Therapeutic Angiogenesis Using Novel Vascular Endothelial Growth Factor-E/Human Placental Growth Factor Chimera Genes

Natsuo Inoue, Takahisa Kondo, Koichi Kobayashi, Mika Aoki, Yasushi Numaguchi, Masabumi Shibuya, Toyoaki Murohara

Background—Vascular endothelial growth factor-A (VEGF-A) promotes angiogenesis but causes adverse side effects such as edema or tissue inflammation. VEGF-E, found in the genome of the Orf virus, specifically binds to VEGF receptor-2 and shows mitotic activity on endothelial cells. Recently, we created two forms of VEGF-E and human placental growth factor (PIGF) chimera genes (VEGF-E chimera #9 and VEGF-E chimera #33), which are humanized genes with VEGF-E function but showing less antigenicity.

Methods and Results—We examined potential proangiogenic activities of these chimera genes. Four types of expression plasmids (pCDNA3.1-LacZ, phVEGF-A, pVEGF-Echimera#9, and pVEGF-Echimera#33) were administered in a rat model of hindlimb ischemia. Either pVEGF-Echimera#9, pVEGF-Echimera#33, or phVEGF-A significantly increased the ratio of ischemic/normal hindlimb blood-flow compared with the control pCDNA3.1-LacZ treated group (by 1.5-fold, 1.5-fold, and 1.4-fold, respectively, \( P < 0.05 \)). Histochemical staining by alkaline phosphatase also revealed that either pVEGF-Echimera#9, pVEGF-Echimera#33, or phVEGF-A increased the capillary density compared with the pCDNA3.1-LacZ treated group (1.4-fold, 1.5-fold, and 1.5-fold, respectively, \( P < 0.05 \)). Furthermore, immunostaining for anti-ED1 revealed that fewer macrophages had infiltrated in both pVEGF-Echimera#9 and pVEGF-Echimera#33 groups compared with the phVEGF-A group (\( P < 0.05 \)).

Conclusions—Novel VEGF-E/human PIGF chimera genes, pVEGF-Echimera#9, and pVEGF-Echimera#33 significantly stimulated angiogenesis in response to tissue ischemia to an almost identical extent to that induced by phVEGF-A with fewer tissue inflammation responses. (Arterioscler Thromb Vasc Biol. 2007;27:99-105.)

Key Words: angiogenesis ■ gene therapy

Local administration of expression genes encoding angiogenic growth factors/cytokines has been performed as one therapeutic modality for severe ischemic disorders such as end-stage myocardial ischemia1,2 or critical limb ischemia,3,4 which were refractory to conventional revascularization. This strategy for the treatment of vascular insufficiency was based on the concept of therapeutic angiogenesis. Studies using animal models of peripheral ischemia have demonstrated that vascular endothelial growth factor (VEGF)-A can stimulate angiogenesis through interaction with its specific receptors, VEGF receptor-1 (VEGFR-1) and VEGFR-2.5 The VEGF family of growth factors presently comprises six members: VEGF-A, placental growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E.6 VEGF-E genes are open reading frames found in the genome of the strains NZ-7, NZ-2, and D1701 of parapox virus, Orf virus.7,8 VEGF-E exclusively binds to VEGFR-2, activates the receptor, and shows almost the same level of mitotic activity on endothelial cells as VEGF-A. Unlike VEGF-A, VEGF-E does not bind to VEGFR-1 and is not expected to cause inflammation via VEGFR-1 expressed on monocytes/macrophages.7 Previously, we reported two types of chimera genes created from VEGF-E and human PIGF (VEGF-E chimera #9 and VEGF-E chimera #33), which are humanized forms of VEGF-E. In keeping with the strong activities of wild-type VEGF-E, they have human PIGF amino acid residues at both the amino and carboxyl ends instead of viral amino acid residues of VEGF-E, resulting in reduced antigenicity.9,10 These synthetic genes are considered useful materials for gene-mediated therapeutic angiogenesis.

Accordingly, the aim of the present study was to investigate the potential proangiogenic efficacies of VEGF-E chimera #9 and VEGF-E chimera #33 genes in rat hind limb ischemia model. Tissue inflammation and edema were also examined and compared between VEGF-E chimera gene and VEGF-A gene transfer groups.

