A Set of Loop-1 and -3 Structures in the Novel Vascular Endothelial Growth Factor (VEGF) Family Member, VEGF-E_NZ-7, Is Essential for the Activation of VEGFR-2 Signaling*

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The vascular endothelial growth factor (VEGF) family plays important roles in angiogenesis and vascular permeability. Novel members of the VEGF family encoded in the Orf virus genome, VEGF-E, function as potent angiogenic factors by specifically binding and activating VEGFR-2 (KDR). VEGF-E is about 45% homologous to VEGF-A at amino acid levels, however, the amino acid residues in VEGF-A crucial for the VEGF-2-binding are not conserved in VEGF-E. To understand the molecular basis of the biological activity of VEGF-E, we have functionally mapped residues important for interaction of VEGF-E with VEGFR-2 by exchanging the domains between VEGF-E_NZ-7 and PI GF, which binds only to VEGFR-1 (Flt-1). Exchange on the amino- and carboxyl-terminal regions had no suppressive effect on biological activity. However, exchange on either the loop-1 or -3 region of VEGF-E_NZ-7 significantly reduced activities. On the other hand, introduction of the loop-1 and -3 of VEGF-E_NZ-7 to placenta growth factor rescued the biological activities. The chimera between VEGF-A and VEGF-E_NZ-7 gave essentially the same results. These findings strongly suggest that a common rule exists for binding with VEGFR-2 through the appropriate interaction between loop-1 and -3 regions.

Vascular endothelial growth factor-A (VEGF-A) plays a pivotal role in vasculogenesis, angiogenesis, and differentiation of hemangioblasts to hematopoietic precursor cells in embryogenesis. VEGF-A is also known to be closely involved in a variety of pathological conditions such as tumor angiogenesis and diabetic retinopathy (1–3).

VEGF-A is a member of the PDGF superfamily because of their structural similarities. VEGF is found to be a dimeric glycoprotein of Mr 34,000–42,000, and have conserved eight cysteine residues in each monomer. These cysteine residues construct a particular folding consisting of two intermolecular and three intramolecular disulfide bonds that generate three loop-like structures, loop-1, -2, and -3. VEGF-A exerts its biological activity by interacting with receptor-type tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) (4–7). Homozygous loss of the VEGFR-1 or VEGFR-2 genes resulted in embryonic lethality between days 8.5 and 9.5, indicating that these VEGF receptors play important roles in vasculogenesis and angiogenesis (8, 9). The different phenotypes of these VEGFR-mice suggest that VEGF-2 is the major positive signal transducer, whereas VEGFR-1 has a negative regulatory role in angiogenesis at early embryogenesis.

The VEGF family in vertebrate genomes includes VEGF-A, PIGF (placenta growth factor), VEGF-B, -C, and -D. PI GF and VEGF-B specifically bind to VEGFR-1 (10–13), whereas VEGF-C and -D bind and activate VEGFR-3 (Flt-4), regulating lymphangiogenesis as well as angiogenesis in the middle stage of embryogenesis (14–18). Amino acids in VEGF-A essential for binding with VEGFR-2 have been studied by the alanine scanning method. Key et al. (19) have reported that Arg-82, Lys-84, and His-86 are indispensable for the interaction between VEGF-A and VEGFR-2.

Recently, we have shown that the VEGF-E_NZ-7 protein, a novel member of the VEGF family could bind specifically to VEGFR-2, activate the receptor, and promote the growth of endothelial cells in vitro and in vivo at a transient condition (20). VEGF-E genes were originally found as an open reading frame in the genome of the NZ-7, NZ-2, and D1701 strains of parapoxvirus, Orf virus (21).

These three genes were structurally very similar to each other compared with VEGF family proteins, and were designated as VEGF-E_NZ-7, ORFV2-VEGF/VEGF-E_NZ-7, and VEGF ED1701 (20, 22, 23). Orf virus causes contagious pustular dermatitis in sheep, goats and, sometimes, humans. Historically, the lesions are highly vascularized and edematous with proliferation of endothelial and inflammatory cells. In addition to VEGF-E_NZ-7, other two VEGF-E members were also shown to specifically bind to VEGFR-2 but not to other receptors.

