Pergamon

European Journal of Cancer Vol. 32A, No. 14, pp. 2413–2422, 1996

Printed in Great Britain
0959-8049/96 $15.00 + 0.00

PII: S0959-8049(96)00387-5

Vascular Endothelial Growth Factor

N. Ferrara

Department of Cardiovascular Research, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080, U.S.A.

INTRODUCTION

THE PROLIFERATION of vascular endothelial cells is a requirement for organ development and differentiation during embryogenesis and for tissue repair and reproductive functions in the adult [1]. Angiogenesis is also implicated in the pathogenesis of a variety of disorders including proliferative retinopathies, age-related macular degeneration, tumours and rheumatoid arthritis [1, 2]. In proliferative retinopathies and age-related macular degeneration, the new blood vessels may cause retinal detachment or irreversible damage to the macula, following cycles of leakage, bleeding and clot organisation [2]. Conversely, tumour-associated neovascularisation, by establishing continuity with the systemic circulation, allows the tumour cells to express their critical growth advantage and also facilitates metastatic spread [1]. A correlation has been observed between the density of microvessels in primary breast carcinoma sections and nodal metastases and survival [3, 4]. Similarly, a correlation has been reported between vascularity and invasive behaviour in several other tumours [5–7]. These findings led several investigators to conclude that the number of vessels in tumour sections is an independent predictor of outcome in a variety of solid tumours [3–7].

The search for potential regulators of angiogenesis has yielded numerous candidates: acidic-fibroblast growth factor (aFGF), bFGF (basic FGF), transforming growth factor-alpha (TGF-α), TGF-β, hepatocyte growth factor, tumour necrosis factor-alpha (TNF-α), prostaglandin E (PGE_2), angiogenin, interleukin-8 (IL-8) etc. [1]. Although these molecules are able to promote angiogenesis in certain model systems, until now it has been difficult to establish a relationship between this activity and the regulation of blood vessel growth.

In 1983, Senger and associates described the partial purification of a protein able to induce vascular leakage in guinea pig skin [8]. This protein was named vascular permeability factor (VPF) and was proposed to be a specific regulator of the hyperpermeability of tumour blood vessels [8, 9]. In 1989, Ferrara and Henzel [10] and Ploüet and associates [11] independently reported the purification (to homogeneity) and sequencing of an endothelial cell-specific mitogen, which they called, respectively, vascular endothelial growth factor (VEGF) and vasculotropin. The subsequent molecular cloning of VEGF and VPF [12–14] unexpectedly revealed that the activities of VEGF and VPF are embodied by the same molecule. The finding that VEGF was potent, diffusible and specific for vascular endothelial cells led to the hypothesis that this molecule might play a unique role in the regulation of physiological and pathological growth of blood vessels [10, 12].

Work done by a number of laboratories all over the world over the last few years has elucidated the role played by VEGF in the regulation of angiogenesis [15]. The recent finding that the loss of even a single VEGF allele results in defective vascularisation and embryonic lethality points to a fundamental role played by this factor in the development of the vascular system [16, 17]. Also, VEGF has been shown to be a key mediator of neovascularisation associated with tumours and intra-ocular disorders [18, 19]. Furthermore, VEGF-induced angiogenesis resulted in a therapeutic effect in animal models of coronary [20] or limb [21] ischaemia and, most recently, in human patients affected by critical leg ischaemia [22].

BIOLOGICAL ACTIVITIES OF VEGF

VEGF is a potent mitogen (ED_{50} 2–10 pM) for micro- and macrovascular endothelial cells derived from arteries, veins and lymphatics, but it is devoid of consistent and appreciable mitogenic activity for other cell types [10, 11, 23]. Consequently, VEGF is regarded as an endothelial cell-specific mitogen. These *in vitro* findings have been corroborated by *in situ* ligand autoradiography studies on tissue sections, which have demonstrated that high-affinity VEGF binding sites are localised on the vascular endothelium of large or small vessels, but not to other types [24, 25]. VEGF promotes angiogenesis in tridimensional *in vitro* models synergistically with bFGF [26]. Also, VEGF induces sprouting from rat aortic rings embedded in a collagen gel [27]. VEGF is also able to induce a strong angiogenic response in a variety of *in vivo* models [11, 12, 28–30]. Furthermore, VEGF induces expression of urokinase-type and tissue-type plasminogen activators (PA) and also PA inhibitor 1 (PAI-1) in cultured endothelial cells [31]. Moreover, VEGF increases expression of interstitial collagenase in human umbilical vein endothelial cells [32]. Other studies have shown that VEGF promotes expression of urokinase receptor in vascular endothelial cells [33]. As previously noted, VEGF is known also as VPF based on its ability to induce vascular leakage in guinea pig skin [8, 9]. Dvorak and colleagues proposed that an increase in micro-

vascular permeability is a crucial step in angiogenesis associated with tumours and wounds [9]. According to this hypothesis, a major function of VEGF in the angiogenic process is the induction of plasma protein leakage. This effect would result in the formation of an extravascular fibrin gel, a substrate of endothelial and tumour cell growth. Recent studies have also suggested that VEGF may be a factor which induces fenestrations in endothelial cells. Topical administration of VEGF resulted in the development of fenestrations in the endothelium of small venules and capillarics, even in regions where endothelial cells are not normally fenestrated [34]. VEGF also induces dose-dependent vasodilatation *in vitro* [35], and produces transient tachycardia and hypotension when injected systemically [36]. Such effects appear to be mediated primarily by endothelial cell-derived nitric-oxide (NO) [35, 36].

ORGANISATION OF THE *VEGF* GENE AND PROPERTIES OF THE VEGF ISOFORMS

The human *VEGF* gene is organised in eight exons, separated by seven introns, and its coding region spans approximately 14 kilobases (kb) [37, 38]. The human *VEGF* gene has been recently assigned to chromosome 6p21.3 [39]. cDNA sequence analysis of a variety of human VEGF clones had initially indicated that VEGF may exist as one of four different molecular species, having, respectively, 121, 165, 189 and 206 amino acids ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) [12, 13, 37, 38]. Alternative exon splicing of a single *VEGF* gene is the basis for this molecular heterogeneity. $VEGF_{165}$ lacks the residues encoded by exon 6, while $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. $VEGF_{189}$ has an insertion of 24 amino acids highly enriched in basic residues and $VEGF_{206}$ has an additional insertion of 17 amino acids. $VEGF_{165}$ is the predominant isoform secreted by a variety of normal and transformed cells. Transcripts encoding $VEGF_{121}$ and $VEGF_{189}$ are detected in the majority of cells and tissues expressing the *VEGF* gene [37, 38]. In contrast, $VEGF_{206}$ is a very rare form [38]. Recently, the genomic organisation of the murine *vegf* gene has been described [40]. Similar to the human gene, the coding region of the murine *vegf* gene encompasses approximately 14 kb and is comprised of eight exons interrupted by seven introns. Analysis of the exons suggests the generation of three isoforms: $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$. However, a fourth isoform comparable to $VEGF_{206}$ is not predicted, since an in-frame stop codon is present in the region corresponding to the human $VEGF_{206}$ open reading frame. Analysis of the 3′ untranslated region of the rat *vegf* gene has revealed a number of sequence motifs that are known to be involved in the regulation of mRNA stability [41].

Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45 kDa [42]. These properties correspond to those of $VEGF_{165}$. $VEGF_{121}$ is an acidic polypeptide that fails to bind to heparin [43]. $VEGF_{189}$ and $VEGF_{206}$ are more basic and bind to heparin with greater affinity than $VEGF_{165}$ [43]. $VEGF_{121}$ is a freely soluble protein; $VEGF_{165}$ is also secreted, although a significant fraction remains bound to the cell surface and the extracellular matrix (ECM). $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the ECM [44], but may be released in a soluble form by heparin or heparinase. Also, these long forms may be released by plasmin following cleavage at the COOH terminus [43, 44]. Plasminogen activation and generation of plasmin have been shown to play an important role in the angiogenesis cascade [45]. Thus, proteolysis of VEGF is also likely to occur *in vivo*. Generation of bioactive VEGF by proteolytic cleavage may be especially important in the micro-environment of a tumour where the increased expression of proteases, including plasminogen activators, is well documented [45]. Recent studies have shown that the bioactive product of plasmin action is comprised of the first 110 NH_2-terminal amino acids of VEGF [46]. However, loss of heparin binding, whether it is due to alternative splicing or plasmin cleavage, results in a substantial loss of mitogenic activity for vascular endothelial cells: compared with $VEGF_{165}$, $VEGF_{121}$ or $VEGF_{110}$ demonstrate 50–100-fold reduced potency when tested in an endothelial cell growth assay [46].

REGULATION OF *VEGF* GENE EXPRESSION

Several mechanisms have been shown to be implicated in the regulation of *VEGF* gene expression. Among these, oxygen tension plays a major role. VEGF mRNA expression is induced by exposure to low pO_2 in a variety of normal and transformed cultured cell types [47–50]. In glioblastoma multiforme and other tumours with a significant necrotic component, VEGF mRNA is highly expressed in ischaemic tumour cells that are juxtaposed to areas of necrosis [47], suggesting that local hypoxia is a major inducer of *VEGF* gene expression within the micro-environment of a tumour. Furthermore, Stone and associates have proposed that hypoxic upregulation of VEGF mRNA in neuroglial cells plays an important physiological role in the development of the retinal vasculature [51].

A 28-base sequence has been identified in the 5′ promoter of the human *VEGF* gene which mediates hypoxia-induced transcription [52]. Such a sequence reveals a high degree of homology and has protein binding characteristics similar to the hypoxia-inducible factor 1 (HIF-1) binding site within the erythropoietin gene [53]. HIF-1 has been purified and cloned as a mediator of transcriptional responses to hypoxia [54]. It has been shown that accumulation of adenosine, which occurs under hypoxic conditions, is involved in the hypoxic induction of the *VEGF* gene [55]. Activation of *C-SRC* has also been shown to participate in the hypoxic upregulation of the *VEGF* gene [56]. However, transcriptional activation is not the only mechanism leading to VEGF upregulation in response to hypoxia [57, 58]. Increased mRNA stability has been identified as an important post-transcriptional component. Sequences that mediate increased stability have been identified in the 3′ untranslated region of the VEGF mRNA. Also, various cytokines or growth factors are able to upregulate VEGF mRNA expression. Epidermal growth factor (EGF), TGF-β or keratinocyte growth factor (KGF) resulted in a marked induction of VEGF mRNA expression in culture keratinocytes [59]. Treatment of quiescent cultures of several epithelial and fibroblastic cell lines with TGF-β resulted in induction of VEGF mRNA and release of VEGF protein in the medium [60], suggesting that VEGF may function as a paracrine mediator for indirect-acting angiogenic agents such as TGF-β [60]. Furthermore, IL-1β induces VEGF expression in aortic smooth muscle cells [61]. Both IL-1α and PGE_2 have

been shown to induce expression of VEGF in cultured synovial fibroblasts, suggesting the participation of such inductive mechanisms in inflammatory angiogenesis [62]. IL-6 has also been shown to induce significantly VEGF expression in several cell lines [63]. Claffey and colleagues have also shown that cell differentiation plays an important role in the regulation of *VEGF* gene expression [64].

Importantly, several specific transforming events result in the upregulation of *VEGF* gene expression. A mutated form of the murine *p53* tumour suppressor gene has been shown to induce VEGF mRNA expression and potentiate phorbol ester stimulated VEGF mRNA expression in NIH 3T3 cells in transient transfection assays [65]. Similarly, oncogenic mutations or amplification of *ras* lead to VEGF upregulation [66, 67]. This effect is blocked by treatment with inhibitors of *ras* farnesyl transferase. Also, overexpression of *v-raf* [67] and *v-Src* [68] leads to *VEGF* upregulation. Therefore, several unrelated alterations in cellular regulatory pathways result in VEGF upregulation, suggesting that this event may be a final common pathway necessary for uncontrolled proliferation *in vivo*.

THE VEGF TYROSINE KINASE RECEPTORS

Two VEGF receptor tyrosine kinases (RTKs) have been identified [69–74]. The Flt-1 and KDR (kinase domain region) receptors bind VEGF with high affinity. Flk-1, the murine homologue of KDR, shares 85% sequence identity with human KDR [72]. Both Flt-1 and KDR/Flk-1 have seven immunoglobulin (Ig)-like domains in the extracellular domain (ECD), a single transmembrane region and a consensus tyrosine kinase sequence which is interrupted by a kinase-insert domain [69–74]. Flt-1 has the highest affinity for $rhVEGF_{165}$, with a K_d of approximately 10–12 pM [69]. KDR has a K_d of 75–125 pM [70]. An alternatively spliced soluble form of Flt-1, lacking the seventh Ig-like domain, transmembrane sequence and cytoplasmic domain, has been identified in human umbilical vein endothelial cells [75]. This soluble Flt-1 receptor is able to inhibit VEGF-induced mitogenesis and may be a physiological negative regulator of VEGF action [75]. Very recent studies have shown that the second Ig-like domain contains major ligand binding determinants in Flt-1 and KDR [76]. Deletion of such a domain in Flt-1 completely abolishes VEGF binding. Introduction of the second domain of KDR in such a mutant restored VEGF binding, but with the ligand specificity of KDR [76].

VEGF has been shown to induce the phosphorylation of at least 11 proteins in bovine aortic endothelial cells [77]. PLC-γ and two proteins that associate with PLC-γ were phosphorylated in response to VEGF [79]. Furthermore, VEGF induces phosphorylation of phosphatidylinositol 3-kinase, ras GTPase activating protein (GAP) and several others. However, these studies did not identify which VEGF receptor(s) are involved in these events.

Several studies have shown that the Flt-1 and KDR proteins have different signal transduction properties [78, 79]. Flk-1/KDR undergoes strong ligand-dependent tyrosine phosphorylation in intact cells [73, 74, 78]. In contrast, Flt-1 reveals a weak or undetectable response [69, 78, 79]. VEGF stimulation results in weak tyrosine phosphorylation that does not generate any mitogenic signal in transfected NIH 3T3 cells expressing Flt-1 [79]. These findings are in agreement with other studies showing that placenta growth factor (PlGF), a VEGF-related molecule (see below), which binds with high affinity to Flt-1 but not to Flk-1/KDR, lacks direct mitogenic or permeability-enhancing properties or the ability to stimulate effectively tyrosine phosphorylation in endothelial cells [80]. Therefore, interaction with Flk-1/KDR is a critical requirement to induce the full spectrum of VEGF biological responses. These findings have been supported by recent studies where receptor-selective VEGF mutants were generated [81]. Those mutants which bind selectively to Flk-1/KDR are fully active endothelial cell mitogens. In contrast, mutants that bind to Flt-1 but not to KDR have a substantially reduced ability to promote endothelial cell growth [81].