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From the Department of Cardiology (N.I., T.K., K.K., M.A., Y.N., T.M.), Nagoya University Graduate School of Medicine, and the Department of Cancer Biology (M.S.), The Institute of Medical Science, The University of Tokyo, Japan.
Correspondence to Takahisa Kondo, MD, PhD, Department of Cardiology, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-8550, Japan. E-mail takahisa@med.nagoya-u.ac.jp
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Methods

Plasmids
The control vector, pcDNA3.1, and pcDNA-LacZ was purchased from Invitrogen. The backbone of pcDNA3.1 is shown in supplemental Figure 1 (available online at http://atvb.ahajournals.org). The cDNA fragment of human VEGF-A cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR), using template mRNA from human glioma U251 cells, and a set of primers (forward, 5'-CCGGGATCCAGCTTCTTTGCTGTCT-3'; reverse, 5'-CCGGGATCTCCGCCTGGCATGCTCA-3'). The cDNA sequence was confirmed and the EcoRI/HindIII fragment of the cDNA was subcloned into the EcoRI/HindIII sites of the pcDNA3.1, resulting in pcDNA3.1.hVEGF-A. The cDNA sequences of VEGF-E chimera #9 and VEGF-E chimera #33 were confirmed, and the EcoRI/BamHI fragment of the cDNA were subcloned into the EcoRI/BamHI sites of the pcDNA3.1, resulting in pcDNAVEGF-E chimera #9 and pcDNA-VEGF-E chimera #33. Plasmids were amplified by Qiagen Mega kit (QIAGEN) according to the manufacturer's instructions.

Rat Ischemic Hind Limb Model
The rat ischemic limb model is a modification of a 2-stage procedures previously described. Male Sprague-Dawley rats (SIC Japan Inc.) weighing 350 to 400 g were anesthetized pentobarbital (50 mg/kg, i.p.), and ischemia was created in the left hind limb; the right leg served as the control. All left-side branches of the aorta distal to the renal arteries and of the iliac artery were ligated. At day 7, the femoral artery was ligated. Animals were randomly divided into 4 groups (n=12 to 14 per group) and treated with 200 μg of pcDNA3.1-LacZ (placZ), pcDNA3.1-hVEGF-A (phVEGF-A), pcDNA3.1-VEGF-E chimera #9 (pVEGF-Echimera #9), and pcDNA3.1-VEGF-E chimera #33 (pVEGF-Echimera #33) on the same day as the second operation. In our preliminary experiments, doze of 200 μg was sufficient to induce angiogenesis in this animal model (supplemental Figure II). All animal protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University.

RT-PCR
Gene expression in the normal rat musculature was evaluated by RT-PCR. Briefly, 200 μg of phVEGF-A, pVEGF-Echimera#9, pVEGF-Echimera#33 genes were injected into the major thigh muscles. Rats were euthanized 4 days after plasmid administration, and the thigh muscles of injected and non-injected limbs were resected for RNA extraction. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected by RT-PCR as an internal control. To detect the contamination of plasmid, PCR without reverse transcription were also performed. All primers were described as follows. The primers were forward, 5'-ATGGCCGGTCTGAGGCTGT-3' and reverse, 5'-AACGCTATGGCCCACCCTC-3' for VEGF-E chimera #9 and #33 respectively, and forward, 5'-GAGAGGGCGAATCAGGAATGAGG-3' and reverse, 5'-GGCTGTCATAGCTCAAGT-3' for hVEGF-A, respectively. The RT-PCR products were 470 bp for hVEGF-A, and 462 bp for VEGF-E chimera #9 and VEGF-E chimera #33. The thermal cycle consisted of a 1-minute denaturation at 94°C, a 1-minute annealing at 55°C, and a 2-minute extension at 72°C.

β-Galactosidase Expression
To document gene transduction, β-galactosidase expression was visualized in muscle sections stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). Frozen tissue sections were incubated in X-gal staining solution (1 mg/mL) for 3 hours at 37°C. Random cross sections taken from the middle portion of the muscles were examined under light microscopy.

