Vascular endothelial growth factor (VEGF) mediates endothelial cell proliferation, angiogenesis, and vascular permeability via the endothelial cell receptors, KDR/Flk-1 and Flt-1. Recently, a gene encoding a polypeptide with about 25% amino acid identity to mammalian VEGF was identified in the genome of Orf virus (OV), a parapoxvirus that affects sheep and goats and occasionally, humans, to generate lesions with angiogenesis. In this study, we examined the biological activities and receptor of OV-derived NZ-7 VEGF (VEGF-E). VEGF-E was found to be a dimer of about 20 kDa with no basic domain and affinity for heparin column, similar to VEGF subtype. VEGF121 has 100-fold less endothelial cell mitotic activity than VEGF165 due to lack of a heparin-binding basic region. Interestingly, however, VEGF-E showed almost equal levels of mitotic activity on primary endothelial cells and vascular permeability activity as VEGF165. Furthermore, VEGF-E bound KDR/Flk-1 (VEGFR-2) and induced its autophosphorylation to almost the same extent as VEGF165, but did not bind Flt-1 (VEGFR-1) nor induced autophosphorylation of Flt-1. These results indicate that VEGF-E is a novel type of endothelial growth factor, utilizing only one of the VEGF receptors, and carrying a potent mitogenic activity without affinity to heparin.

Recently, several VEGF-related genes have been isolated and characterized. Placenta growth factor is suggested to be involved in the growth and maintenance of placental tissue, and VEGF-C was shown to carry a lymphangiogenic activity different from VEGF (6, 7). VEGF-D is also suggested to have an activity similar to VEGF-C (8). However, other genes such as VEGF-B and NZ-type VEGFs encoded in the open reading frames of Orf virus NZ-2 and NZ-7 strains are well characterized (9).

Human VEGF has at least four subtypes due to an alternative splicing, VEGF121, VEGF165, VEGF189, and VEGF206 (10,11). Among these, VEGF165 has the most potent biological activity and is the most abundant subtype in vivo with a few exceptions such as in placenta. VEGF121 is also well expressed in many normal and pathological tissues, however, its biological activity has been shown to be 100-fold weaker than that of VEGF165. The only difference between these two VEGF subtypes is that VEGF165 carries an exon 7-derived 44-amino acid stretch near the carboxyl-terminal region, which is enriched with basic amino acid residues and has affinity for heparin and heparan sulfates (12). Since VEGF165 has little affinity for cell surface and has a low concentration of heparin, could augment the biological activity of VEGF, this basic stretch is considered to be crucial for generation of strong mitogenic signals via VEGF165 (13-15).

VEGF binds two endothelial cell-specific receptors, Flt-1 and KDR/Flk-1 (tyrosine kinases 16–19). In addition, the exon 7-derived basic stretch in VEGF165 has shown to bind to a 130-kDa cell surface molecule (20). Thus, this molecule might also play a role in VEGF signaling, although the mechanism of this action has yet to be elucidated. We are interested in Orf virus NZ strain-encoded VEGFs since the possible gene products have only about 25% amino acid identity with VEGF and have no apparent basic domain, but seem to be involved in the process of pathological angiogenesis in virus-infected lesions.

OV is a linear double-stranded DNA virus and a member of the parapoxvirus genus of the Poxvirus family. It causes contagious pustular dermatitis in sheep and goats and is transmissible to humans by direct contact. Lesions appear after an incubation period of approximately 1 week as hemorrhagic bullae. They may reach several centimeters in diameter, but fade spontaneously after several weeks. Histologically, the lesions are highly vascular and edematous showing an increase in the number of vessels by proliferation of endothelial cells, and contain severe inflammatory infiltrates of mixed character. Viral particles were, however, found only in the cytoplasm of degenerating keratinocytes (21, 22). The lesions induced in sheep and humans after infection with OV show extensive dermal vascular responses which are likely to be a direct effect of the expression of the VEGF-like gene (9,23).
In this study we characterized an OV NZ-7-derived VEGF-like sequence, and found that this gene product utilizes only one of the VEGF receptors, KDR/Flik-1, but shows a potent endothelial cell growth stimulatory activity and vascular permeability activity similar to those of VEGF165 without the heparin-binding region. These results clearly indicate that the NZ-7-derived protein is a new type of VEGF family in the biochemical point of view. Its exonless cDNA structure in the viral genome suggests a phylogenetic origin in vertebrate genome. Since VEGF-A to -D have already been described, we propose the name, “VEGF-E” for this protein.