VEGF-E_NZ-7 has a high affinity to VEGFR-2 at similar levels as VEGF-A, and efficiently competes to VEGF-A (20). This indicates that the binding pocket on VEGFR-2 for VEGF-A and VEGF-E_NZ-7 is significantly overlapped to each other. Interestingly, however, three basic amino acids on VEGF-A essential for the VEGFR-2-binding are not conserved in VEGF-E_NZ-7, and these basic amino acids were changed to hydrophobic or non-charged ones, Val, Gly, and Ser, respectively. These results indicate that the local structure built up by these basic amino acids in VEGF-A are not always required for the ligands that bind to VEGFR-2.

To elucidate a novel rule for the ligand-binding to VEGFR-2, we carried out a series of domain-exchange analysis between

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‡The abbreviations used are: VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cells; PIGF, placenta growth factor; PDGF, platelet-derived growth factor.
VEGF-E and PlGF, or VEGF-E and VEGF-A. Our results clearly indicate that an intimate relationship between the loop-1 and -3 of VEGF-ENZ-7 as well as VEGF-A is crucial for the formation of the three-dimensional structure important for the high-affinity binding to VEGFR-2.

### MATERIALS AND METHODS

**Cells and Culture Conditions**—Spodoptera frugiperda (Sf9) cells and baculovirus transfer vectors (pVL-1393) were purchased from Invitrogen (San Diego, CA) and cultured in EX-CELL 400 medium (JRH Biosciences, Lenexa, KS). A linearized DNA of a mutant Autographa californica nuclear polyhedrosis viral DNA (BaculoGold) was from BD Pharmingen (San Diego, CA). 125I-VEGF-A165 was the product of Amersham Biosciences (Buckinghamshire, UK). NIH3T3 cell lines overexpressing human KDR/VEGFR-2 (NIH3T3-KDR) were previously established and used for signal transduction studies (13). NIH3T3-KDR cells were maintained in Dulbecco's modified Eagle medium (Nissui, Tokyo) supplemented with 10% calf serum, 2 mm l-glutamine, 40 μg/ml kanamycin, and 200 μg/ml G418 sulfate (Geneticin; Invitrogen). Recombinant human VEGF-A165 was prepared as described (13). The enhanced chemiluminescence detection kit was purchased from Amersham Biosciences.

**Polyclonal and Monoclonal Antibodies**—Polyclonal antisera against VEGF-ENZ-7 were raised in rabbits using a 20-amino acid sequence of the carboxyl terminus as antigen (20). Anti-human VEGFR-2 antisera (B2) was prepared previously (24). A monoclonal antibody specific to phosphotyrosine (PY20) was obtained from ICN Biochemicals (Costa Mesa, CA). Secondary antibodies conjugated to horseradish peroxidase were purchased from Amersham Biosciences. Monoclonal neutralizing antibody to VEGF-A165 was purchased from R&D Systems (Minneapolis, MN).

**Construction of VEGF-ENZ-7 Chimeric Mutant**—The synthetic cDNA encoding VEGF-ENZ-7 was cloned to the BamHI and EcoRI restriction sites of pUC18 (20). Histidine residues were introduced by using a double stranded oligonucleotide encoding amino acid residues Cys-130 to Arg-148 of VEGF-ENZ-7 and six histidines followed by stop codon. This oligomer also had the cohesive end for DraiII at the NH2 terminus and for EcoRI at the COOH terminus, and it was ligated to the 3-kb DraiII-EcoRI fragment of the plasmid that contains cDNA of VEGF-E127-7 cloned to pUC18 (pUCE-his). With the same manner, histidine residues
were introduced into the COOH terminus of PlGF-1 cDNA. The double stranded synthetic oligonucleotide encoding Arg-131 to Arg-149 of PlGF-1 was followed by six histidine residues and a stop codon. This oligomer had cohesive ends for the BsmI site at the NH2 terminus and EcoRI site at the COOH terminus. This oligomer was cloned to a 3-kb BsmI-EcoRI fragment of the plasmid (pUCP-his) with full-length cDNA of PlGF-I cloned to BamHI and the EcoRI site of pUC18.