The expression of Flt-1 and KDR is restricted to the vascular endothelium. A 1kb fragment, essential for endothelial-specific expression, was identified in the 5′ flanking region of the Flt-1 prompter [82]. Similar to VEGF, hypoxia has been proposed to play an important role in the regulation of VEGF receptor gene expression. Exposure of rats to hypoxia leads to upregulation of both Flt-1 and KDR genes in the lung vasculature [83]. However, *in vitro* studies have yielded unexpected results. Although hypoxia increases the VEGF receptor number in cultured retinal capillary endothelial cells [84], the expression of KDR is not induced, but paradoxically shows a downregulation [85]. It has been suggested that the hypoxic upregulation of KDR observed *in vivo* is not direct, but requires an unidentified paracrine mediator released from ischaemic tissues [86]. Also, recent studies have shown that both TNF-α [87] and TGF-β [88] have the ability to inhibit expression of the KDR gene in cultured endothelial cells.

ANALYSIS OF THE FLK-1/KDR, FLT-1 AND VEGF GENE KNOCKOUTS

Recent studies have demonstrated that both Flt-1 and Flk-1/KDR are essential for normal development of embryonic vasculature. However, their respective roles in endothelial cell proliferation and differentiation appear to be distinct [89, 90]. Mouse embryos homozygous for a targeted mutation in the Flt-1 locus died *in utero* between day 8.5 and 9.5 [89]. Endothelial cells developed in both embryonic and extra-embryonic sites, but failed to organise in normal vascular channels. Mice, where the *Flk-1* gene had been inactivated, lacked vasculogenesis and also failed to develop blood islands [90]. Haematopoietic precursors were severely disrupted and organised blood vessels failed to develop throughout the embryo or the yolk sac, resulting in death *in utero* between day 8.5 and 9.5 [90]. However, these findings do not necessarily imply that VEGF is equally essential, since other ligands might potentially activate the two receptors and thus replace VEGF action. Very recent studies [16, 17] have provided direct evidence for the role played by VEGF in embryonic vasculogenesis and angiogenesis. Unexpectedly, inactivation of the *vegf* gene resulted in embryonic lethality in heterozygous mouse embryos, between day 11 and 12. The VEGF +/− embryos were growth retarded and also exhibited a number of developmental anomalies. The yolk sac revealed a substantially reduced number of nucleated red blood cells within the blood islands. Significant defects in the vasculature of several other tissues and organs were also observed. *In situ* hybridisation confirmed expression of VEGF mRNA in

heterozygous embryos. Thus, the VEGF +/− phenotype appears to be due to gene dosage and not to maternal imprinting. While several heterozygous phenotypes have been described [91], this may be the first report that the loss of a single allele can be lethal. Therefore, VEGF and its receptors are essential for blood island formation and angiogenesis such that even reduced concentrations of VEGF are inadequate to support a normal pattern of development.

ROLE OF VEGF IN TUMOUR ANGIOGENESIS

Expression of VEGF and its receptors in human tumours

In situ hybridisation studies, performed by several investigators, have demonstrated that the VEGF mRNA is markedly upregulated in the majority of human tumours examined. These include lung [92], thyroid [93], breast [94], gastro-intestinal tract [95, 96], kidney and bladder [97], ovary [98] and uterine cervix [99] carcinomas, angiosarcoma [100] and several intracranial tumours [47, 101–103]. The VEGF mRNA is expressed in tumour cells but not in endothelial cells. In contrast, the mRNAs for Flt-1 and KDR are upregulated in tumour endothelial cells [95, 96, 101]. This distribution is consistent with the hypothesis that VEGF is primarily a paracrine regulator [42]. An exception may be angiosarcoma, where the VEGF and Flt-1 mRNA are found to be co-expressed in angiosarcoma cells, raising the possibility that, in this malignancy, VEGF may be an autocrine factor [100]. Freeman and associates have suggested that lymphocytes infiltrating the tumour may also contribute bioactive VEGF [104]. Immunohistochemical studies have localised the VEGF protein not only to the tumour cells but also to the vasculature [95, 101]. This discrepancy indicates that tumour-secreted VEGF accumulates in the target cells. Ultrastructural studies have localised VEGF bound to tumour endothelial cells to the abluminal plasma membrane and to the recently described vesiculovacular organelles (VVO), cytoplasmic structures which are thought to be involved in macromolecular transport across the tumour endothelium [105].

A correlation has been noted between VEGF expression and microvessel density in primary breast cancer sections [106]. A postoperative survey indicated that the relapse-free survival rate of patients with VEGF-rich tumours was significantly worse than those with VEGF-poor tumours [106]. A similar correlation has been described in gastric carcinoma patients [107]. VEGF-positivity in tumour sections correlated with vessel involvement, lymph node metastasis and liver metastasis. Furthermore, patients with VEGF-positive tumours had a worse prognosis than those with VEGF-negative tumours [107].

Inhibition of VEGF action in vivo

The availability of high-affinity monoclonal antibodies, capable of inhibiting VEGF-induced angiogenesis *in vivo* and *in vitro* [108], made it possible to test the hypothesis that VEGF may play a role in tumorigenesis. In a study published by Kim and associates in 1993, such antibodies were found to exert a potent inhibitory effect on the growth of three human tumour cell lines inoculated subcutaneously in nude mice, including G55 glioblastoma multiforme, A673 rhabdomyosarcoma and SK-LMS-1 leiomyosarcoma [18]. The growth inhibition ranged between 70% and > 95%. The density of blood vessels was significantly lower in sections of tumours from antibody-treated animals compared with controls. Furthermore, neither the antibodies nor VEGF had any effect on the *in vitro* growth of the tumour cells. These findings provided the first direct demonstration that inhibition of the action of an endogenous endothelial cell mitogen may result in suppression of tumour growth *in vivo*. Subsequently, several other tumour cell lines were found to be inhibited *in vivo* by this treatment [109]. Recently, intravital microscopy techniques, by providing a non-invasive imaging of the tumour vasculature, have directly demonstrated a dramatic suppression of tumour angiogenesis in anti-VEGF treated animals [110].

It has been shown that VEGF is a major mediator of the *in vivo* growth of human colon carcinoma HM7 cells in a nude mouse model of liver metastasis [109]. Similar to human tumours, in this model the expression of Flk-1 mRNA was markedly upregulated in the vasculature associated with liver metastases. Treatment with anti-VEGF monoclonal antibodies resulted in a dramatic decrease in the number and size of metastases. Most of the tumours in the treated group were under 1 mm in diameter and all were under 3 mm. Also, neither blood vessels nor Flk-1 mRNA expression could be demonstrated in such metastases. Similarly, administration of anti-VEGF neutralising antibodies inhibited primary tumour growth and metastasis of A431 human epidermoid carcinoma cells in SCID mice [111] or HT-1080 fibrosarcoma cells implanted in BALB/c nude mice [112]. An additional verification of the hypothesis that VEGF action is required for tumour angiogenesis has been provided by the finding that retrovirus-mediated expression of a negative dominant Flk-1 mutant suppresses the growth of glioblastoma multiforme and other tumours *in vivo* [113, 114].