**Experimental Procedures**

**Cells and Culture Conditions**—Sf9 cells were purchased from Invitrogen (California) and cultured in EX-Cell 400 medium (JRH Biosciences, Lenexa, KS). NIH3T3 cells line overexpressing human Flt-1 (NIH3T3-Flt-1) and human KDR/Flik-1 (NIH3T3-KDR) were used for ligand binding and signal transduction studies. Parental NIH3T3 cells were used for PDGF and FGf stimulation assays. NIH3T3-KDR cells and NIH3T3-Flt-1 cells were established as described previously (24). These cell lines as well as parental NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Nissui, Tokyo) supplemented with 10% calf serum and 2 mM l-glutamine. 40 μg/ml kanamycin and 200 μg/ml G418 sulfate (Geneticin; Life Technologies, Inc., Gaithersburg, Maryland, NY). NIH3T3 cell lines overexpressing Flt-4, another member of the VEGF receptor gene family, were kindly provided by Dr. K. Alitalo (University of Helsinki, Finland) and cultured in DMEM containing 10% calf serum and 200 μg/ml geneticin G418 medium. Sinusoidal endothelial cells were isolated from rat liver as described previously (25). Nonparenchymal cells (Kupffer cells) were used for endothelial cell growth-UV medium (Kurabo, Osaka) supplemented with VEGF (10 ng/ml). Recombinant human VEGF165 was prepared as described (24) and recombinant human VEGF121 was obtained from R&D Systems (Minneapolis, MN). Human PDGF B/B was obtained from Boehringer Mannheim (Germany) and recombinant human basic FGF was obtained from R&D.

**VEGF-E cDNA**—The nucleotide sequence of the open reading frame identified in the genome of Orf virus NZ-7 strain (9) was synthesized and used for this study. To facilitate oligonucleotide synthesis, three point mutations without change of amino acid sequences were introduced and used for this study. To facilitate oligonucleotide synthesis, three point mutations without change of amino acid sequences were introduced and used for this study. To facilitate oligonucleotide synthesis, three point mutations without change of amino acid sequences were introduced and used for this study. To facilitate oligonucleotide synthesis, three point mutations without change of amino acid sequences were introduced and used for this study. To facilitate oligonucleotide synthesis, three point mutations without change of amino acid sequences were introduced and used for this study. To facilitate oligonucleotide synthesis, three point mutations without change of amino acid sequences were introduced and used for this study. To facilitate oligonucleotide synthesis, three point mutations without change of amino acid sequences were introduced and used for this study.

**Polyclonal and monoclonal antibodies**—Polyclonal antisera against VEGF-E were raised in rabbits using a carboxyl-terminal 20-amino acid peptide as antigen. The antiserum to peptide specifically recognized radiolabeled ligands, and the binding reactions were allowed to proceed to 4°C for 90 min. All experiments were done in triplicate wells. After incubation, the cells were washed three times with ice-cold binding buffer then twice with ice-cold PBS containing 0.1% BSA. Subsequently, the cells were solubilized by the addition of 0.5 ml of 0.5 N NaOH per well at 37°C for 10 min, followed by an additional wash with 0.5 ml of PBS per well, and the radioactivity was counted in a scintillation counter. Values were analyzed according to Scatchard’s procedure (29, 30).

**Competition assay** was done by incubating the cells with fixed (125I-VEGF165, 1 ng/ml; 125I-VEGF-E 1 to 2 ng/ml) and increasing concentrations of unlabeled ligands. Nonspecific binding was determined by parallel binding assay with NIH3T3-neo cells or by competition assay in the presence of excess unlabeled ligands with NIH3T3-Flt-1 or NIH3T3-KDR/Flik-1.

