Unique signal transduction of the VEGF family members VEGF-A and VEGF-E

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Abstract
Both VEGF (vascular endothelial growth factor)-A and Orf-virus-encoded VEGF-E bind and activate VEGFR (VEGF receptor)-2; however, only VEGF-A binds VEGFR-1. To understand the biological differences between VEGF-A and VEGF-E in vivo, we established transgenic mouse models. K14 (keratin-14)-promoter-driven VEGF-E transgenic mice showed a significant increase in mature blood vessels. However, K14–VEGF-A transgenic mice exhibited severe inflammation and oedema with increased angiogenesis, as well as lymphangiogenesis and lymph vessel dilatation. K14–VEGF-A transgenic mice deficient in VEGFR-1 signalling (K14–VEGF-A-tg/VEGFR-1 TK−/− mice) showed decreases in oedema and inflammation with less recruitment of macrophage-lineage cells, suggesting an involvement of VEGFR-1 in these adverse effects. VEGFE might be more useful than VEGFA for pro-angiogenic therapy.

Introduction
Blood vessels supply nutrients and oxygen to tissues in the body. Their formation involves two major processes: (i) vasculogenesis, which is critical for the differentiation of endothelial progenitor cells into vascular endothelial cells in early embryogenesis; and (ii) angiogenesis, whereby blood vessels develop from a pre-existing vascular network [1]. A variety of factors and their receptors including VEGF (vascular endothelial growth factor)–VEGFR (VEGF receptor), Ang–Tie, Ephrin–Eph, Delta–Notch and the Wnt pathway have been demonstrated to be involved in these processes. The VEGF family and its receptor system appear to play a pivotal role not only in physiological vasculogenesis/angiogenesis, but also in most pathological angiogenesis [2,3]. Vegfa-knockout mice are embryonic lethal due to insufficient formation of vessel network. Even heterozygous Vegfa gene disruption is lethal, indicating that an appropriate amount of VEGF-A protein produced from two Vegfa alleles is necessary for the formation of a well-organized closed circulatory system in the body [2,4].
Most major human diseases are characterized by either excessive angiogenesis, which is common in most malignant tumours, or by poor blood supply, resulting in severe tissue ischaemia or cell death which is observed after strokes in brain and after myocardial infarction. Recent progress in the development of anti-angiogenesis drugs opened a new era in which anti-angiogenic medicine such as anti-VEGF-A neutralizing antibody or receptor tyrosine kinase inhibitors can be used for anticancer therapy. However, it is still not clear whether any of VEGF family members could be useful for pro-angiogenic therapy. In the present article, I summarize biological activities of VEGF-A and VEGF-E in vitro and in vivo (mostly in a transgenic mouse study), and discuss a therapeutic use of VEGF-E in ischaemic diseases.

Structure of VEGF-A and characteristics of its receptors
VEGF-A [or VPF (vascular permeability factor)] was isolated and characterized in the late 1980s, and found to be closely related to the PDGF (platelet-derived growth factor) family. The VEGF-A protein has been shown to be a potent angiogenic growth factor, and its receptor VEGFR-2 is essential for angiogenesis. VEGF-E was first discovered as a virus-encoded gene in the Orf virus, and its role in angiogenesis has been extensively studied in transgenic mouse models.
factor) family (reviewed in [2]). The PDGF/VEGF supergene family carries eight conserved cysteine residues, forming three intramolecular disulfide bonds and two intermolecular disulfide bonds. These intramolecular cross-links generate three loop structures, whereas intermolecular cross-links form and stabilize homodimeric structures.

VEGF-A binds and activates two tyrosine kinase receptors, VEGFR-1 [Flt (Fms-like tyrosine kinase)-1] and VEGFR-2 [KDR (kinase insert domain receptor) in humans/Flik-1 (fetal liver kinase 1) in mice], but not VEGFR-3 [Flt-4] [2,3,5] (Figure 1). When we isolated Flt-1 as a new receptor tyrosine kinase in 1990 [3], on the basis of its structural characteristics, we proposed that Flt-1 belonged to the PDGFR (PDGF receptor) supergene family, but was not a new member of the PDGFR family because of the seven immunoglobulin-like domains in the extracellular domain of Flt-1 instead of five immunoglobulin-like domains in PDGFRs.

