Chimeric VEGF-E$_{NZ7}$/PlGF Promotes Angiogenesis Via VEGFR-2 Without Significant Enhancement of Vascular Permeability and Inflammation

Yujuan Zheng, Masato Murakami, Hiroyuki Takahashi, Mai Yamauchi, Atsushi Kiba, Sachiko Yamaguchi, Naoyuki Yabana, Kari Alitalo, Masabumi Shibuya

Objective—Vascular endothelial growth factor (VEGF) plays critical roles in the regulation of angiogenesis and lymphangiogenesis. However, tissue edema, hemorrhage, and inflammation occur when VEGF-A is used for angiogenic therapy. To design a novel angiogenic factor without severe side effects, we examined the biological function of chimeric VEGF-E$_{NZ7}$/placental growth factor (PlGF), which is composed of Orf-Virus$_{NZ7}$-derived VEGF-E$_{NZ7}$ and human PlGF1, in a transgenic (Tg) mouse model.

Methods and Results—A strong angiogenic response was observed in both VEGF-E$_{NZ7}$/PlGF and VEGF-A$_{165}$ Tg mice. Notably, the vascular leakage of VEGF-E$_{NZ7}$/PlGF-induced blood vessels was 4-fold lower than that of VEGF-A$_{165}$-induced blood vessels. Furthermore, the monocyte/macrophage recruitment in the skin of VEGF-E$_{NZ7}$/PlGF Tg mice was ≈8-fold decreased compared with that of VEGF-A$_{165}$ Tg mice. In addition, the lymphatic vessels in VEGF-E$_{NZ7}$/PlGF Tg mice were structurally normal, whereas they were markedly dilated in VEGF-A$_{165}$ Tg mice, possibly because of the high vascular leakage. Receptor binding assay demonstrated that VEGF-E$_{NZ7}$/PlGF was the ligand only activating VEGF receptor (VEGFR)-2.

Conclusion—These results indicated that neither the hyperpermeability in response to simultaneous stimulation of VEGFR-1 and VEGFR-2 nor VEGFR-1-mediated severe inflammation was associated with VEGF-E$_{NZ7}$/PlGF-induced angiogenesis. The unique receptor binding property may shed light on VEGF-E$_{NZ7}$/PlGF as a novel candidate for therapeutic angiogenesis. (Arterioscler Thromb Vasc Biol. 2006;26:2019-2026.)

Key Words: VEGF-E$_{NZ7}$/PlGF ■ angiogenesis ■ VEGFR-2 ■ permeability ■ inflammation

Angiogenesis is the process of vascular formation from preexisting blood vessels. Vascular endothelial growth factor (VEGF) acts as the crucial and potent angiogenic factor under both physiological and pathological conditions.1 VEGF-A, the best characterized member of the VEGF family, participates in angiogenesis by stimulating both VEGF receptor (VEGFR)-1 and VEGFR-2.2 Notably, most downstream events of VEGF-A are characterized member of the VEGF family, participates in angiogenesis by stimulating both VEGF receptor (VEGFR)-1 and VEGFR-2.2 Notably, most downstream events of VEGF-A are mediated by VEGFR-2 and the subsequent activation of signaling pathways including phospholipase C gamma (PLCγ)-protein kinase C-extracellular signal regulated kinase (PLCγ-PKC-ERK) as well as the production of nitric oxide.3,4

Based on the knowledge of angiogenesis, therapeutic angiogenesis aims at enhancing or promoting neovascularization in chronically ischemic tissue. To date, several growth factors, including VEGF-A, fibroblast growth factor, and hepatocyte growth factor, have entered clinical trials for the treatment of peripheral and myocardial ischemia. Because of its specificity to endothelial cells, VEGF attracts much attention in the field of therapeutic angiogenesis. However, several studies provided important evidence that administration of VEGF-A caused substantial tissue edema, hemorrhage, and inflammation as its side effects in clinical trials.5,6 In addition, many reports strongly suggested that the variation in VEGF concentration under physiological conditions should be tightly controlled. Mouse embryos lacking even a single VEGF allele will result in abnormal blood vessel development and lethality at E9.5.7,8 On the contrary, some investigators also proved that a modest increase in VEGF expression, 2- to 3-fold of the physiological level, resulted in mouse heart development failure and embryonic lethality at E12.5 to 14.5.9 Recently, it was reported that soluble VEGFR-1 (sFlt-1) gene transfer attenuated neointimal formation as well as the upregulation of proinflammatory cytokines after vascular injury, which indicated that endogenous VEGF may promote restenosis, vascular smooth muscle proliferation, and inflammation.10,11 Taken together, these findings suggest that the narrow VEGF-A dose-dependent property and severe side effects may limit its application in angiogenic therapy.

