*Review Article*

# Vascular endothelial growth factor receptor-2: Its unique signaling and specific ligand, VEGF-E

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**Vascular endothelial growth factor receptor-2 (VEGFR-2/KDR/Flk-1) is a high-affinity receptor for vascular endothelial growth factor-A (VEGF-A), and mediates most of the endothelial growth and survival signals from VEGF-A. VEGFR-2 has a typical tyrosine kinase receptor structure with seven immunoglobulin (Ig)-like domains in the extracellular region, as well as a long kinase insert in the tyrosine kinase domain. It utilizes a unique signaling system for DNA synthesis in vascular endothelial cells, i.e. a phospholipase Cγ-protein kinaseC-Raf-MAP kinase pathway. Although VEGF-A binds two receptors, VEGFR-1 and -2, a newly isolated ligand VEGF-E (Orf-virus-derived VEGF) binds and activates only VEGFR-2. Transgenic mice expressing VEGF-$E_{NZ-7}$ showed a dramatic increase in angiogenesis with very few side effects (such as edema and hemorrhagic spots), suggesting strong angiogenic signaling and a potential clinical utility of VEGF-E. VEGF family members bear three loops produced via three intramolecular disulfide bonds, and cooperation between loop-1 and loop-3 is necessary for the specific binding and activation of VEGFR-2 for angiogenesis. As it directly upregulates tumor angiogenesis, VEGFR-2 is an appropriate target for suppression of solid tumor growth using exogenous antibodies, small inhibitory molecules and *in vivo* stimulation of the immune system. (Cancer Sci 2003; 94: 751–756)**

## 1. Background

Angiogenesis is an important biological process not only under physiological conditions but also in a variety of diseases including cancer, diabetic retinopathy and rheumatoid arthritis.[1, 2] Among angiogenic factors reported so far, vascular endothelial growth factor-A (VEGF-A) appears to mediate the basic signaling of angiogenesis, particularly signals for endothelial cell growth and survival *in vivo*.[3] VEGF-A belongs to the platelet-derived growth factor (PDGF) supergene family, and as a homodimer, binds to, and activates two tyrosine kinase receptors, vascular endothelial growth factor receptor (VEGFR)-1 (Flt-1) and VEGFR-2 (KDR/Flk-1 in mice)[4] (Fig. 1). In addition to these tyrosine kinase receptors, a subtype of VEGF-A, VEGF-$A_{165}$, binds another membrane protein, neuropilin-1.[5] Knockout studies indicate that VEGFR-2 is the major signal transducer for the differentiation of endothelial cells from precursor mesodermal cells and the growth of endothelial cells in early embryogenesis, whereas VEGFR-1 plays different roles, negative and regulatory, in angiogenesis at this stage.[6, 7] Questions to answer are (1) what signaling pathways does VEGFR-2 utilize for a variety of VEGF-A-mediated biological activities? and (2) what biological effects are generated after the activation of VEGFR-2 alone without activation of VEGFR-1? Furthermore, (3) which receptor, VEGFR-1 or VEGFR-2, is responsible for stimulating pathological angiogenesis? Accumulating evidence suggests that more than half of pathological angiogenesis involves the activation of VEGFR-2. While VEGFR-1 mediates signaling for less than half, it plays a major role in the inflammatory cell/macrophage-dependent process in a variety of diseases such as rheumatoid arthritis and atherosclerosis.[8, 9] This mini-review focuses on the structure, specific ligand and functions of VEGFR-2.

## 2. Structure and gene expression of VEGFR-2

**a) Domain structure of VEGFR-2.** VEGFR-2 was first isolated by Terman *et al.* in 1991[10] and was named Kinase-insert domain containing receptor (KDR), its overall structure being highly homologous to that of Flt-1/VEGFR-1.[11] The murine *VEGFR-2* gene was independently isolated by two research groups[12, 13] and named *flk-1*. Like VEGFR-1, VEGFR-2 bears an extracellular region with seven immunoglobulin (Ig)-like domains, a transmembrane domain and a tyrosine kinase domain with an about 70-amino-acid insert. The approximately 40-amino-acid stretch at the carboxyl terminus of murine Flk-1 originally reported highly diverged from those of human VEGFR-2 and VEGFR-1. However, this apparent heterogeneity was due to extreme difficulty in nucleotide sequencing of this region, and revision of the amino acid sequence resulted in higher homology to both human VEGFR-2 and -1.