In addition, a ∼60-amino-acid-long tyrosine kinase insert in the PDGFR family also exists in the kinase domain of Flt-1. Not only VEGFR-1 (Flt-1), but also two other VEGFRs conserve these characteristics, i.e. seven immunoglobulin-like domains and a long kinase insert. However, a TyrXaaXaaMet motif within the kinase insert sequence in the PDGFR family important for the activation of the PI3K (phosphoinositide 3-kinase) pathway is not present in any of these VEGFRs, strongly suggesting that intracellular signalling in the PDGFR and VEGFR families is different. We and others demonstrated that VEGFR-2, a major signal transducer for endothelial cell proliferation, utilizes only a little, if any, of the Ras pathway, and VEGFR-1 (Flt-1) is a high conservation of cysteine residues which exist in all PDGF/VEGF supergene family members. Six out of eight cysteine residues form three intramolecular disulfide bonds, making three loop structures. Keyt et al. [17] reported that a portion of loop 3 in VEGF-A (amino acid residues 82–86) is critical for its tight binding with VEGFR-2. Thus we asked whether loop 3 in VEGF-E is necessary and sufficient for the binding and activation of VEGFR-2. On the basis of results using a series of chimaeric constructs between VEGF-E and PDGF (placental growth factor), which only binds VEGFR-1, we showed that not only loop 3, but also loop 1 from the same VEGF species, i.e. a combination between loop 3 and loop 1, is necessary for the binding and activation of VEGFR-2. A combination between loop 3 of VEGF-E and loop 1 of VEGF-A or vice versa did not form any binding molecule. Therefore loop 1 and loop 3 of the same VEGF appear to interact properly with each other, and construct a three-dimensional structure which fits to the pocket on the ligand-binding domain of VEGFR-2 [18,19].

The VEGF gene is not present in the human genome, suggesting that a progenitor of the Orf virus may have captured an original VEGF gene from the genome of an animal, probably an animal other than a mammal, by recombination. An increased blood supply in skin must stimulate the rate of viral replication in the infected cells. Owing to the pro-angiogenic and pro-viral replication activities of VEGF-E, the Orf viral genome is likely to have maintained this gene for a long period of time.

Structure of VEGF-E and its receptor
In 1994, Lyttle et al. [12] reported a gene in the NZ7 Orf viral genome, which is distantly related to the VEGFA gene. The degree of amino acid identity between VEGF-A and NZ7 VEGF-like protein is approx. 29%, whereas that between PDGF and NZ7 protein is approx. 17%. Orf virus, a parapoxvirus, is known to infect sheep, goats and humans, and transiently stimulates angiogenesis at the infected area in the skin, suggesting that the gene product has similar biochemical and biological activities to those of VEGF-A. To clarify these points, we purified the protein and examined its activity. Surprisingly, we found that Orf VEGF-like protein tightly binds and stimulates VEGFR-2 similarly to VEGF-A, but not VEGFR-1 or VEGFR-3 [13]. Since this characteristic is unique among VEGF family members, we proposed the name VEGF-E for Orf-virus-derived protein (Figure 1). Several Orf viral strains such as NZ2, NZ7 and D1701 were reported to carry VEGF-E genes, but each amino acid sequence is a little different from each other [14–16]. Therefore the names VEGF-ENZ2, VEGF-ENZ7 and VEGF-ED1701 have been used for the VEGF-Es from different viral strains.

Although different VEGF-E subtypes differ in their ability to bind neuropilin-1, a common characteristic of the VEGF-E family is strong binding and stimulatory activities to VEGF-R2 without binding with other VEGFRs. Another important common feature between VEGF-Es and VEGF-A is a high conservation of cysteine residues which exist in all PDGF/VEGF supergene family members. Six out of eight cysteine residues form three intramolecular disulfide bonds, making three loop structures. Keyt et al. [17] reported that a portion of loop 3 in VEGF-A (amino acid residues 82–86) is critical for its tight binding with VEGFR-2. Thus we asked whether loop 3 in VEGF-E is necessary and sufficient for the binding and activation of VEGFR-2. On the basis of results using a series of chimaeric constructs between VEGF-E and PDGF (placental growth factor), which only binds VEGFR-1, we showed that not only loop 3, but also loop 1 from the same VEGF species, i.e. a combination between loop 3 and loop 1, is necessary for the binding and activation of VEGFR-2. A combination between loop 3 of VEGF-E and loop 1 of VEGF-A or vice versa did not form any binding molecule. Therefore loop 1 and loop 3 of the same VEGF appear to interact properly with each other, and construct a three-dimensional structure which fits to the pocket on the ligand-binding domain of VEGFR-2 [18,19].