VEGF-E is a group of VEGF-like proteins encoded by several Orf viruses with strong angiogenic activity. Unlike...
VEGF-A165, structurally, VEGF-E has no basic stretch at the carboxy-terminal; functionally, VEGF-E does not use VEGFR-1 to transmit its downstream signaling. Previous findings demonstrate that loop-1 and loop-3 structures of Orf-virusNZ7-derived VEGF-ENZ7 are essential for VEGFR-2 binding. On the other hand, neither edema nor subcutaneous hemorrhage presents in VEGF-ENZ7 transgenic (Tg) mice. However, the mechanism of why VEGF-ENZ7 does not cause edema or hemorrhage is still unclear. Moreover, because VEGF-ENZ7 is only 22.7% and 27.3% identical with VEGF-A165 and human PlGF1, there is a high risk that clinical administration of VEGF-ENZ7 might cause immunorejection. To address these issues, here we characterized 2 humanized VEGF-E molecules denoted as chimeric VEGF-ENZ7/plenta growth factor (PIGF) in a Tg mouse model and clearly proved that the low side effects of both VEGF-ENZ7/PlGF and original VEGF-ENZ7 were attributable to their specificity to corresponding Tg mice at the stage of postnatal day 5 (n=5 per group). Therefore, VEGF-ENZ7 might cause immunorejection. However, the mechanism of why VEGF-ENZ7 does not cause edema or hemorrhage is still unclear. Moreover, because VEGF-ENZ7 is only 22.7% and 27.3% identical with VEGF-A165 and human PlGF1, there is a high risk that clinical administration of VEGF-ENZ7 might cause immunorejection. To address these issues, here we characterized 2 humanized VEGF-E molecules denoted as chimeric VEGF-ENZ7/plenta growth factor (PIGF) in a Tg mouse model and clearly proved that the low side effects of both VEGF-ENZ7/PlGF and original VEGF-ENZ7 were attributable to their specificity to VEGFR-2. Strikingly, in the skin of VEGF-ENZ7/PlGF Tg mice, the vascular leakage was not significantly elevated and the level of inflammatory cell filtration was dramatically decreased.

**Methods**

**Generation of Transgenic Mice**

Animal experiments were performed according to the guidelines of the Institute of Medical Science, the University of Tokyo. The full-length DNA fragments of chimera9, chimera33 (detailed construction is available in the online supplement at http://www.atvb.ahajournals.org), and human VEGF-A165 were cloned into a keratin14 vector (kindly provided by Dr. E. Fuchs, The University of Chicago, III.). EcoRI–HindIII linearized DNA fragment was injected into BDF1 mouse zygotes for the generation of Tg mice (supplemental Figure I). To facilitate comparison of skin phenotypes, the founder mice with a high expression of transgene were crossed with wild type (WT) BALB/c mice. Primers for genomic polymerase chain reaction (PCR) were 5'-TGGGCAACGTGCTGGTTATTGTGCTGTCTC-3’ (forward) and 5’-AGGGTGAAGCAGGGTCCAGCTGTGAAGTG-3’ (reverse).

**ELISA**

The amount of VEGF-A165 in the dorsal skin of postnatal day 5 Tg mice was determined with a human VEGF-A ELISA kit (R&D Systems). All procedures were conducted according to the manufacturer’s direction. The concentration of human VEGF-A165 was presented as the mean of pg VEGF-A/ng total protein in skin lysates. The amount of chimeric VEGF-ENZ7/PIGF in mouse dorsal skin was evaluated with a human PIGF ELISA kit (R&D Systems).

**Histological Analysis**

Tissue samples were fixed in 4% paraformaldehyde (PFA). For hematoxylin/eosin staining, paraffin-embedded specimens were sectioned at 5-μm thickness and stained with hematoxylin/eosin using a standard protocol.

