CELL STRUCTURE AND FUNCTION 26: 25–35 (2001)

REVIEW

Structure and Function of VEGF/VEGF-receptor System Involved in Angiogenesis

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ABSTRACT. **Angiogenesis is an essential biological process not only in embryogenesis but also in the progression of a variety of major diseases such as cancer, diabetes and inflammation. Vascular endothelial growth factor (VEGF) family and its receptor system has been shown to be the fundamental regulator in the cell signaling of angiogenesis. Other systems, Angiopoietin-Tie and EphrinB2-Eph4B etc. are also involved in and cooperate with VEGF system to establish the dynamic blood vessel structures. VEGF receptor belongs to PDGF receptor supergene family, and carries seven Ig-domains in the extracellular region and a tyrosine kinase domain in the intracellular region. Three members of VEGF receptor family, Flt-1, KDR/Flk-1 and Flt-4, have unique characteristics in terms of the signal transduction, and regulate angiogenesis, lymphangiongenesis and vascular permeability. Further studies on VEGF-VEGF receptor system may significantly facilitate our understanding on the physiological as well as pathological vascular systems in the body and the development of new strategies to control and suppress the major diseases in humans.**

Key words: VEGF/angiogenesis/endothelial cells/tyrosine kinase receptor/signal transduction/clinical application

Vertebrates including mammalian species possess a closed circulation system for supplying nutrients and oxygen to the tissues in the body. The large and small blood vessels in this circulation system consist of two major cell types, one is the vascular endothelial cells lining the inside of the vessels as a monolayer, and the other is the smooth muscle cells which regulate the contraction and dilatation of blood vessels. These blood vessels are developed by several steps in embryogenesis, i.e. vasculogenesis (blood vessel formation from precursor cells), angiogenesis (blood vessel formation from pre-existing vessels and vascular endothelial cells), and vascular remodeling (Risau, 1997) (Fig. 1).

Vascular endothelial cells have long been the considered to be the key component for the angiogenesis. However, it remains an open question what kind of molecule(s) is the critical regulator (growth factor, differential factor, etc.) for the endothelial cells. Extensive studies on vascular endothelial growth factor (VEGF) and its receptors during the past 10 years have revealed that this system is the major regulator for vascular endothelial cells and blood vessel formation (Ferrara and Davis-Smyth, 1997; Shibuya, 1995; Mustonen and Alitalo, 1995; Shibuya *et al*., 1999) (Fig. 2).

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Abbreviations: VEGF, vascular endothelial growth factor; Flt-1, Fms-like tyrosine kinase; KDR, kinase insert domain containing receptor.

VEGF

In the 1980s VEGF was identified independently as vascular permeability factor (VPF) and as vascular endothelial cell-specific growth factor in 1980s (Senger *et al*., 1983; Leung *et al*., 1989). Molecular cloning of the genes encoding these "two" proteins clarified that these proteins are essentially the same protein encoded by a single gene (VEGF gene). Therefore, this protein is referred as VEGF, VEGF/VPF or, sometimes, as VPF.

Structurally VEGF belongs to the VEGF-PDGF (platelet-derived growth factor) super-gene family. Among these gene products, 8 cysteine residues are conserved at the same positions, and these products function as a dimer form since 2 out of 8 cysteines generate intermolecular cross-linking, S-S bonds. The other 6 cysteines make 3 intramolecular S-S bonds to form 3 loop structures (Wiesmann *et al*., 1997).

VEGF in humans has at least 3 major subtypes, which are 121, 165 and 189-amino acid proteins based on alternative splicing (Fig. 3). Among them, the 121 and 165-subtypes, or $VEGF_{121}$ and $VEGF_{165}$, are the two representative forms, of which $VEGF_{165}$ bears an additional 44-amino acid basic stretch when compared to $VEGF_{121}$ (Ferrara and Davis-Smyth, 1997). Through this basic stretch, $VEGF_{165}$ associates with heparin or heparin-like molecules in the matrix

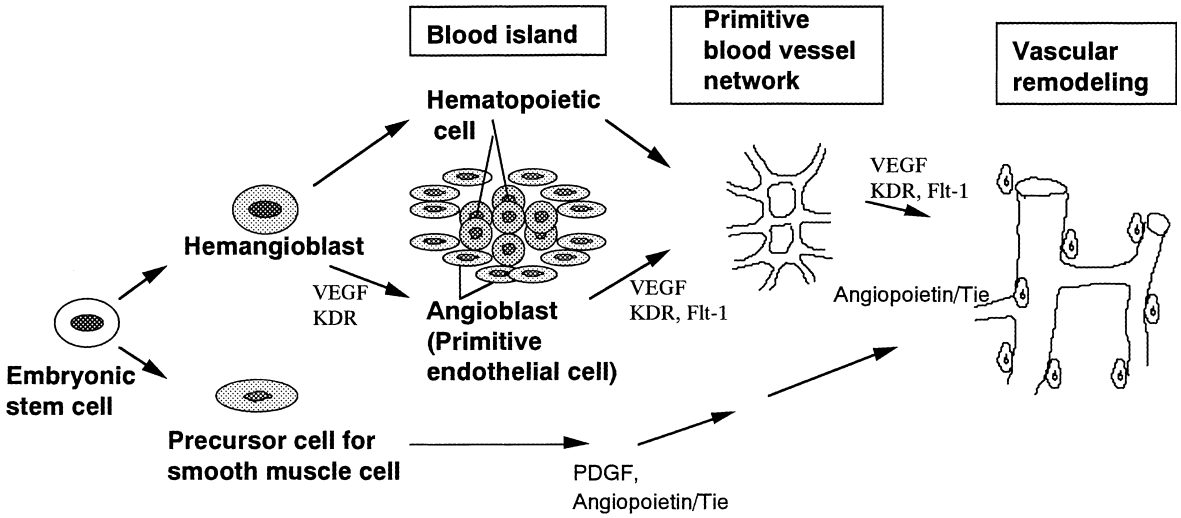

Fig. 1. Multiple steps for blood vessel formation in embryogenesis. At least three steps are thought to be essential: vasculogenesis, angiogenesis and vascular remodeling. VEGF and its receptors as well as other systems such as Angiopoietin-Tie receptors and PDGF-PDGF receptors are intimately involved in these steps.