Non-vertebrate animals such as *Drosophila* carry a single 7-Ig type tyrosine kinase receptor (*D-VEGFR*) gene without the 5-Ig type receptor genes.[14] Therefore, it is likely that all of the 7-Ig/5-Ig tyrosine kinase receptors in mammals, including the three VEGFRs and five PDGFR-related receptors (two PDGFRs and three Fms family members, c-Fms/c-Kit/Flt-3), were phylogenetically generated from a single ancestral 7-Ig tyrosine kinase receptor gene.[15]

Unlike the *VEGFR-1* gene, which encodes a short message for a soluble form in addition to the full-length message,[11, 16] the *VEGFR-2* gene encodes only one message for the full-length receptor of 1357 amino acids. Within the cell, VEGFR-2 protein is produced as a 150-kDa protein without significant glycosylation, then rapidly processed to a protein of about 200 kDa with glycosylation. This 200-kDa molecule is further glycosylated to a mature 230-kDa form, and expressed on the cell surface.[17] The 200-kDa VEGFR-2 is mostly expressed within the cell, possibly in the Golgi apparatus, since exogenously added VEGF-A usually induces autophosphorylation of only the 230-kDa form of VEGFR-2.[17]

**b) Ligand binding region.** Previous studies on the PDGFR family revealed that the first three Ig-like domains constitute the region for direct binding with the ligand, while the fourth Ig-domain is responsible for dimerization of the receptor and an increase in affinity to the ligand. As in the case of PDGFR, deletion analysis of the VEGFR-2 ligand binding domain has shown that the second and third Ig-like domains compose the high-affinity ligand binding region.[18, 19] The first Ig-like do-

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main appears to have a negative regulatory role. Interestingly, the $K_d$ of VEGFR-2 for VEGF-A expressed on the cell surface is about one order of magnitude weaker than that of VEGFR-1. Furthermore, the $K_d$ of artificially created soluble forms of VEGFR-2 such as 7-Ig-sVEGFR-2 without an immunoglobulin Fc region at the carboxyl terminus was much (about 100-fold) lower than that of the soluble form of VEGFR-1. The biological significance of this large difference in the affinity to VEGF-A between VEGFR-2 and -1 is not clear, but one possible explanation is that a third molecule such as neuropilin could up-regulate the affinity between VEGFR-2 and VEGF-A ligand, creating flexibility in the intracellular signaling from VEGFR-2.

**c) Tyrosine kinase domain and carboxyl terminal region.** The structure of the VEGFR-2 tyrosine kinase domain is typical, with about 35% identity at the amino acid level to v-Src and 56% to PDGFR. An important characteristic of VEGFR-2 is the presence of a long kinase insert (KI) in the middle of the tyrosine kinase domain, and the length and position of the KI are very similar to those in PDGFR. However, as Kondo *et al.*[20] first showed for *VEGFR-1*, the exon-intron organization of the KI region in the VEGFR family is different from that in PDGFR family genes. This suggests that significant genomic rearrangement occurred during the formation of 5-Ig tyrosine kinase receptor genes such as *PDGFR* from 7-Ig receptor (*VEGFR*) genes in the phylogenetic development of animals.

In agreement with this hypothesis, the amino acid sequence of the KI in VEGFR-2 is highly divergent from that of the KI in PDGFR. For example, two tyrosine residues with the motif Y-x-x-M or Y-M-x-M in the KI of PDGFR, which are crucial for the binding of the PI3K-p85 subunit and activation of PI3K, do not exist in the KI of VEGFR-2.