The constructs of chimera proteins were produced by exchanging variable regions among VEGF-E NZ-7, VEGF-A, and PlGF. They were achieved by ligating a series of digested fragments of pUCP-his and/or pUCE-his with synthetic double stranded oligonucleotides and/or the fragments produced by the PCR technique. The details of each plasmid construction are available on request.

Each construct contains the following amino acid sequence: 1, Met-1 to Val-40 of PlGF and Asn-35 to the stop codon of VEGF-ENZ-7; 2, Met-1 to Tyr-51 of PlGF and Cys-46 to the stop codon of VEGF-ENZ-7; 3, Met-1 to Glu-64 of PlGF and Tyr-59 to the stop codon of VEGF-ENZ-7; 4, Met-1 to Arg-82 of PlGF and Cys-77 to the stop codon of VEGF-ENZ-7; 5, Met-1 to Glu-98 of PlGF and Thr-93 to the stop codon of VEGF-ENZ-7; 6, Met-1 to Cys-130 of VEGF-ENZ-7 and Glu-129 to the stop codon of PlGF; 7, Met-1 to Cys-88 of VEGF-ENZ-7 and Val-95 to the stop codon of PlGF; 8, Met-1 to Cys-77 of VEGF-ENZ-7 and Thr-84 to the stop codon of PlGF; 9, Met-1 to Cys-88 of VEGF-ENZ-7 and Cys-130 of VEGF-ENZ-7; 10 - 27, shown in Figs. 1 and 4; number 28, Met-1 to Gly-45 of VEGF-ENZ-7, Cys-52 to Cys-77 of VEGF-A, and Val-72 to the stop codon of VEGF-ENZ-7; number 29, Met-1 to Ile-87 of VEGF-ENZ-7, Cys-95 to Cys-128 of VEGF-A, and Asp-131 to the stop codon of VEGF-ENZ-7; number 30, Met-1 to Gly-45 of VEGF-ENZ-7, Cys-52 to Cys-77 of VEGF-A, Val-72 to Ile-87 of VEGF-ENZ-7, Cys-95 to Cys-128 of VEGF-A, and Asp-131 to the stop codon of VEGF-ENZ-7; number 31, Met-1 to Tyr-51 of PlGF, Cys-46 to Cys-72 of VEGF-ENZ-7, and Glu-129 to Arg-131 of PlGF; number 32, Met-1 to His-93 of PlGF, Cys-88 to Cys-130 of VEGF-ENZ-7, and Gln-129 to Arg-131 of PlGF; number 33, Met-1 to Tyr-51 of PlGF, Cys-46 to Cys-72 of VEGF-ENZ-7, Val-78 to His-93 of PlGF.