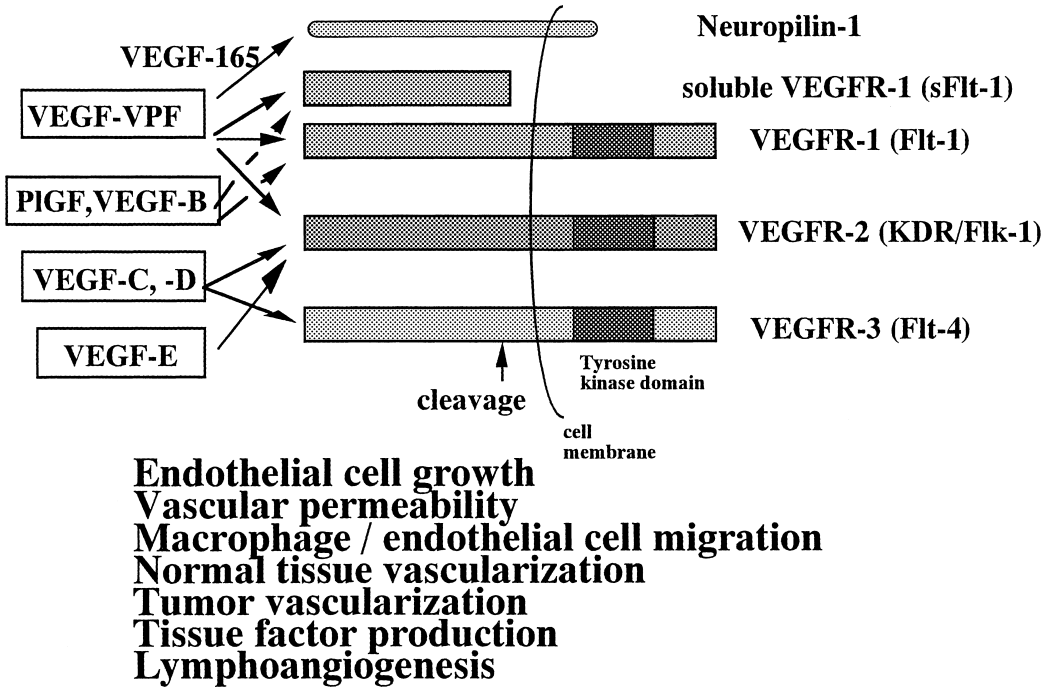

Fig. 2. VEGF and its receptor system. Three structurally related 7-Ig tyrosine kinase receptors and at least 5 ligands are the key regulators for this system. This system is essential for angiogenesis and lymphoangiogenesis in vivo (Shibuya, 2001).

and on the cell surface, and can also associate with the cell surface molecule, neuropilin-1. The association of $VEGF_{165}$ with neuropilin-1 has been reported to increase the affinity of $VEGF_{165}$ with one of the VEGF receptors, KDR (VEGFR-2), to about 10-fold, resulting in $VEGF_{165}$ being the strongest signal transducer among the VEGF subtypes.

Recent studies on knockout mice revealed that the mice lacking $VEGF_{165}$ but still retaining $VEGF_{121}$, or the mice lacking neuropilin-1 gene are both lethal at perinatal stage, due to the dysfunction of the cardiac-large blood vessel system. These results indicate that $VEGF_{165}$ is required at certain critical period during the development of the cardiac-

