized as a preproprotein, which requires proteolytic processing in both the N- and C-terminal regions for activity, and the fully processed growth factor is a noncovalent dimer.

VEGF-E is an Orf virus-encoded VEGF (NZ-strains). The gene products have only 19 to 25% amino acid identity with VEGF, have no apparent basic domain, and seem to be involved in the process of pathological angiogenesis in virus-infected lesions. Its cDNA structure is exonless in the viral genome, suggesting a phylogenetic origin in the vertebrate genome. It has a potent endothelial cell growth stimulatory activity and vascular permeability activity similar to those of VEGF-A<sub>165</sub>, without the heparin-binding region. Histologically, the lesions caused by the Orf virus are highly vascular and edematous, with an increased number of vessels pro-

duced by proliferation of endothelial cells, and contain extensive inflammatory infiltrates of a mixed character. The extensive dermal vascular response seems to be a direct effect of VEGF (Ogawa et al., 1998; Ortega et al., 1999).

### B. Vascular Endothelial Growth Factor Receptors

Three VEGF tyrosine kinase receptors have been identified: The *fms*-like tyrosine kinase Flt-1 (VEGFR-1/Flt-1), the kinase domain region, also referred to as fetal liver kinase (VEGFR-2/KDR/Flk-1), and Flt-4 (VEGFR-3). Each receptor has seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase sequence interrupted by a kinase insert domain (Ortega et al., 1999).

VEGFR-2 appears to be the most important receptor in VEGF-induced mitogenesis and permeability (Waltenberger et al., 1994; Zachary, 1998). It has a lower affinity for VEGF-A ( $K_d = 400\text{--}800\text{ pM}$ ) than VEGFR-1 (Terman et al., 1994). VEGF-C, VEGF-D, and VEGF-E are also ligands for this receptor. Receptor activation during angiogenesis induces the production of platelet-activating factor (PAF) by endothelial cells, stimulates their mitosis and migration, and increases vascular permeability (Joukov et al., 1996; Achen and Stacker, 1998; Ogawa et al., 1998; Ortega et al., 1999; Andre et al., 2000; Partanen et al., 2000; Bernatchez et al., 2002). PAF has many crucial roles in the induction of angiogenesis (Bernatchez et al., 2002). Not only is it involved in inflammatory cell rolling and adhesion (Prescott et al., 1984), but *in vitro* studies indicate that PAF promotes the expression of potent angiogenic factors and chemokines, including acid fibroblast factor, basic fibroblast growth factor (bFGF), and macrophage inflammatory protein 2 (Bussolino et al., 1995). It also potentiates the migration of cultured endothelial cells.

The role of VEGFR-1 in endothelial cell function is less clear. VEGFR-1 has a much weaker kinase activity and is unable to generate a mitogenic response in endothelial cells when stimulated by VEGF, although it has the highest affinity for VEGF-A ( $K_d = 15\text{--}100\text{ pM}$ ) (Terman et al., 1994). It modulates endothelial cell division at the earliest stages of vascular development from the fourth to fifth day of endothelial cell differentiation, just before the formation of the first primitive blood vessels. Embryos and differentiated endothelial cells lacking VEGFR-1 (VEGFR-1<sup>-/-</sup>) show increased vascularization and number of endothelial cells, accompanied by an increased endothelial cell mitotic index. Kearney et al. (2002) suggested that VEGFR-1 modulates the endothelial cell cycle by affecting one or more molecular signaling pathways. Potentially, VEGFR-1 could negatively modulate pathological vascularization and in this way dampen the proangiogenic effects of VEGFR-2 (Dvorak, 2002), but further investigation is needed. VEGFR-1 is also important in cell migration. The binding of VEGF-

A<sub>165</sub> to VEGFR-1 induces directed migration of mononuclear phagocytes across an endothelial cell monolayer as well as their activation, based on the expression of tissue factor procoagulant activity. The interaction between VEGF-A<sub>165</sub> and VEGFR-1 additionally induces a chemotactic response in polymorphonuclear cells. It is significant that monocytes and polymorphonuclear cells only express the gene for VEGFR-1 and not for VEGFR-2 (Berse et al., 1992; Barleon et al., 1996). The relevance of this finding to tumor growth and wound healing will be discussed later. Plouët et al. (1997) showed that the processing of VEGF-A<sub>189</sub> into the 38-kDa homodimer, VEGF-A<sub>110</sub>, or the 34-kDa homodimer, by uPA and plasmin, respectively, is a prerequisite for VEGF-A<sub>189</sub> diffusion toward endothelial cells, activation of the VEGFR-2 signal transduction pathway, and the triggering of endothelial cell proliferation. VEGFR-1 also binds VEGF-B, a process dominated by hydrophobic contacts. However, only a poor mitogenic signal for endothelial cells is induced, and VEGF-B is an inefficient endothelial cell mitogen (Olofsson et al., 1998; Ortega et al., 1999; Andre et al., 2000). Endothelial cells respond to VEGF-B binding to VEGFR-1 by increasing expression and activity of uPA and PAI-1 (plasminogen activator inhibitor 1). The expression of PAI-1 precedes that of uPA and may serve to protect the extracellular matrix from extensive proteolysis (Olofsson et al., 1998).

VEGFR-3 differs from the other two VEGFRs by undergoing proteolytic cleavage in the extracellular domain into two disulfide-linked polypeptides. The 4.5- and 5.8-kb VEGFR-3 mRNAs encode polypeptides, which differ in their C termini, and apparently in their signaling properties, as a result of alternative 3' exon splicing (Joukov et al., 1996; Ortega et al., 1999; Partanen et al., 2000). VEGFR-3 binds only VEGF-C and VEGF-D. Since this receptor is generally restricted to lymphatic endothelial cells, activation stimulates mitosis, migration, differentiation, and survival of these cells (Achen et al., 1998; Partanen et al., 2000).

Neuropilin-1 is a type I transmembrane receptor with a molecular weight of 130 to 135 kDa. It does not function independently but acts as a coreceptor. Although the highly conserved short cytoplasmic domain of neuropilin-1 suggests that this is an important component, no binding partners or obvious protein homology domains have been reported within this portion (Peles et al., 1997; Soker et al., 1998; Latil et al., 2000). However, neuropilin-1 possesses five discrete extracellular domains, and this diversity of protein modules is consistent with the possibility of multiple-binding ligands (Peles et al., 1997; Soker et al., 1998). Neuropilin-1 binds VEGF-A<sub>165</sub> ( $K_d = 200\text{--}300\text{ pM}$ ), and when coexpressed with VEGFR-2, it enhances the binding of VEGF-A<sub>165</sub> to VEGFR-2 4- to 6-fold. The chemotaxis of endothelial cells coexpressing these receptors toward a gradient of VEGF-A<sub>165</sub> is about 2.5 times faster than that of endothelial cells expressing VEGFR-2 alone. VEGF-B<sub>167</sub> can

also bind neuropilin-1 through its heparin-binding domain, encoded by exon 6B. This domain is highly homologous to the neuropilin-1 binding epitopes of VEGF-A<sub>165</sub>. VEGF-B<sub>167</sub> and neuropilin-1 are coexpressed prominently in the smooth muscle cells surrounding larger blood vessels. Furthermore, neuropilin-1 in the endothelial cells of large vessels is located in a juxtacrine relationship with the smooth muscle cell layer producing VEGF-B. Unlike VEGF-A<sub>165</sub>, VEGF-B<sub>167</sub> induces a biological response in cells that express neuropilin-1, but not VEGFRs. The signal transduction pathway is as yet unknown, although Cai and Reed (1999) characterized a neuropilin-1-interacting protein that interacts with the cytoplasmic domain. Neuropilin-1 may also potentiate the effects of VEGF-B in cells where VEGFR-1 is expressed (Makinen et al., 1999; Neufeld et al., 1999). Neuropilin-1 potentiates PAF synthesis; Bernatchez et al. (2002) hypothesized that the pathological role of neuropilin-1, when coexpressed with VEGFR-2, might be a result of increased PAF synthesis by endothelial cells, with sustained vascular permeability, inflammation, and endothelial cell migration.

The second immunoglobulin-like domain in the extracellular domain of VEGFR-1 and VEGFR-3 is responsible for specific ligand recognition. It is incapable of binding any of its ligands in the absence of the other flanking domains 1 and 3. These flanking domains play an important role in maintaining the binding site in a spatial conformation compatible with ligand binding. They do not contribute major binding determinants, since these domains can be switched between VEGFR-1 and 3 with 25 and 32% identity, respectively, without any marked influence on domain 2-mediated ligand binding and subsequent receptor activation. VEGFR-2 also shows specific binding determinants within the second immunoglobulin-like domain, but additional elements are required for full ligand binding. The flanking domain sequences contribute to VEGF binding to this receptor. Immunoglobulin-like domains 4 to 7 are required for maximal transphosphorylation, which initiates the signal transduction cascade (Davis-Smyth et al., 1996).

VEGFR-1 and VEGFR-2 are expressed predominantly by vascular endothelial cells. Their promoters contain a 5' flanking sequence essential for endothelial expression, and the receptors are up-regulated during angiogenesis. There is increasing evidence to support the expression and functional importance of VEGFR-1 and VEGFR-2 in cell types other than endothelial cells. They are present in tumor cells, where they are coexpressed with VEGF, and they are also expressed by smooth muscle cells, pancreatic beta cells, and osteoblasts (Ortega et al., 1999). VEGFR-3 is found mainly in venous endothelium during early embryonic development, together with VEGFR-2, but later in fetal development it becomes mainly confined to lymphatic endothelial cells. Although the lymphatic system seems to develop from large central veins in the embryonic jugular, retroperi-

toneal, and peri-mesonephric regions, very few adult tissues retain VEGFR-3 expression in their venous endothelia. VEGFR-3 is up-regulated in lymphangiogenic vessels but not in angiogenic vessels (Joukov et al., 1996; Lymboussaki et al., 1998; Ortega et al., 1999; Partanen et al., 2000; Karpanen et al., 2001). Neuropilin-1 is expressed in the tips of actively growing axons of certain classes of neuron. It has an important role in axon growth and guidance in the developing embryo. It is also found in the endothelial cells of blood vessels, in the surrounding mesenchymal cells, and in cardiac, placental, pulmonary, hepatic, skeletal, renal, and pancreatic tissues, as well as in a variety of tumor-derived cells (Peles et al., 1997; Soker et al., 1998). Figure 3 gives an overview of the different receptors of the VEGF family.

### C. Signal Transduction

The intracellular signaling pathways governing VEGF expression are still poorly explored.

Binding of VEGF to VEGFR-1 has been implicated in important pleiotropic functions, as discussed above. However, the underlying molecular mechanisms have not yet been resolved. This is at least partly due to the apparent inability of the receptor to respond to VEGF-A by increased kinase activity. It is possible that the low kinase activity is due to a small number of tightly regulated phosphorylation sites. Accordingly, a repressor sequence has been identified in the VEGFR-1 juxtamembrane domain, and replacement of this region by that of VEGFR-2 allows VEGFR-1 to respond to VEGF-A stimulation (Gille et al., 2000). The function of the repressor sequence may be to influence the folding of the receptor, and hence the accessibility of the kinase domain active site. Alternatively, the repressor sequence may regulate interactions with phosphatases (Claesson-Welsh, 2003).

Tyrosine residues known to be phosphorylated are Tyr1213, Tyr1333, Tyr1242, and Tyr1327. Three Src homology 2 domain-containing proteins bind to the phosphorylation site Tyr1213, namely SHP-2, phospholipase C  $\gamma$ 1 (PLC $\gamma$ 1), and growth factor receptor-bound 2. The phosphorylation site Tyr1333 allows binding of PLC $\gamma$ 1 and the adaptor molecules Crk and Nck. Tyr1242 and Tyr1327 are poorly phosphorylated and have not been shown to participate in the binding of signal transduction molecules (Ito et al., 1998).

Binding of VEGF to VEGFR-2 results in autophosphorylation of the following tyrosine residues: Tyr951 and Tyr996, present in the kinase-insert domain; Tyr1054 and Tyr1059, present in the kinase domain; and Tyr1175 and Tyr1214 in the C-terminal tail.

Autophosphorylation of Tyr951 creates a binding site for the VEGFR-associated protein (Wu et al., 2000) and Tyr1175 creates a binding site for Sck (Warner et al., 2000) and PLC $\gamma$ 1 (Takahashi et al., 2001). Binding of PLC $\gamma$ 1 activates protein kinase C, which in turn activates Ras. This pathway induces the activation of the

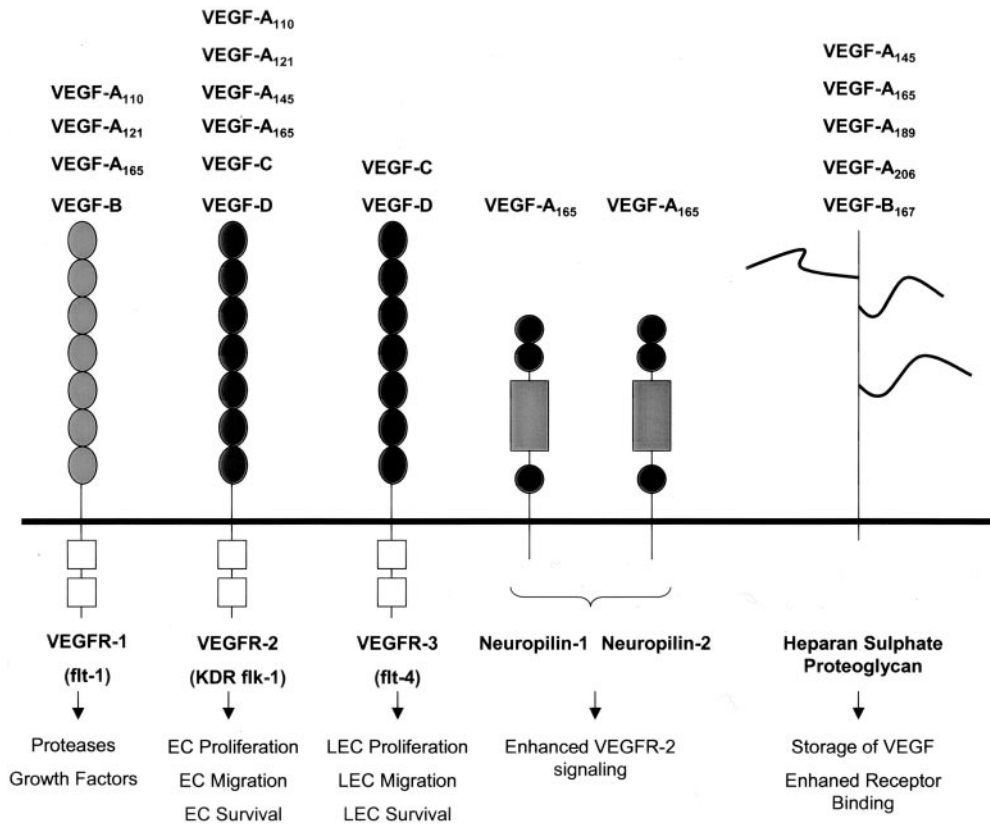


FIG. 3. The VEGF receptor family. The three signaling tyrosine kinase receptors VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1), and VEGFR-3 (flt-4) consist of seven immunoglobulin-like structures in the extracellular domain, a single transmembrane region, and a consensus tyrosine kinase domain interrupted by a kinase insert domain. The accessory receptors neuropilin 1 and neuropilin 2 possess no kinase activity of their own but enhance VEGFR-2 signaling. The heparan sulfate proteoglycans not only enhance VEGFR binding but also serve as a reserve of VEGF.

extracellular regulated kinase (Erk) pathway [p42/44 mitogen-activated protein kinase (MAPK)]. Erk can translocate to the nucleus, where it phosphorylates and activates transcription factors, including c-Jun and the ternary complex factor, which in turn induce immediate transcription of the c-fos gene (Mazure et al., 1997; Rak et al., 2000; Arsham et al., 2002).