Further evidence that VEGF action is necessary for effective tumour angiogenesis has been recently provided in an *in vivo* model of embryonic stem (ES) cell tumorigenesis [17]. ES cells are able to form highly vascularised teratocarcinomas when injected into nude or syngeneic mice. VEGF null ES cells were dramatically impaired in their ability to form tumours into nude mice. Figure 1 illustrates the weight of tumours derived from VEGF +/−, VEGF −/− or ES cells where both alleles of the *c-mpl* gene have been inactivated. Figure 1 documents the substantial difference in the density of CD34-positive vascular elements between *c-mpl* −/− and VEGF −/− tumours. Thus, even in a pluripotent system such as the ES cells, which is expected to have the potential to activate alternative pathways, VEGF is required for *in vivo* growth.

ROLE OF VEGF IN INTRA-OCULAR NEOVASCULAR SYNDROMES

Diabetes mellitus, occlusion of central retinal vein or prematurity with subsequent exposure to oxygen can all be associated with intra-ocular neovascularisation [115]. The new blood vessels may lead to vitreous haemorrhage, retinal detachment, neovascular glaucoma, and eventual blindness [2]. Diabetic retinopathy is the leading cause of blindness in the working population [116]. All of these conditions are known to be associated with retinal ischaemia [115]. In 1948, Michaelson proposed that a key event in the pathogenesis of these conditions is the release by the ischaemic retina of a diffusible angiogenic factor(s) (factor X) respon-

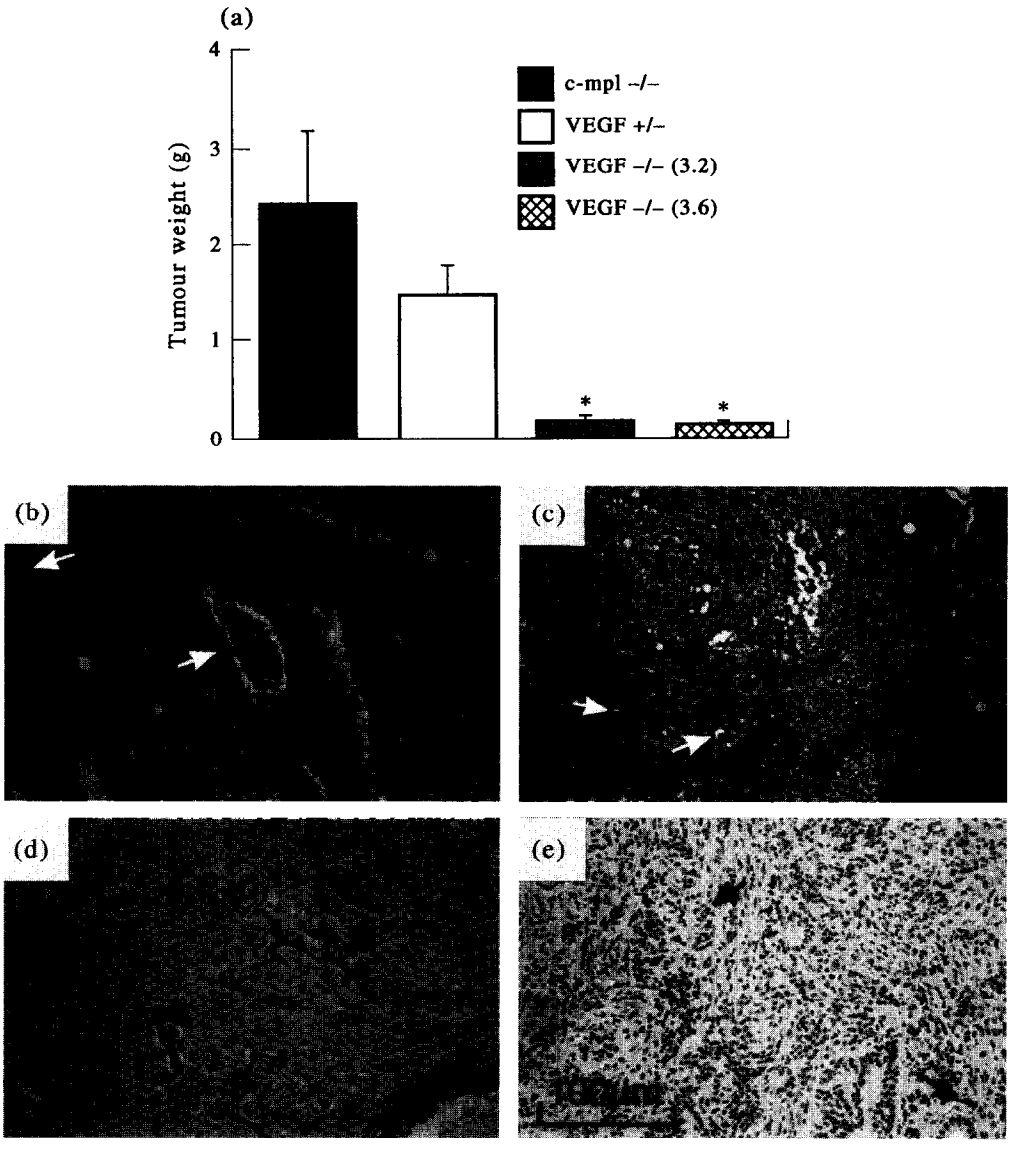

Figure 1. Embryonic stem (ES) cell tumorigenesis in nude mice. (a) Tumour weight; (b), (c), haematoxylin and eosin staining and immunostaining for CD34, (d, e) of tumour sections derived from *c-mpl* − /− (b, d) and VEGF − /− (c, e) ES cells. Histology shows representative sections of teratomas, containing glial tissue, blood vessels (arrowheads) and glandular epithelium. For injection in nude mice, cells were injected in the dorsal area. Values shown in (a) are means ± SEM of tumour weight determined 4 weeks after cell injection. Comparison of tumour weight of *c-mpl* − /− group with those of the VEGF + /+ clones revealed *P* values < 0.005. For immunohistochemistry, a rabbit polyclonal antiserum raised against murine CD34 was used. Primary antibody was detected with streptavidin-biotin. Reprinted with permission from *Nature* 1996, Vol. 380, pp. 439–442. Copyright © 1996 Macmillan Magazines Ltd.

sible for retinal and iris neovascularisation [117]. Recently, elevations of VEGF levels in the aqueous and vitreous humour of eyes with proliferative retinopathy have been described [19, 118, 119]. In a large series, a strong correlation was found between levels of immunoreactive VEGF in the aqueous and vitreous humours and active proliferative retinopathy [19]. Thus, although the involvement of other factors cannot be ruled out, the VEGF levels in eye fluids correlate well with ocular angiogenesis [120]. More direct evidence for a role of VEGF in intra-ocular neovascularisation has been provided in a primate model of iris neovascularisation and in a murine model of retinopathy of prematurity. In the former, intra-ocular administration of anti-VEGF antibodies dramatically inhibits the neovascularisation that follows occlusion of central retinal veins [121]. Similarly, soluble Flt-1 or Flk-1 fused to an IgG suppresses retinal angiogenesis in the mouse model [122].