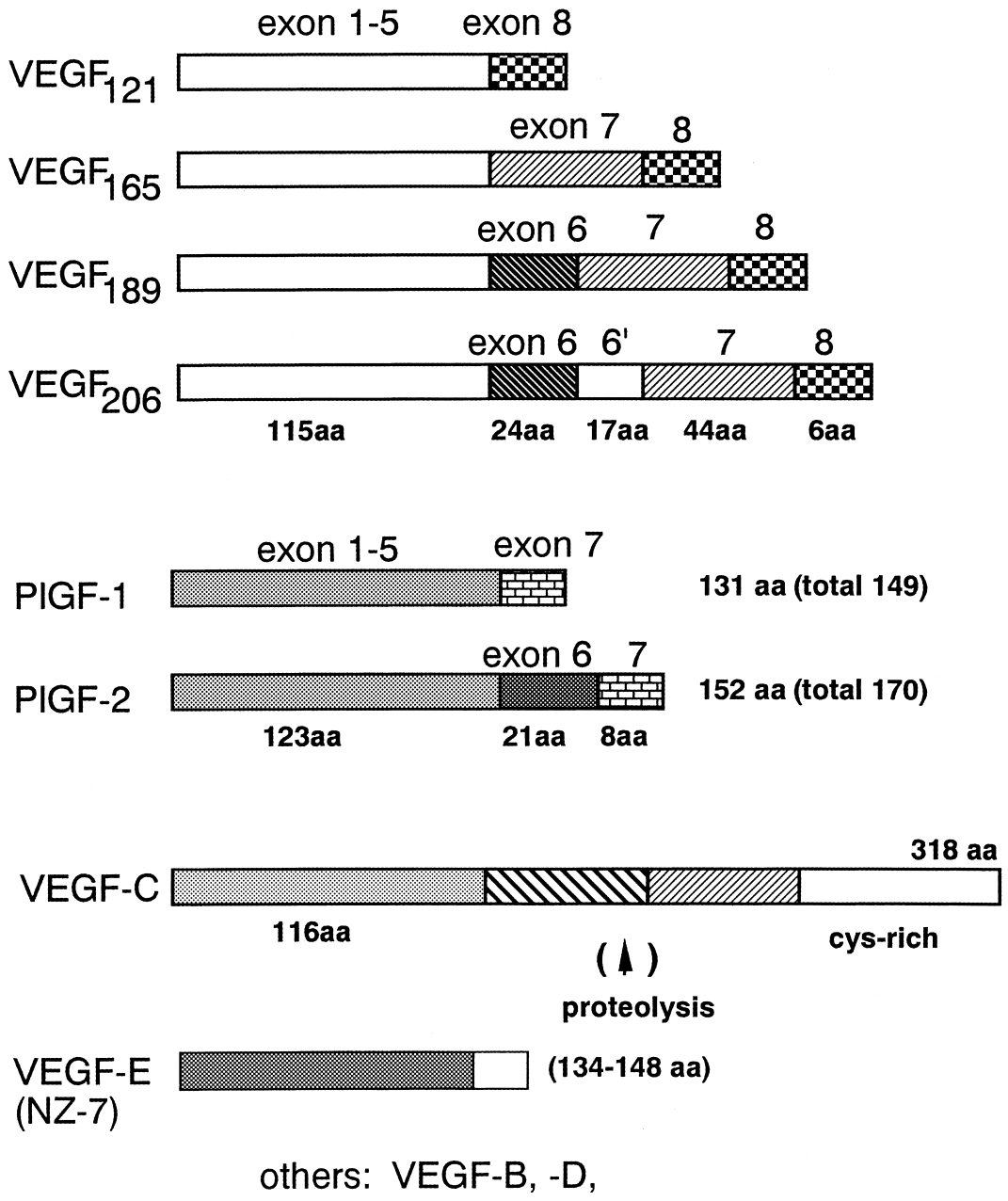

Fig. 3. Comparison of structures of the VEGF family. VEGF-E (NZ-7) which is encoded in the genome of Orf virus NZ-7 strain, binds KDR at high affinity but not Flt-1.

blood vessel system near the neonatal stage (Carmeliet *et al*., 1999; Kawasaki *et al*., 1999).

VEGF-related factors

In addition to VEGF, VEGF family contains several other proteins that are highly related structurally: PlGF, VEGF-B, VEGF-C, VEGF-D and VEGF-E (Fig. 3). As described later, VEGF binds two tyrosine kinase receptors, Flt-1 (VEGFR-1) and KDR (VEGFR-2, also known as Flk-1 in mice) (Shibuya *et al*., 1999). Interestingly, PlGF (placenta growth factor) and VEGF-B bind and activate only Flt-1. On the other hand, VEGF-C and -D have been shown to bind the third receptor Flt-4 (VEGFR-3) and, to some extent, KDR (VEGFR-2). VEGF-C and -D were found to be the important regulators of lymphangiogenesis (Joukov *et al*., 1996; Jeltsch *et al*., 1997).

Although Flt-1-specific ligands exist in the body, such a

ligand which binds and activates only KDR (VEGFR-2) has not been isolated. Very recently, however, we have found that a novel ligand VEGF-E_{NZ-7}, which was originally identified as the product of an ORF (open reading frame) in the genome of Orf virus (NZ-7 strain), specifically binds and activates KDR (VEGFR-2) but not Flt-1 (VEGFR-1) (Ogawa *et al*., 1998). Thus, these receptor-specific ligands, PlGF, VEGF-B and VEGF-E should prove very useful for further studies on the receptor-specific biological functions *in vitro* and *in vivo*.

Regulation of VEGF gene expression

VEGF gene has been shown to be regulated by a variety of stimuli such as hypoxia, growth factors, transformation, p53-mutation, estrogen, TSH, tumor promoters and NO. Although all of the stimuli for VEGF gene upregulation are quite interesting, hypoxia is particularly focused on by many research groups because of its importance and its unique transcriptional regulation.

Under hypoxic conditions, transcriptional factors HIF1α and HIF2α (Hypoxia inducible factor 1) were found to be stabilized and translocated to the nucleus. Within the nuclei, these factors cooperate with another related factor, HIFβ, to activate the VEGF gene via a specific motif HRE (hypoxia response element). For the nuclear translocation of HIF1α and HIF2α, a monopartite-type NLS (nuclear localization signal) was considered to be utilized. However, recently we found that a new type of bipartite NLS is highly conserved at the C-termini of these proteins and is involved in the nuclear translocation of HIF1α and HIF2α (Fig. 4) (Luo and Shibuya, 2001).

VHL protein, the product of the gene responsible for von Hippel Lindau disease, was found to be directly involved in the degradation of VEGF protein under normoxic conditions. Thus, the loss of VHL-function results in the constitutive upregulation of VEGF gene, strongly suggesting that the high susceptibility of brain and renal cancers in VHL patients is due, at least in part, to the abnormal upregulation of the critical angiogenic factor, VEGF.