VEGFR-2 also activates phosphatidylinositol 3'-kinase (PI3K), which results in an increase of the lipid phosphatidylinositol (3,4,5)P<sub>3</sub>, leading to activation of protein kinase B (Akt/PKB), endothelial nitric oxide synthase, and the small GTP-binding protein Rac. Akt/PKB inhibits B-cell lymphoma 2 (Bcl-2)-associated death promoter homolog and caspase-9, thereby promoting cell survival (Gerber et al., 1998). Endothelial nitric oxide synthase generates nitric oxide, resulting in an increase of vascular permeability and cellular migration (Fulton et al., 1999), which is further promoted by Rac (Cross et al., 2003).

VEGF-induced cytoskeletal reorganization and cell migration is also established through interaction of VEGFR-2 with p38MAPK and focal adhesion kinase together with its substrate paxilin (Rousseau et al., 1997). Figure 4 shows several VEGFR signal transduction pathways that are known thus far.

Binding of VEGF to VEGFR-3 results in association of the receptor with the adaptor proteins Shc and growth

factor receptor-bound 2 via Tyr1337 (Pajusola et al., 1994). Activation of the classical Erk pathway is dependent on PLC $\gamma$ /PK-mediated p42/44 MAPK activation and independent of Ras. VEGFR-3 activation leads to induction of PI3K and stimulation of Akt/PKB, which could be important for the survival of blood and lymphatic endothelial cells (Makinen et al., 2001).

#### D. Regulation of Gene Expression

1. *Hypoxia.* Under normal physiological conditions, each of the approximately 10<sup>14</sup> cells in the adult human body is provided with an adequate supply of oxygen to meet its metabolic demands through the concerted function of the pulmonary, hematopoietic, and cardiovascular systems. Oxygen is transported by circulating erythrocytes, the production of which is controlled by the glycoprotein hormone erythropoietin (EPO). EPO-producing cells in the liver and kidneys can sense oxygen concentration and respond to systemic hypoxia by increasing EPO gene transcription. Hypoxia can also be restricted to cells within a localized region of a specific organ, usually as a result of insufficient perfusion. VEGF-A plays a central role in angiogenesis and neo-vascularization, increasing delivery of both oxygen and energy substrates. VEGF-A expression can be induced when cells are subjected to hypoxia or hypoglycemia. This response seems to depend on Hypoxia Regulated/

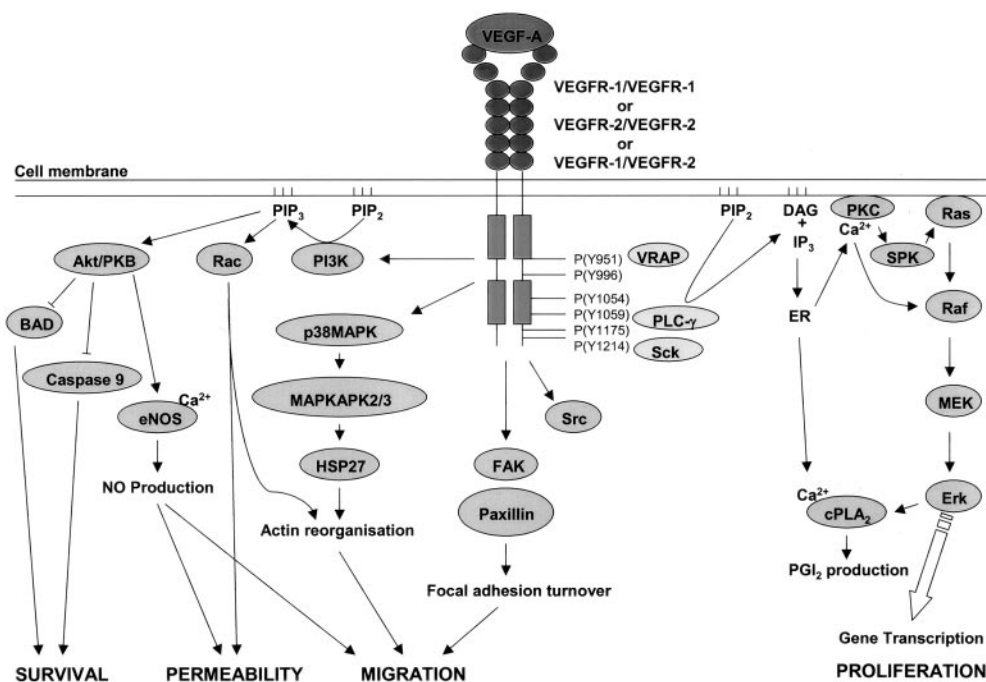


FIG. 4. VEGFR signal transduction. Binding of VEGF to VEGFR causes dimerization and autophosphorylation of the receptor. Intracellular proteins such as VEGFR-associated protein (VRAP), PLC $\gamma$ , and Sck that associate with specific tyrosine residues of VEGFR are phosphorylated upon receptor activation. Several signal transduction pathways are activated by the binding of VEGF to its receptor, leading to increased proliferation, survival, permeability, and migration of cells.

Responsive Element/Enhancer sequences in the 5' and 3' regions of the VEGF-A gene (Dachs and Tozer, 2000; Ryan et al., 2000; Tsuzuki et al., 2000).

The hypoxia-inducible protein complex HIF-1 binds to the enhancer sequences of the VEGF-A gene, EPO gene, and other critical genes, such as those for glycolytical enzymes and glucose transporters. Both transcription and RNA stability can be enhanced (Jones et al., 2001). HIF-1 is a heterodimer, consisting of the HIF-1 $\alpha$  and HIF-1 $\beta$  subunits, or aryl hydrocarbon receptor nuclear translocator, and both are basic-helix-loop-helix per-aryl hydrocarbon receptor nuclear translocator-sim proteins. HIF-1 $\beta$  is constitutively expressed. However, HIF-1 $\alpha$  is degraded under normoxic conditions by ubiquitination (Iyer et al., 1998), which is enhanced by binding on von Hippel-Lindau protein and p53 through the recruitment of ubiquitin ligases (Fig. 5). Hypoxic conditions inhibit the protein ubiquitination and stabilize the HIF-1 $\alpha$  protein. The exact mechanism by which oxygen tension is sensed is unknown. Some authors have postulated that HIF-1 $\alpha$  itself, as an iron-containing protein, can perform this function, whereas others have suggested that a NAPDH-linked oxidase is involved (Forsythe et al., 1996; Ryan et al., 1998; Srinivas et al., 1998; Dachs and Tozer, 2000; Richard et al., 2000; Tsuzuki et al., 2000). In addition to hypoxia, growth factors such as insulin-like growth factors (IGF) 1 and 2 and bFGF have been shown to increase HIF-1 $\alpha$  expression. The modulation of HIF-1 $\alpha$  by growth factors or hypoxia is apparently a parallel-independent pathway (Bermont et al., 2000). Dimerization of HIF-1 $\beta$  and HIF-1 $\alpha$  is required in the

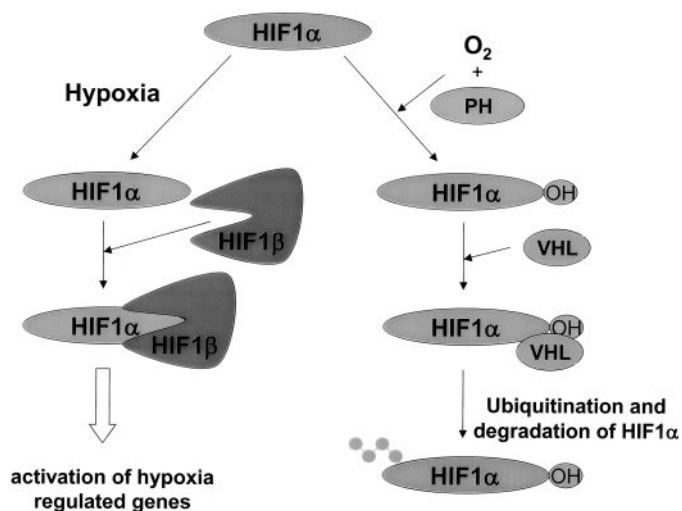


FIG. 5. Regulation of VEGF gene expression. HIF-1, the most important transcription factor for hypoxia-regulated genes, is a heterodimer consisting of HIF-1 $\alpha$  and HIF-1 $\beta$ . HIF-1 $\beta$  is constitutively expressed and stable, but HIF-1 $\alpha$  is only stable under hypoxic conditions. Under normoxic conditions, HIF-1 $\alpha$  is hydroxylated on a proline residue, by an unknown prolyl hydroxylase (PH), which leads to binding of Von Hippel Lindau protein (VHL), ubiquitination, and finally degradation of the protein.

cytosol for a stable association with the nuclear compartment. Steroid receptor coactivator and transcription intermediary factor, both transcriptional coactivators, interact with HIF-1 $\alpha$  to potentiate its activation (Dachs and Tozer, 2000).

The expression of VEGF in hypoxic tumor cells is influenced by the circadian organization of molecular clockwork. Clock genes govern physiologic and behav-



ioral circadian rhythms via an autoregulatory transcription-translation feedback loop. The basic helix-loop-helix per-aryl hydrocarbon receptor nuclear translocator-sim protein domain proteins CLOCK and BMAL 1 form a heterodimer and activate transcription of the Per and Cry genes. At a critical concentration of the PER and CRY proteins, CLOCK/BMAL-mediated activation of the Per and Cry genes is attenuated in a negative feedback loop. VEGF, CLOCK, and BMAL 1 mRNA levels in implanted sarcoma 180 tumors in mice show a circadian oscillation, with high and low levels of VEGF during the light and dark phases, respectively (Koyanagi et al., 2003). The negative component of the molecular loop governs the rhythmic change in hypoxia-induced VEGF gene transcription.

The genes for VEGFR-1 and VEGFR-2 seem to be differentially regulated by hypoxia in cultured endothelial cells. Hypoxia induces an up-regulation of VEGFR-1 mRNA, and an HIF binding site has been described in the promoter region of VEGFR-1, but it is not certain if posttranscriptional mechanisms participate in the regulation of VEGFR-1 expression. VEGFR-2 expression remains unchanged, or moderately down-regulated, in hypoxic conditions in vitro. These findings are inconsistent with the hypoxia-induced up-regulation of both receptors observed in several in vivo models. Since the VEGFR-2 gene does not possess a HIF binding site in its promoter region, the in vivo response to hypoxia could possibly be explained by a post-transcriptional increase in mRNA stability and the stimulatory effects of an as yet unidentified paracrine mediator released by ischemic tissues, which is absent in the supernatant of endothelial cells. One can conclude that both VEGFR-1 and VEGFR-2 are up-regulated in hypoxic conditions (Dachs and Tozer, 2000; Fang et al., 2001). VEGF-B and VEGF-C mRNA are apparently not regulated by hypoxia. It is unclear whether VEGF-D and VEGF-E expression is induced by hypoxia (Enholm et al., 1997).

Reactive oxygen species (ROS) (superoxide, hydrogen peroxide, and their metabolites) are regarded as cytotoxic and mutagenic. Reactive oxygen may play a role in neoplastic growth, because a variety of cell lines derived from human cancers demonstrate significantly elevated hydrogen peroxide. The mechanism of ROS generation in malignant cells is not fully understood but may involve de novo synthesis through defective respiration, byproducts of oxidative metabolism, or the induction of ROS-generating enzymes, such as Nox-1. Nox-1 is a recently identified homolog of gp91*phox*, the catalytic subunit of the phagocyte superoxide generating NADPH-oxidase. Arbiser et al. (2002) showed that Nox-1 expression strongly enhances the tumorigenic potential of DU-145 prostate epithelial cells, with a corresponding increase in tumor vascularity, implying that Nox-1 is angiogenic. Nox-1 signals angiogenic and tumorigenic effects partly through hydrogen peroxide, resulting in an increase of both the synthesis of VEGF

mRNA and the bioactivity of matrix metalloproteinase-9 (MMP-9). It activates the extracellular signal-regulated kinase pathway and NF- $\kappa$ B-dependent transcription, both of which have been implicated in growth and angiogenesis.

2. *Growth Factors and Cytokines.* Tumor necrosis factor-alpha (TNF- $\alpha$ ) is an inflammatory cytokine with a wide spectrum of biological activity, including angiogenesis. It influences the formation of new vessels indirectly, rather than by directly promoting the sprout of endothelial cells and their growth. The release of angiogenic molecules (e.g., bFGF, PAF, VEGF-A, and VEGF-C), and the up-regulation of proteolytic systems (e.g., uPA) are biological events seemingly triggered by TNF- $\alpha$ . Moreover, it has been proven that TNF- $\alpha$  also increases the transcription of the VEGFR-2 gene in vascular endothelial cells and has been postulated that the up-regulation of Sp-1 transcription by TNF- $\alpha$  leads to increased binding of this factor to the Sp-1 binding site of the VEGFR-2 promoter region. This would explain the increase in VEGFR-2 expression. TNF- $\alpha$  probably also increases the transcription of neuropilin-1. The augmented expression of neuropilin-1 and VEGFR-2, after TNF- $\alpha$  stimulation, accounts for the increased migration of endothelial cells and the stimulation of wound repair by VEGF-A<sub>165</sub> (Giraudo et al., 1998; Ristimaki et al., 1998).

Several growth factors, such as tissue growth factor- $\beta$  (TGF- $\beta$ ), epidermal growth factor (EGF), and platelet-derived growth factor BB (PDGF-BB) induce VEGF-A mRNA expression. It has been shown that VEGF-A mRNA is induced in vivo in wounds by PDGF in fibroblasts and by keratinocyte growth factor in epidermal keratinocytes (Enholm et al., 1997). Serum, which contains various growth stimulatory agents, induces an approximately 5-fold increase in VEGF-C mRNA in starved human fibroblasts in vitro. VEGF-C mRNA levels also increased in response to PDGF and to a lesser extent, by EGF and TGF- $\beta$ . Knowing that PDGF is released from platelets following tissue injury, it is possible that VEGF-C is induced in wounds, contributing to the repair of tissue injury. None of these growth factors or serum induces changes in VEGF-B mRNA levels (Enholm et al., 1997). Cytokines such as IL-1 $\alpha$  in human synovial fibroblasts, IL-1 $\beta$  in aortic smooth muscle cells, and IL-6 in tumor cell lines have been shown to stimulate VEGF-A expression. VEGF-C and VEGFR-2 mRNA levels are increased in human umbilical vein endothelial cells (HUVEC) treated with IL-1 $\beta$  (Ristimaki et al., 1998).

The expression of the VEGF-A isoforms 121 and 165 can also be regulated by IGF-1. The intrinsic activity of this growth factor has been investigated in endometrial adenocarcinoma cells devoid of estrogen receptors. IGF-1 fails to increase the transcription of the VEGF-A gene. There is a delayed increase of VEGF-A mRNA, so the regulation of mRNA occurs at a posttranscriptional

level. In colorectal carcinoma cell lines, IGF-1 appears to operate at both transcriptional and posttranscriptional levels (Akagi et al., 1998). Therefore, the regulation level of VEGF-A expression appears to be tissue specific, or it may depend on the profile of oncogene and antioncogene mutations (Bermont et al., 2000). It is not known whether this growth factor plays a significant role in the VEGF regulation of nontumoral angiogenic processes.