**Fig. 2.** Amino- and carboxyl-terminal regions of the VEGF-E are not essential for the biological activity. A, a variety of constructs for the replacement of amino- and carboxyl-terminal regions in VEGF-ENZ-7 (orange) with the corresponding regions of PlGF (green). a, VEGFR-2 (KDR) autophosphorylation was measured by using NIH3T3-KDR cells. The cells were stimulated with chimera mutant proteins (4–120 ng/ml), lysed, and then subjected to SDS-PAGE for Western blotting with an anti-phosphotyrosine antibody (α-PY) or with an anti-VEGFR-2 antibody (α-KDR IK-5). The activity was indicated by +, ++, equal to wild type VEGF-ENZ-7; ++, 2-3-fold weaker than wild type VEGF-ENZ-7; +, 3-10-fold weaker than wild type VEGF-ENZ-7; −, phosphorylation was not detected in this concentration range. b, proliferation of HUVEC. Quiescent HUVEC were stimulated with 1, 10, and 100 ng/ml chimera mutant proteins. After 4 days, cells were stained and the cell number was determined by averaging the counting of five spots to wild type VEGF-ENZ-7; +, 2-fold weaker than wild type VEGF-ENZ-7; at 100 ng/ml; +, 4-fold weaker than wild type VEGF-ENZ-7; −, HUVEC proliferation was not detected. B, binding activities of the chimeric proteins between VEGF-ENZ-7 and PlGF to VEGFR-2. The activities were measured by competition experiments using 125I-VEGF-A and VEGFR-2 binding systems (see "Materials and Methods"). C, the activities of chimeric proteins for stimulation of HUVEC growth.
NIH3T3-KDR (VEGFR-2) cells were grown to semiconfluence and stimulated with 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, and 10 mM endothelial cells and fibroblasts were co-cultured in 24-well plates at 1,000 cells/well on 24-well collagen-coated plates with a fixed concentration of 125I-VEGF-A and increasing concentrations of non-radiolabeled chimera mutant proteins for 3 h at 37 °C. The plates were washed twice and blocked with binding buffer (1% BSA, 1% non-fat milk, 0.05% Tween 20) and probed with the primary antibody diluted in blocking solution. The signal was visualized using peroxidase-conjugated goat anti-rabbit IgG antibody (Qiagen) followed by chemiluminescence (ECL, Amersham) according to the manufacturer’s instructions. The blots were incubated with a blocking solution (5% bovine serum albumin, 5% non-fat milk, and 0.1% Tween 20) and probed with mouse anti-human CD31 for 60 min at 37 °C in blocking buffer (1% bovine serum albumin in phosphate-buffered saline) and washed with blocking buffer. Secondary, alkaline phosphatase-conjugated goat anti-mouse IgG was added to the cells followed with incubation for 60 min at 37 °C. After washing, 5-bromo-4-chloro-3-indolyl phosphate was used to develop a color to visualize endothelial cells. Tubules were analyzed under a bright-field microscope and total length of branching in a fixed area (mm/mm²) of the randomly chosen 5 spots per well.

RESULTS

Production and Dimer Formation of VEGF-E<sub>NZ.7</sub> Mutants—Alanine scanning analysis of VEGF-A has shown that basic amino acids at 82, 84, and 86 as well as other amino acids in loop-3 are essential for VEGF-2 binding, however, the VEGF-E family that binds to VEGF-2 at a similar affinity as VEGF-A does not conserve these amino acids (Fig. 1). To examine which region(s) in VEGF-E is crucial for the binding and activation of VEGF-2, we constructed a series of chimeric mutants by exchanging the variable region of VEGF-E<sub>NZ.7</sub> with that of PIGF and/or VEGF-A. The carboxyl terminus of all chimeric mutants had an insertion of six histidines as a tag sequence for the convenience of protein purification. This His tag did not suppress the activity of VEGF-E<sub>NZ.7</sub>.

VEGF-E<sub>NZ.7</sub> was exchanged with PIGF from the NH₂-terminal, and the exchanging region was extended toward the COOH-terminal, serially (Figs. 2 and 3). Secondary, exchanging was performed from the COOH terminal towards serially. Loop-wise exchanging on loop-1, -2, and -3 was produced by both PIGF and VEGF-A amino acid sequences (Fig. 4A). The site-directed mutagenesis was also performed by exchanging only 4 amino acid residues (Fig. 4B). Finally, loop-1 and -3 regions of VEGF-E<sub>NZ.7</sub> were introduced to PIGF as the gain of function mutants (Fig. 5) (see “Materials and Methods”).

The molecular size of mutant proteins in non-reducing conditions were in the range of M<sub>r</sub> 40,000 to 45,000, and 20,000 to 25,000 in reducing conditions, consistent with wild type VEGF-E<sub>NZ.7</sub> and PIGF (Fig. 3, data not shown). These results indicate that all mutants did not show any disruption in dimerization.

**NH₂- and COOH-terminal Sequences in VEGF-E<sub>NZ.7</sub> Are Replaceable with the Corresponding Sequence in PIGF without a Significant Loss of Biological Functions**—To evaluate the
affinity of each mutant to receptor, a binding assay was carried out by using the extracellular domain of VEGFR-2 tagged with the Fc portion of IgG (see “Materials and Methods”). Because VEGF-ENZ-7 could compete with VEGF-A in binding to VEGFR-2, chimeric proteins were tested for their ability to compete with 125I-labeled VEGF-A 165 for interaction to VEGFR-2-Fc immobilized onto 96-well plates.