ELISA
To detect VEGF-E chimera protein in the serum of rats, levels of human PGF were measured with specific ELISA kit (R&D Systems) before and 4 and 14 days after pVEGF-Echimera#9 treatment. This antibody can detect the epitope of VEGF-E chimera protein.

Laser Doppler Blood Flowmetry
We measured the ratio of ischemic (left)/normal (right) hind limb blood flow using laser Doppler blood flowmetry (LDBF; moorLDI, Moor Instrument) as described previously. Low to no flow is displayed as dark blue, whereas high blood flow is displayed as red to white. At 4 predetermined time points (immediately after second surgery and on postoperative days 7, 14, and 21), we performed 2 consecutive laser scanings over the same region of interest (legs and feet). The average flow of the ischemic and nonischemic legs was calculated on the basis of histograms of the colored pixels. To minimize variations due to ambient light, blood flow was expressed as the ischemic (left)/normal (right) limb flow ratio.

Histological Analysis
At day 4, four animals per group were euthanized and adductor muscles were removed from ischemic limbs for histological analysis. For immunohistochemistry, an anti-ED1 antibody (BMA) was used as a primary antibody and detected using the ABC Kit (Vector laboratories) with a biotinylated anti-mouse serum and peroxidase-conjugated streptavidin. The density of ED1-positive cells was expressed as the mean number of ED1-positive cells per field of view. At day 21, all animals were euthanized and adductor muscles were removed from ischemic limbs for histological analysis. Frozen sections were subjected to alkaline phosphatase (AP) staining by the indoxyl-tetrazolium method. AP staining turns capillary endothelial cells a dark blue color only when they are viable and when the intracellular enzyme activity remains intact. For capillary counts, to ensure that the capillary densities were not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was determined.

In Vitro HUVEC Proliferation Assay
We examined the effects of recombinant VEGF-E chimera #33 protein on HUVEC proliferation by MTT assay as previously described. Briefly, 1×10^5 of HUVECs (CAMBREX) were added to 200 μL of cell culture medium containing 2% FBS in 96-well plate (1000 cells per well). Construction of VEGF-E chimera #33 protein was reported previously. In the presence or absence of 10 ng/mL of VEGF-E chimera #33 protein, and to ensure that the effects of VEGF-E chipera#33 were solely mediated by VEGFR-2, 10 ng/mL of VEGF-E chimera #33 protein and 0, 1, and 10 μmol/L of SU1498 (Calbiochem), a VEGFR-2-specific receptor tyrosine kinase inhibitor, were also added in each group. Recombinant human VEGF-A (rhVEGF-A; 10 ng/mL) (R&D Systems) and recombinant human PGF (rhPGF; 10 ng/mL) (RELICATech GmbH) were also examined. The plates were incubated for 72 hours at 37°C, and 20 μL of CellTiter 96 Solution Agent (Promega) was added to each well. After incubation for 3 hours at 37°C, the absorbance at 490 nm were measured using a 96-well plate reader (BIORAD).

In Vitro Chemotaxis Assay
U937 cells (human histiocyte lymphoma cells) were cultured in RPMI1640 containing 10% FBS. Chemotaxis was assayed in 48-well chamber plates (Becton Dickinson Labware) with 8 μmol/L porosity polycarbonate filter membrane. U937 (5×10^3) cells were washed with PBS and serum starved for 2 hours, then resuspended in
50 µL of RPMI medium containing 0.1% FBS in each well; 30 µL of RPMI medium containing various densities of rhVEGF-A and VEGF-E chimera #33 protein (0, 1, 10, and 100 ng/mL) was placed in each bottom well. After incubation at 37°C for 2 hours, the cells on the upper surface of the filters were removed, and the cells which migrated to the lower surface were fixed in 100% methyl alcohol and stained with Giemsa solution (Sigma) and May-Grunwald eosin methylene blue solution (Merck). For quantitative assessment, the mean number of migrated cells in four randomly chosen high-power fields was counted under a microscope.

Quantification of Chemokines
U937 cells (1×10⁶/mL) were incubated in RPMI1640 with rhVEGF-A (100 ng/mL) or VEGF-E chimera #33 protein (100 ng/mL) containing 0.1% FBS for 3 hours at 37°C. The medium was collected and cell debris removed by low-speed centrifugation at 1500 rpm for 10 minutes. Levels of inflammatory chemokines (interleukin [IL]-8 and tumor necrosis factor [TNF]-α) were assayed using specific ELISA kit.