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In vitro and in vivo angiogenic activities associated with VEGF-A and VEGF-E
Since VEGF-E and VEGF-A have a high affinity for VEGFR-2 which is a major positive signal transducer towards DNA synthesis, it was not surprising that both proteins showed a similar stimulatory activity in the proliferation of primary vascular endothelial cells in culture [13]. However, since VEGF-A, but not VEGF-E, binds and activates VEGFR-1, it is open to question whether these two proteins have a similar activity in vivo.
Using a K14 (keratin-14)-promoter-driven transgenic mouse system, we found that K14–VEGF-ENZ7 transgenic mice had a slight reddish skin colour and 5–10-fold increase in angiogenesis in subcutaneous tissues. Angiogenesis was basically well organized, and pericytes were closely attached with endothelial cells [20]. Since several groups reported that K6 (keratin-6)- or K14-driven VEGF-A transgenic mice show severe oedema, inflammation and even angiomata-like lesions as well as angiogenesis [21–23], it is of interest to see whether another VEGFR-2-stimulatory ligand, VEGF-E, has a different phenotype in vivo compared with VEGF-A.

To avoid any minor differences at the expression plasmid vector, we simply replaced VEGFE cDNA with human VEGFA165 cDNA in the same K14 vector, and made another transgenic mouse in which the product is changed from VEGF-E to VEGF-A. Surprisingly, K14–VEGF-A transgenic mice had various adverse effects: perinatal death, a lower growth rate in a high-VEGF-A-expressing mouse, local inflammation, tissue oedema and leakiness of blood vessels in association with an increased dermal capillary network [24] (Figure 2). These results are consistent with those reported by others [21–23]. In addition, lymphangiogenesis and lymph vessel dilatation were observed in VEGF-A transgenic mice, but not in VEGF-E mice [25]. As a control, K14–PlGF transgenic mice showed a minor, 2–3-fold, increase in angiogenesis in the skin [20].

In histological section, macrophage-lineage cells were infiltrated in subcutaneous tissues in VEGF-A transgenic mice, and mRNA levels of inflammatory cytokines such as TNFα (tumour necrosis factor α) and IL-6 (interleukin 6) were up-regulated at the mRNA level in the skin [24]. Levels of these cytokines were very low, if any, in VEGF-E transgenic mice. Since the major difference in biochemical activities between VEGF-A and VEGF-E is that VEGF-A alone can activate VEGFR-1, these adverse effects seen in VEGF-A transgenic mice may be, at least partly, derived from activation of VEGFR-1.

Vascular permeability activity of VEGF-A, VEGF-E and Trimeresurus flavoviridis snake-venom-derived VEGF

VEGF-A transgenic mice showed a hypervascular permeability, whereas VEGF-E transgenic mice did not. Several snake venoms were reported to have a vessel-targeting molecule and stimulate vascular permeability. To know which molecule has this activity, we attempted to purify it from the venom of habu snakes living in southern islands in Japan. We isolated a protein and its cDNA from snake-venom tissue, and found that it is another VEGF-like protein which bears a strong vascular permeability activity but a weak endothelial cell-proliferation activity [26]. This molecule, named Trimeresurus flavoviridis snake-venom-derived VEGF (T.f. svVEGF) tightly binds and activates human VEGFR-1, but VEGFR-2 only weakly. Since PlGF, which activates VEGFR-1, has only one-tenth the permeability-inducing activity of VEGF-A, a strong

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permeability signal is thought to be generated from a combination of highly activated VEGFR-1 and weakly activated VEGFR-2.

Thus hypervascular permeability in VEGF-A transgenic mice appears to be derived from a combined activation of VEGFR-1 and VEGFR-2 which does not occur in VEGF-E transgenic mice.

