Angiogenic Gene Therapy for Experimental Critical Limb Ischemia

Acceleration of Limb Loss by Overexpression of Vascular Endothelial Growth Factor 165 but not of Fibroblast Growth Factor-2

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Abstract—Recent studies suggest the possible therapeutic effect of intramuscular vascular endothelial growth factor (VEGF) gene transfer in individuals with critical limb ischemia. Little information, however, is available regarding (1) the required expression level of VEGF for therapeutic effect, (2) the related expression of endogenous angiogenic factors, including fibroblast growth factor-2 (FGF-2), and (3) the related adverse effects due to overexpression of VEGF. To address these issues, we tested effects of overexpression of VEGF165 using recombinant Sendai virus (SeV), as directly compared with FGF-2 gene transfer. Intramuscular injection of SeV strongly boosted FGF-2, resulting in significant therapeutic effects for limb salvage with increased blood perfusion associated with enhanced endogenous VEGF expression in murine models of critical limb ischemia. In contrast, VEGF165 overexpression, 5-times higher than that of baseline on day 1, also strongly evoked endogenous VEGF in muscles, resulting in an accelerated limb amputation without recovery of blood perfusion. Interestingly, viable skeletal muscles of either VEGF165 or FGF-2–treated ischemic limbs showed similar platelet-endothelial cell adhesion molecule–1–positive vessel densities. Maturation of newly formed vessels suggested by smooth muscle cell actin–positive cell lining, however, was significantly disturbed in muscles with VEGF. Further, therapeutic effects of FGF-2 were completely diminished by anti-VEGF neutralizing antibody in vivo, thus indicating that endogenous VEGF does contribute to the effect of FGF-2. These results suggest that VEGF is necessary, but should be delicately regulated to lower expression to treat ischemic limb. The therapeutic effect of FGF-2, associated with the harmonized angiogenic effects seen with endogenous VEGF, provides important insights into therapeutic angiogenesis.

Key Words: angiogenesis ▪ vascular endothelial growth factor ▪ fibroblast growth factor-2 ▪ limb ischemia ▪ limb salvage

Early clinical studies on gene transfer of angiogenic growth factors, including vascular endothelial growth factor (VEGF), suggest a possible strategy to treat critically ischemic limbs.1,2 Related adverse effects of intramuscular gene transfer of VEGF, however, have been given less attention. Recent reports indicated that transgenic3 or adenoviral4 overexpression of VEGF resulted in formation of leaky vessels in laboratory animals, and that plasmid-based intramuscular VEGF gene transfer led to transient edema in human subjects.1,2 Other unfavorable effects of VEGF overexpression include formation of “angioma-like” fragile capillaries, without connections to preexisting vessels, probably due to leading imbalance of angiogenic signals.5 To our knowledge, our previous report on VEGF165 gene transfer to the vessel wall is the first demonstration of in vivo angiomatic endothelial proliferation via VEGF in the severe neointimal formation associated with extravasation of red blood cells,6 and more recently, Lee et al7 also reported a similar pathology via retrovirus-mediated overexpression of VEGF in the myocardium.

In addition to VEGF, an earlier study also demonstrated similar angioma-like capillary formations without significant increase in collateral blood flow by continuous release of the protein form of acidic fibroblast growth factor (aFGF, FGF-1) in the ischemic myocardium.8 These experimental studies thus suggest that increase in local concentrations of angio-
genic factors does not always contribute to forming functional neovascularization, probably due to the imbalance in angiogenic action of each polypeptide.

Angiogenesis, including its physiological maturation associated with recruitment of smooth muscle cells (SMCs), is a well-harmonized process, and not only VEGF but also other factors including the FGF family, hepatocyte growth factor, and angiopoietins are likely to play significant and harmonized roles in angiogenic processes. Although direct gene transfer studies using these have potent angiogenic ability in vivo, little information is now available regarding both the best gene to treat limb ischemia and whether more transgene expression is more efficient. Clearly, it would be favorable to make use of a gene that would lead to more efficient blood perfusion, as well as more blood vessels.

To determine if a higher expression of transgene would lead to better results, we used recombinant Sendai virus (SeV)–mediated gene transfer to boost therapeutic genes. This approach showed highly efficient transgene expression in various organs. We here used 2 independent SeVs expressing human VEGF165 and murine FGF-2, an alternative angiogenic mitogen, to treat hind limb ischemia.