**Whole-Mount Immunofluorescence Staining**

Mouse ears were fixed in 4% PFA and blocked in TNB buffer (NEN Life Science Products) containing 0.3% TritonX-100. Primary antibodies, rat anti-mouse platelet/endothelial cell adhesion molecule-1 (lPECAM-1; BD Pharmingen), rabbit anti-mouse lymphatic vessel endothelial hyaluronan receptor 1 ([LYVE-1] from Dr Kari Alitalo, University of Helsinki, Finland) or anti-mouse α-smooth muscle actin (Sigma) were diluted in TNB buffer, and ear samples were incubated with primary antibodies at 4°C overnight. Then samples were washed 3 times, followed by incubation with secondary antibodies anti-rat IgG Alex 546, anti-rabbit IgG Alex 488, or anti-mouse IgG Alex 488 (Molecular Probes).

**In Vivo Vascular Permeability Assay**

Eight-week-old mice were injected with Evans blue dye at a dose of 30 mg/kg from the tail vein. For quantification of the vascular leakage, free intravascular dye was removed by PBS perfusion 30 minutes after injection of Evans blue. Then the ears and dorsal skin were dissected, and the Evans blue dye was extracted with formamide overnight at 55°C. The concentration of dye was measured spectrophotometrically at 620 nm and expressed as μg of Evans blue/g of tissue according to a standard curve.

**VEGFR Autophosphorylation Assay**

NIH3T3-VEGFR-1, NIH3T3-VEGFR-2, and NIH3T3-VEGFR-3 cell lines were seeded to semiconfluence on collagen-coated multiple-well plates in DMEM containing 10% calf serum (CS) or 10% PBS for NIH3T3-VEGFR-3 cell line. Cells were stimulated with 100 ng/mL of different growth factor at 37°C for 5 minutes, washed by ice-cold PBS with 0.1 mmol/L Na2VO4 twice and lysed in...
1% Triton X-100 lysis buffer. The same amount of protein from each sample was used for Western blotting analysis. Phosphorylated VEGFRs were detected with the antibody against phosphorytrosine, PY20 (BD Pharmingen).

Neuropilin-1 Immunoprecipitation
Four hundred nanograms of VEGF-A165, human VEGF-C (R&D Systems), VEGF-ENZ7, chimera9, and chimera33 (expressed in baculovirus system and purified by VEGFR-2/Fc affinity chromatography) were incubated with 2 μg neuropilin-1/Fc (R&D Systems) in the presence of heparin, respectively. Immunocomplexes binding to protein G-Sepharose beads (Pharmacia Biotech) were washed 5 times gently. Immunoprecipitates were resolved on a 12% SDS-PAGE gel.

Western Blotting
Proteins separated by SDS-PAGE were transferred to Immobilon P membrane. The membrane was blocked with 5% BSA in PBST buffer and subjected to primary antibody incubation for 2 hours. The primary antibodies were VEGF C-1 against human VEGF-A165, PI GF C-20 against chimeric VEGF-ENZ7/PI GF (Santa Cruz Biotechnology), anti-human VEGF-C antibody (R&D System), anti-VEGF E9252, anti-human VEGFR-1 and VEGFR-2 antibody (generated by our laboratory), and anti-human VEGF-3 antibody (from Dr. Kari Alitalo). Corresponding horseradish peroxidase-conjugated secondary antibodies were used for detection of the primary antibody and developed by chemiluminescent reagent.

RT-PCR Analysis of Angiogenic Factors and Proinflammatory Cytokines
Total RNA was extracted from the ears of 8-week-old mice. The RT reaction was performed with random hexanucleotide primers and superscript cDNA synthesis kit (Invitrogene). The primers used were as follows: for VEGF-C, 5'-CCAGCACAGGTTACCTCAGCAA-3' (forward) and 5'-TGGACAGTGAGGCCAGGATG-3' (reverse); for VEGF-D, 5'-GCCTCAAAAAAGCTTGGCAGATTTG-3' (forward) and 5'-ATCTGGGCCATCTCCGACTT-3' (reverse); for angiotatin-1 (Ang 1) 5'-CAGTTGGAGCCGGAATTTCT-3' (forward) and 5'-ATCTGGGCATCTCCGACTT-3' (reverse); for tumor necrosis factor (TNF), 5'-AGGCTGTGAGCCCACTGTT-3' (forward) and 5'-CAGTTGGAGCCGGAATTTCT-3' (reverse); for β-actin, 5'-TCGTGCGTGACATCAAAGAG-3' (forward) and 5'-TGGACAGTGGAGCCCAAGAG-3' (reverse).