The biological activity of many cytokines and growth factors is mediated by activation of a signal transducer and activator of transcription (Stat). Niu et al. (2002) showed that constitutively activated Stat3 is capable of activating VEGF expression. Moreover, Stat3-induced VEGF-A up-regulation requires the Stat3 binding site at position -848 in the VEGF-A promoter region, providing evidence that VEGF-A is a direct target gene of Stat3. They demonstrated that the interruption of Stat3 signaling inhibits VEGF-A expression, identifying Stat3 as a promising molecular target for antiangiogenic therapy. Since Stat3 is downstream of several important angiogenic tyrosine kinases, such as Src and EGFR, blocking Stat3 may inhibit the neovascularization mediated by multiple angiogenic signaling pathways. For example, targeting Stat3 in tumors may potentially inhibit VEGF-A expression induced by hypoxia and oncogenic tyrosine kinases, both of which are dependent on Src activation. The constitutive activation of Stat3 may also stimulate tumor angiogenesis by down-regulating the expression of angiostatic mediators, as disrupting Stat3 signaling in tumor cells activates the expression of IP-10 and IFN- $\beta$ , inhibitors of angiogenesis.

Cyclooxygenase (COX)-1 and -2 enzymes convert arachidonic acid to prostaglandins and thromboxanes. COX-1 is constitutively expressed and is responsible for normal kidney and platelet function, as well as the maintenance of the gastrointestinal mucosa. The COX-2 enzyme can be induced by a variety of proinflammatory cytokines (e.g., IL-1), growth factors (e.g., EGF), TGF- $\beta$ , inducible nitric oxide synthetase, and ultraviolet B radiation (Gately, 2000). The role of COX-2 in cancer promotion has been demonstrated experimentally in a model of human familial adenomatous polyposis. Oshima et al. (1996) showed that the tumor burden was significantly reduced by the genetic knockout of COX-2. COX-2 expression has been reported in 80% of gastrointestinal adenocarcinomas (Eberhart et al., 1994). It is localized to the neoplastic epithelium, stromal cells, and new angiogenic endothelial cells, whereas COX-1 is distributed widely within mature blood vessels (Masferrer et al., 1999). COX-2 overexpression results in dedifferentiation, adhesion to extracellular matrices, and inhibition of programmed cell death in intestinal cells (Tsuji et al., 1997). Although the exact mechanisms by which COX-2 promotes tumor cell growth are unclear, it stimulates endothelial motility and tube formation in Caco-2 and HCA-7 cells by increasing the production of proangiogenic factors such as VEGF-A. Using an MKN45

xenograft model, Sawaoka et al. (1999) demonstrated that COX-2 inhibition by NS-398, a selective COX-2 inhibitor, suppresses protein levels of VEGF-A and bFGF, and that the angiogenic and apoptotic indices are significantly associated (Sawaoka et al., 1999). They postulated that apoptosis was induced by suppressing angiogenesis. Celecoxib, a potent and selective COX-2 inhibitor, induces apoptosis by blocking Akt activation (Hsu et al., 2000). Nonsteroidal anti-inflammatory drugs (NSAID) inhibit the activity of mitogen-activated protein kinase, an important intermediate in several signaling pathways involved in the activation of transcription factors and early response genes leading to cell proliferation and differentiation and essential for the induction of angiogenesis. These nonselective COX inhibitors also inhibit mitogen-activated protein kinase translocation into the nucleus (Jones et al., 1999). Furthermore, NSAIDs increase the expression of the von Hippel Lindau tumor suppressor, which leads to increased ubiquitination of total protein. This results in decreased HIF-1 $\alpha$  accumulation, hypoxia-induced VEGF/VEGFR-1 expression, and the inhibition of hypoxia induced angiogenesis (Jones et al., 2002).

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production has been shown to be selectively linked to COX-2 activity through the inducible PGE<sub>2</sub> synthase pathway in both inflammatory and aberrant cell growth processes (Murakami et al., 2000). Experimental studies have shown that PGE<sub>2</sub> production, or the addition of PGE<sub>2</sub> to cell cultures, can mediate important carcinogenic mechanisms. It has been suggested that PGE<sub>2</sub> stimulates angiogenesis by increasing Bcl-2 levels and inhibiting apoptosis. However, the precise link between prostaglandin production and Bcl-2 synthesis has not been elucidated. PGE<sub>2</sub> also seems to inhibit the immune response against cancer (Kambayashi et al., 1995) and perhaps enhances the invasiveness of neoplastic cells by increasing matrix metalloproteinase-2 (MMP-2) activation (Tsuji et al., 1997). PGE<sub>2</sub>, a potent stimulator of osteogenesis in vivo, causes rapid induction of VEGF-A mRNA and VEGF-A production in osteoblastic cells in vitro (Harada et al., 1994). The mechanisms of PGE<sub>2</sub> up-regulation of VEGF-A still have to be elucidated. Since angiogenesis is associated with the early phase of bone formation, the induction of VEGF-A may play a role in PGE<sub>2</sub> stimulation of bone formation in vivo. Further studies on how the products of COX-2 mediate the transcriptional regulation of the VEGF-A gene are needed.

3. *Hormonal Regulation.* Estrogens stimulate VEGF-A gene transcription and stabilize VEGF-A mRNA, prolonging the half-life of the transcripts (Shweiki et al., 1993; Hyder et al., 1996; Ruohola et al., 1999). This interaction has been studied in human breast cancer (MCF-7) and uterine cell lines. MCF-7 cells express both estrogen receptors  $\alpha$  and  $\beta$ , which normally cause transactivation by binding an estrogen response element in the promoter region of certain

genes. The 5' regulatory regions of VEGF-A have not been found to contain any classical estrogen response element. However, the regulatory regions contain several AP-1 and Sp1 sites, which are known to mediate estrogen action. The exact mechanisms remain to be determined (Ruohola et al., 1999). Progestins have been reported to increase VEGF-A expression in the human uterus and in human breast cancer cells (T47-D), associated with transcriptional activation of the VEGF gene (Hyder et al., 1996). The antiestrogens tamoxifen and toremifene, both of which are used in the treatment of breast cancer, do not inhibit the estrogen-induced increase of mRNA expression. On the contrary, they increase VEGF-A mRNA. It has been postulated that the increase in VEGF-A expression is also mediated by an AP-1-related mechanism, but this also requires further study (Gagliardi and Collins, 1993; Hyder et al., 1996; Adams et al., 2000). The clinical impact of these findings will be discussed later.

The effect of testosterone on VEGF-A expression has been studied in the androgen-dependent S115 mouse breast cancer cell line and human prostatic tissue. Transcriptional activation causes an increase in VEGF-A expression, as well as stabilization of the mRNA. The upstream regulatory region of the VEGF gene does not seem to contain any androgen or gonadotrophin response elements. However, the ligand-bound androgen receptor has been found to modulate transcription indirectly via other transcription factors, such as the AP-1 complex (Ruohola et al., 1999).

VEGF-B and VEGF-C are also expressed in MCF7 and S115 breast cancer cells, but they are not affected by hormonal treatment to the same extent as VEGF-A. VEGF-D mRNA is expressed only in S115 cells and is not regulated by testosterone (Ruohola et al., 1999).

#### *E. Physiological Regulation of Vascular Endothelial Growth Factor in Normal Adults*

Physiological angiogenesis is mainly restricted to wound healing and the female reproductive cycle.

**1. Wound Healing.** Wound healing can be subdivided into four phases: acute inflammation, re-epithelialization, granulation tissue formation, and tissue remodeling.

Following cutaneous injury, hemorrhage is stopped by platelet activation and the initiation of the clotting cascade. This results in the formation of a clot, consisting of platelets, embedded in a mesh of cross-linked fibrin fibers, together with small amounts of plasma fibronectin, vitronectin, and thrombospondin. The activated platelets release several cytokines and growth factors stored in their granules. One of these growth factors is VEGF-A, which attracts circulating neutrophils and monocytes. This chemotactic response is mediated by VEGFR-1, expressed on the inflammatory cells (Schaffer and Nanney, 1996; Martin, 1997). Wound healing experiments in VEGF-A transgenic mice, described by Det-

mar et al. (1998), showed increased leukocyte rolling and adhesion in the postcapillary venules at the wound site. This can be explained by the molecular changes in the surface of the endothelial cells, such as the expression of E- and P-selectin. The exact function of VEGF-A in this process is not clear. As well as phagocytosing particles and releasing toxic metabolites and enzymes, the recruited neutrophils and monocytes also produce a series of proinflammatory cytokines, including IL-1 $\beta$  and TNF- $\alpha$ . The leukocyte-derived cytokines, together with TGF- $\beta$  released from platelets, and serum derived factors, are able to induce VEGF-A gene expression in the keratinocytes at the wound margins. VEGF-A expression in these cells is also regulated by HIF-1. This heterodimer is formed in hypoxic regions in the healing wound or after stimulation by IGF-1 and IGF-2, as described above (Frank et al., 1995; Senger and van de Water, 2000). It is notable that only keratinocytes express VEGF-A during this process, with the exception of a few mononuclear cells.

During re-epithelialization, the activated keratinocytes migrate along the interface between the clot and the underlying healthy dermis. This movement is only possible when the fibrin barrier of the clot is dissolved. The most important fibrinolytic enzyme is plasmin, which is derived from plasminogen within the clot itself, and can be activated either by tPA or uPA. Both of these activators are up-regulated in the migrating keratinocytes. VEGF-A regulates the production of plasminogen activators and PAI-1 in endothelial cells. It is unclear whether the increased VEGF-A expression during re-epithelialization also stimulates the production of uPA and tPA by keratinocytes in an autocrine fashion (Schaffer and Nanney, 1996; Martin, 1997). Several matrix metalloproteinases, each of which cleaves a specific subset of matrix proteins, are also up-regulated by wound-edge keratinocytes (Lohi et al., 2001; Mirastschijski et al., 2002; Saarialho-Kere et al., 2002).

An ingrowth of new blood vessels and fibroblasts, together with the synthesis of the components of connective tissue matrix (collagen type I and III), transform the fibrin-fibronectin stroma into the highly cellular and vascular granulation tissue of healing wounds. Endothelial cell migration requires freedom from the constraints of the basement membrane. VEGF-A, released by keratinocytes, monocytes, and endothelial cells, acts in a paracrine and autocrine manner on the capillaries at the wound edge. VEGFR-1 and VEGFR-2 are up-regulated in these endothelial cells, and their activation by VEGF-A results in the generation of proteases such as uPA, tPA, plasmin, and collagenase, which are capable of digesting basal lamina components. VEGF-A also induces an increase in permeability (Dvorak et al., 1995b) by disorganizing the endothelial junction proteins (VE-cadherin and occludin). This process results in gap formation and decreased barrier properties between adjacent endothelial cells. It is proposed that this enhances

the supply of proteins and cells needed to form granulation tissue. Furthermore, receptor activation leads to the up-regulation of  $\alpha_{v\beta_3}$  integrins, which are expressed transiently at the tips of sprouting capillaries and enhance endothelial cell migration. Finally, receptor activation results in endothelial cell migration (VEGFR-1) and proliferation (VEGFR-2), as mentioned earlier.

In addition to endothelial migration from adjacent pre-existing blood vessels, another source of endothelialization is the recruitment of bone marrow-derived endothelial progenitor cells (EPC) from the peripheral circulation (Takahashi et al., 1999). EPCs can be considered to be embryonic angioblasts, which are VEGFR-2+/AC133+ (a hematopoietic stem cell marker, with an as yet unrecognized function), and migrate, proliferate, and capable of differentiating into mature endothelial cells, which are VEGFR-2+/AC133- (Caprioli et al., 1998). Hattori et al. (2001) showed that high plasma levels of VEGF promoted the mobilization of EPCs through activation of metalloproteinases, adhesion molecules, and remodeling of the extracellular matrix within the bone marrow. EPC mobilization promotes an up-regulation of Id1 and Id3, induced by the raised plasma VEGF-A (Benezra et al., 2001). Id proteins (Id1, 2, 3, and 4) contain a highly conserved dimerization motif known as the helix-loop-helix (HLH) domain, which mediates its interaction with other proteins. Their primary targets are the basic HLH transcription factors controlling cell type-specific gene expression and the expression of cell cycle regulatory genes. Heterodimerization between Id and bHLH proteins prevents transcription factors from binding DNA (Lassar et al., 1994). The exact pathways activated by VEGF-induced Id up-regulation need further elucidation. There is comobilization of VEGFR-1+ myeloid cells and EPCs, suggesting that these hematopoietic precursor cells are associated with the newly formed vessels and may be essential for the incorporation of EPCs (Benezra et al., 2001).

Endothelial cells assemble as solid cords, which subsequently acquire a lumen. VEGF-A<sub>121</sub>, VEGF-A<sub>165</sub>, and their receptors on endothelial cells (VEGFR-1 and VEGFR-2) enhance and increase lumen formation, in addition to increasing vessel length. Other molecules affecting lumen formation are the integrins  $\alpha_{v\beta_3}$ . Intercalation or thinning of endothelial cells and fusion of pre-existing vessels is a third mechanism which allows newly formed vessels to increase their diameter and length (Schaffer and Nanney, 1996; Martin, 1997).

VEGF-A also acts on pericytes in an autocrine and paracrine manner to stimulate their proliferation and migration. In the normal retina, the recruitment of pericytes is significantly delayed and lags behind the formation of the endothelial plexus. The time interval during which the vasculature is maintained without a pericyte coating determines a window of plasticity for the vasculature to be remodelled and adjusted to the physiological

needs of the tissue. It is proposed, but not yet proven, that this mechanism is also important during wound healing (Benjamin et al., 1998; Yonekura et al., 1999). In a study of wound healing in pig skin, Paavonen et al. (2000) observed that VEGFR-3-positive lymphatic vessels appear in the wound simultaneously with blood vessels but regress sooner. Angiogenic blood vessels are negative for VEGFR-3 during normal blood vessel regeneration in wounds. Lymphangiogenesis may be an important phenomenon in wound healing, but it needs further investigation.

Granulation tissue is eventually remodeled, as blood vessels are resorbed and fibroblasts disappear. The end result is a scar composed largely of dense type I collagen, with occasional widely dispersed fibrocytes and blood vessels.

**2. The Female Reproductive Cycle.** The ovarian vasculature is not distributed equally; primordial and pre-antral follicles do not have their own vascular supply but are supported by vessels in the surrounding stroma. As the antrum develops in the follicle, the thecal layer acquires a vascular sheath, consisting of two capillary networks, one in each of the theca interna and externa. The capillary networks are connected, and all capillary blood passes from the theca interna into small vessels, which become continuous with the ovarian stromal veins. All capillaries remain outside the basement membrane of the follicle, so that the granulosa layer remains avascular until the time of ovulation. The vascular density increases during follicle development from the pre-antral to the antral stage (Gordon et al., 1995). One of the earliest signs of follicular atresia is the reduced vascularity of the follicle. This limits the access to nutrients, substrates, and trophic hormones (Hazzard and Stouffer, 2000). At ovulation, the follicle is converted into the corpus luteum. During ovulation in the rat, the inner capillary plexus adjacent to the basement membrane expands by sprouting into the avascular granulosa layer to form a dense network of sinusoidal capillaries. The outer capillary plexus delays sprouting but eventually becomes interconnected in the corpus luteum. This sinusoidal meshwork differentiates into arterial and venous systems. A decrease in the density of blood vessels during early luteolysis has been observed, and this decline may be related to morphological changes in endothelial cells, including apoptosis. During luteolysis, the larger microvessels are maintained, probably to assist in resorption of the luteal mass (Hazzard and Stouffer, 2000).