**Ligand binding and competition assay**—NIH3T3-Flt-1 and NIH3T3 KDR/Flik-1 cells were seeded at 1 × 10^4 cells/well on 24-well collagen-coated plates 24 h prior to experimental use. The cells were preincubated with binding buffer (DMEM, 10 mM HEPES (pH 7.2), 0.1% BSA) at 4°C for 30 min. Then the medium was replaced with 0.3 ml of binding medium (DMEM, 10 mM HEPES (pH 7.2), 0.5% BSA) containing radiolabeled ligands, and the binding reactions were allowed to proceed to 4°C for 90 min. All experiments were done in triplicate wells. After incubation, the cells were washed three times with ice-cold binding buffer then twice with ice-cold PBS containing 0.1% BSA. Subsequently, the cells were solubilized by the addition of 0.5 ml of 0.5 N NaOH per well at 37°C for 10 min, followed by an additional wash with 0.5 ml of PBS per well, and the radioactivity was counted in a scintillation counter. Values were analyzed according to Scatchard’s procedure (29, 30).

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**Autophosphorylation of Receptors Flt-1 and KDR/Flik-1**—For in vivo phosphorylation, NIH3T3-Flt-1 cells were grown to near confluence, and starved overnight in 0.1% serum-containing medium and stimulated at 37°C with 10 ng/ml VEGF165, VEGF-E, or BSA for 5 min. In the case of NIH3T3-KDR/Flik-1, the cells were stimulated after being starved overnight in 0.5% serum medium because of the relatively strong autophosphorylation of KDR/Flik-1. The cells were washed in ice-cold PBS with 0.1 mM NaVO₄, twice and lysed in 1% Triton X-100 lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 2% aprotinin, 1 mM PMSF, 50 mM sodium fluoride, 10 mM Na₄P₂O₇, and 2 mM Na₃VO₄). The lysates were lyophilized and centrifuged (15,000 rpm × 10 min). Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad). Various amounts of protein of each sample were used for analysis. For immunoblotting, the cell lysates were subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose sheet. The blots were incubated with a blocking solution (5% BSA containing washing buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.3% Tween 20)) and probed with the primary antibody diluted in the blocking solution. The signal was visualized...
A New Type Endothelial Growth Factor VEGF-E

RESULTS

Homodimer Structure of VEGF-E (NZ-7 VEGF) with No Affinity to Heparin

To examine the biological and biochemical features of VEGF-E, we synthesized its cDNA as described under "Experimental Procedures," and purified its gene product on a large scale using Baculovirus system. Detection of the proteins was carried out using a rabbit antisera against the carboxy-terminal peptide of VEGF-E (Fig. 1B).

VEGF-E is predicted to be a 149-amino acid protein including a signal peptide of about 20 amino acids, and its overall structure without the basic amino acid cluster region is similar to the shortest subtype of VEGF, VEGF₁₂₁ (Fig. 1A). As expected from the structural similarity to VEGF₁₂₁, VEGF-E did not show any affinity for the heparin column (data not shown). In addition, VEGF-E had only a weak affinity for the cation exchange column, thus, we purified it by using S-P column under acidic (pH 6.0) conditions as shown in Fig. 2. The elution pattern of VEGF-E showed a double band of 22 and 24 kDa, the former was 2–3-fold more intense than the latter based on immunoblotting. The difference of these two forms could be due to a minor difference of glycosylation. The silver staining for the 24-kDa VEGF-E was very faint. Repeated experiments suggest that this form is rather resistant to this staining.

The VEGF family belongs to the VEGF-PDGF supergene family, all the members of which have a dimer structure under natural conditions. Using reducing and nonreducing conditions, we found that VEGF-E exists as a homodimer of 44 kDa in the absence of reducing reagent, while it is separated to the 22–24-kDa monomer in the presence of a high concentration of dithiothreitol (Fig. 1B). These results indicate that the basic structure of VEGF-E is consistent with that of a member of the VEGF-PDGF supergene family.