In the carboxyl-terminal region, 6 tyrosine residues are present in VEGFR-2, and most of them are conserved among the vertebrates (Fig. 2). As discussed below, at least one of the tyrosines is essential for the critical signaling for DNA synthesis.

**d) Gene expression of VEGFR-2.** The gene expression of VEGFR-2 in mice starts around E7.0 in the posterior mesodermal area of the embryo, appearing as a broad band, then a part of the VEGFR-2-expressing cells migrate into the yolk sac to form blood islands.[6,21] The primitive endothelial cells surrounding hematopoietic cells in each blood island express a high level of VEGFR-2, and start to form a primitive blood vessel network with a tubular structure. VEGFR-2 is specifically expressed on the endothelial cells, endothelial cell progenitor cells at embryonal stages and a variety of vascular endothelial cells at adult stages.[22,23] Furthermore, the endothe-

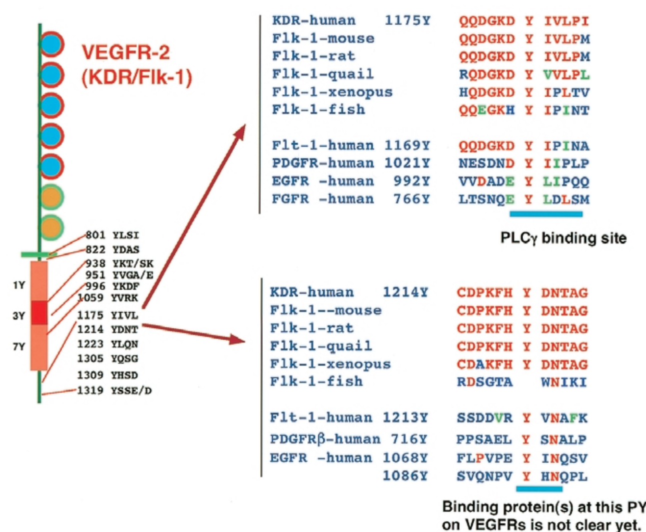


**Fig. 2.** Structure of VEGFR-2 and phylogenetic conservation of the 1175-tyrosine-containing region among vertebrates except for fish. The 1175-tyr and 1214-tyr are two major autophosphorylation sites on human VEGFR-2. The 1175-PY region is the PLCγ binding site, but the protein(s) that binds to the 1214-PY region is unknown.

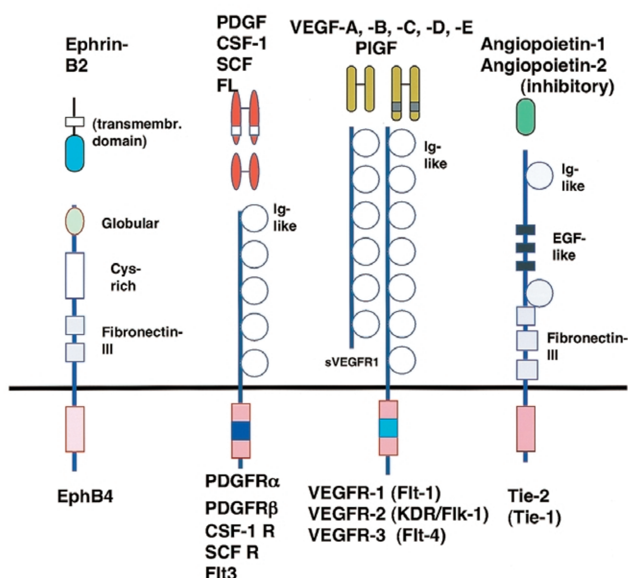


**Fig. 1.** A schematic representation of major receptor-type tyrosine kinases and their ligands related to angiogenesis. VEGF-A binds VEGFR-1 and -2, VEGF-C and -D bind VEGFR-3 (and weakly bind VEGFR-2), whereas VEGF-E specifically binds VEGFR-2. PlGF and VEGF-B are ligands specific to VEGFR-1.