VEGF-A is important in luteal angiogenesis. VEGF-A mRNA or protein is detectable in the granulosa cells of primordial and primary follicles, as they progressively become localized to the granulosa surrounding the oocyte and theca cells of the preovulatory follicle. After ovulation, VEGF-A mRNA and protein expression are observed in granulosa-derived luteal cells. VEGF-A expression in the corpus luteum appears highest early in

the luteal phase and declines after the mid-luteal phase, with little or no expression in the late corpus luteum (Fraser and Lunn, 2000). Neutralization of VEGF-A during the early luteal phase inhibits the development of the normally extensive capillary bed. Luteal function, reflected by the secretion of progesterone, is reduced by 60%. Whether oxygen tension is a primary regulator of VEGF-A expression in ovarian tissues is unknown. Follicles with a high content of dissolved oxygen also contain the highest follicular fluid concentration of VEGF-A. These follicles display better fertilization rates and embryo development than do oocytes from severely hypoxic follicles (Hazzard and Stouffer, 2000).

Gonadotrophic hormones, particularly luteinizing hormone (LH), appear to be major regulators of angiogenesis in the ovary (Hyder and Stancel, 1999). The LH-stimulated luteinization of granulosa cells at the time of ovulation is associated with enhanced VEGF-A expression. Further studies are needed to determine whether the action of LH is critical for vascularization of the theca in the developing antral follicle, and whether the rescue of the corpus luteum by human chorionic gonadotrophin (hCG) in early pregnancy involves modification of the luteal vasculature. It is speculative whether the decline in VEGF-A expression in the late luteal phase of the menstrual cycle is related to reduced sensitivity of the corpus luteum to LH. Laitinen et al. (1997) showed that LH/hCG decreased the expression of VEGF-C in luteinized granulosa cells. VEGF-B expression is not affected. This implies different roles for the VEGF species in the developing corpus luteum (Laitinen et al., 1997; Hazzard and Stouffer, 2000).

Shifren et al. (1996) described the cellular localization and abundance of both VEGF-A mRNA and protein in the cycling human endometrium. VEGF-A mRNA was observed in the cytoplasm of glandular epithelial cells, accompanied by diffuse stromal staining. VEGF-A expression increased in the secretory phase throughout the endometrium. Both estradiol and progesterone induced the expression of VEGF-A (Shifren et al., 1996).

### III. Vascular Endothelial Growth Factor in Tumor Growth

#### A. Vascular Endothelial Growth Factor and Tumorigenesis

All solid tumors are composed of two compartments: the malignant cells and the stroma. The latter is composed of many elements, which can be grouped into four categories: new blood vessels, inflammatory cells, connective tissues, and a fibrin-gel matrix. Tumor fibrin originates from the extravasation and extravascular clotting of plasma fibrinogen by tumor cell prothrombinase and is dependent on increased vascular permeability. Hyperpermeability and macromolecular transvascular transport are facilitated by the existence of interendothelial junctions, caveolae (vesiculo-vacuolar

organelles), and fenestrations, all of which are induced by VEGF-A produced by tumor cells (Hobbs et al., 1998; Monsky et al., 1999). The increased permeability and decreased selectivity of neovascular fenestrated endothelium, compared with the naturally occurring fenestrated endothelium, results from the absence of the basement membrane and the decreased anionic glycolyx on the luminal surface of the fenestral diaphragms. The mechanism by which VEGF-A modulates the changes leading to an increase in capillary permeability is unknown.

The transformation from provisional matrix (fibrin-fibronectin gel) to the mature collagenous tumoral stroma requires the following events: an influx of monocytes that differentiate into macrophages, the replication of fibroblasts and endothelial cells, the migration of macrophages, endothelial cells, and fibroblasts into the fibrin-fibronectin gel, and finally, the degradation of this provisional matrix and progressive replacement by matrix proteins, proteoglycans, and glycosaminoglycans produced by fibroblasts (Dvorak et al., 1995a). The mature tumoral stroma resembles the granulation tissue of healing wounds. There are only small differences between the formation of tumoral stroma and the process of wound healing. First, wound healing is preceded by tissue injury. Such an insult is generally not associated with tumoral stroma generation, which is initiated by the increased permeability of local blood vessels. Second, platelets, which are important in wound healing, have not been found outside blood vessels in solid tumors. However, tumor cells themselves express clotting factors and secrete growth factors, which function as fibroblast mitogens and chemoattractants. Third, fibrin and fibronectin persist in tumoral stroma, whereas both appear only transiently in wounds that heal normally. Fourth, the abundant fibrin deposited around some tumors may serve as a barrier to lymphocytes, macrophages, and other inflammatory cells (Dvorak, 1986). The persistence of fibrin and fibronectin in tumoral stroma is probably due to the fact that tumors produce VEGF-A constitutively. This explains the expression: "Tumors are wounds that do not heal".

Blood vessels in the core and periphery of developing tumors differ. Irrespective of the host or tumor type, core vessels have certain common features: open interendothelial junctions, fenestrated endothelium, a discontinuous or absent basement membrane, and abnormally convoluted lumina. In contrast, peripheral vessels are large and venular and are formed by endothelial cells characterized by fenestrations (formed by fusion of intracellular caveolae) and attenuation. Open interendothelial junctions are not as prevalent as in core vessels (Roberts and Palade, 1997). It is notable that vessels in the tumor periphery represent a true transition from normal nonproliferating host vessels to tumor vessels generated in response to VEGF-A, without the further complicating influences such as tumor secreted pro-

teases, necrosis, hypoxia, and increased interstitial pressure, present in the tumor core (Dvorak, 1986; Nagy et al., 1989). Tsuji et al. (2002) performed morphometric studies on resected specimens of human colorectal carcinoma. They described a significant increase in the mean microvessel diameter as the pathological process progressed from normal ( $7.03 \mu\text{m}$ ), to localized carcinoma ( $7.99 \mu\text{m}$ ), and then to metastatic carcinoma ( $9.75 \mu\text{m}$ ). The risk of regional or distant metastasis increased as the microvessel diameter increased. Giant capillaries ( $>60 \mu\text{m}$ ) have been identified as a site for cancer cell intravasation. Whether VEGF is involved in the genesis of enlarged microvessels remains to be determined. The number of VEGF-positive cells failed to correlate with the microvessel diameter (Tsuji et al., 2002).

During tumorigenesis, neoplastic lesions initially undergo an avascular growth phase to a size not much greater than 2 to 3  $\text{mm}^3$ . This phase is followed by a second event that distinguishes a growing tumor from one that is dormant, the switch from the avascular to vascular phenotype, or "the angiogenic switch". This initiates a cascade of events that results in the expansion of tumor volume and subsequent metastasis. The formation of new blood vessels provides a mechanism by which an in situ tumor lesion can circumvent the critical limitations of the oxygen diffusion distance and restrictions on nutrient exchange (Folkman, 1995; Carmeliet, 2000; Carmeliet and Jain, 2000; Kerbel, 2000). The angiogenic switch is regulated by the net balance between positive and negative regulators of new capillary growth (Folkman, 1986; Hanahan, 2002). Figure 6 gives an

overview of the stages of tumor development, growth, and metastasis, in which angiogenesis plays an important role.

An important inhibitor of angiogenesis is the p53 tumor suppressor gene. p53 arrests cell cycle progression under nonviable conditions and mediates hypoxia-induced apoptosis. The p53 gene product promotes the expression of inhibitors of angiogenesis, such as thrombospondin, and inhibits the expression of VEGF-A, but not VEGF-B or VEGF-C (Fontanini et al., 1998; Ravi et al., 2000). p53 is mutated in 50% of all cancers and is also inactivated by viral oncoproteins (Yuan et al., 2002). HIF-1 $\alpha$  has been found to be overexpressed in most human cancers, since active tumor growth is accompanied by hypoxia (Graeber et al., 1996; Elson et al., 2000). Hypoxic conditions lead to the up-regulation of both p53 and HIF-1 $\alpha$  (Carmeliet et al., 1998; Wenger et al., 1998). HIF-1 $\alpha$  binds to and stabilizes p53, provided the latter is not mutated (Zhong et al., 1999). This association initiates apoptosis: The angiogenic switch is maintained in the off position, since positive factors are overruled by the angiogenesis inhibitors. The loss of p53 expression enhances the heterodimerization of HIF-1 $\alpha$  with HIF-1 $\beta$  and up-regulates the expression of VEGF-A in tumor cells (Blagosklonny et al., 1998; Zhong et al., 1999; Blancher et al., 2000). In this situation, there is a net balance of activators over inhibitors, and new blood vessels will be formed (Dachs and Tozer, 2000; Ryan et al., 2000). In human tumors, the p63/p51/p73L/p40/KET gene, a new p53 family member (p63 gene), is not mutated as frequently as p53, but certain isoforms are

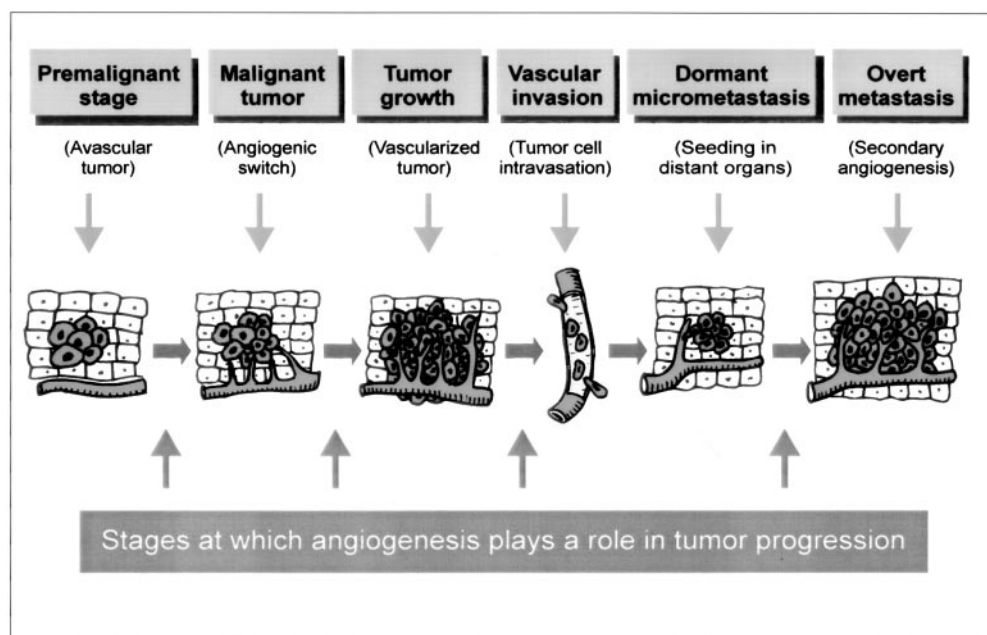


FIG. 6. Angiogenesis in cancer development, growth, and metastasis. Tumors are diffusion limited in their growth without their own blood supply. The activation of the angiogenic switch allows the tumor to recruit surrounding blood vessels and stimulate them to form new vessels in the tumor. The tumor vasculature supplies the tumor with oxygen and nutrients, which enables further growth. In the next stage, the tumor can invade the vessel wall and spread to distant organs to form dormant micrometastases. Finally, secondary angiogenesis can occur, allowing these dormant micrometastases to grow into overt metastases.

occasionally lacking or overexpressed. TAp63 $\gamma$  and dNp63 $\alpha$ , the two major isoforms of p63, bind to the VEGF-promotor region, and respectively, down- and up-regulate VEGF gene expression. The modulation of VEGF expression is closely correlated with the distinct roles of both isoforms in the regulation of HIF-1 $\alpha$  stability. Senoo et al. (2002) showed that the degradation of HIF-1 $\alpha$  is stimulated by TAp63 $\gamma$ . The other isoform, dNp63 $\alpha$ , stabilizes HIF-1 $\alpha$ . Both isoforms are involved in the posttranslational regulation of HIF-1 $\alpha$ , but not its transcription (Senoo et al., 2002).

The importance of the matrix metalloproteases (MMP) in the induction of the angiogenic process has recently been described. MMPs, which are produced by microvascular endothelial cells, break down the extracellular matrix. This is one of the earliest and sustained events in the process of new capillary formation. VEGF-A has been shown to modulate the production and activity of these proteins (Grunstein et al., 1999). Fang et al. (2000) reported that the suppression of MMP-2 alone inhibits the transition from the prevascular to the vascular stage during tumor development. They showed that MMP-2 is required to switch to the angiogenic phenotype during the development of chondrosarcoma and during the progression of plasma cell tumors. Bergers et al. (2000) showed that MMP-9 is a functional component of the angiogenic switch during multistage pancreatic angiogenesis. Activation of MMP-9 induces an up-regulation of VEGF-A. Loss of MMP-9 function in several solid tumors, however, cannot block tumor growth completely, suggesting either alternative pathways or a broader redundancy in mobilizing proteinases (Bergers et al., 2000). Since MMP-9 bioactivity, as well as VEGF-A mRNA synthesis, is increased by Nox1, ROS are important triggers of the angiogenic switch (Arbiser et al., 2002).

Nör et al. (2001) demonstrated that VEGF-A, expressed by tumor cells, up-regulates expression of the antiapoptotic protein Bcl-2 in endothelial cells in vitro, and overexpression of Bcl-2 is sufficient to enhance endothelial cell survival and protect against apoptosis induced by growth factor deprivation. Bcl-2 acts by preserving mitochondrial integrity (Adams and Cory, 1998). Furthermore, potentiation of angiogenesis, associated with endothelial Bcl-2 overexpression, is not only attributable to enhanced survival of these cells but is also mediated by the resulting synthesis of endothelial-derived proangiogenic IL-8 (Nör et al., 2001).

Survivin, a member of the inhibitor of apoptosis proteins family, is undetectable in normal adult tissue but is expressed abundantly in most human cancers in vivo (Mesri et al., 2001). Survivin is up-regulated by VEGF-A and inhibits endothelial cell apoptosis. The mechanism by which survivin influences apoptosis is still to be elucidated but involves the suppression of caspase activity (LaCasse et al., 1998; Deveraux and Reed, 1999). Since both Bcl-2 and survivin are up-regulated by VEGF-A,

O'Conner et al. (2000) suggested that inhibition of endothelial cell apoptosis during angiogenesis may occur simultaneously through parallel and nonoverlapping pathways.

It is now accepted that platelets play an important role in hematogenous tumor cell metastasis. Some tumor cells induce platelet aggregation, and this correlates directly with their metastatic potential (Tang and Honn, 1994). Several studies show that platelets cover tumor cells, offering protection from cytolysis by natural killer cells (Nieswandt et al., 1999; Borsig et al., 2001). It has been hypothesized that the tumor cell-platelet aggregates lodge in the microcirculation, adhere to the vascular endothelium, and facilitate tumor cell aggregation. The initial tumor cell-platelet bridging involves binding between platelet GpIIb/IIIa and tumor cell  $\alpha_{v\beta 3}$ , requiring the expression of endothelial tissue factor and subsequent generation of thrombin from prothrombin by the activated coagulation cascade. This binding results in a rapid induction of platelet aggregation and the secretion of angiogenic factors, such as VEGF-A, stored in platelet granules (Tripathi and Nakada, 2002). Selheim et al. (2002) recently showed that endogenously secreted platelet VEGF-A may function as a positive feedback regulator during platelet activation, in addition to its major role as an angiogenic factor. They also demonstrated that platelets, VEGFR-1, and VEGFR-2 seem to activate the same signaling pathways as those in endothelial cells. In conclusion, it appears that platelets can stimulate tumor growth, angiogenesis, and metastasis.