Biological Activity of VEGF-E on Endothelial Cells

Stimulation of Endothelial Cell Growth—Since an important characteristic of VEGF is endothelial cell-specific growth stimulatory activity, we examined the effect of VEGF-E on the proliferation of HUVEC as well as rat liver sinusoidal endothelial cells (25). Although the structural similarity between VEGF-E and VEGF₁₂₁ is only 25% at the amino acid level, unexpectedly, VEGF-E showed a potent mitotic activity and stimulated the growth of these two types of endothelial cells to almost the same degree as VEGF₁₆₅ (Fig. 3). Cell numbers at 1 ng/ml VEGF-E corresponded to those at 0.8 ng/ml VEGF₁₆₅ and at 10 ng/ml VEGF₁₂₁. The maximum number of cells stimulated with VEGF₁₂₁ was one-half that with VEGF₁₆₅ and VEGF-E. Furthermore, VEGF-E induced an elongated morphology of rat sinusoidal endothelial cells to a degree comparable to that with VEGF₁₆₅ (Fig. 4).

Enhancement of Vascular Permeability—To examine the effect of VEGF-E upon vascular permeability, we carried out the Miles assay using guinea pigs and Evans Blue dye as a marker. As shown in Fig. 5, 20 ng of VEGF₁₆₅ was sufficient to saturate
the release of dye from the capillary. The same concentration of VEGF-E exerted comparable vascular permeability factor activity as VEGF165.  

Since the full-length Flt-1 and KDR/Flk-1 receptors separately expressed in culture cells are better materials for binding competition experiments, we used human Flt-1-overexpressing NIH3T3 cells (NIH3T3-Flt-1), human KDR/Flk-1-overexpressing cells (NIH3T3-KDR/Flk-1), and control cells carrying the vector alone (NIH3T3-neo).

As indicated in Fig. 6A, VEGF-E competed the binding of 125I-VEGF to NIH3T3-KDR/Flk-1. Competition of VEGF-E for binding of 125I-VEGF to Flt-1 receptor (Fig. 6B). As a positive control, unlabeled VEGF competed the binding of 125I-VEGF to NIH3T3-Flt-1 and NIH3T3-KDR/Flk-1 (Fig. 6, A and B). These results strongly suggest that VEGF-E can bind to KDR/Flk-1 but not to Flt-1.

VEGF-E Binds to KDR/Flk-1 but Not to Flt-1

To further examine KDR/Flk-1 as the receptor for VEGF-E, we tested whether VEGF-E could stimulate intracellular signaling from KDR/Flk-1. The first event for the signal transduction from receptor tyrosine kinase is the autophosphorylation of the receptor molecules on tyrosine. NIH3T3-KDR/Flk-1 cells were serum-starved overnight and then stimulated with VEGF165, VEGF-E, or fetal bovine serum. As shown in Fig. 7A, both VEGF165 and VEGF-E clearly stimulated autophosphorylation of KDR/Flk-1 based on the immunoprecipitation with anti-KDR/Flk-1 antiserum, followed by Western blotting using an anti-phosphotyrosine antibody. Addition of serum to the culture medium to 5% did not stimulate any KDR/Flk-1 autophosphorylation (data not shown), indicating that the serum does not contain VEGF, VEGF-E, or related growth factors at sufficient levels for activation of KDR/Flk-1.

On the other hand, VEGF-E could not activate Flt-1 overexpressed on NIH3T3 cells, although VEGF induced autophosphorylation of Flt-1 (Fig. 7B). In both cases of VEGF and VEGF-E, the autophosphorylation of KDR/Flk-1 occurred only on the upper molecule of the two major bands of KDR/Flk-1 (Fig. 7A). We have recently shown that the largest KDR/Flk-1,
230 kDa in size, is the mature form expressed on the cell surface (26).