Neovascularisation is also a major cause of visual loss in age-related macular degeneration (AMD), the overall leading cause of blindness [123]. A significant percentage of AMD patients (approximately 20%) manifest the neovascular (exudative) form of the disease. In this condition, new vessels stem from the extraretinal choriocapillary. Leakage and bleeding from these vessels lead to damage to the macula and ultimately to loss of central vision. Due to proximity to the macula, laser photocoagulation or surgical therapy are of very limited value. Very recent studies have documented the immunohistochemical localisation of VEGF in surgically resected choroidal neovascular mem-

Figure 2. Amino acid sequence of $VEGF_{165}$ and VEGF-related molecules: VEGF-B, VEGF-C, $PlGF_{152}$ and one of the two VEGF-like sequences identified in the genome of the parapoxvirus *orf* virus. The conserved cysteine residues are boxed.

branes from AMD patients [124], raising the possibility that VEGF is also the mediator of neovascularisation in AMD.

VEGF-RELATED GENES

Over the last few years, three VEGF-related genes have been identified from mammalian sources. The encoded factors are known as placenta growth factor (PlGF), VEGF-B and VEGF-C/VRP. In addition, two sequences in the genome of the parapoxvirus *orf* virus show homology to VEGF. Figure 2 shows the alignment of the amino acid sequences of these molecules with the sequence of $VEGF_{165}$. The structural homology to VEGF suggests that these molecules may play a role in the regulation of blood vessel growth. The first VEGF-related factor identified was PlGF. This factor may exist as one of two isoforms which arise from alternative splicing of mRNA [125]. These isoforms are known as $PlGF_{131}$ and $PlGF_{152}$, respectively. It has been shown that PlGF binds to high-affinity Flt-1 but not KDR [80]. PlGF demonstrated minimal or absent activity when tested in vascular endothelial cell growth assays, further supporting the hypothesis that binding to KDR is a requirement for mitogenesis. However, PlGF was able to potentiate the bioactivity of low, marginally efficacious, concentrations of VEGF [80]. Interestingly, naturally occurring heterodimers between VEGF and PlGF have been identified in the conditioned medium of a rat glioma cell line [126]. In agreement with previous studies, the PlGF homodimer demonstrated minimal activity on endothelial cells. However, the VEGF:PlGF heterodimer was active, although its potency was approximately 7-fold lower than the VEGF homodimer [126].

Similar to the VEGF receptors, Flt-4 is a RTK with seven Ig-like domains in the ECD [127]. The expression of Flt-4 mRNA, which is initially localised to angioblasts and venules in the early embryo, becomes restricted to the lymphatic endothelium at later stages of development, thus suggesting a role for Flt-4 in the regulation of lymphangiogenesis [128]. A ligand selective for Flt-4 has been recently identified by two groups and has been named VEGF-C [129] or VEGF-related peptide (VRP) [130]. VEGF-C/VRP is a secreted protein with 399 amino acid residues and has a 32% identity to VEGF. VEGF-C/VRP has been reported to stimulate the growth of human lung endothelial cells, albeit at 100-fold less potency than $VEGF_{165}$ [130].

A newly identified member of the VEGF gene family is VEGF-B [131]. This molecule consists of 188 amino acids, including the signal peptide. VEGF-B has been reported to stimulate the growth of human and bovine vascular endothelial cells. Interestingly, VEGF-B is distributed primarily in the skeletal muscle and myocardium and is co-expressed with VEGF [131]. VEGF-B and VEGF are also able to form heterodimers, when co-expressed [131].

Two sequences having homology to VEGF have been identified in the genome of two different strains of *orf* virus, a parapoxvirus that affects goats, sheep and occasionally humans [132]. The lesions of goats and humans following *orf* virus infection are characterised by extensive microvascular proliferation in the skin, raising the possibility that the product of the viral VEGF-like gene is responsible for such lesions.

CONCLUSION

The recent findings that heterozygous mutations inactivating the *VEGF* gene and homozygous mutations inactivating the Flt-1 or Flk-1/KDR genes result in profound deficits in vasculogenesis and blood island formation, leading to early intra-uterine death, emphasise the pivotal role played by the VEGF/VEGF-receptor system in the development of the

vascular system. Knockout mouse studies of the VEGF-related genes may elucidate whether these molecules also play an important role.

Recombinant VEGF or gene therapy with the *VEGF* gene may be used to promote endothelial cell growth and collateral vessel formation. This would represent a novel therapeutic modality for conditions that are frequently refractory to conservative measures and unresponsive to pharmacological therapy. The high expression of VEGF mRNA in human tumours, the presence of the VEGF protein in ocular fluids of individuals with proliferative retinopathies as well as the localisation of VEGF and AMD lesions, strongly supports the hypothesis that VEGF is a key mediator of angiogenesis associated with various pathological conditions. Therefore, anti-VEGF antibodies or other inhibitors of VEGF action may be of therapeutic value for a variety of malignancies as well as for other angiogenic disorders. Although the safety of such treatment has not yet been established, it is tempting to speculate that an anti-VEGF therapy may have low toxicity, possibly limited to inhibition of wound healing and ovarian and endometrial function, since endothelial cells are essentially quiescent in most adult tissues. Recently, a humanised version of a high-affinity anti-VEGF monoclonal antibody, which retains the same affinity and efficacy as the original murine antibody, has been generated and may be used in future clinical trials for several possible indications (L. Presta, Genentech, U.S.A.).