Results
High Perinatal Mortality and Inflammation Phenotypes Present in VEGF-A165 but Not VEGF-ENZ7/PIGF Tg Mice
A keratin14 promoter expression cassette, specifically driving its downstream gene expression in the basal epidermal keratinocytes, was used to generate Tg mice. Here we used 2 chimeric VEGF-E genes denominated chimera9 and chimera33 (supplemental Figure I, available online at http://atvb.ahajournals.org). Of the VEGF-A165 Tg mouse founders, 43.8% died by postnatal day 22, whereas the mortality of chimera9 and chimera33 Tg founders was ≈7%, which was normal for mice in the case of more than 8 pups in 1 litter (Figure 1A). The high perinatal mortality suggests that exogenous expression of VEGF-A165 over the physiological dose is lethal to mice. The protein level of VEGF-A165 and VEGF-ENZ7/PIGF in the dorsal skin of Tg pups at postnatal day 5 was examined by ELISA. On average, 124 pg of VEGF-A165, 575 pg of chimera9, and 421 pg of chimera33 per mg of total lysate protein were determined in the corresponding Tg mice (Figure 1B). Despite the lower transgene expression, severe inflammation developed around the snout of VEGF-A165 Tg mice from age 6 weeks (supplemental Figure II). Immunoprecipitation results indicated that no antibody in serum of Tg mice could be coprecipitated with VEGF-A165, chimera9, or chimera33 as expected from the early expression of keratin14 promoter (supplemental Figure III).

VEGF-ENZ7/PIGF Induces Potent Angiogenesis but Not Lymphangiogenesis
Angiogenesis and lymphangiogenesis in the ear skin of Tg mice were examined by whole-mount immunostaining with blood vessel–specific marker PECAM-1 and lymphatic endothelial–specific marker LYVE-1. Based on the property of the keratin14 promoter, the blood vessel density in dermis was dramatically increased because of the angiogenic response to chimeric VEGF-ENZ7/PIGF, VEGF-E9252, and VEGF-A165, respectively (Figure 2A, left panels, and 2B). However, the lymphatic vessels of VEGF-A165 Tg mice were markedly dilated and accompanied by a significant increase in lymphatic vessel area percentage. In contrast, in the ear specimens of VEGF-E9252/PIGF and VEGF-ENZ7/PIGF Tg mice, most lymphatic vessels were collapsed or slightly dilated and the percentage of lymphatic vessel area varied almost in the same range as in WT mice (Figure 2A, right panels, and 2C). The extensively diluted lymphatic vessels in VEGF-A165 Tg mice might be caused by the accumulation of tissue fluid leaking from blood vessels.

Severe Inflammation Develops Only in VEGF-A165 Tg Mice
We next examined the histological changes in lip skin. Different from WT mice of the same age, the specimen from a VEGF-A165 Tg mouse showed keratinocyte hyperplasia, vessel dilation, inflammatory cell infiltration, and tissue edema (Figure 3A). In addition, by immunofluorescence staining, numerous CD11b/Gr-1 positive monocytes and F4/80/Gr-1 positive macrophages were found in the ear skin of VEGF-A165 Tg mice. On the contrary, monocyte/macrophage rarely presented in the ear specimens of VEGF-E9252/PIGF Tg mice, which were only 1.5 to 2-fold of that in WT mice (Figure 3B). Quantification showed that an 8.2-fold increase in CD11b positive monocyte and an 8.9-fold increase in F4/80 positive macrophage were detected in VEGF-A165 Tg mice; also, macrophage numbers were increased 4.2-fold in VEGF-ENZ7 Tg mice (Figure 3C). RT-PCR analysis indicated that the expression levels of endogenous VEGF-A164, VEGF-B, FGF2, Ang2, PDGF-A and PDGF-B were stable in all Tg mice and their WT littermates (data not shown). However, the expression of endogenous VEGF-C and VEGF-D was upregulated in VEGF-A165 Tg mice, which might be related to VEGF-A165-recruited monocyte/macrophage. On the other hand, significant Ang1 upregulation was found in VEGF-E927, chimera9, and chimera33 Tg mice (Figure 3D). Furthermore, except for a significant upregulation of IL-6 and a slight upregulation of TNF expression in the skin of VEGF-A165 Tg mouse, other proinflammatory cytokines, including IL-1α, IL-1β, IFN-γ, and matrix metalloproteinase type 9 (MMP9) had the same expression level in all Tg mice and their WT littermates (data not shown). These results strongly suggest that inflammatory condition is
closely correlated with the expression of exogenous VEGF-A165 but not VEGF-ENZ7/PlGF.