To further confirm no detectable binding of VEGF-E to Flt-1, we tested whether the soluble form of Flt-1 could inhibit the biological activity of VEGF-E on endothelial cell culture. We and others have shown that soluble Flt-1 naturally expressed in vivo carrying about 80% of the extracellular domain of Flt-1, efficiently blocks the biological activity of VEGF at more than 20-fold the molar ratio to VEGF (33–35). However, a 50-fold excess of soluble Flt-1 could not suppress the endothelial cell culture.

![Fig. 4. Mitotic and morphological activity of VEGF-E on rat liver sinusoidal endothelial cells.](image1)

![Fig. 5. Vascular permeability activity of VEGF-E.](image2)
In addition to Flt-1 and KDR/Flk-1, the VEGF receptor family includes Flt-4 as a third member which specifically binds VEGF-C and -D but not VEGF (8, 36, 37). However, VEGF-E did not induce any autophosphorylation of the processed 125-kDa form of Flt-4 in the Flt-4-overexpressing NIH3T3 cells (data not shown).

Furthermore, we examined the autophosphorylation of other possible receptors such as PDGF receptor and FGFR receptor by VEGF-E, since VEGF-E shows 15% amino acid identity with PDGF. However, these receptors endogenously expressed on NIH3T3 cells were not autophosphorylated with VEGF-E, although the control PDGF and FGF stimulated the autophosphorylation of their receptors (data not shown) and induced cell proliferation (Fig. 8A), as well as morphological change (Fig. 8B). Therefore, from these results we concluded that VEGF-E binds and activates only one of the VEGF receptors, KDR/Flk-1.

**Signal Transduction of VEGF-E**

Recently we found that the major signal transduction pathway from KDR/Flk-1 overexpressed in NIH3T3 cells and in primary endothelial cells after stimulation with VEGF is through the PLCγ-protein kinase C-mitogen-activated protein kinase pathway (26). Therefore, we examined the signal transduction of VEGF-E on rat primary sinusoidal endothelial cells. As shown in Fig. 7C, VEGF-E rapidly induced tyrosine phosphorylation of PLCγ followed by the activation of mitogen-activated protein kinase. The time course of the phosphorylation of PLCγ was quite similar to that of autophosphorylation of KDR/Flk-1 receptor, and the degree of the phosphorylation was essentially the same as that in the presence of VEGF165 (Fig. 7, A and C) (26). The highest level of autophosphorylation of KDR/Flk-1 was observed 5 min after stimulation with VEGF-E. Based on these results, we conclude that the signal transduction of VEGF-E via KDR/Flk-1 is very similar to that of VEGF165.

**Histological Analysis of Angiogenesis Induced by VEGF-E in Vivo**

Various samples were mixed with Matrigel and implanted subcutaneously into the flanks of the mice. Four days after injection the animals were sacrificed and the gels were obtained for analysis. Two strains of mice, BALB/c nude mice (Fig. 9, A-G) and ICR mice (Fig. 9, H and I) were used in these experiments. Macroscopically, Matrigels containing VEGF165 or VEGF-E were hypervascularized, compared with the control Matrigel with PBS alone (Fig. 9, A, B, and C). Under stereoscopic microscope, Matrigel containing VEGF-E showed many newly formed microvessels invading into Matrigel from pre-existing diluted vessels (Fig. 9G). Sections of gel were stained with hematoxylin and eosin and examined under a light microscope (Fig. 9, D, E, F, and G). The control Matrigel without growth factor was not vascularized. On the other hand, several diluted vessels, newly formed microvessels, and hemorrhages were detected within the Matrigels mixed with VEGF165 or VEGF-E. Microvessels close to the ligand-containing Matrigels were frequently congested with red blood cells. Essentially the same histological changes were observed in these two strains of mice except for inflammatory cells surrounding vessels which were detected only in ICR mice.