Materials and Methods

Gene Transfer Agents

Virus titer was determined by hemagglutination assay using chicken red blood cells and was kept at −80°C until use. Human VEGF165 cDNA expression plasmid vector was previously obtained, and murine FGF-2 cDNA was a kind gift from Dr T. Imamura (National Institute of Bioscience and Human-Technology, Tsukuba, Japan).

Animals and Surgical Procedures

Male C57BL/6 (6 weeks old) and BALB/c nu/nu mice (5 weeks old) were from KBT Oriental Co Ltd (Charles River Grade, Tosu, Saga, Japan). All animal experiments were done under approved protocols and in accordance with recommendations for the proper care and use of laboratory animals by the Committee for Animal, Recombinant DNA, and Infectious Pathogen Experiments at Kyushu University and according to The Law (No. 105) and Notification (No. 6) of the Japanese Government.

Under sufficient anesthesia with an intraperitoneal injection of sodium pentobarbital, the entire left superficial femoral artery and vein (from just below of deep femoral arteries to popliteal artery and vein) was ligated, cut, and excised for the mild ischemia model. For the severe ischemia model, additional excision of left external iliac artery and vein with deep femoral and circumflex arteries and veins was also made. Consistency of limb prognosis of these models was confirmed by 5 repeated pilot experiments using more than 10 animals/model at once and by the same operator (I.M.). For gene therapy intervention, 25 μL of vector solutions were injected into each port (2 for thigh and 2 for calf) soon after the surgical procedures.

Immunossays

Recombinant protein in tissue lysate was determined using Quantikine Immunoassay systems for human and murine VEGFs and murine FGF-2 (R&D Systems Inc) according to the manufacturer’s instructions. Each figure for protein measurements was obtained in separate experiments, and pooled samples were never used to confirm the consistency. For value standardization, the wet weight (expressed as pg/g muscle) or total protein concentration was measured by a commercially available protein assay system (Bio-Rad Laboratories).

Laser Doppler Perfusion Images

Measurements of the ischemic (left)/normal (right) limb blood flow ratio were performed using a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments). To minimize data variables due to ambient light and temperature, the LDPI index was expressed as the ratio of left (ischemic) to right (nonischemic) limb blood flow.

Histological Analysis and Immunohistochemistry

All thigh muscles of the limb salvage model at 2 or 10 days after the surgical procedure were subjected to quantification of squares of the histological category, namely, muscle necrosis without repair response, granulation tissue, muscle regeneration, and undamaged muscles, was done using MacScope software. The histological findings were evaluated by a blinded examiner (Y.Y.).

Vessel densities of both ischemic and nonischemic skeletal muscle tissues at 10 days after surgery for the limb salvage model were immunohistochemically determined, using anti-murine platelet-endothelial cell adhesion molecule (PECAM)-1 monoclonal antibody (B&D Pharmingen). A mirror section was simultaneously stained with anti–α-smooth muscle cell actin (SMDA) monoclonal antibody (Sigma). Digital images of 10 microscopic fields from 3 different sections from each animal were stored, and PECAM-1–positive vessels with morphological circumference of staining of 1 or more endothelial cells or those with positive for SMA vessels were counted by 2 independent pathologists (I.M. and Y.Y.). PECAM-1 stain with single dot was not counted as capillary vessels. Mean values independently expressed by these examiner were used. Vessel density labeled with PECAM-1 or with both was expressed as the number of capillaries per mm².

Inhibition of VEGF Activity

In vivo suppression of endogenous VEGF activity was done using VEGF-specific neutralizing rabbit polyclonal IgG (cross-reactive to human and murine VEGF, NeoMarkers Co, Fremont, Calif). One day before surgery, the disposable microosmotic pump (Model 1007D, ALZA Co) with 200 μL of either nonimmunized rabbit IgG or anti-VEGF IgG (1 mg/mL, respectively) was implanted into the peritoneal cavity. This pump continuously releases these solutions at the rate of 0.5 to 1.0 μL/h for around 7 days. Soon after the ischemia-related surgery, additional bolus administration of these antibodies (100 μg, respectively) was given via the pineal vein.

Statistical Analysis

All data were expressed as mean±SD, and the data, except for those related to limb survival, were analyzed by 1-way ANOVA with Scheffe’s adjustment. For survival analysis, survival rate expressed by limb salvage score (LSS) was analyzed using Kaplan-Mayer’s method. The statistical significance of the survival experiments was determined using the log-rank test, and P<0.05 was considered to be statistically significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Characterization of Hind Limb Ischemia Models

Before assessing the therapeutic and adverse effects of angiogenic factors, we first tested 3 models of limb ischemia using 2 different surgical approaches: (1) via excisions of whole femoral and saphenous arteries and vein of C57BL/6 mice to induce mild limb ischemia (mild ischemia model), (2) via excisions of whole external iliac artery and vein, femoral artery and vein, and all related branches of C57BL/6 mice, and (3) via the same surgical procedure as (2) using BALB/c nu/nu mice. Our pilot study demonstrated that the 2nd model mice had no limb necrosis, occasionally showing toe insufficiency (limb salvage model), and approximately 60% of animals in the 3rd model resulted in limb amputation, at least...
above knee level (autoamputation model), 10 days after the initial surgery.