Statistical Analysis
All values are presented as mean±SEM. Statistical significance was evaluated by one-way ANOVA followed by Dunnett modified test. Differences were considered statistically significant at a probability value of P<0.05.

Results
Confirmation of Gene Expression
Human-specific VEGF-A, VEGF-E chimera #9, and VEGF-E chimera #33 mRNA expression was confirmed in the thigh muscles injected with phVEGF-A, pVEGF-E chimera #9 and pVEGF-E chimera #33, respectively (Figure 1A). The specific PCR product for the hVEGF-A gene corresponding to 470 bp was detected in the tissue RNA from pVEGF-A injected limbs 4 days after plasmid injection. The specific PCR products for the VEGF-E chimera #9 and VEGF-E chimera #33 gene, corresponding to 462 bp, were also detected in the RNA from the limbs 4 days after administration of pVEGF-E chimera #9 and pVEGF-E chimera #33. Neither hVEGF-A, VEGF-E chimera #9, or VEGF-E chimera #33 specific mRNA were detected in the samples from contralateral nonischemic limbs, nor in the samples without reverse transcription. β-galactosidase expression was documented in the muscles treated with pLacZ after 4 days. Gene transduction was limited to the skeletal myocytes (Figure 1B). The level of immunoreactive human PlGF was increased in the pVEGF-E chimera #9 group compared with the pLacZ group (P<0.05) (supplemental Figure III).

Laser Doppler Blood Flowmetry
Microangiographic and vessels density measurements were associated with changes in blood perfusion (Figure 2). At day 21 of the treatment, the ischemic/normal hind limb LDBF ratio was increased 1.5-fold in pVEGF-E chimera #9-treated and 1.4-fold in pVEGF-E chimera #33-treated rats compared with the control pLacZ group (P<0.001). phVEGF-A treatment also induced a 1.5-fold increase in the ischemic/normal hind limb ratio (P<0.01 versus pLacZ).

Histological Assessment
Histochemical staining by alkaline phosphatase also revealed that the pVEGF-E chimera #9, pVEGF-E chimera #33, and
phVEGF-A–treated groups had increased the capillary/muscle fiber ratio compared with the pLacZ-treated group (1.4-, 1.5-, and 1.5-fold, respectively, \( P < 0.05 \); Figure 3). Furthermore, immunostaining for anti-ED1 revealed that lower number of macrophages was infiltrated in both pVEGF-E chimera #9 and pVEGF-E chimera #33-treated groups than in the pLacZ-treated control group. Values are mean±SEM (n=8 to 10) at each time point. *\( P < 0.05 \), **\( P < 0.01 \) vs pLacZ.

In Vitro HUVEC Proliferation Assay
We examined the effects of exogenous VEGF-E chimera #33 protein on the proliferative activity of HUVECs. VEGF-E chimera #33 protein significantly enhanced the proliferation of HUVECs in a dose-dependent manner (Figure 5B). Otherwise rhPlGF did not increase proliferation compared with control (data not shown). The VEGF-E chimera#33–induced proliferation was completely abolished with SU1498, a VEGFR-2–specific receptor tyrosine kinase inhibitor (Figure 5B).

Chemokinetic Response of U937 to rhVEGF-A
We observed that rhVEGF-A caused a dose-dependent increase in the migrations of U937 cells. On the other hand, VEGF-E chimera #33 protein did not show a significant difference compared with the control medium (Figure 6A). In the rhVEGF-A–treated group, the production of the inflammatory cytokines, IL-8 and TNF-\( \alpha \), was significantly increased compared with the VEGF-E chimera #33–treated group (Figure 6B and 6C).