VEGF-ENZ7/PlGF Tg Mice Possess Nearly Normal Vascular Permeability

To facilitate the investigation of vascular leakage in vivo, a small molecular dye, Evans blue, was administrated to mice via tail vein injection. The ears of VEGF-A165 Tg mouse became quite blue because of the severe vascular leakage during the 30 minutes after the injection of Evans blue, whereas the ear specimens from other Tg mice became only slightly blue (Figure 4A). For accurate quantification of vascular permeability, leaked Evans blue dye was extracted from both the dorsal skin and ear. The amount of dye leakage in VEGF-ENZ7 and VEGF-ENZ7/PlGF Tg mice was slightly higher (2.6- and 1.5-fold increase, respectively) than that in WT mice, probably because of the increased vessel numbers in skin. In contrast, the vascular dye leakage of VEGF-A165 Tg mice was increased 6.1-fold in the ear and 6.6-fold in the skin (Figure 4B). The ratio between Evans blue leakage and blood vessel area percentage indicates that VEGF-ENZ7/PlGF and VEGF-ENZ7 are not involved in the enhancement of vascular permeability (Figure 4C). These findings strongly suggest that severe leakage from blood vessels is one of the reasons resulting in lymphatic vessel dilation in VEGF-A165 Tg mice.

VEGF-ENZ7/PlGF-Induced Blood Vessels Are Well Coated With Vascular Pericytes

Following the signs of Ang1 upregulation and nearly normal vascular permeability in VEGF-ENZ7 and VEGF-ENZ7/PlGF Tg mice, vascular pericytes coating in the ear skin was assessed by whole-mount immunostaining of α smooth muscle actin (αSMA) to visualize smooth muscle cell (SMC). The results clearly showed that in the skin of VEGF-ENZ7 and VEGF-ENZ7/PlGF Tg mice the surface of either arteriole or venule was coated with well organized αSMA-positive SMCs. In contrast, SMCs loosely attached to arteriole, and pore structures primarily because of disorganized-SMC were present in the surface of venule in VEGF-A165 Tg mice (Figure 5). These results indicate that both VEGF-ENZ7 and VEGF-ENZ7/PlGF-induced blood vessels are mature.

VEGF-ENZ7/PlGF Is VEGFR-2–Specific Ligand Without Binding to Neuropilin-1

To address the cause of the different phenotypes exhibited by Tg mice at the molecular level, we next analyzed the receptor binding properties of VEGF-A165, VEGF-ENZ7, and VEGF-ENZ7/PlGF. NIH3T3 cell lines, which overexpressed human VEGFR-1, VEGFR-2, and VEGFR-3, respectively, were used for VEGFR autophosphorylation assay. The results indicated that VEGF-A165 induced both VEGFR-1 and VEGFR-2 autophosphorylation, whereas under the same conditions the phosphorylated VEGFR-2 signal was detected by stimulation with chimera9, chimera33, and VEGF-ENZ7 (Figure 6B). Moreover, VEGF-3 autophosphorylation assay revealed that except for VEGF-C, none of ligands, including VEGF-A165, VEGF-ENZ7, chimera9, and chimera33, could stimulate VEGFR-3 (Figure 6B). In addition, neuropilin-1/Fc immunoprecipitation assay demonstrated that only VEGF-A165 could be coprecipitated by neuropilin-1/Fc in the presence of heparin (Figure 6C). These results proved that although VEGF-ENZ7 was partially substituted by the
VEGFR-1–specific ligand PlGF1, VEGF-ENZ7/PlGF still retained VEGFR-2 specific binding ability.