**Effect of Plasmid-Mediated VEGF165 Gene Transfection on Autoamputation Model**

Using the autoamputation model, we first tested the therapeutic effect of VEGF165 gene transfer via plasmid-based intramuscular expression. We presently categorized the degree of limb necrosis for 4 salvage scores for evaluation of therapeutic effects of gene transfer (Limb Salvage Score [LSS]): LSS=4, complete limb salvage; LSS=3, limb necrosis below heel; LSS=2, limb necrosis below knee; LSS=1, limb necrosis above knee; and LSS=0, total autoamputation around the inguinal ligament. As shown in Figure 1, pCAG-VEGF165 (human VEGF165 cDNA driven with the CAG-hybrid promoter: cytomegalovirus immediate early enhancer+chicken β-actin promoter) showed a mild limb salvaging effect, however, was not statistically significant in comparison with that of phosphate-buffered saline (PBS: dilution buffer)–injected mice. An additional experiment for quantification of plasmid-based VEGF165 expression in this model showed 42.1% of exogenously transferred human VEGF (mean=102.0 pg/g muscle, n=8) compared with that of endogenous baseline (murine VEGF) with limb ischemia (mean=242.5 pg/g muscle, n=12).

**SeV-Mediated Expression of Angiogenic Factors In Vivo**

To achieve an increased transepigen expression in muscles, we constructed SeVs encoding human VEGF165 (SeV-VEGF165) or murine FGF-2 (SeV–FGF-2). Although in vitro gene expression studies using these viruses showed similar levels of recombinant protein secretion (see the online data supplement available at http://www.circresaha.org), the in vivo expression study of angiogenic factors yielded paradoxical results. SeV–FGF-2–mediated protein synthesis increased in a dose-dependent manner, similar to findings with SeV-luciferase (see online data supplement), reaching 100-fold over the endogenous expression at the highest titer (Figure 2A). In contrast, a dose-dependent increase of human VEGF165 protein was far less and did not reach 2-fold at 107

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**Figure 1.** Limb prognosis curve according to the limb salvage score after plasmid-based intramuscular gene transfer of VEGF165 or phosphate buffered saline (PBS) injection. Each group included 8 mice with autoamputation model. Curve was obtained using Kaplan-Mayer's method, and data were analyzed using log-rank test.

**Figure 2.** A through C, Protein expression after SeV-mediated intramuscular gene transfer of angiogenic factors in ischemic or nonischemic muscles. Values were standardized wet weight of each muscle and expressed as mean±SD. A and B, Bar graphs indicating in vivo expression of exogenously transferred FGF-2 (A) and VEGF (B) gene in thigh and calf muscles of C57BL/6 with mild limb ischemia. Two days after surgery and gene transfer, all thigh and calf muscles (n=6, each) were subjected to ELISA for murine FGF-2 (A) and murine or human VEGF (B), respectively. Mean values (pg/g muscle) are shown as a bar graph, and error bars indicate SD. B, Endogenous murine VEGF expression was assessed using the murine-specific ELISA system as indicated “murine,” whereas exogenous “human” VEGF165 was assessed using the human VEGF–specific system. Note log scale. C, Bar graphs indicating time-dependent changes of exogenous transgene expression after FGF-2 (left, day 1: n=12 and day 2: n=16, respectively) or VEGF165 (right, day 1: n=12 and day 2: n=12, respectively) gene transfer to C57BL/6 mice with severe limb ischemia. Note that transgene expression of VEGF165 markedly decreased at day 2, whereas FGF-2 showed an apparent increase in a time-dependent manner. Baseline indicates baseline endogenous expression of severely ischemic muscles without gene transfer (n=6, each). *P<0.01, analyzed by 1-way ANOVA. D, Bar graphs indicating gene transfer–mediated enhancement of endogenous murine VEGF expression in limb muscles of C57BL/6 mice without operation (open bars), or severe ischemia (solid black bars). Two days after the surgery and gene transfer, all thigh and calf muscles (n=6 each, total n=12 muscles) were subjected to murine VEGF–specific ELISA.
pfu (plaque forming units); furthermore, VEGF165 transgene expression was undetectable at $10^8$ pfu (Figure 2B).