Structure of VEGF receptors

In 1990, we isolated a novel type tyrosine kinase receptor

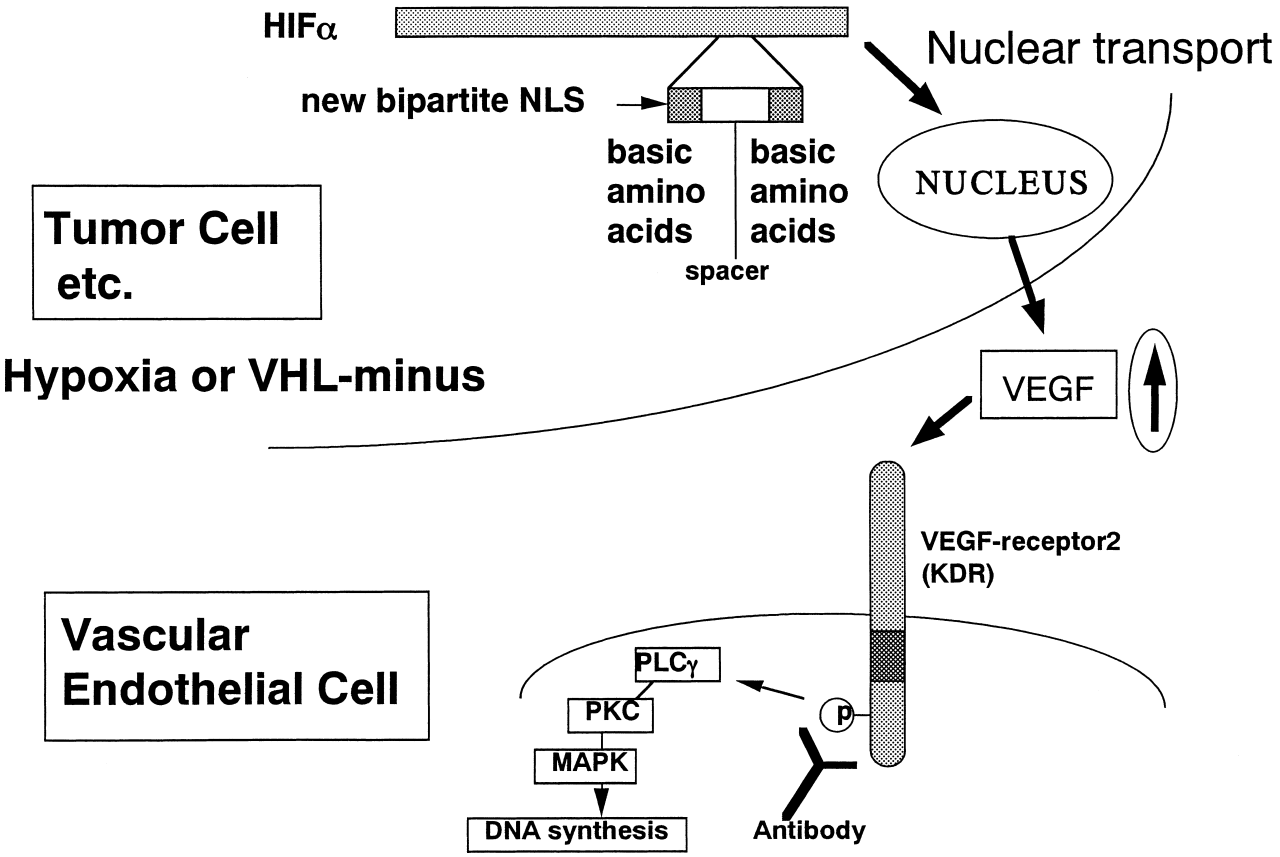

Fig. 4. Regulation of VEGF gene expession and signal transduction of KDR (VEGF receptor-2). One major regulatory system of VEGF gene expression is the transcription factor HIF-dependent system. HIFα family utilizes a novel type bipartite NLS for nuclear translocation (Luo and Shibuya, 2001). KDR mediates endothelial cell proliferation signal via the PLCγ-PKC-MAP kinase pathway but not the Ras-dependent pathway (Takahashi *et al*., 1999).

gene from the placental cDNA library, and based on its structural similarity we named it as Fms-like tyrosine kinase-1 (Flt-1) (Shibuya *et al*., 1990). We also showed that the *flt-1* mRNA is specifically expressed in vascular endothelial cells. A few years later, Flt-1 was found to tightly bind to VEGF, thus, it was the first high affinity receptor for VEGF (VEGFR-1) to be identified (De Vries *et al*., 1992). The second high affinity receptor, KDR (Flk-1 in mice) has also been molecularly cloned (Fig. 2) (Terman *et al*., 1992). These receptors carry 7 immunoglobulin-like domains in the extracellular region, whereas Fms (M-CSF receptor), Kit (Stem cell factor receptor) and PDGF receptors have 5 Ig-like domains within the extracellular region. Therefore, it seems clear that these two receptor groups, 7-Ig receptors (VEGFRs) and 5-Ig receptors (Fms/Kit/PDGFR), belong to the same super-gene family. Furthermore, the third member of the 7-Ig receptors, Flt-4 (VEGFR-3) gene, was isolated by the Alitalo group and Birnbaum group, who showed that, although VEGF does not bind to Flt-4, Flt-4 and its ligand VEGF-C and -D are intimately involved in lymphangiogenesis as well as angiogenesis (Pajusola *et al*., 1992; Galland *et al*., 1993).

Two VEGF receptors, Flt-1 and KDR, have interesting biochemical characteristics. Flt-1 has the highest affinity to VEGF (Kd=1−10 pM) among the VEGF receptors, but its tyrosine kinase activity is very weak. On the other hand, KDR carries one-order weaker affinity to VEGF (Kd=about 200 pM), however, its kinase activity is as strong as other representative tyrosine kinase receptors such as EGF receptor (Seetharam *et al*., 1995; Sawano *et al*., 1996).