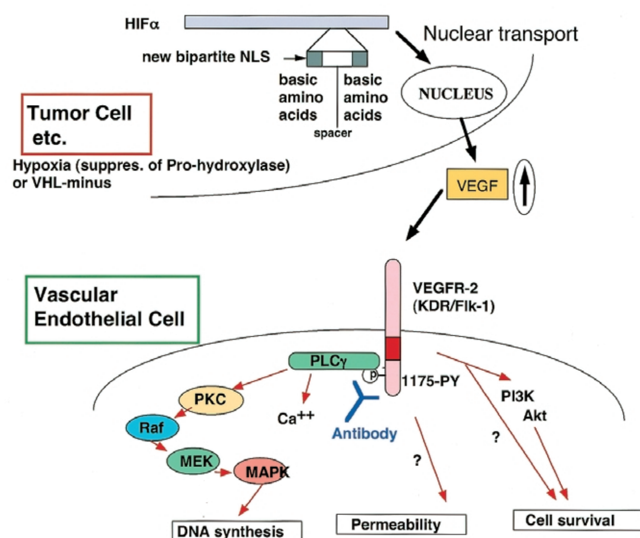


**Fig. 3.** Signal transduction of VEGFR-2 for endothelial cell proliferation via the PLCγ-PKC-MAP kinase pathway. Inhibitors specific to VEGFR-2 tyrosine kinase or antibodies which block the PLCγ binding to 1175-PY region efficiently suppress this signaling pathway. In a normoxic condition, HIFα proteins (HIF1α, 2α, 3α) are structurally modified by prolyl hydroxylase, and degraded via the ubiquitin ligase pathway in a VHL-dependent manner. In a hypoxic condition, which suppresses prolyl hydroxylase activity, or in VHL-minus genetic background (von Hippel-Lindau disease), HIFα proteins are stabilized, resulting in nuclear localization and induction of hypoxia-responsive genes such as VEGF-A.

lial cells with filopodia guiding the neovascularization in murine neonatal retina also appear to express VEGFR-2, even though these cells have yet to form a typical tubular structure.[24) In hematopoietic cells, Eichmann *et al*. demonstrated that hematopoietic stem cell-like cells budding from endothelial cells in chicken embryo express VEGFR-2.[25) Similar phenomena were reported in mouse embryo.

At adult stages, endothelial cell progenitor cells were reported to exist among bone marrow cells which express VEGFR-2. However, it is not yet clear what percentage of bone marrow cells is endothelial cell progenitor cells, or how strongly these cells express VEGFR-2.[26)

### 3. Signal transduction via VEGFR-2

The tyrosine kinase receptor family has about 50 members, including Insulin R/IGF-1R, the EGFR family, 7-Ig/5-Ig receptors (VEGFRs and the PDGFR family), FGFRs, HGF-Rs, NGFRs, and Eph receptors. Most of these tyrosine kinase receptors, except for VEGFRs and Eph receptors, have the potential to transform non-transformed cells, such as NIH3T3 cells. Also, basically all the transforming tyrosine kinases utilize the Ras activation pathway for cell transformation.

Since overexpression of VEGFR-1 or VEGFR-2 in NIH3T3 cells failed to transform these cells even in the presence of a high concentration of VEGF-A,[27–29) we hypothesized that VEGFRs, particularly the major positive signal transducer VEGFR-2, utilize a unique signaling pathway for endothelial cell proliferation. Xia *et al*. suggested the possible involvement of a protein kinase C pathway downstream from VEGF-A based on experiments using a PKC inhibitor.[30) We have directly shown that the binding with VEGF-A and activation of VEGFR-2 induce the binding of phospholipase Cγ (PLCγ) to VEGFR-2 via its carboxyl-terminal SH2 domain, as well as strong phosphorylation at tyrosine.[31) Then, the activated PLCγ efficiently activates protein kinase C (particularly PKCβ), and stimulates the Raf-MEK-MAP kinase pathway[31) (Fig. 3).