**Relationship between inflammation and VEGF**

Barleon et al. [27] and Clauss et al. [28] reported that VEGFR-1 mRNA is expressed in monocyte/macrophage-lineage cells, and it was up-regulated in LPS (lipopolysaccharide) activation. The VEGFR-1-specific ligand PlGF stimulated migration of these cells. Sawano et al. [29] clearly showed that the VEGFR-1 protein is expressed as a cell-surface molecule by using a monoclonal antibody against human VEGFR-1. This indicates that the VEGFR-1 signal is important for recruitment of inflammatory cells such as macrophages and stimulation of inflammatory response. A mouse model for rheumatoid arthritis demonstrated that chronic inflammation, including macrophage recruitment to the knee-joint region, was significantly suppressed when the arthritis model mice were crossed with VEGFR-1 signal-deficient genetic mice, i.e. VEGFR-1 (Flt-1) tyrosine-kinase-minus (VEGFR-1 TK\(^{-/-}\)) mice [30].

**Involvement of VEGFR-1 in lymphangiogenesis via recruitment of macrophages**

Lymphangiogenesis is mainly regulated by the VEGF-C/VEGF-D and VEGF-3 system. Angiopoietin1-Tie2 is also shown to involve this process. Although VEGF-A does not bind VEGFR-3, K14–VEGF-A transgenic mice showed lymphangiogenesis in the skin [25]. One possible mechanism for that is an effect of the VEGF-A–VEGFR-2 system, since VEGFR-2 is expressed at lower levels in LECs (lymphatic endothelial cells). However, this seems unlikely because an overexpression of a VEGF-2-specific ligand, VEGF-E, did not induce lymphangiogenesis at detectable levels in K14 transgenic mice. Another possibility is an indirect effect of the activation of VEGFR-1, since VEGFR-1 is not expressed in LECs. A significant decrease in lymphangiogenesis as well as lymph vessel dilatation in K14–VEGF-A-tg/VEGFR-1 TK\(^{-/-}\) transgenic mice supports this possibility [25].

Macrophages and vascular endothelial cells are two major cell types which express VEGFR-1, and macrophages are known to secrete lymphangiogenic ligands VEGF-C/VEGF-D [31,32]. Thus the question arises of whether bone-marrow-derived macrophage-lineage cells and VEGFR-1 signal in these cells play an important role in lymphangiogenesis in VEGF-A transgenic mice. Transplantation of bone marrow cells obtained from VEGFR-1 TK\(^{-/-}\) mice into irradiated K14–VEGF-A transgenic wild-type mice demonstrated a decrease in lymphangiogenesis as well as less recruitment of macrophages into subcutaneous tissues [25]. These results indicate that the VEGFR-1 signal is involved in lymphangiogenesis indirectly via recruitment of macrophages.

**Therapeutic use of VEGF-E for ischaemic diseases**

Severe ischaemia and low oxygen/nutrition occur mainly in heart and brain in humans, after myocardial infarction and brain stroke. To rescue such pathological conditions, pro-angiogenesis therapy using members of the VEGF family is promising. However, the use of VEGF-A is associated
with risk of increased oedema and leakiness of blood vessels, resulting in high interstitial fluid pressure. Furthermore, VEGF-A frequently stimulates inflammation by promoting macrophage migration (Figure 2). VEGF-E (or a humanized form with lower antigenicity) may be better able to stimulate the formation of mature blood vessels and well-organized vessel networks, while promoting less vessel permeability and only a minor inflammatory response [33,34].

Conclusion and future prospects

In physiological angiogenesis, VEGF-A binds both VEGFR-1 and VEGFR-2, regulating organized blood vessel formation. However, in therapeutic angiogenesis, VEGF-A may induce critical problems such as oedema and inflammation mostly via activation of VEGFR-1. VEGF-E family members bind VEGFR-2, but not VEGFR-1, thus exhibiting very low levels of adverse effects such as oedema and inflammation. Further studies on this new member of the VGF family with regard to its clinical applications are warranted. In addition, other angiogenic molecules such as modified angiopoietin, FGF (fibroblast growth factor) and HGF (hepaticocyte growth factor) in combination with VEGF-E should also be studied with the aim of developing a better strategy for pro-angiogenic therapy [35,36].

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