Discussion
Patients with severe myocardial or limb ischemia who are not optimal candidates for percutaneous or surgical revascularization have been increasing worldwide. Although therapeutic angiogenesis using VEGF-A and other growth factors were expected to solve these problems, clinical efficacies were disappointing in several recent clinical trials,\(^{16–18}\) suggesting the need for alternative growth factor therapies. In the present study, we investigated the angiogenic efficacies of two types of VEGF-E chimera genes. The major findings of this study were as follows: (1) Intramuscular administration of two types of VEGF-E chimera genes, in the expression
plasmid form, promoted neovascularization in the ischemic hind limb to the level similar to that of human VEGF-A; (2) In pVEGF-E chimera #9 and #33 treated animals, fewer infiltrated macrophages were observed compared with pVEGF-A treated muscles; (3) Recombinant VEGF-E chimera #33 had dose-dependent proliferative activity on HUVECs via VEGFR-2; Finally, (4) VEGF-A-stimulation increased migration of monocytic cells and inflammatory cytokines such as IL-8 or TNF-α.

VEGF-A binds to both VEGFR-1 and VEGFR-2, whereas VEGF-E and VEGF-E chimeras bind to only VEGFR-2. Proangiogenic effects of VEGF-E chimeras are mediated solely by VEGFR-2 signaling, inducing migration and proliferative effects on vascular endothelial cells, formation of microcapillary vessels,7,19 and recovery of peripheral blood perfusion. There is a difference in the efficacy of the signal transduction of VEGFR-1 and VEGFR-2. VEGFR-1 undergoes weak tyrosine autophosphorylation in response to VEGF, almost one-order of magnitude weaker than that of VEGFR-2. In this study, VEGF-E chimera #33 protein induced HUVEC proliferation and its effect was completely abolished by SU1498, a VEGFR-2 specific tyrosine kinase inhibitor. On the other hand, VEGFR-1 ligand PIGF was ineffective, suggesting that VEGFR-2 signaling as the major regulator of endothelial cell proliferation and VEGFR-1 signaling alone may not be sufficient to initiate endothelial cell proliferation.19,20 Accumulating evidence suggests that more than half of pathological angiogenesis involves the activation of VEGFR-2. Although VEGFR-1 mediates signaling for less than half of such cases, it plays a major role in the inflammatory cell/macrophage-dependent process in a variety of diseases such as rheumatoid arthritis and atherosclerosis.21

In line with these previous studies, phVEGF-A administration caused varying number of macrophages to infiltrate the ischemic muscle in this study, whereas in pVEGF-E chimera #9 and #33 treated muscles, only a few macrophages were observed. One putative explanation for this discrepancy.
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inflammatory cytokine secreted by monocyte/macrophage lineage and has been shown to induce the actin fiber formation and intercellular gap formation of endothelial cells.\textsuperscript{25} TNF-\(\alpha\), also released by macrophages, regulates microcirculatory systems via relaxation of vascular pericytes and increases vascular permeability.\textsuperscript{26} Furthermore, administration of rVEGF-A mobilizes macrophages and monocytes in peripheral blood while simultaneously increasing atherosclerotic plaque formation and progression.\textsuperscript{27} Macrophages also contribute to plaque instability through elaboration of matrix metalloproteinases and other hydrolytic enzymes as well as tissue factor, thus increasing risk of thrombotic occlusion in the event of plaque rupture. Taken together, VEGF-E families, selective VEGFR-2 ligands, can induce angiogenesis via endothelial cell proliferation without stimulating monocyte/macrophage lineage. Therefore, VEGF-E chimeras could avoid the excessive tissue inflammation, edema, or atherosclerotic plaque development, which are often observed in VEGF-A–induced therapeutic angiogenesis. Inflammation is one of the major factors to promote angiogenesis. In the present study, VEGF-E chimera genes did cause little inflammation, but promoted angiogenesis to the similar level as did VEGF-A. Then why VEGF-E chimera genes could promote ischemia induced-angiogenesis without mobilizing inflammatory cells? One possible explanation is that angiopoietin-1 was upregulated in VEGF-E chimera #9 and #33 transgenic mice.\textsuperscript{10} Although the mechanisms by which VEGF-E chimeras increases angiopoietin-1 levels are not clear so far, angiopoietin-1 might promote mature vessel growth in concert with VEGF-E chimera proteins.