When VEGF-ENZ-7 was exchanged with PlGF from the NH2-terminal toward COOH-terminal serially (Fig. 2A, chimera 1), 34 amino acids of the NH2-terminal region in VEGF-ENZ-7 were replaceable without any reduction of affinity to VEGFR-2 (Fig. 2, chimera 1). An extension of the PlGF region to the first conserved cysteine residue resulted in a minor reduction of the affinity (Fig. 2B, 2). However, as shown by chimeras 3, 4, and 5, the PlGF amino acid sequence close to and over loop-1 resulted in the loss of affinity similar to intact PlGF (Fig. 2B).

Secondary exchanging was performed from the COOH terminus toward the NH2 terminus serially (Fig. 2A). Among the mutants 6, 7, and 8, chimera 6 with 18 amino acids of the COOH-terminal exchange from the 8th conserved cysteine to the COOH-terminal end remained its affinity. However, the replacement over loop-3 such as chimeras 7 and 8 had lost their binding ability. Taken together, NH2 and COOH termini were not critical for VEGF-ENZ-7 to interact with VEGFR-2.

To confirm this result, a chimera with simultaneous exchanging of both NH2 and COOH termini were evaluated (Fig. 2A, chimera 9), and showed no significant suppression in its affinity. These results may imply that at least loop-1 and -3 are very critical for VEGF-ENZ-7, whereas the importance of loop-2 remained unclear.

Loop-2 but Not Loop-1 and -3 in VEGF-ENZ-7 Is Exchangeable to Either PlGF or VEGF-A Sequence—Keyt et al. (27) reported that several amino acids in loop-3 are essential for the binding of VEGF-A to VEGFR-2 (27). To analyze the importance of each loop region of VEGF-ENZ-7, a variety of chimera mutants with loop region-specific replacement were tested (Figs. 4 and 5). These exchanged regions do not contain β-strands that flank the loop site. Therefore, the basic architecture of these chimeric proteins would not be disrupted.

As shown in Fig. 4A, chimeras 10 and 11 had an exchange on loop-1 (Tyr-54 to Asn-68 between β1 and β2 strands), 12 and 13 on loop-2 (Gly-78 to Ile-87 between β3 and β4), 14 and 15 on the loop-3 containing region (Val-98 to Ser-123 between β5 and β8), and 16 and 17 on the loop-3 short region (Val-105 to Asn-118 between β6 and β7) with the corresponding regions of VEGF-A or PlGF (Fig. 1A).
We found that the loop-2 region in VEGF-ENZ-7 is exchangeable to that of PlGF or VEGF-A (Fig. 4, chimera 12 and 13), whereas loop-1 and -3 specific exchanges to PlGF caused a significant defect (Fig. 4, chimera 11, 15, and 17). These results indicate that the important regions for VEGF-ENZ-7 to interact to VEGFR-2 are not only loop-3 but also loop-1. Along with these PlGF replacements, the VEGF-A region was also introduced into loop regions of VEGF-ENZ-7 as a reference. Surprisingly, independent introduction of loops of VEGF-A, which is a strong ligand for VEGFR-2, also resulted in significant reduction in biochemical and biological activities (Fig. 4, chimeras 10, 14, and 16). Therefore, both loop-1 and -3 regions are equally important in VEGF-A as well as in VEGF-ENZ-7. This result implies that VEGF-ENZ-7 and VEGF-A may have a common mechanism in the interaction to VEGFR-2, by having critical regions in loop-1 and -3.

Loop-1 but Not the Adjacent Short Sequences Are Important for Biological Function—More detailed mutations were introduced to identify important amino acid residues. The region for loop-1 exchange was divided into 3 parts, which were composed of YLGE, ESTN, and LQYN. These residues were changed to PlGF counterparts, DVVS, SEVE, and HMFS (Figs. 1A and 4B). Among these mutants, a chimera with ESTN exchanged with SEVE severely lost the affinity, and exchange of YLGE to DVVS had minor loss in affinity, whereas no effect in another exchange mutant (chimeras 20, 19, and 21, respectively). These results suggest that the affinity reduction conferred by the exchange of the loop-1 region in chimera 11 was mainly caused by the replacement of ESTN on VEGF-ENZ-7 to SEVE in PlGF. In the loop-1 of VEGF-ENZ-7, accumulation of negatively charged residues might have disrupted the binding action of VEGF-ENZ-7. In addition, regions upstream from loop-1 (chimera 22 and 23) and a region downstream of loop-3 (number 26) were replaceable to the corresponding regions of PlGF, however, short stretches upstream from loop-3 were not (numbers 24 and 25).