In the extracellular domain of these VEGF receptors, the second and third Ig domains were found to be the VEGF binding region (Keyt *et al*, 1996; Tanaka *et al*., 1997). We also showed that the 4th to 7th Ig regions are essential for the receptor dimerization (Tanaka *et al*., 1997; Shinkai *et al*., 1998).

Interestingly, the *flt-1* but not the *KDR* gene encodes two polypeptides. One is the full length tyrosine kinase receptor, and the other is the soluble form of Flt-1 which encodes only the 1st to 6th Ig region without tyrosine kinase domain. This soluble form is generated by a premature termination of the *flt-1* mRNA because of the aberrant polyadenylation

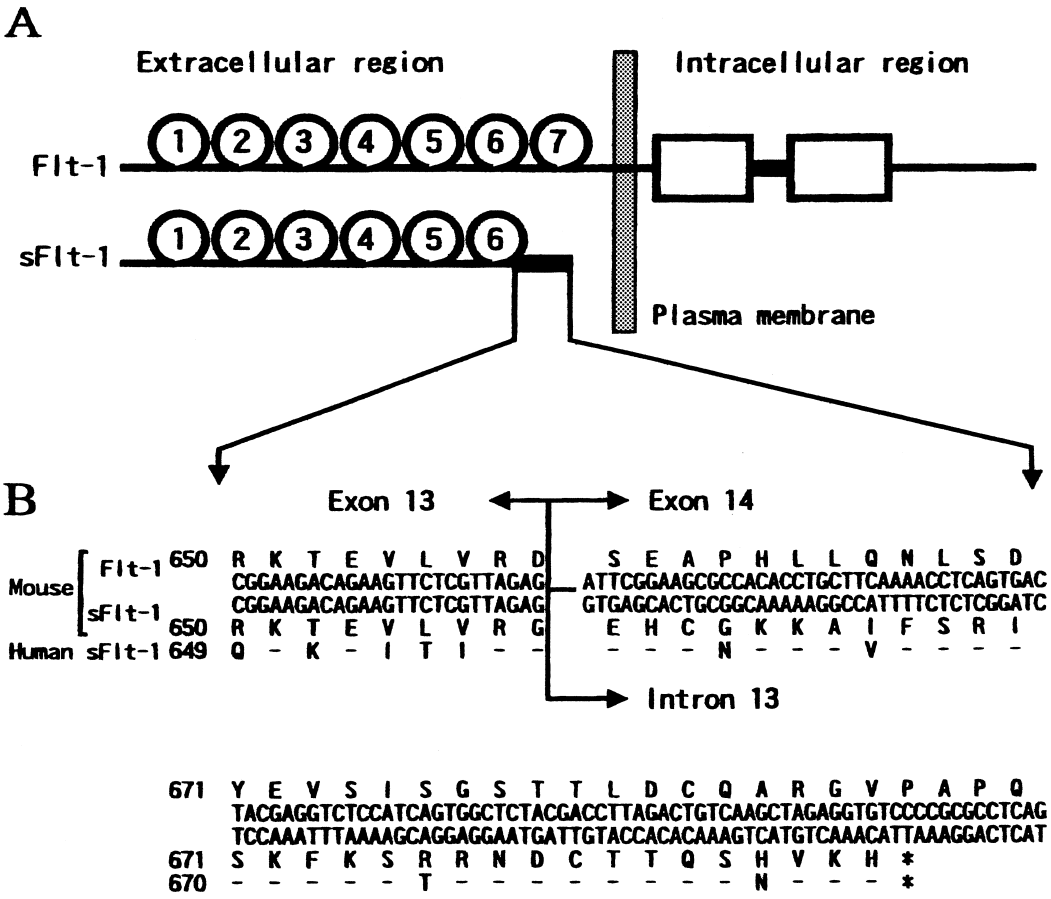

Fig. 5. Soluble form of Flt-1. Soluble Flt-1 carries 1st to 6th Ig domains and 31-amino acid stretch encoded by the intron 13 (Kendall and Thomas, 1993; Kondo *et al*., 1998; Shibuya, 2001).

in intron-13 (Kendall and Thomas, 1993). Therefore, the soluble Flt-1 peptide carries about 670-amino acid sequence derived from the 1st to 6th Ig domains and additional 31-amino acid stretch which is encoded in the 5'-region of intron-13. Surprisingly, this intron-13-encoded 31-amino acid sequence is not a meaningless one, since this sequence is highly conserved among mammalian species (Kondo *et al*., 1998) (Fig. 5). Based on these findings, the *flt-1* gene is considered to be phylogenetically set up to encode two polypeptides, the regular receptor and the ligand-bining soluble peptide.

Another important characteristic of the VEGF receptors is the kinase-insert region within the tyrosine kinase domain. Both Flt-1and KDR bear a long kinase-insert of about 70 amino acids, the length of which is very similar to the 5-Ig type receptors. However, the motifs within the kinase-insert region among 7-Ig and 5-Ig receptors were quite different. The 5-Ig receptors such as PDGF receptors were shown to have strong autophosphorylation tyrosine residues within this K-I region, which are Tyr-X-X-Met motifs important for the PI3-kinase activation. However, none of the VEGF receptors have these autophosphorylation tyrosine residues (Tyr-X-X-Met) within the K-I region or in other portions in the intracellular domain (Shibuya *et al*., 1990). This clear structural difference strongly suggests that the signal transduction pathway used by the VEGF receptors may be quite different from that by 5-Ig receptors. This point will be further described in the ne

xt section.