Figure 6. A, U937 chemotaxis assay with various density of rhVEGF-A and recombinant VEGF-E chimera #33 protein. For quantitative assessment, the mean number of migrated cells in four randomly chosen high-power fields were counted under a microscope. Values are mean\(\pm\)SEM. *\(P<0.05\) vs control. B and C, Cytokine quantification. U937 cells (1\(\times\)10\(^6\)/ml) were treated with 100 \(\mu\)g/mL of rhVEGF-A and VEGF-E chimera #33 protein. IL-8 and TNF-\(\alpha\) were measured using specific ELISA after 3 hour incubation. *\(P<0.05\) vs control and VEGF-E chimera #33. Values are mean\(\pm\)SEM.

would be that VEGFR-1 is, in fact, the only VEGF receptor of monocyte/macrophage lineages. Furthermore, in this study, U937 cells of human myelomonocytic cell line were stimulated and released inflammatory cytokines such as IL-8 and TNF-\(\alpha\) by rhVEGF-A but not by rVEGF-E. Our data are consistent with the phenotypic features of VEGF-A and VEGF-E chimera #9 and #33 gene transgenic mouse, previously reported. VEGF-A transgenic mouse showed severe edema, hemorrhage, and inflammatory cell infiltration in addition to an angiogenic response,\textsuperscript{22,23} whereas no leaky lesions or hemorrhagic spots were observed in VEGF-E transgenic mice.\textsuperscript{24} Moreover, VEGF-E chimera gene transgenic mouse did not increase vascular permeability like VEGF-A transgenic mouse.\textsuperscript{10} Recent clinical trials using VEGF-A failed to show clinical benefit compared with placebo control.\textsuperscript{16,17} In the RAVE trial, administration of adenoviral VEGF-A increased leg edema nearly 30%.\textsuperscript{13} This result may be related to VEGF-A–induced vascular permeability via recruitment of inflammatory cells and release of various cytokines such as IL-8 and TNF-\(\alpha\). IL-8 is an inflammatory cytokine secreted by monocyte/macrophage lineage and has been shown to induce the actin fiber formation and intercellular gap formation of endothelial cells.\textsuperscript{25} TNF-\(\alpha\), also released by macrophages, regulates microcirculatory systems via relaxation of vascular pericytes and increases vascular permeability.\textsuperscript{26} Furthermore, administration of rVEGF-A mobilizes macrophages and monocytes in peripheral blood while simultaneously increasing atherosclerotic plaque formation and progression.\textsuperscript{27} Macrophages also contribute to plaque instability through elaboration of matrix metalloproteinases and other hydrolytic enzymes as well as tissue factor, thus increasing risk of thrombotic occlusion in the event of plaque rupture. Taken together, VEGF-E families, selective VEGFR-2 ligands, can induce angiogenesis via endothelial cell proliferation without stimulating monocyte/macrophage lineage. Therefore, VEGF-E chimeras could avoid the excessive tissue inflammation, edema, or atherosclerotic plaque development, which are often observed in VEGF-A–induced therapeutic angiogenesis. Inflammation is one of the major factors to promote angiogenesis. In the present study, VEGF-E chimera genes did cause little inflammation, but promoted angiogenesis to the similar level as did VEGF-A. Then why VEGF-E chimera genes could promote ischemia induced-angiogenesis without mobilizing inflammatory cells? One possible explanation is that angiopoietin-1 was upregulated in VEGF-E chimera #9 and #33 transgenic mice.\textsuperscript{10} Although the mechanisms by which VEGF-E chimeras increases angiopoietin-1 levels are not clear so far, angiopoietin-1 might promote mature vessel growth in concert with VEGF-E chimera proteins.

Drawbacks of gene therapy using VEGF-E chimera #9 and #33 are that VEGF-E gene was derived from Orf virus. However, in VEGF-E chimera #9 and #33, the only remaining structures of VEGF-E are binding sites for VEGFR-2. The amino- or carboxyl-terminal peptide sequences whose regions are known to be highly immunogenic, were replaced with those of human PlGF. Therefore, these “humanized VEGF-E family genes” are expected to be less immunogenic compared with the wild-type VEGF-E.

In conclusion, we found that gene therapy using novel VEGF-E/human PlGF chimera genes promoted angiogenesis without inflammatory cell infiltration in the setting of hindlimb ischemia. These novel genes may become one of the therapeutic options for patients refractory to conventional revascularization.

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Disclosure

None.

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Supplemental Figure I
Supplemental Figure II
human PIGF (pg/mL)

Supplemental Figure III