VEGF-ENZ-7 have its specific insertion-like stretch with 8 amino acid residues in the loop-3 region (Fig. 1A). This stretch

Fig. 5. Functional analysis of VEGF-ENZ-7/PlGF chimera mutant proteins, 28–33. A, the construct map of chimera mutants and their activities for VEGFR-2 autophosphorylation and HUVEC growth (see Fig. 2, legend). B, binding activities of the chimeric proteins to VEGFR-2. C, the activities of chimeric proteins for stimulation of HUVEC growth.
was not found in other VEGF-E members or in the VEGF family. Interestingly, the flanking region of this stretch showed similarities in amino acid sequence to other VEGF-E genes. This stretch was deleted to test its significance for the VEGF-E_NZ-7 protein. The deletion resulted in a complete loss of biological activities, indicating that this short stretch is indispensable for VEGF-E_NZ-7 (Fig. 4B, chimera 27).

Cooperation between a Proper Set of Large Loop-1 and Large Loop-3 Is Crucial for Restoration of Biological Activity in VEGF-E_NZ-7—To examine the regions in VEGF-E_NZ-7 necessary and sufficient for the activation of KDR/VEGFR-2, we further constructed chimeric molecules between VEGF-E_NZ-7 and PlGF. Under the background of the PlGF sequence, the loop-1-containing sequence and the loop-3-containing sequence in VEGF-E_NZ-7 were introduced to the corresponding regions (Fig. 5A, 31–33). For a control experiment, under the background of VEGF-E_NZ-7, the loop-1 and -3 regions in VEGF-A were replaced to the corresponding regions (Fig. 5A, 28–30). As shown in chimera 33, both loop-1 and -3-containing regions were found to be required for the activation of VEGFR-2 kinase.

Although VEGF-E_NZ-7 and VEGF-A are able to stimulate VEGFR-2, to our surprise, a single replacement of either loop-1 or -3 in VEGF-A to the corresponding region in VEGF-E_NZ-7 strongly suppressed the biological activity (Fig. 5, chimeras 28 and 29). Both loop-1 and -3 of VEGF-A are required for a partial recovery of the activation of VEGFR-2 (chimera 30). Taken together, these results strongly suggest that an appropriate pair of loop-1 and -3 is essential for the construction of threedimensional structure for the binding and activation of VEGFR-2.

The Binding Ability of Mutants to VEGFR-2 Correlates Well with the Activity of Receptor Autophosphorylation Assay and HUVEC Proliferation Assay—These mutant proteins were tested for their ability to induce autophosphorylation of VEGFR-2 using a cell line, NIH3T3-KDR (see “Materials and Methods”) (Fig. 6, data not shown). The ability was highly correlated with the affinity of mutant proteins to the receptor (Figs. 2, 4, and 5). The mutants with high affinity, as the wild type VEGF-E_NZ-7 protein to receptor, demonstrated a strong activity in induction of autophosphorylation of KDR/VEGFR-2, whereas the mutants with no affinity did not in a concentration range up to 150 ng/ml in the final medium. These results indicate that the critical regions of VEGF-E_NZ-7 for binding to VEGFR-2 are also important for inducing autophosphorylation of VEGF-2.

VEGF-A-induced signal transduction for the proliferation of endothelial cells is mainly mediated by VEGFR-2. To evaluate the relationship of the abilities to induce autophosphorylation of VEGF-2 and to induce a proliferation of endothelial cells, mutant proteins were tested for their activity to induce proliferation of HUVEC. As expected, mutant chimeras 1, 2, 6, 9, 12, 13, 19, 21–23, 26, 30 (weak), and 33 with high affinity for VEGF-2 could lead the proliferation of HUVEC, whereas mutants with low receptor affinity facilitated weak mitogenesis (Figs. 2, 4, and 5). Those mutants with no affinity failed to show such activity, with one exception, number 18. Mutant number 18 did not show detectable affinity to VEGF-2, but it partially induced mitogenesis of endothelial cells. These results suggest that the affinity strength of mutant proteins mostly correlates with their activity to induce mitogenesis of HUVEC.