### B. Lymphangiogenesis and Tumor Growth

A number of recent reports have described a correlation between VEGF-C expression, tumor lymphangiogenesis, and the formation of metastases in regional lymph nodes (Akagi et al., 2000; Saaristo et al., 2000). Since experiments with lymph vessels are difficult to conduct, a direct causal role for VEGF-C-mediated lymphangiogenesis in the dissemination of tumor cells was demonstrated only in 2001 using a transgenic mouse model of nonmetastatic  $\beta$ -cell carcinogenesis (Mandriota et al., 2001). VEGF-C is a ligand for VEGFR-2 and VEGFR-3 (Lymboussaki et al., 1998), and the latter is more associated with lymph vessels. Valtola et al. (1999) observed an increased expression of VEGFR-3 in breast cancer xenografts but could not distinguish if these were blood or lymph vessels. A low VEGF-D to VEGF-C ratio in lung adenocarcinoma correlates with both lymph node metastasis and lymphatic invasion by cancer cells (Niki et al., 2000). The down-regulation of VEGF-D in the process of lymphangiogenesis has not yet been explained, and contradictions exist in the literature concerning the expression and role of VEGF-D. The up-regulation of VEGF-C and VEGF-A possibly promotes lymph node metastasis by different mechanisms, the former facilitating the entry of tumor cells into the lymph

phatic vasculature, and the latter stimulating tumor growth at metastatic sites by angiogenesis.

The correlation of VEGF-D and intratumoral lymphangiogenesis was further investigated in VEGF-D overexpressing breast cancer xenografts by Stacker et al. (2001), who observed well defined lymphatic vessels throughout the tumor at a much higher density than in non-VEGF-D expressing tumors, in addition to enlargement of peritumoral lymphatic vessels. They hypothesized that the intratumoral lymphatic network promotes metastatic tumor spread by increasing opportunities for metastatic tumor cells to leave the primary network. It is not yet clear whether VEGF-D also promotes molecular interactions between tumor cells and lymphatic endothelial cells to facilitate tumor cell entry into the lymphatics (Stacker et al., 2001). A significant correlation has also been described between VEGF-C levels in thyroid, prostate, gastric, colorectal, breast, and lung primary tumors, and lymphangiogenesis and lymph node metastasis. Despite these highly suggestive correlative clinical findings, a direct role for VEGF-C in tumor lymphangiogenesis and subsequent metastasis has yet to be demonstrated, and to date there is no animal model in which these phenomena can be explored.

Karpanen et al. (2001) suggested that inhibition of tumor-associated lymphangiogenesis (for example, by using soluble VEGFR-3 proteins) could inhibit tumor metastasis. However, caution is warranted, since destruction of the lymphatic vessels could elevate the already increased interstitial fluid pressure inside the tumor, thereby further impairing the delivery of other anticancer drugs (Alitalo and Carmeliet, 2002). Increased fluid pressure could also result in closer contact between tumor cells, leading to a compensatory increase in lymphangiogenic growth factor levels (Orlandini and Oliviero, 2001). Greater intratumoral pressure may also increase the likelihood of hematogeneous spread (Carmeliet and Jain, 2000).

### C. The Role of Vascular Endothelial Growth Factor in the Impaired Host Antitumor Immune Response

A defective host antitumor immune response is an important mechanism allowing tumors to evade control by the immune system. Induction of an effective antitumor response requires the active participation of host bone marrow-derived antigen-presenting cells, responsible for the presentation of tumor-specific antigens. Dendritic cells (DC) are the most potent APC. They play a central role in antitumor immunity by taking up tumor antigens and stimulating antigen-specific T cells. A population of DC isolated from the peripheral blood of patients with breast, head, and neck cancer had a significantly reduced ability to cluster and stimulate allogeneic and antigen-specific T cell responses (Gabrilovich et al., 1996). They had a substantially lower level of expression of major histocompatibility complex class II and costimulatory molecules than DC from control donors. Tu-

mors may affect DC maturation from progenitors, with the population of DC in tumor-bearing hosts being functionally defective (Gabrilovich et al., 1996; Oyama et al., 1998). Almand et al. (2000) described a decrease in the numbers of DC in patients with early stage cancer and even more profound changes in those with advanced cancer. This decrease can be explained by the presence of immature cells lacking the typical specific markers of the normal DC. The production of soluble tumor-derived factors is critical for the generation of these immature cells. Removal of the source of these factors by surgical excision restores the process of DC differentiation and results in higher numbers of DC in the circulation (Almand et al., 2000).

VEGF is one of the tumor-derived factors implicated in these defects (Adams and Cory, 1998). The mRNA for the VEGFR-1 and -2 have been detected in human hemopoietic progenitor cells, suggesting that VEGF may act directly on these cells (Gabrilovich et al., 1996; Ohm et al., 1999). VEGF-A binding to progenitor cells via the VEGFR-1, with inhibition of nuclear factor- $\kappa$ B activation and the signal transduction pathway, has been described (Dikov et al., 2001). Nuclear factor- $\kappa$ B regulates the transcription of many genes involved in the immune response, including cytokines and growth factors. Inhibition leads to defective functional maturation of the DC and may contribute to suboptimal induction of immunity in cancer patients (Oyama et al., 1998; Gabrilovich et al., 1999). The inhibition of VEGF-A by anti-mouse VEGF-A neutralizing antibody in tumor-bearing mice decreases plasma VEGF-A and enhances the function of DC by improving their differentiation (Gabrilovich et al., 1999). Takahashi et al. (2002) investigated the importance of VEGF-C expression and DC infiltration in resected gastric carcinoma specimens. They showed that the density of DC correlates significantly with the depth of invasion, tumor size, and extent of lymphatic involvement. A high density of DC is associated with a better prognosis. There was a negative correlation between DC density and VEGF-C expression, suggesting that VEGF-C may also affect the distribution or functional maturation of DC in the tumor microenvironment (Takahashi et al., 2002).

### D. Vascular Endothelial Growth Factor as a Prognostic Factor or an Indicator of Angiogenesis

An adequate assessment and measurement of tumor angiogenic activity has not been established, and thus far, no simple reliable methods are available. Many groups have attempted to quantify angiogenesis and provide prognostic information by the measurement of tumor microvessel density, intratumoral VEGF determination, or monitoring circulating VEGF. Although the majority of studies confirm the importance of these tumor angiogenic features (Dvorak, 2002), they are rarely used in routine clinical practice, and no guidelines are



available. More efforts at including angiogenic markers in clinical practice are warranted.

**1. Microvessel Density.** Microvessel density (MVD), determined immunohistochemically, is thought to be a marker representing the effect of angiogenesis and often correlates with intratumoral VEGF mRNA levels. It is not only important for the oxygen and nutritional supply of proliferating tumor cells, but it also reflects the potential for invasion and metastasis, because newly generated microvessels are an initial target of tumor cell invasion. Hundreds of reports have examined the prognostic value of MVD in breast, prostate, and other cancers (Fox et al., 2001; Gasparini, 2001; Hlatky et al., 2002). Most of these studies report positive correlations between MVD and tumor recurrence, although some report lack of, or even negative, correlations between these endpoints. Although MVD is a useful prognostic marker, it is not, by itself, an indicator of vascular function or therapeutic efficacy, nor should it be used to guide the stratification of patients for therapeutic trials (Hlatky et al., 2002).

**2. Intratumoral Vascular Endothelial Growth Factor Determination.** Intratumoral VEGF-A content can be measured using several methods, such as immunohistochemistry, in situ hybridization, quantitative immunoassays, Western blotting, or the reverse-transcriptase polymerase chain reaction. Recent advances in mass spectrometry have enabled the analysis of intratumoral VEGF-A protein isoforms (Landuyt et al., 2003). Although it is clear that the amount of VEGF-A expressed by tumors affects clinical outcome, none of the techniques used to quantitate tumor VEGF-A expression in solid tumors are routine, and at present it is uncertain if such studies were widely undertaken that they would be clinically useful or cost-effective for predicting outcomes in individual patients (Dvorak, 2002).

VEGF plays an important role in the neovascularization of primary breast cancer. There is a very close association between VEGF-A expression, assessed by immunocytochemistry and enzyme-linked immunosorbent assay in tumor cytosols and an increase in MVD (Toi et al., 1995). At least 12 retrospective studies, involving 3688 cases, have assessed the association between VEGF and clinical outcome in breast cancer, and most found VEGF to be a significant and independent prognostic indicator for relapse-free survival. A thorough analysis of all these studies is beyond the scope of this article and is discussed elsewhere (Linderholm et al., 2000; Fox et al., 2001; Gasparini, 2001). The observation that VEGF is critical for the initial subcutaneous growth of breast carcinoma cells, whereas other factors can compensate for the loss of VEGF after the tumors have reached a certain size, is also of interest (Yoshiji et al., 1997).

Several studies have also demonstrated a correlation between the up-regulation of total VEGF-A mRNA expression and a poor prognosis in nonsmall cell lung

cancer (Giatromanolaki et al., 1996; Bunn, 2001; Fox et al., 2001). A high VEGF-A<sub>189</sub> mRNA isoform expression ratio as well as total VEGF mRNA in the tumor is associated with a high intratumoral microvessel count and a shorter time to relapse and survival (Yuan et al., 2001). A low VEGF-D to VEGF-C ratio in tumors correlates with both lymph node metastasis and lymphatic invasion by cancer cells (Niki et al., 2000). The concentration of VEGF in bronchial lavage fluid could have a potential role as a predictive variable, but this needs to be confirmed in larger trials (Ohta et al., 2002).

The prognostic role of angiogenesis in colorectal cancer has been reviewed recently (Papamicheal, 2001). There is a stepwise up-regulation of VEGF-A and MVD during carcinogenesis (Kondo et al., 2000). VEGF-C tissue expression correlates with the extent of lymph node metastases but not with hematogenous metastasis or with overall survival (Akagi et al., 2000). VEGF-D is an independent prognostic factor for survival (White et al., 2002), but a significant correlation between VEGF-D expression and either MVD or vascular invasion has not been identified, suggesting that VEGF-D does not act as an angiogenic factor in colorectal carcinoma but may play a role in the development and function of lymphatic vessels, thereby promoting lymphatic metastases. VEGF-A expression is also a prognostic indicator in most studies of gastric cancer (Maeda et al., 1996; Fox et al., 2001).

Prostate carcinogenesis appears to be a multistep process, often progressing from low- to high-grade prostatic intraepithelial neoplasia, then advancing to focal carcinoma, and finally to invasive carcinoma and metastasis. Metastasizing human xenograft prostate cancer cells in rats express higher VEGF-A levels and more tumor vascularization compared with cells of a lower metastasizing capacity (Joseph et al., 1997). Human prostate cancer tissue stains more intensely with VEGF-A than benign hyperplastic and normal prostate tissue, and VEGF-A expression increases with malignant transformation (Balbay et al., 1999). VEGFR-1 is apparently expressed uniformly in prostate cancer tissue, while VEGFR-2 is more heterogeneously distributed and related to tumor grade, with greater expression in higher grade tumors (Weidner et al., 1993). VEGF-A expression in the human prostate is regulated by sex steroids, and castration reduces tumor VEGF-A levels and inhibits tumor growth (Stewart et al., 2001). This is due partly to marked stabilization of VEGF mRNA transcripts. VEGF-A is a factor in the development and progression of malignant prostatic disease. Androgen ablation can curtail disease progression by inhibiting angiogenesis through a reduction of VEGF-A levels within these cancers and activating programmed cell death of androgen-dependent cells (Joseph et al., 1997). Unfortunately, androgen-dependent cells eventually become androgen independent, with two distinct subtypes. One retains expression of the androgen receptor and a degree of

growth stimulation by androgen, although the cells can continue to grow after androgen ablation. Therefore, these cells are androgen independent because they are not susceptible to androgen ablation, but they remain androgen sensitive, as exposure to androgens stimulates their growth. The demonstration that VEGF-A levels in these cells are regulated by androgen explains how these cells remain sensitive to androgen stimulation in the absence of androgen sensitivity (Joseph et al., 1997). The second, most lethal, androgen-independent subtype is completely insensitive to androgen-induced growth stimulation and does not express the androgen receptor. VEGF-A is constitutively expressed and up-regulated in these cells, not by androgens, but by cellular hypoxia. There seems to be a coordinated loss of androgen regulation of VEGF-A expression associated with progression of prostate cancer to an androgen-independent state.

3. *Monitoring Circulating Vascular Endothelial Growth Factor.* Data in the literature suggest that the circulating VEGF-A level is a useful marker of tumor status and prognosis in most types of human cancer (Toi et al., 1995; Chin et al., 2000; Linderholm et al., 2000, 2001; Poon et al., 2001). A high serum VEGF-A level in cancer patients is generally associated with unfavorable clinical parameters such as disease progression, lack of response to chemotherapy, and poor survival. Increased serum VEGF levels may therefore be clinically useful for the prediction of increased tumor growth, recurrence, or metastatic spread in individual patients. In contrast, VEGF-A serum measurements cannot be expected to be helpful in patients with minimal disease, and therefore have no role as a screening tool (Dvorak, 2002).

The exact origin of free-circulating VEGF-A remains uncertain. Most of the serum VEGF-A (VEGF-A<sub>S</sub>) is derived from the  $\alpha$ -granules of platelets during coagulation (Mohle et al., 1997; Verheul et al., 1997). VEGF-A expressed by inflammatory cells infiltrating tumor and circulating peripheral white blood cells may also contribute to the VEGF-A<sub>S</sub> (Salven et al., 1999). Several authors have suggested that it would be more accurate to use plasma VEGF-A, which is much lower than VEGF-A<sub>S</sub>, when considering tumor status because plasma VEGF-A concentration reflects normal physiological angiogenesis and any disease-related overspill (Banks et al., 1998; Kraft et al., 1999; Wynendaele et al., 1999; Fuhrmann-Benzakein et al., 2000). A circadian variation in the plasma concentration of VEGF has been described in tumor-bearing mice (Koyanagi et al., 2003) and humans (Endo et al., 2002). However, the majority of studies of circulating VEGF have been performed using serum, showing a positive correlation between VEGF-A<sub>S</sub> and tumor stage or patient survival, thereby supporting the clinical relevance of VEGF-A<sub>S</sub> in cancer patients. Moreover, platelets are thought to serve as a reserve of biologically active VEGF in the circulation by endocytosing and concentrating secreted VEGF from the

tumor rather than actively producing VEGF themselves (Poon et al., 2001). Both serum and plasma VEGF-A measurements are not entirely satisfactory as indicators of clinically relevant parameters for the reasons mentioned above, and the search for reliable and clinically useful angiogenic markers continues. In a study of VEGF in human surgical wounds, tissue concentrations of VEGF did not correlate with those in plasma (Hormbrey et al., 2003).

### *E. Potential Therapeutic Applications Related to Vascular Endothelial Growth Factor*

The concept of treating tumors by inhibiting their ability to recruit blood vessels is based on the premise that tumors are dependent on angiogenesis and remain dormant if neovascularization is prevented. Remarkable and very promising tumor growth suppression has been observed in animal models with several antiangiogenic approaches. The tumor vasculature can be attacked in various ways by different agents: blockade of activators of angiogenesis, direct inhibition of endothelial cells, or prevention of matrix breakdown or integrin/survival signaling (Fox et al., 2001; Kerbel and Folkman, 2002). The discussion will focus on VEGF-A, as this is a central target for therapies inhibiting the tumor vasculature.