To our surprise, given the general pathway of cell growth signaling via tyrosine kinases (but consistent with the lack of transforming activity of VEGFR-2), after stimulation with VEGF-A, Ras was activated only very weakly, if at all, in both

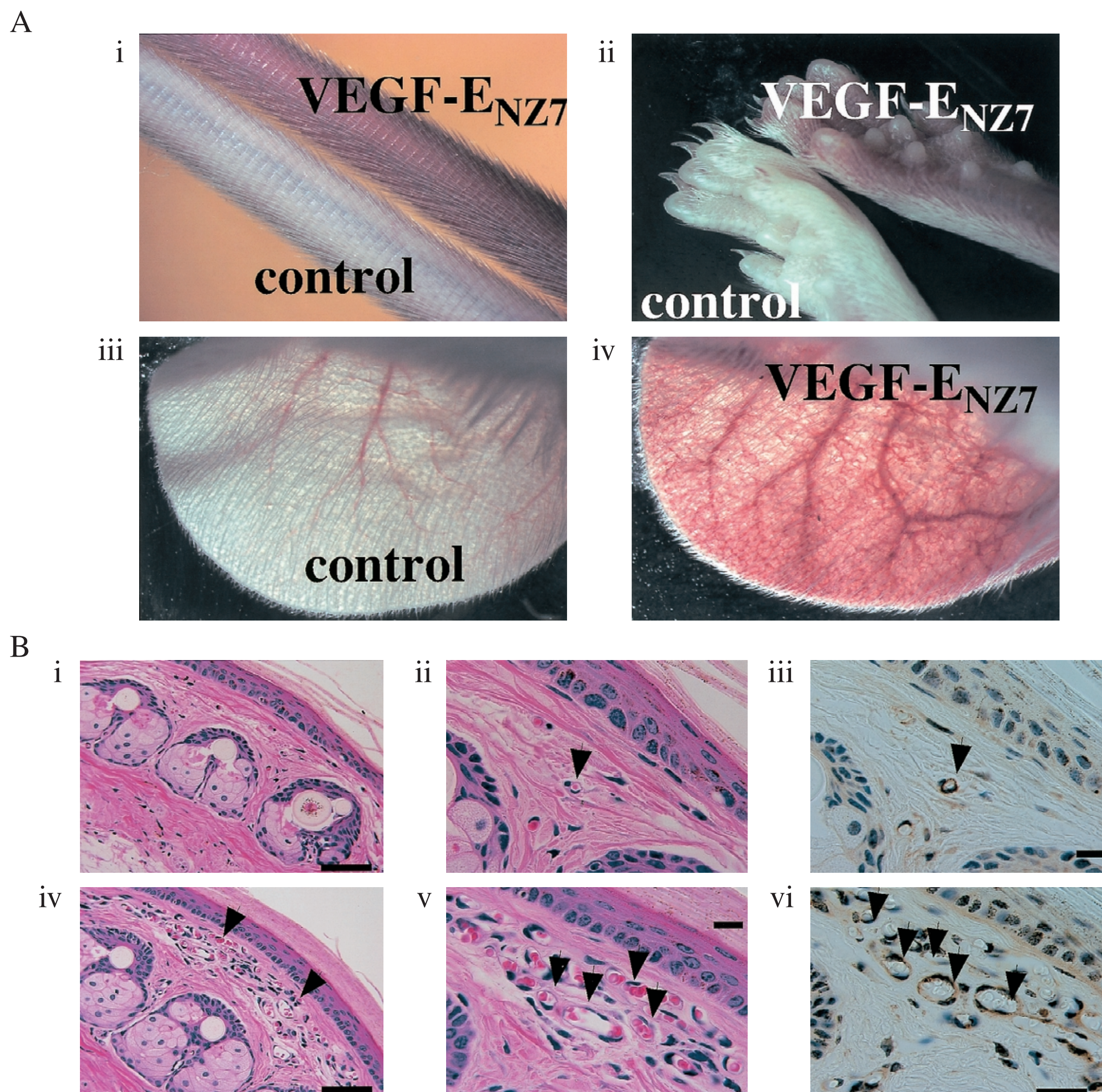


**Fig. 4.** VEGF-E$_{NZ-7}$ transgenic mice show significant neovascularization in the skin without clear side effects. A. Tail (i), toes (ii), and ear (iv) of VEGF-E$_{NZ-7}$ transgenic mice exhibit significant neovascularization without clear edema or hemorrhagic spots. B. Microscopic analysis of the tail in VEGF-E$_{NZ-7}$ transgenic mice. About a 10-fold increase in angiogenesis was observed in the transgenic mice (iv–vi) compared with control mice (i–iii). i, ii, iv and v, H-E staining; iii and vi, immunostaining for vWF.[40)

primary endothelial cells and VEGFR-2-overexpressing NIH3T3 cells.[32] In general when tyrosine kinases activate Ras, the tyrosine kinase itself or a tyrosine kinase-adaptor complex recruits Grb2 or Shc-Grb2 complex, which further recruits Sos, a nucleotide exchange factor for Ras, to stimulate Ras to GTP-bound form. However, after binding with VEGF-A, the activated VEGFR-2 did not efficiently recruit Grb2 or Shc-Grb2 to the receptor. This is consistent with the very weak activation of Ras downstream of VEGF-A signaling.[32]

A PI3K inhibitor, wortmannin, did not significantly suppress VEGF-dependent DNA synthesis of endothelial cells in a 20-h culture system. However, treatment of cells with PI3K inhibitor for longer than 48 h suppressed endothelial cell proliferation, most likely due to its strong inhibition of the survival signal from focal adhesion.

In a series of analyses on the effects of tyrosine to phenylalanine mutation in VEGFR-2 and tryptic phosphotyrosine-containing peptides, we have determined that a single autophosphorylation site, the 1175-tyrosine residue in human VEGFR-2, is the major site for the activation of the PLCγ-PKC-MAP kinase pathway[31] (Fig. 3). As expected, 1175-tyr or the corresponding tyrosine (for example, 1173-tyr in mice) is well conserved in VEGFR-2 in vertebrates (except for fish Flk-1). On the other hand, one report suggested that Ras is activated downstream from VEGFR.[33] The reason for this discrepancy is not clear.