Evaluation of Mutants in Tubular Formation Assay—VEGF-A is known to stimulate the endothelial cells to form a tubule-like structure in vitro and in vivo. The chimeric mutants of VEGF-E_NZ-7 were tested for their activity for tubular formation in a recently developed co-culture system between HUVEC and human diploid fibroblasts (see “Materials and Methods”) (Fig. 7). To decrease a background tube formation in this system detectable without any exogenous angiogenic factors, we added anti-VEGF-A neutralizing antibody into culture medium.

At first, we demonstrated that VEGF-E_NZ-7 could induce the tubular formation in vitro (Fig. 7A). Next we examined tubular formation by chimera mutants. Representative results and quantitative analysis are shown in Fig. 7, B and C. The strength of chimera proteins for tubular-forming activity was correlated with the affinity to the receptor, and with the abilities to autophosphorylate the receptor and induce the proliferation of endothelial cells. Interestingly, the tubules induced by VEGF-E_NZ-7 were morphologically distinguished from that of VEGF-A, where VEGF-E_NZ-7-induced tubules were slightly thicker. Chimera number 18, which showed only weak HUVEC proliferation without the significant affinity to receptor and the receptor autophosphorylation ability in vitro could induce the tubular formation as effectively as VEGF-A and VEGF-E_NZ-7. The morphology induced by number 18 was closely related to that of tubules induced rather by VEGF-A than VEGF-E_NZ-7. This result suggests that this tubular formation system is more sensitive than other assays such as the binding assay, receptor
In this study, the important regions of VEGF-ENZ-7 for binding to VEGFR-2 were analyzed by using domain exchanging with PlGF and site-directed mutagenesis. PlGF does not bind to VEGF-2 but only to VEGF-1 (12, 13, 28). VEGF-ENZ-7 and PlGF have a similar amino acid composition, and they also completely conserve the critical cysteine-knot motif that was composed of eight cysteine residues (see Fig. 1B). Recently, the crystal structure study for PlGF (29) revealed that PlGF actually conserves the tertiary structure with VEGF (30, 31) and biological activities. This result indicates that the amino and carboxyl termini of VEGF-ENZ-7 are not involved directly in binding with VEGFR-2. As shown in mutants 3–5, 7, and 8 (Fig. 2), extending the exchange region into the core region and over loop-1 and -3 regions resulted in a significant defect in biological and biochemical activities. These results suggest that the regions containing loop-1 and -3 are critical for VEGF-ENZ-7 to be functionally intact. As expected, exchanging the narrowed regions of VEGF-ENZ-7 only containing loops-1 and -3 (mutant
10 and 11 for loop-1, 14–17 for loop-3) also produced functionally
defective proteins, whereas the loop-2 exchange did not reduce activity (mutants 12 and 13). Mutant proteins with low
affinity to VEGFR-2 could not induce autophosphorylation of
VEGFR-2, proliferation of HUVEC, or tubular formation by
endothelial cells except for chimera 18. These results suggest that
the affinity strength to the receptor basically reflect their
effectiveness in biological response.
In addition to the replacement by PlGF, VEGF-A regions
were also introduced to VEGF-ENZ-7, in a loop-specific manner,
as a reference (Figs. 4A and 5A). Surprisingly, these mutants
with VEGF-A loops also had significant reduction in biochem-
ical and biological activities. It could suggest that a single loop
of VEGF-A is not enough to be biologically functional. A pair of
loop-1 and -3 of VEGF-ENZ-7, or VEGF-A may be required to
build up the receptor-binding determinant for VEGFR-2. Mu-
tants 10 and 14 did not bind to VEGFR-2 and lost biological
activity. Mutant 18, which is a combination of numbers 10 and
14 with regions of loops-1 and -3 of VEGF-A onto VEGF-ENZ-7,
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Atsushi Kiba, Naoyuki Yabana and Masabumi Shibuya

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