Discussion

We have shown here that VEGF-ENZ7/PlGF is a potent angiogenic factor without a high vascular permeability and inflammatory response. It was reported that the vascular leakage of K14-Ang1/VEGF double Tg mice was significantly lower than that of K14-VEGF Tg mice.19 Interestingly, Ang1 upregulation was found in VEGF-ENZ7/PlGF and VEGF-ENZ7 Tg mice. Ang1 is predominantly secreted by periendothelial cells, including vascular SMCs and others.20 Consistent with the sign of Ang1 upregulation, we proved that VEGF-ENZ7/PlGF and VEGF-ENZ7–induced blood vessels were well coated with SMC, and the vascular leakage in these Tg mice was significantly suppressed compared with that in VEGF-A165 Tg mice. In addition to leakage-resistance, Ang1 was also reported to have anti-inflammatory effects under certain conditions.21 Indeed, except for VEGF-A165 Tg mice, we did not detect IL-6 and TNF upregulation in VEGF-ENZ7/PlGF or VEGF-ENZ7 Tg mice.

Figure 3. VEGF-ENZ7/PlGF induces angiogenesis with low inflammation. A, Histological analysis of the inflammatory condition around the lower lip of a WT mouse and VEGF-A165 Tg mouse. Specimen from VEGF-A165 Tg mouse showed keratinocyte hyperplasia (red arrowhead), papilloma (black arrowhead), vessel dilation (black arrow), inflammatory cell infiltration (white arrowhead), and tissue edema (red arrow). B, Inflammatory cell infiltration in the ear skin of Tg mice examined by immunofluorescence staining. Left panels show the staining results of CD11b (green) merged with Gr-1 (red), and right panels are the staining of F4/80 (green) merged with Gr-1 (red). Magnification is 400×. Scale bar is 50 μm. C, Quantification of monocyte/macrophage recruitment. *P<0.005, **P<0.0002 vs WT. Inflammatory cells with double positive staining signal were calculated from 5 different regions for each sample. D, RT-PCR analysis of the expression level of proangiogenic factors and proinflammatory cytokines. The lane “WT” following each Tg mouse was the corresponding WT littermate.
In the case of VEGF-A165, receptor-binding complexity may be the major mechanism for severe vascular leakage. According to the unique properties of VEGF-like protein purified from snake venom (svVEGF), which was recently characterized by Takahashi et al., we consider that VEGFR-1 signaling also plays an important role in vascular permeability. The strong activation of VEGFR-1 in association with the mild activation of VEGFR-2 yields higher vascular permeability than the activation of VEGFR-1 or VEGFR-2 alone by a VEGFR-1–specific ligand PlGF or a VEGFR-2–specific ligand VEGF-ENZ. In addition, neuropilin-1 is known to mediate angiogenesis by enhancing the binding stability and signaling potency of VEGF-A165 with VEGFR-2. Several VEGF-like proteins from Orf virus, such as VEGF-ENZ2 and VEGF-ED1701, have the properties of both VEGFR-2 and neuropilin-1 binding. However, in the present study, our results demonstrated that both VEGF-ENZ/PlGF and VEGF-ENZ are VEGFR-2–specific ligands, neither binding to neuropilin-1 nor to heparin. Therefore, VEGF-A165–dependent stimulation of VEGFR-2 with the enhancement of neuropilin-1 might yield much stronger downstream signaling and result in relatively higher vascular permeability than stimulation with VEGFR-2–specific ligands.

Severe inflammation around the lips and skin inflammatory cell infiltration were very typical phenotypes presented in VEGF-A165 Tg mice but not in VEGF-ENZ/PlGF Tg mice. The major reason to induce inflammation in VEGF-A165 Tg mice might be that VEGF-A165 is a proinflammatory cytokine with a strong chemotactic property via VEGFR-1 signaling. Since VEGFR-1 was found in the surface of the monocyte/macrophage lineage, it has been proven that most of the chronic inflammatory diseases, including rheumatoid arthritis, were mediated by VEGFR-1 signaling. So far there is no report about the inflammatory conditions in K14-Ang1/VEGF double Tg mice. Therefore, we could not predict the result of synergistic administration of VEGF-A and Ang1 in angiogenic therapy. However, our results showed that a single molecule, VEGF-ENZ/PlGF, does not induce severe monocyte/macrophage recruitment. Furthermore, VEGF-ENZ/PlGF expression via plasmid DNA in the ischemic muscle of adult mice also showed a potent angiogenic response as well as significantly improved blood perfusion with less
infiltration of inflammatory cells compared with VEGF-A165 (Murohara T. and Shibuya M., unpublished results, 2006).