One should point out, however, that primary endothelial cells do contain Ras protein. It is well known that Ras is deeply involved in the focal adhesion-FAK/c-Src/actin pathway in many cell types. Thus, it is reasonable that Ras in endothelial cells functions in the cell-to-matrix and cell-to-cell attachment and signaling of cell migration. Survival signaling for endothelial cells from the VEGF receptor is reported to involve the PI3K-Akt pathway.[34] Since the signal to activate PI3K by VEGFR-2 is usually not very strong, it is possible that another pathway is also used for this signal.

## 4. Biological functions of VEGFR-2

VEGF-A which activates both VEGFR-1 and -2 stimulates (1) endothelial cell proliferation, (2) tubular formation, (3) endothelial cell survival, (4) endothelial cell migration, (5) vascular permeability *in vivo*, (6) gene expression of Ets-1, tissue factor, PAI-1, matrix metalloproteinases, etc., and (7) monocyte/macrophage migration. Based on the results obtained using VEGF-E (see below), receptor-specific VEGF-A mutants and VEGFR-1-specific ligand PlGF and VEGF-B, part of the signaling for (1) to (6) and most of that for (7) appear to be mediated by VEGFR-1, while most of the signaling for (1) to (6) is mediated through VEGFR-2.[35]

## 5. VEGFR-2-specific ligand, VEGF-E

To clarify the biological functions influenced by each VEGFR, we searched for a novel type of VEGF, and found that the Orf virus genome-encoded VEGF-like gene[36] encodes a protein (designated VEGF-$E_{NZ-7}$) that specifically binds at high affinity and activates VEGFR-2.[37] Consistent with the lack of ability of radio-labeled VEGF-$E_{NZ-7}$ to bind VEGFR-1 overexpressed in NIH3T3 cells, soluble VEGFR-1 (sFlt-1), a potent inhibitor of VEGF-A, failed to suppress the biological activity of VEGF-$E_{NZ-7}$. Interestingly, VEGF-$E_{NZ-7}$ does not bear a typical heparin-binding domain with a basic amino acid stretch, or bind neuropilin, but it still exhibits high-affinity binding to VEGFR-2. VEGF-$E_{NZ-7}$ showed activity to promote endothelial cell growth and survival at similar levels to VEGF-$A_{165}$, and had a slightly less potent effect on vascular permeability than VEGF-$A_{165}$.