Immunostaining of VEGF-E-containing Matrigel with antivon Willebrand factor antibody confirmed that the inner layer of the enlarged and microvessel-like structures consisted of vascular endothelial cells (Fig. 9). A clear difference in histology between VEGF-E-induced and VEGF165-induced vessels in

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2 T. Takahashi and M. Shibuya, unpublished data.
Matrigel was not detected. The pathological characteristics induced with VEGF-E were quite similar to those reported in Orf virus-infected lesions. These results indicate that VEGF-E bears a potent angiogenic activity similar to VEGF165 not only in *in vitro* cell culture but also in *in vivo* experimental conditions.

**DISCUSSION**

In this study we have examined the characteristics of VEGF-E (NZ-7 VEGF) which was originally identified as an open reading frame in the genome of the Orf virus NZ-7 strain (9), and demonstrated that VEGF-E is a novel type of VEGF family in several ways. First, although VEGF-E is only 19–25% identical at the amino acid level to VEGF (Fig. 1), it binds KDR/Flk-1 (VEGFR-2) at a high affinity similar to VEGF165. Second, unlike any of the VEGF subtypes, VEGF-E cannot bind Flt-1 nor transduce signals from Flt-1. Third, although VEGF-E does not bear a heparin-binding basic region, its biological activities for endothelial cell growth and vascular permeability are almost equal to those of the potent angiogenesis and vascular permeability factor, VEGF165. Therefore, VEGF-E is unique in that it carries a simple structure and utilizes only one of two VEGF receptors but shows significant biological activities on vascular endothelial cells.

The region on Flt-1 for binding to VEGF has been shown to be localized on the second Ig domain in the extracellular domain, however, a portion of the third Ig domain downstream is required for high affinity binding (35, 38, 39). A similar region of the second Ig domain on KDR was also shown to be important for binding with VEGF, but the binding affinity is still low, suggesting the surrounding portion is also important. The region for binding VEGF-E on KDR/Flk-1 has yet to be studied, however, the efficient suppression of VEGF binding to KDR/Flk-1 by VEGF-E and vice versa strongly suggests that the binding regions for both ligands on KDR/Flk-1 overlap at least in part.

A series of point mutational analysis of VEGF 165 by Keyt et al. (12) have revealed that 3 basic amino acid residues, Arg-82, Lys-84, and His-86, on VEGF play a role in the interaction of VEGF with KDR/Flk-1. Interestingly, a region on VEGF-E corresponding to these 3 amino acids on VEGF does not contain any basic amino acid residues. Therefore, although a hydrophilic interaction between this basic 82–86 stretch on VEGF and the binding site on the second and third Ig domains of KDR/Flk-1 is critical for high affinity binding of VEGF to KDR/Flk-1, this interaction model may not be generalized to other ligands such as VEGF-E. Since VEGF-E can bind KDR/Flk-1 at almost the same affinity as VEGF165 without the
82–86 basic stretch, a different binding motif with rather hydrophobic residues may exist on VEGF-E. Furthermore, since VEGF-E induces a strong activation of KDR/Flk-1, the interaction of this putative motif on VEGF-E with KDR/Flk-1 is thought to be sufficient for inducing dimerization of the receptor. It seems important to elucidate the motif critical for interaction between KDR/Flk-1 and this new type of ligand, VEGF-E.