It was reported that adenoviral PlGF gene transfer induced arteriogenesis (ie, large and stable blood vessels at ischemic regions). However, we suggest that the amino- and carboxy-terminal sequences derived from human PlGF1 do not play a major role in stable angiogenesis induced by VEGF-ENZ7/PlGF because the specificity to VEGFR-2 of these chimeric proteins was not changed from the original VEGF-ENZ7. The Tg mouse system, plasmid DNA transfer, and adenoviral transfer may be different to each other in terms of the onset and the time course of gene expression. Thus, VEGF-A165, VEGF-ENZ7/PlGF, and PlGF should be further compared using the same gene/protein transfer methods in a variety of experimental animal models.

Our experiments also showed that in VEGF-A165 Tg mice not only was the lymphatic vessel dramatically dilated but also the percentage of lymphatic vessel area was significantly increased. Under physiological conditions, lymphatic vessels should keep in collapse despite being able to drain extracellular fluid. It is most likely that VEGF-C and VEGF-D, which were secreted by VEGF-A165-recruited monocyte/macrophage, induced lymphangiogenesis. Another possibility for lymphangiogenesis is the direct stimulation of VEGFR-2, which is also expressed on the surface of lymphatic endothelial cells. However, our data indicated that VEGFR-2 might not be directly involved in lymphangiogenesis or involved only at a minor level because the lymphatic vessel area percentage in VEGF-ENZ7/PIGF and VEGF-ENZ7 Tg mice was not significantly increased.

In this present study, we examined the angiogenic activity and side effects of 2 chimeric VEGF-ENZ7/PIGF molecules, chimera9 and chimera33. The identity of chimera9 and chimera33 with human PIGF1 is 50.3% and 61.8% at the amino acid level, respectively, whereas the original VEGF-ENZ7 is only 27.3% identity with human PIGF1. Therefore, considering the antigenicity, we consider that VEGF-ENZ7/PIGF is better than VEGF-ENZ7 in therapeutic angiogenesis. The mechanism of VEGF-ENZ7/PIGF with lower side effects could be explained as follows (supplemental Figure IV). Because of VEGFR-2-specific binding, VEGF-ENZ7/PIGF induces angiogenesis without high vascular permeability and severe inflammation. As a strong chemotactic cytokine, VEGF-A165 recruits monocyte/macrophage via VEGFR-1 signaling, which consequently leads to the severe inflammation in vivo. Moreover, VEGF-A165-recruited monocyte/macrophages secrete VEGF-C and VEGF-D, which subsequently induce lymphangiogenesis via VEGFR-3 signaling. In addition, minor direct stimulation of the lymphatic endothelial cell surface receptor, VEGFR-2, with VEGF-A165, VEGF-ENZ7/PIGF, or VEGF-ENZ7 may also partially contribute to lymphangiogenesis. With the stimulation of VEGFR-1 and VEGFR-2 simultaneously, blood vessels induced by VEGF-A165 have much higher vascular permeability, and serious leakage eventually leads to the dramatic dilation of lymphatic vessels. Therefore, all the findings presented here clearly indicate that the humanized VEGF-ENZ7 could be a novel factor with very low side effects for angiogenesis therapy.

Sources of Funding
This work was supported by Grant-in-Aid Special Project Research on Cancer-Bioscience 17014020 from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a grant from the program “Research for the Future” of the Japan Society for Promotion of Science; and the program “Promotion of Fundamental Research in Health Science” of the Organization for Pharmaceutical Safety and Research.

Disclosures
None.

References


18. Whitaker GB, Limberg BJ, Rosenbaum J. Vascular endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF(165) and VEGF(121) J Biol Chem. 2001;276:25520–25531.


Chimeric VEGF-ENZ/PiGF Promotes Angiogenesis Via VEGFR-2 Without Significant Enhancement of Vascular Permeability and Inflammation
Yujuan Zheng, Masato Murakami, Hiroyuki Takahashi, Mai Yamauchi, Atsushi Kiba, Sachiko Yamaguchi, Naoyuki Yabana, Kari Alitalo and Masabumi Shibuya

Arterioscler Thromb Vasc Biol. 2006;26:2019-2026; originally published online June 22, 2006; doi: 10.1161/01.ATV.0000233336.53574.a1
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/26/9/2019

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2006/06/22/01.ATV.0000233336.53574.a1.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental data