Two other VEGF-E family members, VEGF-$E_{NZ-2}$ and VEGF-$E_{D1701}$ were subsequently isolated,[38, 39] both with essentially the same activity as VEGF-$E_{NZ-7}$. However, VEGF-$E_{NZ-2}$ was reported to have heparin-binding activity, although it is not yet clear which amino acid stretch is responsible for this activity. It should be noted that VEGF-E family members show only 20 to 25% amino acid identity with VEGF-A, though the $K_d$ for VEGFR-2 is almost the same as that of VEGF-$A_{165}$ for VEGFR-2.

To further compare the biological activities of VEGF-E and VEGF-A, we generated K14-promoter driven VEGF-$E_{NZ-7}$ transgenic (Tg) mice.[40] Gene expression of VEGF-$E_{NZ-7}$ in the basal cell layer of the skin of these mice induced a strong angiogenic response, about a 10-fold increase compared to the control mice. To our surprise, edematous lesions and hemorrhagic spots on the ear and other dermal tissues which were reported as side effects in VEGF-A Tg mice[41] were not detectable in VEGF-E Tg mice (Fig. 4). The endothelial cell-cell junction as well as the recruitment of pericytes to endothelial cells was well organized. The mechanisms behind the strong angiogenic activity with fewer side effects of VEGF-$E_{NZ-7}$ *in vivo* are worthy of further study. At least two explanations are possible: (1) without the support of neuropilin, VEGF-$E_{NZ-7}$ binds at high affinity with and continuously activates VEGFR-2, resulting in a tight endothelial cell-to-cell junction with less leakage, and (2) the functions of lymphatic vessels such as the absorption of fluid in tissues are well maintained in VEGF-E