1. Folkman J, Shing Y. *Angiogenesis. J Biol Chem* 1992 **267**, 10931–10934.
2. Garner. A. Vascular diseases. In Garner A, Klintworth GK, eds. *Pathobiology of Occular Disease. A Dynamic Approach.* 2nd Edition, New York, Marcel Dekker, 1994, 1625–1710.
3. Weidner N, Semple P, Welch W, Folkman J. Tumor angiogenesis and metastasis. Correlation in invasive breast carcinoma. *N Engl J Med* 1991, **324**, 1–6.
4. Weidner N, Folkman J, Pozza F, *et al.* Tumor angiogenesis: a new significant and independent progonostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992, **84**, 1875–1888.
5. Wakui S, Furusato M, Sasaki H, *et al.* Tumor angiogenesis in prostatic carcinoma with and without bone metastasis: a morphometric study. *J Pathol* 1992, **168**, 257–262.
6. Macchiarini P, Fontanini G, Hardin MJ, Squartini F, Angeletti CA. Relation of neovascularization to metastasis of non-small cell lung carcinoma. *Lancet* 1992, **340**, 145–146.
7. Smith-McCune KS, Weidner N. Demonstration and characterization of the angiogenic properties of cervical dysplasia. *Cancer Res* 1994, **54**, 804–808.
8. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983, **219**, 983–985.
9. Dvorak HF. Tumours: wounds that do not heal. Similarity between tumor stroma generation and wound healing. *N Engl J Med* 1986, **315**, 1650–1658.
10. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 1989, **161**, 851–859.
11. Ploüet J, Schilling J, Gospodarowicz D. Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT20 cells. *EMBO J* 1989, **8**, 3801–3807.
12. Leung DW, Cachianes G, Kuang W-J, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989, **246**, 1306–1309.
13. Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT. Vascular permeability factor, an endothelial cell mitogen related to platelet derived growth factor. *Science* 1989, **246**, 1309–1312.
14. Conn G, Bayne M, Soderman L, *et al.* Amino acid and cDNA sequence of a vascular endothelial cell mitogen homologous to platelet-derived growth factor. *Proc Natl Acad Sci USA* 1990, **87**, 2628–2632.
15. Ferrara N. Vascular endothelial growth factor. *Trends Cardiovasc Med* 1993, **3**, 244–250.
16. Carmeliet P, Ferreira V, Breier G, *et al.* Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996, **380**, 435–439.
17. Ferrara N, Carver-Moore K, Chen H, *et al.* Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996, **380**, 439–442.
18. Kim KJ, Li B, Winer J, *et al.* Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* 1993, **362**, 841–844.
19. Aiello LP, Avery R, Arrigg R, *et al.* Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 1994, **331**, 1480–1487.
20. Pearlman JD, Hibberd MG, Chuang ML, *et al.* Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. *Nature Medicine* 1995, **1**, 1085–1089.
21. Takeshita S, Zhung L, Brogi E, *et al.* Therapeutic angiogenesis: a single intra-arterial bolus of vascular endothelial growth factor augments collateral vessel formation in a rabbit ischemic hindlimb model. *J Clin Invest* 1994, **93**, 662–670.
22. Isner JM, Pieczek A, Schainfeld R, *et al.* Clinical evidence of angiogenesis following arterial gene transfer of phVEGF$_{165}$ in patient with ischaemic limb. *Lancet* 1996, **348**, 370–374.
23. Pepper MS, Wasi S, Ferrara N, Orci L, Montesano R. *In vitro* angiogenic and proteolytic properties of bovine lymphatic endothelial cells. *Exp Cell Res* 1994, **210**, 298–305.
24. Jakeman LB, Winer J, Bennett GL, Altar CA, Ferrara N. Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rate tissues. *J Clin Invest* 1992, **89**, 244–253.
25. Jakeman LB, Armanini M, Phillips HS, Ferrara N. Developmental expression of binding sites and mRNA for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. *Endocrinology* 1993, **133**, 848–859.
26. Pepper MS, Ferrara N, Orci L, Montesano R. Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis *in vitro*. *Biochem Biophys Res Commun* 1992, **189**, 824–831.
27. Nicosia RF, Nicosia SV, Smith M. Vascular endothelial growth factor, platelet-derived growth factor and insulin-like growth factor-1 promote rat aortic angiogenesis *in vitro*. *Am J Pathol* 1995, **145**, 1023–1029.
28. Phillips GD, Stone AM, Jones BD, Schultz JC, Whitehead RA, Knighton DR. Vascular endothelial growth factor (rhVEGF$_{165}$) stimulates direct angiogenesis in the rabbit cornea. *In Vivo* 1995, **8**, 961–965.
29. Tolentino JM, Miller JW, Gragoudas ES, Chatzistefanou K, Ferrara N, Adamis AP. VEGF is sufficient to produce iris neovascularization and neovascular glaucoma in a non-human primate. *Arch Ophthalmology* 1996, **114**, 964–970.
30. Connolly DT, Heuvelman DM, Nelson R, *et al.* Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 1989, **84**, 1470–1478.
31. Pepper MS, Ferrara N, Orci L, Montesano R. Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor type 1 in microvascular endothelial cells. *Biochem Biophys Res Commun* 1991, **181**, 902–908.
32. Unemori E, Ferrara N, Bauer EA, Amento EP. Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. *J Cell Physiol* 1992, **153**, 557–562.
33. Mandriota S, Montesano R, Orci L, *et al.* Vascular endothelial growth factor increases urokinase receptor expression in vascular endothelial cells. *J Biol Chem* 1995, **270**, 9709–9716.
34. Roberts WG, Palade G. Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J Cell Sci* 1995, **108**, 2369–2379.