Expanded methods

Construction of Chimeric VEGF-ENZ7/PIGF Gene

Two chimeric VEGF-ENZ7/PIGF molecules were constructed as follows: Chimera 9 was composed of three DNA fragments, corresponding to Met1-Val40 of human PI GF1, Asn35-Cys130 of Orf virusNZ7-derived VEGF-ENZ7 and Glu129-Arg149 of human PI GF1. Chimera 33 was composed of five DNA fragments, corresponding to Met1-Tyr51 of human PI GF1, Cys46-Cys71 of VEGF-ENZ7, Val78-His93 of human PI GF1, Cys88-Cys130 of VEGF-ENZ7 and Glu129-Arg149 of human PI GF1.
Supplemental Figure I. Schematic representing the construction of the keratin14 expression cassette.

Supplemental Figure II. Photographs of mouse snout showed that severe inflammation only developed around the lips of VEGF-A165 Tg mice

Supplemental Figure III. Immunoprecipitation of VEGF-A165, chimera9 and chimera33 with the serum from corresponding Tg mice revealed that no antibody in serum of Tg mice could be coprecipitated with VEGF-A165, chimera9 or chimera33 as expected from the early expression of keratin14 promoter

Supplemental Figure IV. Schematic explanation of the different angiogenic mechanism utilized by VEGF-E_{NZ7}/PIGF and VEGF-A_{165}. Due to VEGFR-2 specific binding property, VEGF-E_{NZ7}/PIGF induced angiogenesis without high vascular permeability and severe inflammation. (Green arrows indicate VEGF-E_{NZ7}/PIGF and VEGF-E_{NZ7}-induced downstream events via VEGFR-2). On the contrary, as a strong chemotactic cytokine, VEGF-A_{165} recruits monocyte/macrophage via VEGFR-1 signaling and therefore resulting in severe inflammation in vivo. Meanwhile, VEGF-A_{165} recruited Monocyte/macrophage subsequently secreted VEGF-C and VEGF-D, which consequently stimulated lymphangiogenesis directly (pink arrow). Furthermore, because of activating VEGFR-1 and -2 simultaneously as well as neuropilin-1 enhancement, blood vessels induced by VEGF-A_{165} have much higher vascular permeability. The serious leakage eventually leads to a dramatic dilation of lymphatic vessels. In addition, direct stimulation (dashed) of the lymphatic endothelial cell surface receptor VEGFR-2, with VEGF-A_{165}, VEGF-E_{NZ7}/PIGF or VEGF-E_{NZ7} might have made a minor contribution to lymphangiogenesis. (Blue arrows indicate VEGF-A_{165}-induced downstream events via both VEGFR-1 and -2.)
Supplemental Figure I  Zheng et al
Supplemental Figure II  Zheng et al

WT  VEGF-A_{165}  VEGF-E_{NZ7}  chimera9  chimera33
Supplemental Figure III Zheng et al
Supplemental Figure IV  Zheng et al

- VEGFR-1
- VEGFR-3
- Neuropilin1
- VEGF-A/PlGF
- VEGF-C/VEGF-D
- Angiogenesis
- Lymphangiogenesis
- Monocyte/macrophage
- VEGFR-1: Recruit
- VEGFR-2: Induce inflammation
- Angiogenesis with normal vascular permeability
- Angiogenesis with high vascular permeability
- Lymphatic vessel dilation

VEGF-A Recruited monocyte/macrophage secrete:

- VEGF-A
- VEGF-D
- Angiogenesis
- Lymphangiogenesis

VEGFR-1:

- Endothelium
- Monocyte/macrophage
- VEGFR-1
- VEGFR-3
- Neuropilin1
- VEGF-A/PlGF
- VEGF-C/VEGF-D
- Angiogenesis
- Lymphangiogenesis
- Recruit
- Induce inflammation

VEGFR-2:

- Endothelium
- Monocyte/macrophage
- VEGFR-1
- VEGFR-3
- Neuropilin1
- VEGF-A/PlGF
- VEGF-C/VEGF-D
- Angiogenesis
- Lymphangiogenesis
- Recruit
- Induce inflammation

Angiogenesis:

- Endothelium
- Monocyte/macrophage
- VEGFR-1
- VEGFR-3
- Neuropilin1
- VEGF-A/PlGF
- VEGF-C/VEGF-D
- Angiogenesis
- Lymphangiogenesis
- Recruit
- Induce inflammation

Lymphatic vessel dilation