Signal transduction of VEGF receptors

In general, signal transduction mediated by tyrosine kinase receptors has several biochemical steps: (1) ligand binding to the receptor, (2) receptor dimerization, (3) activation of tyrosine kinase, (4) autophosphorylation of the receptor, (5) binding and activation of adaptors at the autophosphorylation sites (Schlessinger, 2000). VEGF receptors are known to follow essentially the same processes as above.

Since the major positive signal of VEGF such as endothelial cell growth is generated by KDR, we, as well as others, have been primarily focusing on signal transduction from KDR. Using NIH3T3 cells overexpressing KDR and primary vascular endothelial cells, KDR was found to have an interesting signal transduction pathway. Most of the representative tyrosine kinase receptors such as EGF receptor are known to utilize Ras-activation pathway toward MAP kinase activation (Schlessinger and Ullrich, 1992). For such Ras-activation, Shc-Grb2-Sos or Grb2-Sos pathway is directly involved in this system. However, KDR was not found to utilize the Ras-activation pathway, or only very little of it, if any, and most of the MAP kinase activation was mediated via the PLCγ-PKC pathway. PLCγ was directly bound to the autophosphorylated KDR, then tyrosine-phosphorylated and activated. The activated PLCγ stimulated the PKC activation, particularly the PKCβ form, and activated Raf-1 to MAP kinase cascade (Takahashi and Shibuya, 1997; Takahashi *et al*., 1999).

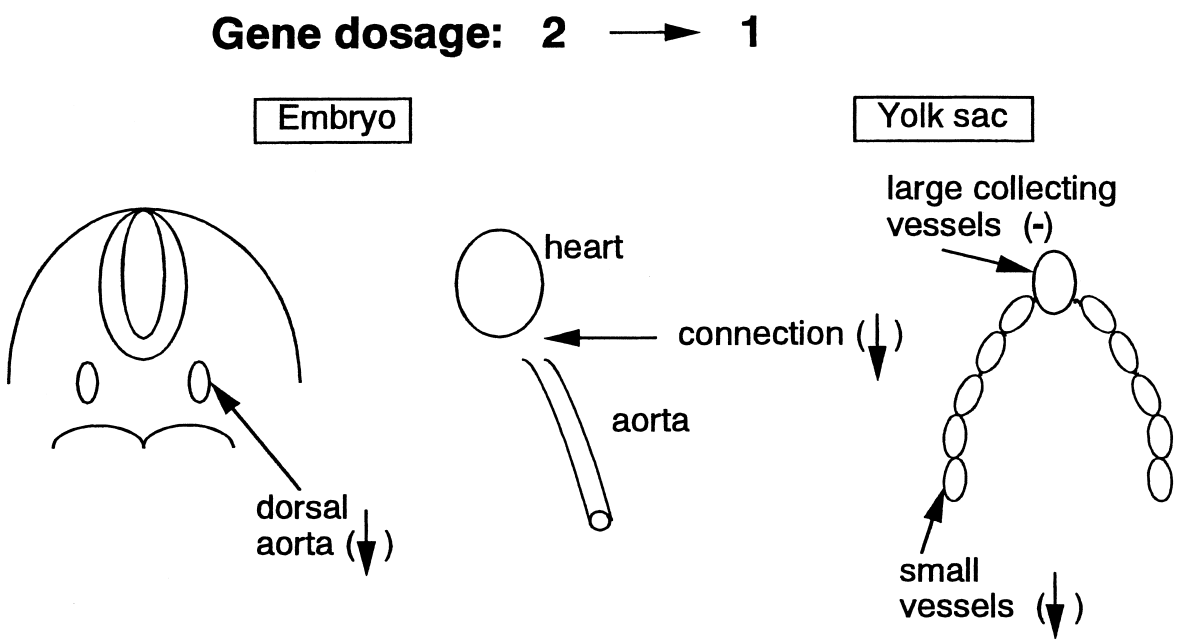

Fig. 6. VEGF (+/–) heterozygote mice are embryonic lethal due to abnormality in circulation system. Even the loss of a single allele of the VEGF gene results in multiple defects in the cardiovascular system (Carmeliet *et al*., 1996; Ferrara *et al*., 1996).

This unique signaling pathway from the KDR was further supported by the following evidence: (1) almost no detectable formation of Ras-GTP after stimulation of vascular endothelial cells with VEGF; (2) the introduction of a dominant negative form of Ras by adenovector to vascular endothelial cells could not efficiently block the VEGF-dependent MAP kinase activation and DNA synthesis, although this DN-Ras adenovector strongly suppressed the Ras-dependent MAP kinase activation and DNA synthesis in NIH3T3 cells stimulated with PDGF; (3) the Shc-Grb2-Sos complex which is important for the activation of Ras is not activated in KDR-mediated signaling pathway (Takahashi *et al*., 1999).

Taken together, VEGF receptor utilizes a unique signaling pathway for endothelial cell proliferation. It is raises questions as to what other kinds of signaling pathways are involved in the survival signal and morphogenesis of the vascular endothelial cells mediated by VEGF and its receptors (Fig. 4).

Knockout studies on VEGF and its receptor genes

VEGF and its two receptor genes were found to be essential for the development of vascular system in embryogenesis. Furthermore, even heterozygote mice for VEGF gene deficiency were embryonic lethal, strongly suggesting that the local concentration of VEGF in the embryonal tissues should be adjusted between certain critical levels (Carmeliet *et al*., 1996; Ferrara *et al*., 1996) (Fig. 6).