1. *Antibodies.* VEGF-A-neutralizing antibodies, such as A4.6.1 and MV833, have been developed to block VEGF-A from binding to its receptors and have shown impressive preclinical antitumor activity (Kim et al., 1993; Folkman, 1995; Presta et al., 1997; Kanai et al., 1998; Ferrara and Alitalo, 1999). Other monoclonal antibodies, for example 2C3 (Brekken et al., 2000), DC101 (Prewett et al., 1999), and IMC-1C11 (Hunt, 2001), block the binding of VEGF to VEGFR-2 but not to VEGFR-1. These antibodies cause apoptosis of the endothelial cells of newly formed immature vessels, which are dependent on VEGF-A to maintain cell adhesion to a provisional extracellular matrix until periendothelial cells facilitate a more permanent mode of adhesion. Consequently, the integrity of mature vessels is not influenced by antiangiogenic therapy (Benjamin et al., 1999; Brekken et al., 2000). Anti-VEGF antibody treatment generally converts fast-growing, angiogenesis-dependent, human tumor xenografts transplanted subcutaneously in nude or severe combined immune deficiency mice into small, avascularized microcolonies. A VEGFR-1 antibody has recently been shown to inhibit tumor growth almost as effectively as a VEGFR-2 antibody (Luttun et al., 2002), indicating that the poorly understood VEGFR-1 might be more important than previously thought.

Blocking the VEGFR-1 signal transduction pathway can significantly enhance the inhibitory effect of anti-VEGFR-2 antibodies, and a combination of anti-VEGFR-1 and anti-VEGFR-2 antibodies is more effective in inhibiting endothelial cell proliferation. This suggests that the VEGFR-2 homodimer and the VEGFR-2/VEGFR-1 heterodimer, but not the VEGFR-1 ho-

modimer, are responsible for VEGF-mediated signal transduction. A bifunctional antibody has been designed using the variable domains from both IMC-1C11 (an anti-VEGFR-2 antibody) and FBK612 (an anti-VEGFR-1 antibody) as building blocks. The resultant antibody is capable of binding to both VEGFR-1 and VEGFR-2 and of blocking the interaction between the receptors and their respective ligands, VEGF and PlGF. It is a more potent inhibitor of VEGF-stimulated endothelial cell mutagenesis than either of its parent antibodies and seems promising (Lu et al., 2001). Leenders et al. (2002) created a heterodimeric VEGF variant with one wild-type and one mutated receptor-binding interface by exchanging loops 1, 2, and 3 of VEGF with the corresponding domains of PDGF-B. The variant retains the ability to bind both VEGFR-1 and VEGFR-2 at one pole, which is the wild-type binding interface. It not only inhibits the activity of VEGF-A but also that of other VEGFR-1 and VEGFR-2 ligands. Further preclinical studies to test its efficacy as a therapeutic antiangiogenic agent are needed, and the potential problems of immunogenicity and half-life must be solved before clinical trials can commence.

Of these different antibodies, most clinical experience has been obtained with A4.6.1, also called bevacizumab (Avastin; Genentech, San Francisco, CA). Generally, it is well tolerated in humans, although unexpected side effects such as bleeding, hypertension, or asymptomatic proteinuria can occur (Bunn, 2001; Margolin et al., 2001). In a recent double-blinded phase II trial of 116 patients with metastatic renal cell carcinoma, presented at the American Society of Clinical Oncology in 2002, bevacizumab approximately doubled the median time to progression (Yang et al., 2002).

*2. Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibition.* VEGF function can also be inhibited by small molecules that block or prevent activation of VEGF-A receptor tyrosine kinases (Schlaeppli and Wood, 1999) and consequently interfere with the VEGF signal transduction pathway. Several drugs have been developed and are being tested in clinical trials.

SU5416, a potent VEGFR-2 inhibitor (SUGEN, Inc., South San Francisco, CA), was one of the first of this category to be explored clinically. Parenteral administration of SU5416 is necessary in contrast to the new generation of drugs. Adverse effects, particularly headaches and thromboembolic events in phase I trials in combination with chemotherapy, have dampened initial enthusiasm (Kuenen et al., 2002; Stopeck et al., 2002). Moreover, antitumor activity is limited.

Several new small, often orally available, tyrosine kinase inhibitor molecules are in clinical development. They mainly block several subtypes of the VEGFR and also other tyrosine kinase receptors such as KIT, PDGF receptor, fibroblast growth factor receptor 1, Tie-2, and FLT3 (Laird et al., 2000, 2002; Wedge et al., 2000; Wood

et al., 2000). The results of ongoing clinical trials are eagerly awaited.

### 3. Other Strategies

*a. Toxins.* Tumor cell-specific cytotoxic conjugates containing bacterial or plant toxins can inhibit the stimulating effect of VEGF-A on tumor angiogenesis. VEGF-A<sub>165</sub>-DT385 conjugates (diphtheria toxin domains fused or chemically conjugated to the VEGF-A<sub>165</sub> isoform) efficiently inhibit tumor growth in vivo (Olson et al., 1997). This treatment results in vascular damage to the tumor without any apparent toxicity to normal tissues. Since VEGF-A<sub>165</sub> binds to neuropilin-1 (NP-1), which is highly expressed on neuronal cells, the VEGF-A<sub>165</sub> conjugates may exert some underlying neurotoxic effects, such as the motor degeneration observed with reduced VEGF expression (Oosthuysen et al., 2001). Therefore, a VEGF-A<sub>121</sub>-DT385 conjugate, which selectively binds to VEGFR-2 with no toxin delivery to NP-1-positive cells, has been developed. Administration of the VEGF-A<sub>121</sub> toxin conjugate inhibits proliferating endothelial cells in fast-growing tumors. Localized apoptosis of tumor cells surrounding the disrupted immature blood vessels is subsequently seen (Wild et al., 2000). In conclusion, the VEGF-A toxin conjugates produce a significant inhibition of tumor growth and temporary stabilization but do not induce regression.

*b. Apoptosis-Induced Drug Delivery.* Apoptosis-induced drug delivery (AIDD) is a new strategy using apoptosis as a biological control mechanism to deliver drugs from carrier cells to tumor cells. The tumor cells phagocytose apoptotic bodies loaded with drugs, and drug transport through the more permeable apoptotic membrane is enhanced, thereby increasing drug delivery. Since VEGF-A is overexpressed in many solid tumors, Ma et al. (2002) developed endothelial cells expressing a VEGFR-2-FAS fusion protein that induces apoptosis on binding VEGF-A. Paclitaxel was chosen as the cytotoxic, which was loaded into the cells by cocultivation. The technique is probably also applicable to other lipophilic drugs. AIDD offers three fundamentally unique features that may result in selective drug delivery to tumors and produce an antitumor response. First, the apoptotic triggering mechanism can promote and direct drug uptake by tumor cells from apoptotic carrier cells. Second, AIDD may interfere with tumor angiogenesis if fusion proteins expressed on carrier cells serve as decoy receptors for growth factors secreted by tumors. Third, genetic manipulation of carrier cells offers many opportunities to optimize selective drug delivery to tumors, such as the use of enzyme activated prodrug systems, minimizing nonspecific apoptosis (Ma et al., 2002).

*c. Rapamycin.* One of the most serious complications of generalized immunosuppression following organ transplantation is the high risk of recurrence of neoplastic tumors or the development of de novo cancer. Rapamycin is a bacterial macrolide with antifungal and immunosuppressant properties capable of retarding tumor

growth in mice by inhibiting angiogenesis (Guba et al., 2002). Tumor vascularization is impaired by diminishing tumor cell VEGF-A secretion. Serum VEGF-A levels are decreased in tumor-bearing mice, and VEGF-A mRNA concentrations are reduced in vitro. Since VEGF-A production by tumor cells is only modestly decreased, Guba et al. (2002) suggested that the inhibition of the PI3K-p70S6 kinase pathway, activated by VEGF receptor binding, accounts for abrogated VEGF-induced human HUVEC proliferation and tubule formation. Rapamycin might prove to be an effective alternative to conventional cyclosporin-based therapies, with fewer de novo and recurrent neoplasms following transplantation.

*d. Antitumor Strategies with a Secondary Effect on Vascular Endothelial Growth Factor.* Many antitumor therapies not directly targeted against VEGF or its receptors can have secondary effects on VEGF expression and activity. For example, neovastat inhibits metalloproteinases (Gingras et al., 2001), but it can also block the action of VEGF-A and inhibit VEGF-A-mediated biological actions, including proliferation, migration, cell survival, and vascular permeability (Beliveau et al., 2002). C225, an anti-epidermal growth factor receptor antibody, decreases angiogenic factors including intratumoral VEGF (Perrotte et al., 1999).

*e. DNA Vaccination.* Flk-1 DNA vaccination 10 days after injection of mice with CT-26 colon carcinoma cells can inhibit the growth of metastases established prior to vaccination. Vascularization is markedly reduced in tumors of immunized mice compared with the control animals. The vaccine probably triggers a T-cell-mediated immune response against proliferating endothelial cells that overexpress Flk-1 (Niethammer et al., 2002).

*4. Combination Therapy.* VEGF blocking therapy has considerable antitumor efficacy in the preclinical setting, but several studies have demonstrated significant synergism when these new drugs are combined with conventional anticancer treatments.

Irradiation of human tumor xenografts can induce expression of VEGF, fibroblast growth factor, and PDGF (Gorski et al., 1999). The VEGF-VEGFR-2 complex abrogates the killing of endothelial cells by irradiation through increased expression of the antiapoptotic protein Bcl-2. The disruption of the paracrine relationship between tumor and endothelium by VEGFR-2 antagonists enhances the vasculitic effects of ionizing radiation (Lee et al., 2000a; Geng et al., 2001). Ning et al. (2002) assessed the antitumor effect of radiotherapy plus SU5416 or SU6668 compared with radiotherapy alone. Enhanced tumor kill was achieved by combining radiotherapy with the antiangiogenic agents. SU6668 was more effective than SU5416 because it targets multiple tyrosine kinase receptors.

As antiangiogenic treatments can only reduce the tumor mass to an avascular angiogenic, VEGF-independent microcolony and tumor growth resumes when they

are discontinued, combinations of both angiostatic and cytotoxic agents may be required to affect a cure. The addition of paclitaxel enhances the antitumor effect of Mab DC101, a monoclonal anti-VEGFR-2 antibody, in metastatic human transitional cell carcinoma growing within the bladder of athymic nude mice and improves their survival (Inoue et al., 2000). This improved response seems to result from a better induction of apoptosis. Paclitaxel increases microtubule stability, resulting in cell cycle arrest and apoptosis of proliferating tumor and endothelial cells, while DC101 induces apoptosis of proliferating endothelial cells by inhibiting the expression of the antiapoptotic proteins Bcl-2 and MCL1. The clinical use of this novel combination is appropriate in advanced bladder cancer. Klement et al. (2000) tested the combination of low-dose vinblastine and Mab DC101 in poor prognosis human neuroblastoma cell lines grown in SCID mice. A complete regression was induced and maintained while the combination therapy was administered, but either agent alone caused only partial and temporary regressions. They concluded that the blockade of VEGF function may render surviving endothelial cells more vulnerable to chemotherapy, especially if given at low doses, by the possible down-regulation of multiple antiapoptotic proteins normally induced by VEGF (Klement et al., 2000). Margolin et al. (2001) assessed the combination of rhuMab-VEGF, a recombinant humanized monoclonal antibody, with either doxorubicin, carboplatin plus paclitaxel, or fluorouracil plus leucovorin, administering each of the three combinations to four patients. RhuMabVEGF can be combined safely with chemotherapy, without synergistic toxicity, at doses associated with VEGF blockade. Further evaluation in randomized trials is required (Margolin et al., 2001). Some chemotherapeutic agents given in low doses are not effective alone, and as in the case of cisplatin, actually stimulate tumor growth (Klement et al., 2002). Low-dose cisplatin can cause an increase in endothelial cell VEGF-A or enhanced expression of tumor cell epidermal growth factor receptors, either of which can promote tumor growth. However, when anti-VEGFR-2 antibody is given concurrently, growth stimulation is eliminated, and the antitumor effects are greater compared with those observed when the antibody is given alone. Improved antiangiogenic therapy and lower toxicity may be achieved when cytotoxic chemotherapy, delivered on an antiangiogenic schedule of low frequent doses over a prolonged period, is combined with an angiogenesis inhibitor. Bevacizumab is the most clinically studied anti-VEGF agent in both mono and combination therapy. When combined with irinotecan, 5-fluorouracil, and leucovorin in metastatic colorectal cancer, overall survival was prolonged by approximately five months compared with chemotherapy alone. This is the first time an antiangiogenic agent has been shown to increase survival (Hurtwitz et al., 2003). This synergistic effect may be greatly

dependent on the “normalization” of tumor vessels by anti-VEGF therapy, leading to increased tumor perfusion and oxygenation (Jain, 2001) and better delivery and efficacy of chemotherapy (Wildiers et al., 2003).

#### IV. Vascular Endothelial Growth Factor in Nonmalignant Disease

##### A. Rheumatoid Arthritis and Osteoarthritis

Rheumatoid arthritis (RA) is an autoimmune, polyarticular disease characterized by proliferation of the synovial lining cells, thickening of the synovial membrane (an increase in thickness of six to eight cell layers), villus projections, and dense infiltration of the synovia with macrophages, lymphocytes, and plasma cells. Pannus formation over the articular surfaces erodes cartilage, resulting in progressive destruction and ankylosis of the joint. The additional demand for oxygen and nutrients made by the infiltrating leukocytes and the proliferating synovial lining cells leads to areas of hypoxia within the synovium. The increase in intra-articular pressure, caused by the large volume of synovial fluid, temporarily obliterates capillary flow in the synovium, contributing to the low oxygen level. Osteoarthritis (OA) is a non-inflammatory, degenerative disease affecting the hyaline cartilage of the articular surfaces and directly related to aging and mechanical damage. Key pathological features include degeneration of cartilage, irregular thickening and remodelling of subchondral bone with sclerosis and cyst formation, together with marginal soft tissue changes and osteophyte growth. Eventually, ankylosis of the joint is seen. Hypoxia, possibly a result of reduced subchondral blood flow, may play a role in the development of tissue damage. Inflammatory synovitis can occur in the late stages of OA.

Microvessel density is increased significantly in both RA and OA (Walsh, 1999), and the VEGF-A<sub>121</sub> isoform is constitutively expressed in both conditions. Expression may be necessary for the normal functioning of synovial vessels, the endothelial cells of which are permeable by virtue of fenestration, allowing nutrients to diffuse into the joint fluid and nourish the articular cartilage. VEGF-A<sub>165</sub>, VEGFR-2, and neuropilin-1 all correlate closely with synovial angiogenesis in RA. VEGF-A is chemotactic for monocytes. It has been suggested that VEGF-A is involved in the formation of synovial lesions, not only through angiogenesis, but also by direct enhancement of inflammation by recruitment of monocytes to the synovia (Ikeda et al., 2000). VEGF-A induces the expression of uPA and tPA, as well as PAI-1, resulting in the generation of plasmin. This in turn activates matrix metalloproteinases, which are the key enzymes involved in the destruction of arthritic joints.

The activation pathways in OA and RA differ. The VEGF-A/VEGFR-2 pathway is activated in RA but not OA; therefore, angiogenesis follows different pathogenic pathways in these two diseases. VEGF-A inhibits apo-

ptosis of endothelial cells through VEGFR-2 activation, positively influencing the remodeling of new vasculature in RA. VEGFR-2 is not activated in OA, so the synovium has an impaired capacity to establish a viable vasculature. This difference has therapeutic implications. Inhibitors of VEGF-A, or the interaction between VEGF-A and VEGFR-2, may be useful in the treatment of RA. In OA, VEGF-A cannot maintain the angiogenic homeostasis of the synovium. Therapy should stimulate VEGF-A expression or enhance VEGF-A angiogenicity, using factors with synergic angiogenic activity (Giatromanolaki et al., 2001). Further studies are needed to determine the molecular mechanisms activating the angiogenic switch in RA, which results in an up-regulation of VEGF-A<sub>165</sub> as well as its receptors VEGFR-2 and neuropilin-1 (Ikeda et al., 2000).