35. Ku DD, Zaleski JK, Liu S, Brock T. Vascular endothelial growth factor induces EDRF-dependent relaxation of coronary arteries. *Am J Physiol* 1993, **265**, H586–592.
36. Yang R, Thomas GR, Bunting S, *et al.* Effects of VEGF on hemodynamics and cardiac performance. *J Cardiovasc Pharmacol* 1996, **27**, 838–844.
37. Tisher E, Mitchell R, Hartmann T, *et al.* The human gene for vascular endothelial growth factor. *J Biol Chem* 1991, **266**, 11947–11954.
38. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 1991, **5**, 1806–1814.
39. Vincenti V, Cassano C, Rocchi M, Persico G. Assignment of the vascular endothelial growth factor gene to the humam chromosome 6p21.3. *Circulation* 1996, **93**, 1493–1495.
40. Shima DT, Kuroki M, Deutsch U, Ng Y-S, Adamis AP, D'Amore PA. The mouse gene for vascular endothelial growth factor. *J Biol Chem* 1996, **271**, 3877–3883.
41. Levy AP, Levy NS, Wegner S, Goldberg MA. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 1995, **270**, 13333–13340.
42. Ferrara N, Houck K, Jakeman L, Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocrinol Rev* 1992, **13**, 18–32.
43. Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* 1992, **267**, 26031–26037.
44. Park JE, Keller G-A, Ferrara N. The vascular endothelial growth factor (VEGF) isoforms. Differential deposition into the subepithelial extracellular matrix and bioactivity of ECM-bound VEGF. *Mol Biol Cell* 1993, **4**, 1317–1326.
45. Rifkin DB, Moscatelli D, Bizik J, *et al.* Growth factor control of extracellular proteolysis. *Cell Differ Devel* 1990, **32**, 313–318.
46. Keyt B, Berleau L, Nguyen H, *et al.* The carboxyl-terminal domain (111–165) of VEGF is critical for mitogenic potency. *J Biol Chem* 1996, **271**, 7788–7795.
47. Shweioki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 1992, **359**, 843–845.
48. Shima DT, Adamis AP, Ferrara N, *et al.* Hypoxic induction of vascular endothelial cell growth factors in the retina: identification and characterization of vascular endothelial growth factor (VEGF) as the sole mitogen. *Mol Med* 1995, **2**, 64–71.
49. Brogi E, Wu T, Namiki A, Isner JM. Indirect angiogenic cytokines upregulate VEGF and bFGF expression in vascular smooth muscle cells, whereas hypoxia upregulates VEGF expression only. *Circulation* 1994, **90**, 649–652.
50. Goldberg MA, Schneider TJ. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J Biol Chem* 1994, **269**, 4355–4361.
51. Stone J, Itin A, Alon T, *et al.* Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression in neuroglia. *J Neurosci* 1995, **15**, 4738–4747.
52. Liu Y, Cox SR, Morita T, Kourembanas S. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5′ enhancer. *Cir Res* 1995, 77, 638–643.
53. Madan A, Curtin PT. A 24-base pair sequence 3′ to the human erythropoietin contains a hypoxia-responsive transcription enhancer. *Proc Natl Acad Sci USA* 1993, **90**, 3928–3932.
54. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor-1 is a basic helix-loop helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci USA* 1995, **92**, 5510–5514.
55. Takagi H, King GL, Ferrara N, Aiello LP. Hypoxic induction of VEGF is mediated by adenosine through A_2 receptors and elevation of cAMP in retinal microvascular pericytes and endothelial cells. *Invest Ophthalmol Vis Sci*, in press.
56. Mukhopadhyay D, Tsilokas L, Zhou X-M, Foster D, Brugge JS, Sukhatme VP. Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. *Nature* 1995, **375**, 577–581.
57. Ikeda E, Achen MG, Breier G, Risau W. Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *J Biol Chem* 1995, **270**, 19761–19766.
58. Stein I, Neeman M, Shweiki D, Itin A, Keshet E. Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. *Mol Cell Biol* 1995, **15**, 5363–5368.
59. Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. Regulation of VEGF expression in cultured keratinocytes. Implications for normal and impaired wound healing. *J Biol Chem* 1995, **270**, 12607–12613.
60. Pertovaara L, Kaipainen A, Mustonen T, *et al.* Vascular endothelial growth factor is induced in response to transforming growth factor-β in fibroblastic and epithelial cells. *J Biol Chem* 1994, **269**, 6271–6274.
61. Li J, Perrella MA, Tsai JC, *et al.* Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells. *J Biol Chem* 1995, **270**, 308–312.
62. Ben-Av P, Crofford LJ, Wilder RL, Hla T. Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett* 1995, **372**, 83–87.
63. Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ. Interleukin 6 induces the expression of vascular endothelial growth factor. *J Biol Chem* 1996, **271**, 736–741.
64. Claffey KP, Wilkinson WO, Spiegelman BM. Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathways. *J Biol Chem* 1992, **267**, 16317–16322.
65. Kieser A, Weich H, Brandner G, Marme D, Kolch W. Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. *Oncogene* 1994, **9**, 963–969.
66. Rak J, Mitsuhashi Y, Bayko L, *et al.* Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res* 1995, **55**, 4575–4580.
67. Grugel S, Finkenzeller G, Weindel K, Barleon B, Marme D. Both v-Ha-Ras and v-Raf stimulate expression of vascular endothelial growth factor in NIH 3T3 cells. *J Biol Chem* 1995, **270**, 25915–25919.
68. Mukhopadhyay D, Tsiokas L, Sukhatme VP. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. *Cancer Res* 1995, **55**, 6161–6165.
69. deVries C, Escobedo JA, Ueno H, Houck KA, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 1992, **255**, 989–991.
70. Terman BI, Vermazen MD, Carrion ME, *et al.* Identification of the KDR tyrosine kinase as a receptor for vascular endothelial growth factor. *Biochem Biophys Res Commun* 1992, **34**, 1578–1586.
71. Shibuya M, Yamaguchi S, Yamane A, *et al.* Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase (flt) closely related to the *fms* family. *Oncogene* 1990, **8**, 519–527.
72. Matthews W, Jordan CT, Gavin M, Jenkins NA, Copeland NG, Lemischka IR. A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit. *Proc Natl Acad Sci USA* 1991, **88**, 9026–9030.
73. Quinn T, Peters KG, deVries C, Ferrara N, Williams LT. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci USA* 1993, **90**, 7533–7537.
74. Millauer B, Wizigmann-Voos S, Schnurch H, *et al.* High affinity binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 1993, **72**, 835–846.
75. Kendell RL, Thomas KA. Inhibition of vascular endothelial growth factor by an endogenously encoded soluble receptor. *Proc Natl Acad Sci USA* 1993, **90**, 10705–10709.

76. Davis-Smyth T, Chen H, Park J, Presta L, Ferrara N. The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade. *EMBO J* 1996, **15**, 4919–4927.
77. Guo D, Jia Q, Song HY, Warren RS, Donner DB. Vascular endothelial growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. *J Biol Chem* 1995, **270**, 6729–6733.
78. Waltenberger J, Clasesson-Welsh L, Siegbahn A, Shibuya M, Heldin C-H. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 1994, **269**, 26988–26995.
79. Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shibuya M. A unique signal transduction pathway for the FLT tyrosine kinase, a receptor for vascular endothelial growth factor. *Oncogene* 1995, **10**, 135–147.
80. Park JE, Chen H, Winer J, Houck K, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem* 1994, **269**, 25646–25654.
81. Keyt B, Nguyen H, Berleau L, Duarte C, Park J, Chen H, Ferrara N. Identification of VEGF determinants for binding Flt-1 and KDR receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis. *J Biol Chem* 1996, **271**, 5638–5646.
82. Morishita K, Johnson DE, Williams LT. A novel promoter for vascular endothelial growth factor receptor (flt-1) that confers endothelial-specific gene expression. *J Biol Chem* 1995, **270**, 27948–27953.
83. Tuder RM, Flook BE, Voelkel NF. Increased gene expression of VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or chronic hypoxia. *J Clin Invest* 1995, **95**, 1798–1807.
84. Thieme H, Aiello LP, Ferrara N, King GL. Comparative analysis of VEGF receptors on retinal and aortic endothelial cells. *Diabetes* 1995, **44**, 98–103.
85. Takagi H, King GL, Ferrara N, Aiello LP. Hypoxia regulates VEGF receptor KDR-Flk-1 gene expression through adenosine A_2 receptors in retinal capillary endothelial cells. *Invest Opthalmol Vis Sci* 1996, **37**, 1311–1321.
86. Brogi E, Schatteman G, Wu T, *et al.* Hypoxia-induced paracrine regulation of vascular endothelial growth factor receptor expression. *J Clin Invest* 1996, **97**, 469–476.
87. Patterson C, Perrella MA, Endege WO, Yoshizumi M, Lee M-E, Haber E. Downregulation of vascular endothelial growth factor receptors by tumor necrosis factor-α in cultured human vascular endothelial cells. *J Clin Invest* 1996, **98**, 4900–4960.
88. Mandriota SJ, Menoud PA, Pepper MS. Transforming growth factor beta 1 down-regulates vascular endothelial growth factor receptor 2/flk-1 expression in vascular endothelial cells. *J Biol Chem* 1996, **271**, 11500–11505.
89. Fong G-H, Rassant J, Gertenstein M, Breitman M. Role of Flt-1 receptor tyrosine kinase in regulation of assembly of vascular endothelium. *Nature* 1995, **376**, 66–67.
90. Shalabi F, Rossant J, Yamaguchi TP, *et al.* Failure of blood island formation and vasculogenesis in Flk-1 deficient mice. *Nature* 1995, **376**, 62–66.
91. Brandon EP, Idzerda RL, McKnight GS. Targeting the mouse genome: a compendium of knockouts (part III). *Curr Biol* 1995, **5**, 635–654.
92. Mattern J, Koomagi R, Voom M. Association of vascular endothelial growth factor expression with intratumoral microvessel density and tumour cell proliferation in human epidermoid lung carinoma. *Br J Cancer* 1996, **73**, 931–934.
93. Viglietto G, Maglione D, Rambaldi M, *et al.* Upregulation of vascular endothelial growth factor (VEGF) and downregulation of placenta growth factor (PlGF) associated with malignancy in human thyroid tumors and cell lines. *Oncogene* 1995, **11**, 1569–1579.
94. Brown LF, Berse B, Jackman RW, *et al.* Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Human Pathol* 1995, **26**, 86–91.
95. Brown LF, Berse B, Jackman RW, *et al.* Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Res* 1993, **53**, 4727–4735.
96. Suzuki K, Hayashi N, Miyamoto Y, *et al.* Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. *Cancer Res* 1996, **56**, 3004–3009.
97. Brown LF, Berse B, Jackman RW, *et al.* Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. *Am J Pathol* 1993, **143**, 1255–1262.
98. Olson TA, Mohanraj D, Carson LF, Ramakrishnan S. Vascular permeability factor gene expression in normal and neoplastic human ovaries. *Cancer Res* 1994, **54**, 276–280.
99. Guidi AJ, Abu-Jawdeh G, Berse B, *et al.* Vascular permeability factor (vascular endotheilal growth factor) expression and angiogenesis in cervical neoplasia. *J Natl Cancer Inst* 1995, **87**, 12137–12145.
100. Hashimoto M, Ohsawa M, Ohnishi A, *et al.* Expression of vascular endothelial growth factor and its receptor mRNA in angiosarcoma. *Lab Invest* 1995, **73**, 859–863.
101. Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth is a potential tumour angiogenesis factor *in vivo*. *Nature* 1992, **359**, 845–847.
102. Phillips HS, Armanini M, Stavrou D, Ferrara N, Westphal M. Intense focal expression of vascular endothelial growth factor mRNA in human intracranial neoplasms: association with regions of necrosis. *Int J Oncol* 1993, **2**, 913–919.
103. Berkman RA, Merrill MJ, Reinhold WC, *et al.* Expression of the vascular permeability/vascular endothelial growth factor gene in central nervous system neoplasms. *J Clin Invest* 1993, **91**, 153–159.
104. Freeman MR, Schneck FX, Gagnon ML, *et al.* Peripheral blood T lymphocytes and lymphocytes infiltrating human cancers express vascular endothelial growth factor: a potential role for T cells in angiogenesis. *Cancer Res* 1995, **55**, 4140–4145.
105. Qu-Hong J, Nagy JA, Senger DR, Dvorak HF, Dvorak AM. Ultrastructural localization of VPF/VEGF to the abluminal plasma membrane and vesiculovacuolar organelles of tumor microvascular endothelium. *J Histochem Cytochem* 1995, **43**, 381–389.
106. Toi M, Hoshima S, Takayanagi T, Tominaga T. Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. *Jpn J Cancer Res* 1994, **85**, 1045–1049.
107. Maeda K, Chung YS, Ogawa Y, *et al.* Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. *Cancer* 1996, 77, 858–863.
108. Kim KJ, Li B, Houck K, Winer J, Ferrara N. The vascular endothelial growth factor proteins. Identification of biologically relevant regions by neutralizing monoclonal antibodies. *Growth Factors* 1992, **7**, 53–64.
109. Warren RS, Yuan H, Matli MR, Gillett NA, Ferrara N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 1995, **95**, 1789–1797.
110. Borgström P, Hillan KJ, Sriramao P, Ferrara N. Complete inhibition of angiogenesis and growth of micro tumors by anti-VEGF neutralizing antibodies. Novel concepts of angiostatic therapy from intravital microscopy. *Cancer Res* 1996, **56**, 4032–4039.
111. Melnyk O, Schuman MA, Kim KJ. Vascular endothelial growth factor promotes tumor dissemination by a mechanism distinct from its effect on primary tumor growth. *Cancer Res* 1996, **56**, 921–924.
112. Asano M, Yukita A, Matsumoto T, Kondo S, Suzuki H. Inhibition of tumor growth and metastasis by an immunoncutralizing monoclonal antibody to vascular endothelial growth factor/vascular permeability factor. *Cancer Res* 1995, **55**, 5296 5301.
113. Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A. Glioblastoma growth is inhibited in vivo by a negative dominant Flk-1 mutant. *Nature* 1994, **367**, 576–579.
114. Millauer B, Longhi MP, Plate PH, Shawver LK, Risau W, Ullrich A. Dominant-negative inhibition of Flk-1 suppresses