Heparin is known to enhance the biological activity of VEGF at lower concentrations, but is inhibitory at higher concentrations (14). Heparin interacts not only with the basic region of VEGF165 but also with two VEGF receptors, although its interaction with KDR/Flk-1 is not very strong. Therefore, the effects of heparin or heparan sulfate-containing molecules on the endothelial cell surface appear complicated and might differ with biological conditions. However, that VEGF165 has 10–100-fold more biological activity than VEGF121 clearly indicates that the interaction of VEGF165 with heparin-like molecules through the basic stretch or the existence of this basic stretch itself is important for a higher affinity binding of VEGF165 to KDR/Flk-1 and for the generation of strong cytoplasmic signals. Recently, a 130-kDa membrane-associated molecule named neuropilin-1 was reported to bind the basic stretch on VEGF165 (20, 40). This molecule might also be involved in VEGF-KDR/Flk-1 signaling. Interestingly, however, VEGF-E does not carry the basic stretch corresponding to exon 7 on VEGF165 nor show any affinity to heparin column at detectable levels. Furthermore, in a cross-linking study using 125I-VEGF165 and HUVEC, VEGF-E strongly competed the binding of VEGF165 with KDR but not the binding of VEGF165 with 130-kDa molecule (data not shown). Therefore, the interaction of VEGF-E with heparin-like molecules or with the 130-kDa molecule may not be necessary for generation of a strong mitotic and permeability signal via KDR/Flk-1. A possible explanation for the strong biological activity of VEGF-E is that high affinity of a ligand is sufficient for a strong activation of KDR/Flk-1. A possible explanation for the strong biological activity of VEGF-E is that high affinity of a ligand is sufficient for a strong activation of KDR/Flk-1 and that the basic stretch of VEGF165 is simply functional for the increase in the affinity of VEGF165 for KDR/Flk-1. In addition, the diffuse margin of the blue spots generated with VEGF-E might be due to no affinity of VEGF-E to Flt-1 or neuropilin-1. Further studies are required to understand the molecular mechanism of VEGF-E activity and the function of exon 7-derived basic stretch on VEGF165.

VEGF binds both KDR/Flk-1 and Flt-1, whereas VEGF-E binds and stimulates only KDR/Flk-1. Flt-1 as well as KDR/Flk-1 is expressed at higher levels in vascular endothelial cells.
during embryogenesis and most of the pathological angiogenesis. Flt-1 carries about a 10-fold higher binding affinity for VEGF but much weaker tyrosine kinase activity than KDR/Flk-1. Furthermore, Flt-1 knockout mice studies have shown that flt-1(-/-) homozygous mice are embryonic lethal at embryonic day 8.5–9.0 due to an overgrowth of endothelial-like cells and disorganization of a variety of blood vessels (41). These results suggest that Flt-1 functions as a negative regulator of VEGF signaling at least in embryogenesis. Since VEGF utilizes both receptors, VEGF might control a balance between positive and negative signals (42). On the other hand, VEGF-E utilizes only KDR/Flk-1 which is a positive regulator of endothelial proliferation. This feature of VEGF-E might be sufficient to generate pathological angiogenesis as shown in Fig. 9, but not be appropriate for regulating the fine architecture of the physiological blood vessel network in embryogenesis.

An interesting possibility is that the cellular VEGF-E gene exists in the vertebrate genome similar to the case of parapox-virus OV IL-10 gene (43), and is utilized at certain periods or in a specific tissue in vivo at the embryonic or postnatal/adult stage. The KDR/flk-1 gene was shown to be essential for the establishment of the blood vessel network and for hematopoiesis at early embryogenesis by gene targeting (44). Thus, VEGF-E gene may be a candidate gene for regulating a part of these processes in cooperation with the classical VEGF.

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Fig. 9. VEGF-E-induced in vivo angiogenesis in mice. Angiogenic response to various growth factors was examined using Matrigel (see “Experimental Procedures”). A and D, Matrigel with PBS alone; B and E, Matrigel with recombinant human VEGF165 (400 ng/0.5 ml). C, F, G, H, and I, Matrigel with purified VEGF-E (400 ng/0.5 ml). A–C, Matrigels containing VEGF165 or VEGF-E were hypervascularized. D–F, the samples were stained with hematoxylin and eosin (× 200). Newly formed microvessels were detected within the Matrigels mixed with VEGF165 or VEGF-E (arrowheads). G, magnification of a Matrigel containing VEGF-E with stereoscopic microscope. Many newly formed microvessels are invading into Matrigel from pre-existing dilated vessels. H and I, the samples were stained with hematoxylin and eosin, and anti-von Willebrand factor antibody (× 200). Several dilated vessels were also found in the Matrigels with VEGF-E (× 200). The samples in A–G and in H and I were obtained using BALB/c nude mice and ICR mice, respectively. The experiments were repeated at least three times, with essentially the same results obtained.
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