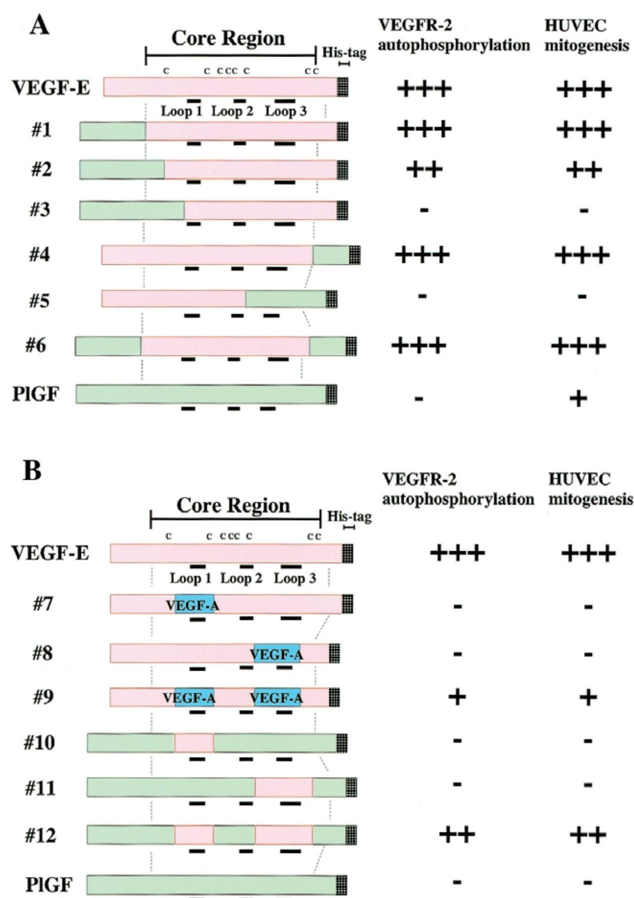


**Fig. 5.** The set of loop-1 and -3 structures in VEGF-$E_{NZ-7}$ is essential for the activation of VEGFR-2 signaling. A. The amino-terminal and carboxyl-terminal regions can be replaced with the corresponding sequences of PlGF without suppression of VEGFR-2-stimulatory activity. B. Cooperation between loop-1 and -3 in VEGF-E (chimera #12) or VEGF-A (chimera #9) is crucial for the binding and activation of VEGFR-2.[44] Sequences derived from VEGF-E, PlGF and VEGF-A are indicated by light-brown, green and blue color, respectively.

Tg mice, leading to an appropriate balance between leaks from newly formed blood vessels and absorption by lymph vessels.

One significant characteristic of VEGF-$E_{NZ-7}$ is its very strong affinity to VEGFR-2 despite a low amino acid homology to VEGF-A. Thus, we attempted to clarify which domain in VEGF-E is essential for the construction and organization of a high-affinity tertiary structure for the ligand-binding region of VEGFR-2. All of the VEGF/PDGF supergene family products have eight cysteines in a monomer polypeptide. Six cysteines are used for intramolecular S-S bonds, and the other two for inter-molecular S-S bonds to form a dimer structure.[42] Thus, three loops are formed within a monomer, loop-1, loop-2 and loop-3.

Keyt *et al*. showed that a few basic amino acids in loop-3 of VEGF-A are essential for binding to VEGFR-2 since mutation of these amino acids removed the binding activity.[43] However, none of these basic amino acids is conserved at the same position in the VEGF-E family, suggesting that they are not an absolute requirement for ligand-VEGFR-2 binding. Using a series of chimeric constructs of VEGF-E, VEGF-A and PlGF, we found that cooperation between loop-1 and loop-3 in VEGF-E or VEGF-A is crucial for the appropriate tertiary structure needed for high-affinity binding to VEGFR-2.[44] A combination of loop-1 from VEGF-E and loop-3 from VEGF-A or *vice versa* did not work, suggesting that combining loop-1 and loop-3 from the same molecule, VEGF-E or VEGF-A, is important (Fig. 5).

Since VEGF-$E_{NZ-7}$ induces significant angiogenesis *in vivo* with few side effects, VEGF-E family members should be carefully studied as candidates for a potential angiogenic factor for clinical use in pro-angiogenic therapy.

### 6. VEGFR-2 as a target for suppression of solid tumor growth

A large body of evidence indicates that the VEGF-VEGFR system is involved in the pathological angiogenesis which promotes the malignancy of solid tumors, diabetic retinopathy and rheumatoid arthritis. VEGFR-1 (Flt-1) is involved in inflammatory diseases and tissue-specific metastasis of cancer,[45,46] while the VEGF-A and VEGFR-2 system is a direct target in the suppression of pathological angiogenesis. Anti-VEGF-A monoclonal antibody is now under phase III clinical trial, and monoclonal antibody against VEGFR-2 also has a suppressive effect on solid tumor growth in mice.[47] Many pharmaceutical companies are developing small molecules which specifically block the tyrosine kinase activity of VEGFR-2,[48,49] although most compounds block in parallel the tyrosine kinase activity of VEGFR-1 due to the high structural homology between the two receptors.

Another target for regulating VEGFR-2 signaling could be the critical autophosphorylation site on the receptor. Downstream signaling is an important target for the suppression of endothelial cell proliferation via the VEGF-A-VEGFR-2 pathway.[31] Furthermore, a recent report suggests that oral administration of a bacterial-type vector (non-toxic *Salmonella typhimurium*) containing VEGFR-2-expressing vector DNA induces an immune response to VEGFR-2, leading to an efficient inhibition of solid tumor growth in the immunized animals.[50] In the near future we may have an appropriate tool or strategy for regulating the activity of VEGFR-2 and thereby blocking severe pathological angiogenesis.

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