KDR/flk-1 (–/–) mice were embryonic lethal at E8.5-E9.0 due to no development of blood vessels as well as very low hematopoiesis (Shalaby *et al*., 1995). *flt-1* (–/–) mice were also embryonic lethal at the same stage E8.5, but due rather to overgrowth and dysorganization of endothelial cells (Fong *et al*., 1995). Therefore, although both receptors are crucial for embryogenesis, KDR is thought to be the strong positive signal transducer, whereas Flt-1 has a negative regulatory function for vascular formation.

Since Flt-1 has a quite strong affinity to VEGF but its kinase activitiy is very weak, we hypothesized that the

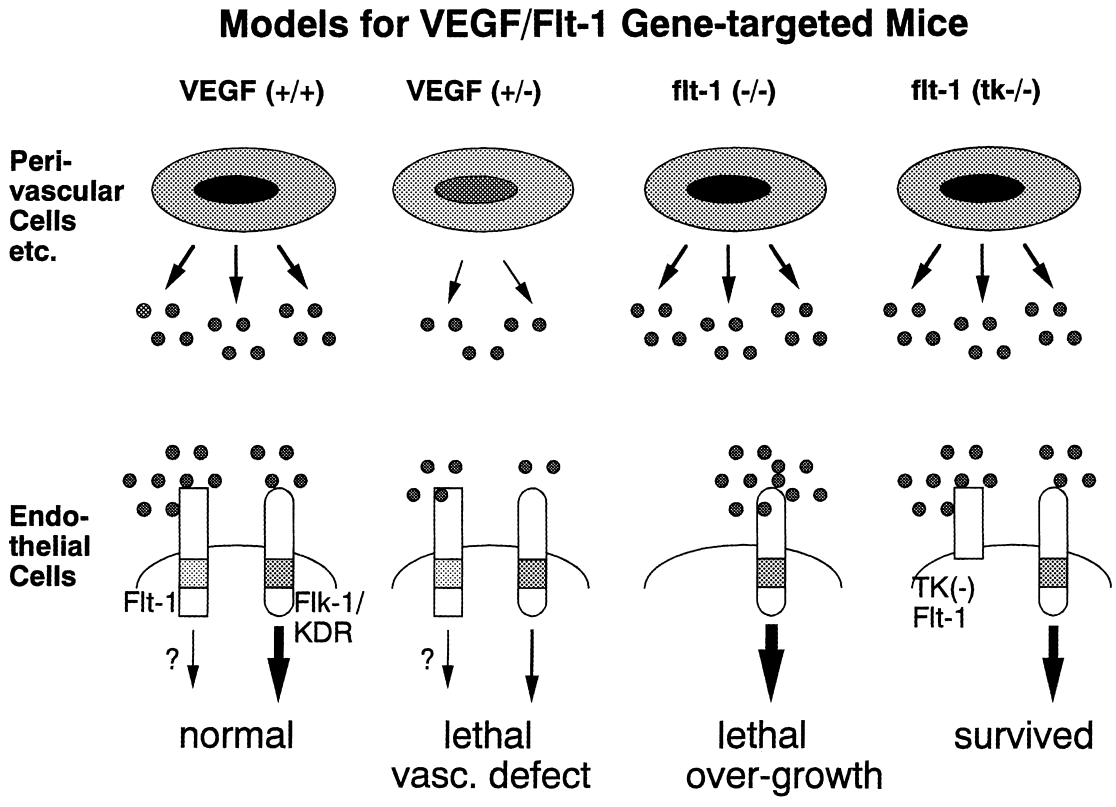

Fig. 7. The extracellular domain of the Flt-1 is sufficient for the negative regulatory role of *flt-1* gene in embryogenesis. The *flt-1* gene (–/–) mice are embryonic lethal due to overgrowth and dysorganization of endothelial cells (Fong *et al*., 1995; Hiratsuka *et al*., 1998). Tyrosine kinase-minus mice can survive with almost normal circulation system, suggesting that the VEGF-trapping activity of the ligand binding domain of Flt-1 is crucial to embryogenesis (Shibuya, 2001).

Mouse VEGFs in Tumor-induced Ascites

Ascites sample	characteristics	VEGF (ng/ml)
BP-8 (sarcoma)	bloody	850
Ehrlich (mammary ca)	bloody	600
SR-C57Bl/6 (sarcoma)	bloody	560
SR-DDD (sarcoma)	bloody	520
MH134 (hepatoma)	bloody	350
S37 (sarcoma)	bloody	310
S180 (sarcoma)	bloody	168
MM2 (mammary ca)	bloody	83
X5563 (plasmacytoma)	bloody	38
L1210 (leukemia)	bloody	23
6C3HED-OG (lymphoma)	nonbloody	20
P815 (mastocytoma)	nonbloody	18
6C3HED-RG (lymphoma)	nonbloody	14
normal mouse serum		2