In patients with early stage RA, serum VEGF-A levels are most often profoundly elevated in those who later develop chronic synovitis (Ballara et al., 2001; Lee et al., 2001). High serum levels in early stage disease correlate with the erythrocyte sedimentation rate, C-reactive protein, and serum rheumatoid factor levels, as well as the number of tender and swollen joints and the subsequent radiological joint damage. These observations support the introduction of a more aggressive therapeutic regimen early in the disease if serum VEGF-A levels are very high. A reduction in serum VEGF-A levels is associated with a symptomatic improvement in the arthritis (Lee et al., 2001). Ballara et al. (1999) described elevated sFlt-1, the naturally occurring VEGF antagonist, in both early and late RA but probably not at levels which inhibit angiogenesis. An anti-TNF $\alpha$  monoclonal antibody, which decreases VEGF-A levels, has been found to reduce joint vascularity and swelling and ameliorate symptoms. A reduction in VEGF-A correlates with decreased C-reactive protein levels and swollen joint scores. Since angiogenesis is important in the development of the invasive joint pannus seen in RA, direct suppression of VEGF-A activity may be effective in reducing the pain, swelling, and destruction of joints. Thalidomide and paclitaxel are currently in clinical trials, and the benefits of VEGF-targeted therapy in arthritis have still to be proven (de Brandt et al., 2000).

Wauke et al. (2002) recently showed that the VEGF-C protein is localized on synovial lining cells, stromal cells, and endothelial cells in RA synovial. However, VEGF-C mRNA is only expressed in synovial lining cells and stromal cells. Conversely, in OA synovial tissues, the VEGF-C protein is only expressed weakly in these cells. VEGF-C is probably up-regulated by the numerous cytokines and growth factors produced by the inflamed synovium in RA, such as IL-1 $\beta$  and TNF- $\alpha$ . The extracellular domain of VEGFR-3 in synovial tissues is also more strongly expressed in RA than in OA. These VEGFR-3-expressing vessels are also positive for VEGFR-2, PAL-E, and laminin and are therefore blood vessels (Paavonen et al., 2002). The functional signifi-

cance of ectopic VEGFR-3 expression in synovial blood vessel endothelium is unknown, but it may be involved in the maintenance of blood vessel fenestrations and in the formation of synovial fluid. Wilkinson and Edwards (1991) described the presence of lymphatic vessels in the normal synovium and a lack of lymphatics in the synovium of rheumatoid. The increased blood vessel permeability in inflamed joints could lead to an increased accumulation of interstitial fluid, as could the relative lack of lymphatic vessels. VEGF-D is expressed in the lining cell layer of the normal synovium but is seen only in a few cells, or entirely absent, in rheumatoid synovial lining (Paavonen et al., 2002).

### B. Cardiovascular Ischemia

The development of collateral vessels after coronary occlusion has been familiar to clinicians for many years. They appear at the interface between normal and ischemic tissue, as a result of a gradually developing high-grade stenosis or occlusion. Lee et al. (2000b) showed that one of the first genes up-regulated by hypoxia is that encoding HIF-1. They detected increased steady-state levels of HIF-1 $\alpha$  mRNA in the first 24 h after an acute myocardial infarction, or during acute myocardial ischemia, in the region of affected myocardium. HIF-1 protein was not found in the peripheral blood. Since VEGF-A is also up-regulated in cultured hypoxic myocytes, and since the VEGF-A gene has a hypoxia responsive element, an increase in myocardial HIF-1 protein in ischemic tissue may be necessary, at least in part, for the enhanced expression of myocardial VEGF-A. VEGF-A protein is found only in the endothelium-lined medium and small arterioles and in capillaries, in contrast to HIF-1 protein, which is expressed in both vascular endothelial cells and myocardial cells. Following the onset of myocardial ischemia or infarction, VEGF-A expression persists longer than that of HIF-1 $\alpha$ . Lee et al. (2000b) have suggested that the response of HIF-1 $\alpha$  to ischemia occurs early and is transient, whereas the VEGF-A response is of longer duration and is probably necessary for the preservation of the myocardium and limitation of hypoxic cellular destruction.

As patients can present with persistent disabling angina, the natural compensatory angiogenesis is not always sufficient. There are two possible explanations: the production of angiogenic cytokines is inadequate, or the response to them is attenuated. Angiogenesis secondary to reduced VEGF expression is impaired in older animals, and similar reductions have been observed in diabetic and hypercholesterolemic mice of normal weight. Patients with advanced coronary disease are often older and are more likely to have diabetes, hypercholesterolemia, or other undetermined characteristics that limit the up-regulation of angiogenic cytokines by ischemia, but nevertheless they may respond to exogenous angiogenic cytokines (Freedman and Isner, 2002). Individual differences in cytokine expression may be another rea-

son for the variation in collateral development (Schultz et al., 1999).

Angiogenic cytokines can be administered as a natural recombinant human protein or by gene transfer to promote the development of collateral blood vessels, which may constitute endogenous bypass conduits around occluded native arteries (Helisch and Ware, 1999; Bing, 2001). The recombinant protein shows a more precise dose-response relationship in comparison to gene transfer. The protein is usually administered systemically and is limited by the potential adverse effects of the high plasma concentrations required to achieve adequate myocardial uptake, including hypotension and edema. Intravenous delivery is unlikely to result in sufficient myocardial uptake or residence time to achieve important biological effects. It has also been injected into the pulmonary artery or the left atrium transeptically, periaortally (during bypass surgery or via a thoracotomy), transendocardially (using an electromechanical catheter) into myocardial tissue, or directly into the coronary artery. A phase I trial of intravenous or intracoronary VEGF-A<sub>165</sub> showed a significant improvement in exercise time and perfusion with less angina compared with preintervention baseline values (Freedman and Isner, 2002). The vascular endothelial growth factor in ischemia for vascular angiogenesis study compared two doses of VEGF-A protein with placebo (178 patients), given as a single intracoronary infusion followed by three separate intravenous infusions. Adverse effects such as hypotension and edema were not observed. However, the exercise duration, grade of angina, quality of life, and angiographic or nuclear perfusion at 60 days were not significantly different between the groups. This negative result reinforces the concern that the pharmacokinetics of recombinant protein administered into the vascular space may lead to inadequate local delivery of angiogenic growth factor to ischemic myocardium, as suggested by studies using labeled ligand.

Angiogenesis can also be induced by gene therapy. Naked DNA and adenoviral vectors produce only transient transfection, which is suitable for the time frame required for angiogenesis. Intracoronary administration may be sufficient for adenoviral gene transfer, but naked plasmid DNA requires direct intramuscular injection because of degradation by circulating nucleases (Isner, 2002). In the largest phase I trial, 30 patients were given naked DNA encoding the VEGF-A<sub>165</sub> gene intramyocardially through a mini-thoracotomy. Improvements in symptoms, exercise time, and time to angina were maintained one year after the procedure. Sequential single photon emission computed tomography demonstrated partial or complete resolution of rest defects after gene transfer. This is consistent with the concept that these defects are foci of hibernating viable myocardium, which resume or improve their contractile activity after neovascularization. The first clinical study of percutaneous,

catheter-based, myocardial gene transfer was designed by Vale et al. (2001) to establish the safety and feasibility of a nonsurgical needle injection. There were no complications, nor deaths, while the symptomatic improvement was accompanied by improved perfusion and electromechanical function. Studies have been performed with adenoviral-mediated transfection of VEGF-A<sub>121</sub>. A first generation adenoviral vector was administered to 15 patients undergoing bypass graft surgery and to six patients as sole therapy via a mini-thoracotomy (Rosengart et al., 1999). Symptoms improved in both sets of patients, although stress-induced perfusion images were unchanged. The use of adenoviral vectors has its limits. They are immunoreactive, and the expression is lost a few weeks after gene transfer. Too high a dose of adenovirus may result in toxicity and the loss of myocardial contractility.

One of the main concerns of therapeutic angiogenesis is the potential risk of pathological angiogenesis. This occurs in tumor growth, diabetic proliferative retinopathy, macular degeneration, rheumatoid arthritis, osteomyelitis, and neovascularization of atherosclerotic plaques. Recent reports have suggested that VEGF-A may increase atherogenesis by inducing activation and migration of monocytes via VEGFR-1, intracellular and vascular adhesion molecules, and monocyte chemoattractant protein-1, or by inducing intraplaque angiogenesis (Barleon et al., 1996; Marumo et al., 1999; Kim et al., 2001). Endothelial cell dysfunction, with subsequent infiltration of circulating monocytes and lymphocytes, may be the primary event for atherogenesis (Ross, 1993). Inoue et al. (1998) hypothesized that VEGF-A expression by endothelial cells in atherosclerotic lesions can be considered as an integral part of the change in endothelial cell function in atherogenesis, since endothelial cells in normal human coronary arteries do not stain immunologically for VEGF-A. Their experiments also demonstrated that activated macrophages, accumulating in the atherosclerotic lesions, show positive immunostaining for VEGF-A. VEGF-A staining is also positive in less differentiated smooth muscle cells. Locally produced VEGF-A mobilizes macrophages and monocytes (Celletti et al., 2001; Zhao et al., 2002). Activated macrophages secrete growth factors and cytokines, which mediate hyperplasia and atherogenesis. Macrophages also express matrix metalloproteinases and other hydrolytic enzymes, increasing the likelihood of plaque rupture. If this occurs, the risk of thrombotic occlusion is increased by the expression of tissue factor in the macrophages (Eisenstein, 1991; Celletti et al., 2001). The microvascular network is more abundant and extends into the intima of atherosclerotic lesions, while in normal vessels the vasa vasorum is confined to the adventitia and outer media (Moulton et al., 1999). VEGF-A may be one of the growth factors involved in the development of intraplaque microvessels (Inoue et al., 1998).

Further studies are required to confirm preliminary suggestions that the currently achievable levels of gene expression with plasmid DNA is sufficient for therapeutic angiogenesis, while avoiding adverse effects. The choice, dose, and route of administration of angiogenic growth factors also require optimization. Clinical trials will be required to test growth factor combinations, and attempt to reproduce the cascade evolved by nature to maintain vascular networks.

### C. Peripheral Vascular Disease

Despite major advances in both surgical and percutaneous revascularization techniques, therapeutic options for patients with peripheral vascular disease of the lower limbs are limited. Conventional drug therapy is of no benefit. When vascular obstruction is extensive, percutaneous revascularization may not be feasible. Surgical therapy is complicated by a variable morbidity and mortality and is dependent on long-term graft patency. Therapeutic angiogenesis is another way to affect revascularization but is more difficult in older patients with endothelial dysfunction secondary to diabetes or hyperlipidemia (Rissanen et al., 2001).

Most therapeutic angiogenesis in peripheral vascular disease has focused on gene therapy. Work with angiogenic gene therapy has almost exclusively used adenoviruses and naked DNA, because they can transduce nondividing cells and generate transient expression. However, adenoviruses can cause similar problems to those seen with adenoviral vectors employed in the therapeutic angiogenesis of cardiac ischemia. Intramuscular administration is the most popular route for gene transfer, but vectors given intra-arterially lead to a more extensive biodistribution (Springer et al., 1998). The transfection efficiency in skeletal myocytes is low, even through healthy nonatherosclerotic arteries. A more local, efficient, and prolonged effect occurs after intramuscular injection (Takeshita et al., 1996). To date, clinical trials of angiogenic therapy using recombinant proteins or genes have been aimed mainly at the treatment of inoperable chronic critical ischemia. Baumgartner et al. (1998) gave two intramuscular injections of naked-plasmid encoding VEGF-A<sub>165</sub> to nine patients suffering from critical limb ischemia. VEGF concentrations in the circulation increased, collateral flow improved on angiography, and clinical status improved, with healing of ischemic ulcers, limb salvage, a reduction in analgesic consumption, and the resolution of rest pain. Similar results were achieved in thrombangitis obliterans with intramuscular gene transfer (Isner et al., 1998). In a randomized, double-blind study of catheter-mediated VEGF gene transfer with plasmid or adenovirus to the femoral arteries after percutaneous transluminal angioplasty, vasculature was improved in the VEGF treatment groups three months later (Mack et al., 1998). Further evaluation in larger randomized, double-blind clinical trials is needed. Improvements in gene transfer

and the efficacy, specificity, and regulation of gene expression are needed to optimize therapy in the target area, and avoid side effects in ectopic tissues.

Iwaguro et al. (2002) recently demonstrated that gene transfer of vectors encoding VEGF-A into EPCs *ex vivo*, and then injection of these modified EPCs *in vivo*, increases neovascularization in an animal model of hind-limb ischemia. The proliferation rate of VEGF-A expressing EPCs is higher than that of nontransduced EPCs. Moreover, the former show enhanced adhesion and incorporation in activated and inactivated HUVEC monolayers, suggesting that modulation of adhesion molecule expression after VEGF-A gene transfer may promote homing of EPCs to foci of ischemia. EPCs may induce vasculogenesis, the *in situ* formation of blood vessels from endothelial cells or angioblasts, initially with the formation of cell clusters or blood islands. In addition, VEGF-A-transduced EPCs may activate native endothelial cells, enhancing angiogenesis in the native vasculature. A likely hypothesis is that both mechanisms, vasculogenesis and angiogenesis, are at play simultaneously (Iwaguro et al., 2002). Further investigation is required to completely understand the biological activity of gene-transduced EPCs *in vivo*. These studies were performed in small animals lacking an intact immune system and need to be repeated in a larger animal model, which more closely resembles a human.

#### D. Diabetes Mellitus

Diabetes mellitus is an endocrine disease characterized by a generalized microangiopathy, particularly affecting the retina, kidneys, and nervous and vascular systems. Proliferative diabetic retinopathy is a major cause of adult blindness. Retinal capillary closure, a hallmark of the disease, leads to tissue hypoxia and ischemia. Neovascularization and increased vascular permeability develop adjacent to nonperfused areas. Subsequent bleeding, fibrosis, and tissue edema frequently result in visual loss. VEGF has emerged as a major mediator of intraocular neovascularization. VEGF-A not only stimulates the development of new retinal vessels but also plays an important role in the early stages of diabetic retinopathy. It stimulates microaneurysm formation and capillary occlusion with ischemia, as well as promoting increased vascular permeability (Duh and Aiello, 1999; Chakrabarti et al., 2000). Many cell types in the eye produce VEGF-A, including retinal pigment cells, retinal capillary pericytes, endothelial cells, glial cells, Mueller cells, and ganglion cells (Aiello et al., 1994). VEGF expression can be increased more than 30-fold in ischemic conditions (Duh and Aiello, 1999). Furthermore, retinal microvascular endothelial cells express many high-affinity VEGF receptors, with an affinity and molecular size similar to those of nonocular endothelial cells. Several authors have described a vitreous to aqueous VEGF-A gradient in the eye. This gradient-driven diffusion of angiogenic

factors from the posterior to the anterior chamber of the eye may account for the neovascularization of the anterior chamber associated with retinal ischemia (Aiello et al., 1994). Since there is no significant correlation between the serum and vitreous humor concentrations of VEGF-A in patients with diabetes mellitus, the intraocular synthesis of VEGF-A is probably the main contributing factor to the high ocular fluid levels in patients with progressive diabetic retinopathy (Shinoda et al., 1999).