the growth of many tumor types *in vivo*. *Cancer Res* 1996, **56**, 1615–1620.
115. Patz A. Studies on retinal neovascularization. *Invest Ophthalmol Vis Sci* 1980, **19**, 1133–1138.
116. Olk RJ, Lee CM. *Diabetic Retinopathy: Practical Management*. Philadelphia, J.B. Lippincott Co., 1993.
117. Michaelson IC. The mode of development of the vascular system of the retina with some observations on its significance for certain retinal disorders. *Trans Ophthalmol Soc UK* 1948, **68**, 137–180.
118. Adamis AP, Miller JW, Bernal M-T, *et al.* Increased vascular endothelial growth factor in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol* 1994, **118**, 445–450.
119. Malecaze F, Clemens S, Simorer-Pinotel V, *et al.* Detection of vascular endothelial growth factor mRNA and vascular endothelial growth factor-like activity in proliferative diabetic retinopathy. *Arch Ophthalmol* 1994, **112**, 1476–1482.
120. Ferrara N. Vascular endothelial growth factor—The trigger for neovascularization in the eye (Editorial). *Lab Invest* 1995, **72**, 615–618.
121. Adamis AP, Shima DT, Tolentino M, *et al.* Inhibition of VEGF prevents retinal-ischemia associated iris neovascularization in a primate. *Arch Ophthalmol* 1996, **114**, 66–71.
122. Aiello LP, Pierce EA, Foley ED, *et al.* Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. *Proc Natl Acad Sci USA* 1995, **92**, 10457–10461.
123. Bressler NM, Fine SL, Bressler SB. Angiographic interpretation of classic and occult choroidal neovascularization. In Lewis H, Ryan SJ, eds. *Medical and Surgical Retina*. St. Louis, Mosby, 1994, 3–11.
124. Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest Ophthalmol Vis Sci* 1996, **37**, 855–868.
125. Maglione D, Guerriero V, Viglietto G, Delli-Bovi P, Persico MG. Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci USA* 1991, **88**, 9267–9271.
126. DiSalvo J, Bayne ML, Conn G, *et al.* Purification and characterization of a naturally occurring vascular endothelial growth factor placenta growth factor heterodimer. *J Biol Chem* 1995, **270**, 7717–7723.
127. Pajusola K, Aprelikova O, Korhonen J, *et al.* FLT4 receptor tyrosine kinase contains seven immunoglublin-like loops and is expressed in multiple human tissues and cell lines. *Cancer Res* 1992, **52**, 5738–5743.
128. Kaipainanen A, Korhonen K, Mustonen V, *et al.* Expression of the FLT4 receptor tyrosine kinase becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci USA* 1995, **92**, 3566–3570.
129. Joukov V, Pajusola K, Kaipainen A, *et al.* A novel vascular endothelial growth factor, VEGF-C, is a ligand for the FLT4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996, **15**, 290–298.
130. Lee J, Gray A, Yuan J, Luoh S-M, Avraham H, Wood WI. Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4. *Proc Natl Acad Sci USA* 1996, **93**, 1988–1992.
131. Olofsson B, Pajusola K, Kaipaineen A, *et al.* Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci USA* 1996, **93**, 2576–2581.
132. Lyttle DJ, Fraser KM, Flemings SB, Mercer AA, Robinson AJ. Homologs of vascular endothelial growth factor are encoded by the poxvirus *orf* virus. *J Virol* 1994, **68**, 84–92.