Ehrlich, MM2: similar levels of VEGF in *in vitro* culture media

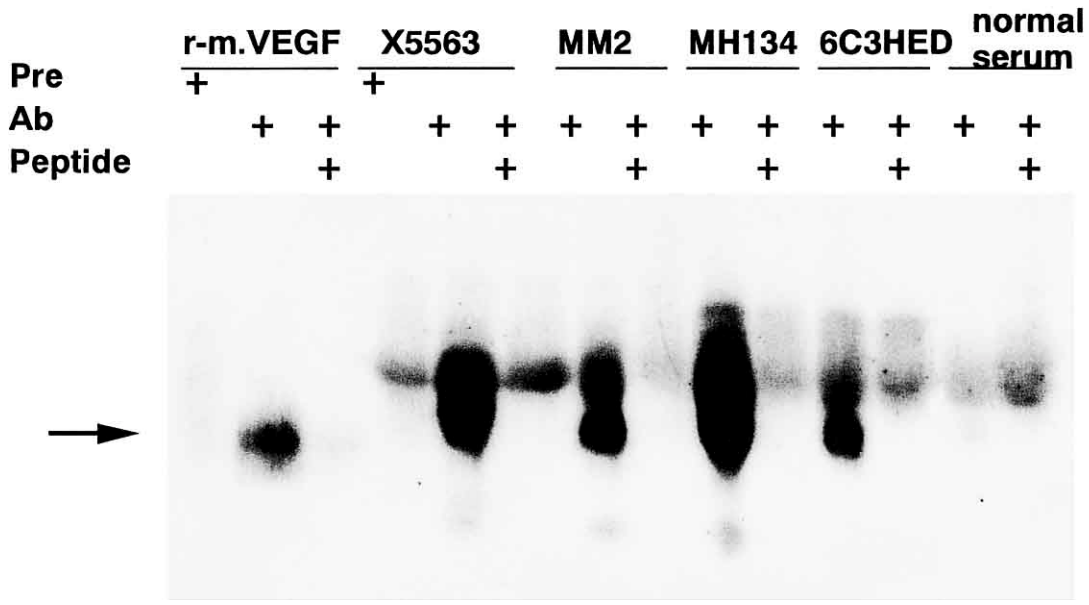

Fig. 8. VEGF levels in the ascites fluid of ascites tumor-bearing mice. Carcinoma- and sarcoma-derived ascites tumors secrete large amounts of VEGF into ascites fluid. The major subtype of VEGF accumulated in the ascites fluids is the 164-amino acid subtype (Luo *et al*., 1998b).

negative regulatory role of Flt-1 is due to its VEGF trapping activity. To prove this hypothesis, we generated a mice strain which lacks the tyrosine kinase domain of Flt-1. We found that such mice developed almost normal circulation system and survived (Hiratsuka *et al*., 1998). Therefore, the simplest explanation for this result is that in the early stages

of embryogenesis, the major role of Flt-1 is to bind VEGF and suppress the physiological levels of VEGF via the ligand binding domain (Fig. 7).

Involvement of VEGF and its receptors in pathological angiogenesis and hyperpermeability

A number of reports have indicated that the upregulation of VEGF is well correlated with the progression of diseases such as diabetic retinopathy, rheumatoid arthritis and solid tumors (Ferrara and Davis-Smyth, 1997; Shibuya, 1995). Furthermore, not only in pathological angiogenesis but also the abnormal hyperpermeability, VEGF was found to be deeply involved. POEMS (Crow-Fukase) syndrome, in which the patients have general edema, was closely related with VEGF. The Maruyama group showed that VEGF levels in the serum of patients with this syndrome were more than 10-fold higher than those in healthy people (Watanabe *et al*., 1996).

We have examined the accumulation of VEGF in the ascites fluid of ascites tumor-bearing mice. VEGF levels in ascites fluids were very high, 50 to over 500 ng/ml (Luo *et al*., 1998b) (Fig. 8). Quite interestingly, treatment of ascites-bearing mice with anti-VEGF neutralizing antibody significantly suppressed the volume of ascites and the number of ascites tumor cells. Furthermore, this treatment strongly inhibited the bleeding tendency of ascites (Luo *et al*., 1998a). Thus, an anti-VEGF therapy may be a powerful procedure to suppress the progression of ascites tumors at the clinical level.

Angiogenic and anti-angiogenic therapy via VEGF and its receptors

Since the VEGF and its receptor system is considered to be the fundamental regulator for blood vessel formation, it seems important to apply this system for clinical use both in the stimulation of normal angiogenesis and in the suppression of pathological angiogenesis.

For the stimulation of angiogenesis in patients with cardiac ischemia and with blood flow failure, a number of trials using VEGF protein or VEGF expression vectors have already been initiated (Isner and Asahara, 1999). So far, VEGF expression vector system is thought to be hopeful, although some side effects such as edema are reported (Suri *et al*., 1998). More recently, precursor cells which could differentiate to vascular endothelial cells were found in peripheral blood and in bone marrow-derived cells (Asahara *et al*., 1999). These precursor cells also may be useful for supporting the new blood vessel formation *in vivo*.

For the suppression of pathological angiogenesis such as tumor angiogenesis, many approaches have been under investigation. The reagents of anti-VEGF and its receptor system so far developed include anti-VEGF neutralizing monoclonal antibody, soluble Flt-1, anti-KDR kinase (SU5416 and SU6668) and anti-KDR or anti-Flt-1 neutralizing antibody (Kim *et al*., 1993; Kondo *et al*., 1993; Aliello *et al*., 1995; Kong *et al*., 1998; Fong *et al*., 1999; Klement *et al*., 2000; Sawano *et al*., 2001). The molecular mechanisms for the blocking of VEGF-receptor system by these reagents differ from one to another. Thus, it is important to clarify which reagent is the best treatment for controlling which pathological angiogenesis in different diseases, and what kind of combination therapy, such as radiation plus anti-angiogenic therapy versus chemotherapy plus anti-angiogenic therapy, is most efficient for the suppression of solid tumor growth in cancer patients.

Acknowledgments. I thank all the colleagues in my laboratory, the Institute of Medical Science, University of Tokyo for the studies on VEGF and its receptors.

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(*Received for publication, Februrary 9, 2001*
and accepted, Februrary 15, 2001)