Panretinal photocoagulation induces the regression of ocular neovascularization. Aiello et al. (1994) found that the concentration of VEGF-A in ocular fluid declined in all patients who had decreased neovascular activity following laser photocoagulation. Presumably the reduction in retinal ischemia decreases the production of angiogenic factors, suppressing neovascularization. Anti-VEGF antibodies reduce the *in vitro* stimulation of endothelial cell growth in samples of vitreous fluid from patients with active proliferative diabetic retinopathy and high concentrations of VEGF-A by more than 65%. The incomplete inhibition of growth stimulatory activity suggests that simultaneous activity of other proliferative factors, such as bFGF and IGF, is also important (Chiarelli et al., 2000). Since VEGF-A plays a major role in diabetic ocular disease, several VEGF inhibitory agents have been investigated in animal models of ischemic retinopathy. Soluble VEGF receptor chimeric proteins, which bind VEGF-A, reduce neovascularization by 50% when injected into the vitreous humor of mice (Aiello et al., 1995). Similarly, VEGF-A-neutralizing antibodies have been used in a primate model of ischemic iris neovascularization, with a significant inhibition of angiogenesis. Two different antisense molecules reduced retinal neovascularization in the ischemic mouse retina model by 25 and 31% (Duh and Aiello, 1999). Ozaki et al. (2000) described complete inhibition of retinal neovascularization after oral administration of PTK787 and PKC412 (*N*-benzoyl staurosporine), which inhibit VEGF receptor tyrosine kinase activity. This suggests that despite the possible contribution of other growth factors, blockade of VEGF receptor signaling is sufficient to completely inhibit retinal neovascularization. The relative lack of efficacy of intraocular injections of VEGF receptor chimeric proteins or antisense RNA may reflect difficulties with delivery, or inhibition of VEGF receptor kinase activity may be a more effective way to block VEGF signaling than decreasing the amount of VEGF available to receptors (Ozaki et al., 2000).

Evidence that VEGF is involved in diabetic renal disease is less compelling. VEGF-A has been detected in the epithelial cells of the glomerulus, podocyte, and collecting ducts. Angiotensin II increases VEGF-A in human mesangial cells through the AT1 receptor. As recent studies have suggested that the inhibition of the renin-angiotensin system can delay the development and progression of diabetic nephropathy and retinopathy, a po-



tential role of VEGF-A in diabetic nephropathy is suspected. Elevated VEGF-A expression has been observed by immunohistochemistry in the sciatic nerves and dorsal root ganglia of diabetic rats, suggesting that VEGF-A also has a potential role in diabetic neuropathy (Duh and Aiello, 1999).

Patients with diabetes have a two to five times greater risk of cardiovascular disease. They have a greater morbidity and mortality after a myocardial infarction and experience more congestive heart failure than nondiabetic patients with the same size of infarct. Revascularization procedures such as percutaneous transluminal coronary angioplasty, stenting, and coronary artery bypass grafting are less effective in patients with diabetes. Since peripheral vascular disease is also a major problem, therapeutic angiogenesis is an important approach to enhance the growth of collateral vessels, as described previously. Duh and Aiello (1999) concluded that although anti-VEGF therapies appear very promising in reducing microvascular complications such as diabetic retinopathy, pro-VEGF therapies have the potential to increase collateral formation and reduce macrovascular complications associated with myocardial infarction and peripheral limb ischemia. VEGF-A agonists require direct administration into the coronary vessels or muscles of an ischemic limb to maintain a local effect and to prevent an exacerbation of microvascular complications.

It is not known whether VEGF-A antagonists increase the risk of macrovascular disease. Local delivery of VEGF-A inhibitors to the eye should cause fewer side effects than systemic administration. High intraocular concentrations can be achieved by intravitreal injections, but repeated injections for the treatment of a chronic disease such as diabetic retinopathy is not ideal, because of the risk of endophthalmitis, vitreous hemorrhage, and retinal detachment. The localized expression of sFlt-1, a soluble truncated form of the VEGF receptor VEGFR-1/Flt-1, following gene transfer using adenovirus and adenovirus-associated virus vectors consistently inhibits experimental neovascularization by approximately 50% (Bainbridge et al., 2002). It is still unclear whether the residual neovascularization is due to incomplete inhibition of the VEGF response or the uninhibited activity of an alternative angiogenic pathway. Long-term effects need further evaluation in appropriate models and clinical studies. It is thought that the use of systemic VEGF-A antagonists is usually safe, but problems may arise in situations when an endogenous neovascular response is desired, such as when aiding wound healing after a myocardial infarction, or in peripheral arterial insufficiency (Duh and Aiello, 1999). Treatment with VEGF-A agonists and antagonists may not necessarily be mutually exclusive. Further clinical trials are needed to examine the efficacy and safety of these treatment modalities and to resolve the apparent paradox.

### *E. Endometriosis, Preeclampsia, and Ovarian Hyperstimulation Syndrome*

Angiogenesis is important in the pathophysiology of endometriosis. According to the transplantation theory of Sampson (Oosterlynck et al., 1993), viable endometrial cells that reach the peritoneal cavity by retrograde menstruation could implant, forming endometriotic lesions. It is possible that further outgrowth of these ectopic endometrial implants, also found in well vascularized sites such as lung, skin, and muscle, depends on new capillary growth. Shifren et al. (1996) identified VEGF-A in peritoneal fluid. Concentrations were significantly higher in women with moderate to severe endometriosis in comparison to those with minimal to mild or no disease. VEGF-A is localized predominantly in the glandular epithelium of endometriosis lesions. Pelvic implants and peritoneal fluid macrophages are the most likely source of VEGF-A in peritoneal fluid. McLaren et al. (1996) showed that peritoneal fluid macrophages express receptors for steroid hormones and secrete VEGF-A in response to ovarian steroids. The VEGF receptors VEGFR-1 and VEGFR-2 were also detected, suggesting an autocrine regulation. Further investigation is needed to determine the therapeutic relevance of antiangiogenic treatment in endometriosis.

Preeclampsia is associated with endothelial damage and dysfunction. There is a deficiency of antithrombotic and vasodilator factors such as prostacyclin, increased concentrations of vasoconstrictor products such as endothelin, and an excess of pro-thrombotic products such as von Willebrand factor and the cell adhesion molecules vascular cell adhesion molecule-1 and E-selectin. Further evidence of endothelial cell damage is elevated fibronectin concentrations, increased capillary permeability, elevated concentrations of thrombomodulin, and characteristic lesions such as those seen in glomerular capillary endothelial cells. The endothelial damage is also associated with neutrophil and platelet activation, which promote further vascular destruction through a positive feedback loop (Lyll et al., 1997). The trigger that initiates endothelial damage in preeclampsia is unclear. However, there is considerable evidence linking it with impaired trophoblast invasion of the maternal placental bed spiral arteries. It has been postulated that this defect may result not only in poor fetoplacental perfusion but also in the release of factors into the maternal circulation, leading to widespread maternal endothelial damage and the subsequent clinical symptoms (Roberts and Palade, 1997). Lyll et al. (1997) observed that serum VEGF-A concentration is significantly lower in pregnant women and even further reduced in preeclampsia. Conversely, Roberts and Palade (1997) found that serum VEGF-A levels in women with preeclampsia were significantly higher than in normal pregnant women. This discrepancy may be caused by the antibodies used in the assays, as there is a possible cross reac-

tion between VEGF-A and platelet-derived growth factor or placental growth factor (Baker et al., 1995; Roberts and Palade, 1997). Further investigation is needed to elucidate the exact role of VEGF-A in pre-eclampsia and the diagnostic relevance of serum VEGF-A.

The ovarian hyperstimulation syndrome (OHSS) is the most serious complication arising from the use of gonadotropins to induce ovulation. The major components in the development of the syndrome are ovarian enlargement and increased vascular permeability. The latter leads to the extravasation of protein-rich fluid from the intravascular space into the peritoneal, pleural, and pericardial cavities, edema, and hypovolemia, leading to hypotension and anuria in the most severe cases (Levin et al., 1998). The syndrome is more likely to occur when a good ovarian response to gonadotrophic hormones is seen, with large numbers of stimulated follicles and an increase in serum estradiol. Several studies have reported a substantial rise in serum VEGF, but it is not yet certain whether VEGF is truly involved in the pathogenesis of OHSS, or whether the increase is only an epiphenomenon of other regulatory mechanisms (Lee et al., 1997; Artini et al., 1998; Ferrara et al., 1998). However, even if the raised VEGF serum concentration is only circumstantial, it is apparently a good marker to predict the onset of OHSS. Ludwig et al. (1999) showed that a cut-off concentration of 200 pg/ml free serum VEGF on the day of hCG administration was a good indicator (negative predictive value 92% and positive predictive value 75%) of individual risk in patients undergoing ovarian hyperstimulation for in vitro fertilization (Ludwig et al., 1999). A larger prospectively designed study is needed to further evaluate this new screening tool and the proposed cut-off value.

#### F. Psoriatic Skin Disease

Psoriasis is a common chronic skin disease characterized by recurrent erythematous skin plaques with epidermal hyperplasia, a variable inflammatory cell infiltrate, and proliferation of blood vessels and neovascularization. The increased number of hyperpermeable blood vessels is required to meet the greater nutritional need of the hyperplastic psoriatic epidermis. In a study of psoriatic plaques, Bhusan et al. (1999) showed that VEGF and its receptors VEGFR-1 and VEGFR-2 are overexpressed by keratinocytes in the suprabasal epidermis and fibroblasts, implying a dermally derived influence on neovascularization. They emphasized that even clinically normal skin in patients with psoriasis is preset to initiate angiogenesis if the correct stimuli are present, or if there is a local reduction in angiogenesis inhibitors. Detmar et al. (1994) concluded that VEGF-A synthesis and secretion by keratinocytes in psoriasis is stimulated by TGF- $\alpha$  and EGF, since receptors for these factors are overexpressed in psoriatic skin. Serum VEGF-A concentrations are increased in

patients with psoriasis (Bhusan et al., 1999), and this may reflect overproduction, either in the skin with overflow into the circulation or as a primary genetic aberration. Antiangiogenesis treatment may be a novel selective therapeutic strategy. Psovascar (AE-941) is currently being assessed in patients with severe psoriatic skin lesions.

### V. Conclusions and Future Directions

VEGF occupies a central position in the process of angiogenesis. Advances in the understanding of its contribution to the angiogenesis balance have been made in recent years. The identification of different isoforms has raised the possibility of more detailed prognostic profiling and follow-up of cancer patients. The development of new agents capable of modifying the function of VEGF has led to the use of novel treatments in both malignant and nonmalignant diseases, although clinical trials are still at an early stage.

VEGF-blocking therapy appears very promising in the treatment of cancer, since it is directed only against migrating and proliferating capillary endothelial cells at a site of angiogenesis. The mode of action and toxicity profile of these drugs facilitates their use with chemotherapy and radiotherapy. Combination therapy seems more effective, because both the endothelial cell and tumor cell compartments of a tumor are targeted, and blocking angiogenesis may decrease the interstitial pressure in tumors, leading to a greater penetration of the cytotoxic drugs. As well as potentiating standard treatment, these inhibitors could be used in patients following conventional therapy or curative resection to maintain the dormancy of micrometastases.

In diseases such as rheumatoid arthritis, the vasculature promotes the delivery of inflammatory mediators, cells, and nutrients potentiating ongoing disease. VEGF-blocking therapy may be a useful adjunct to existing anti-inflammatory approaches. Some antirheumatic drugs, such as anti-TNF $\alpha$ , methotrexate, indomethacin, and corticosteroids may exert effects on the vasculature by modulating VEGF-A levels. Promising results have been obtained in preclinical trials of anti-VEGF products in animals with proliferative retinopathy and macular edema. Since microvascular and macrovascular pathologies frequently exist simultaneously in diabetic patients, the most important therapeutic challenge is to identify methods of combining the paradoxical pro- and antiangiogenic treatment strategies. VEGF-manipulating drugs will probably become popular in benign ophthalmologic, dermatologic, and rheumatologic conditions characterized by angiogenesis, in which prolonged suppression of pathologic neovascularization is needed.

The early results of clinical trials of VEGF-blocking therapies in advanced cancer have been rather disappointing. Why tumors fail to respond as anticipated from

promising preclinical studies is unclear. First, it is questionable whether tumors grown subcutaneously in preclinical animal studies can adequately predict the response of a spontaneously occurring human tumor, which is influenced by the environment of the host organ. Second, many animal studies use cancer cell lines with a high proliferative rate. Since most of the spontaneously developing human cancer cell lines are slow growing, a particular therapeutic strategy may result in a significantly different response rate, depending on the cell line. Third, as tumors grow they produce a wide array of angiogenic molecules in addition to VEGF. It is therefore possible that when VEGF is blocked, the angiogenic process is maintained by the up-regulation of other growth modulators. Perhaps a combination of antibodies and inhibitors is required to control tumor growth. Furthermore, it is debatable whether current study designs utilize therapeutic components in adequate doses for a sufficient period of time. It appears that angiogenesis inhibitors must be administered continuously and for longer than is usual with conventional cytotoxic agents, as the regression or involution of a vigorously growing capillary bed is slower than the lysis of tumor cells. The ability of several quantitative methods to monitor the efficacy of antiangiogenic therapy, and to aid prognosis, is currently under investigation. The microvessel count is a good prognostic factor for many human tumors, but it cannot predict vascular function. In fact, vessels become more efficient during the early phase of some antiangiogenic therapies (Jain, 2001).

Initial long-term animal studies and clinical trials, e.g., with interferon (Ezekowitz et al., 1992) reported that proliferating endothelial cells develop little or no resistance to angiogenesis inhibitors, making long-term treatment feasible. However, relapse and progression during antiangiogenic therapy have been observed, and several possible mechanisms have been stated (Kerbel et al., 2001). It is important to realize that VEGF-blocking therapy might have several as yet unknown immediate and long-term dose-limiting side effects. Anti-VEGF therapy clearly interferes with the coagulation system, as both life threatening bleeding (Bunn, 2001) and thromboembolic events (Kuenen et al., 2002) have been observed. There is also the fear of peripheral and central nervous system toxicity, given the observation of progressive motor neuron degeneration reminiscent of amyotrophic lateral sclerosis in VEGF defective mice (Oosthuysen et al., 2001), and the unexpected dose-limiting central neurotoxicity of other angiogenesis inhibitors (Logothetis et al., 2001). Although it has not yet been documented, there is also the potential risk of aggravating ischemic heart disease by inhibiting the formation of a collateral circulation.

The first placebo-controlled clinical trials of VEGF therapy in cardiovascular ischemia have led to a more realistic assessment of the potential of therapeutic an-

giogenesis and raised a number of questions. For example, how can one explain the discrepancy between the considerable efficacy in animal models of coronary or limb ischemia of even very small amounts of growth factors and the rather disappointing clinical results? An essential difference may be that young and otherwise healthy animals are able to mount an effective endogenous angiogenic response that can be maximized by the additional stimulus provided by recombinant protein or gene therapy. It is possible, however, that more persistent exposure to growth factors, either individually or in combination, may be effective. Furthermore, it is not known whether the increased systemic levels of angiogenic cytokines resulting from these therapies will alter the expression of adhesion molecules in the systemic circulation, triggering dormant tumors or exacerbating proliferative and hemorrhagic retinopathy. Improvements in imaging technologies are needed to better evaluate the formation of vessels smaller than 200  $\mu\text{m}$  after initiating angiogenic therapy.

More insight is needed into the exact role of various growth mediators, the pathways they initiate, and if these are inhibited, the scavenger mechanisms which can maintain angiogenesis. Further preclinical and clinical trials are required to test various treatment strategies and to determine their toxicity profiles. Nevertheless, antiangiogenic therapy is a promising approach, particularly in cancer treatment. It has the potential to transform cancer into a chronic, nonlethal disease.

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