In our several years of experience, SeV-mediated gene expression gradually increased in a time-dependent manner, and usually reached a peak 2 days after gene transfer in several organs. Thus, we asked if a vector-mediated transgene expression would in muscles show a similar pattern to that seen in other organs by assessing exogenous protein levels on day 1. SeV–FGF-2 showed an expected increase in transgene expression (Figure 2C, right graph, day 1: $n=110$, day 2: $n=16$), whereas SeV–VEGF165 showed paradoxically a time-dependent decrease (left graph, days 1 and 2: $n=110$, respectively).

Ischemia-Induced Endogenous VEGF Expression Is Enhanced by Angiogenic Gene Transfer

To explain the incomparable time-course expression patterns between VEGF165 and FGF-2, we hypothesized that overexpression of exogenous VEGF165 might accelerate tissue ischemia via a “permeability” action at day 1 and might inversely downregulate the SeV-dependent transcription at day 2. Further, FGF-2 partly shares angiogenic activity with VEGF by enhancement of endogenous VEGF expression, which suggests that transgene-related modulation of endogenous VEGF expression may be a clue to explain these paradoxical findings. We thus examined the endogenous VEGF protein synthesis in skeletal muscles followed by gene transfer. Neither VEGF165 nor FGF-2 gene transfer at $10^7$ pfu significantly enhanced endogenous VEGF levels in muscles under normal circulation (Figure 2D), whereas both dramatically enhanced endogenous murine VEGF expression under conditions of severe limb ischemia ($P<0.001$).

We then histologically assessed effects of gene transfer of angiogenic factors using the C57BL/6 severe ischemia model. Ischemic surgery with luciferase transfection showed diffusely damaged muscle fibers associated with intracellular edema and inflammatory infiltrate 2 days later (data not shown). These findings were markedly enhanced by VEGF165 gene transfer (left graph), yet inhibited by FGF-2 gene transfer (data not shown).

Exogenously Overexpressed VEGF165 Leads Ischemic Limb to Damage Rather Than to Salvage

Based on these findings, we tested the deleterious or therapeutic effects of gene transfer of angiogenic factors in vivo using the limb salvaging model (C57BL/6) or autoamputation model (balb/c nu/nu), respectively.

In assessment of deleterious effect, some mice treated with VEGF165 lost limbs (5/10 lost limbs, %LSS = 52.5%, $P<0.0001$ compared with other groups), whereas all mice of other groups, including FGF-2, showed almost complete limb salvage (FGF-2, %LSS = 100%; luciferase, %LSS = 98%), thereby suggesting the limb damaging effect of VEGF165 gene transfer (Figure 3A, left). To test whether VEGF-mediated unfavorable effects were due to the biological action of VEGF or to inflammatory responses against VEGF protein derived from different species, we blocked the biological function of VEGF using an anti-VEGF neutralizing antibody (Figure 3A, middle).
antibody in another experiment. The antibody-treated group partly, but significantly, attenuated the limb prognosis under VEGF overexpression (Figure 3A, middle), suggesting that the biological function is important in the deleterious effect of VEGF gene transfer. To exclude a possibility that early overexpression of VEGF might affect postoperative wound healing, we did an additional experiment regarding later vector injection protocol. Three days later VEGF165 gene transfer still resulted in the poorer limb prognosis (%LSS=65.6%) than other control groups (Figure 3A, right). Further, in assessment of therapeutic effects (Figure 3B), SeV-VEGF165 did not improve hind limb prognosis (8/10 lost limbs, %LSS=15.0%), similar to findings in the luciferase-injected mice (5/6 lost limbs, %LSS=16.7%), whereas FGF-2 significantly obviated amputation in autoamputation model (2/10 lost limbs, %LSS=77.5%), thereby indicating apparent limb salvaging effects of FGF-2 gene transfer.

Muscle Regeneration Is Disturbed in VEGF-Treated Limb

We next examined the histopathology of muscular tissue of these limb salvage model mice. Typical microscopic findings of the proximal half portion of the posterior group of thigh muscles are shown in Figures 4a through 4c.

In general, all tissue sections were roughly categorized into 4 different histopathological areas, namely, (1) granulation tissue area, (2) muscle regenerating area, (3) undamaged muscular area, and (4) necrotic area without repair response. In case of luciferase-treated muscles as a control (n=13), granulation tissue areas contained necrotic muscle fibers (Figure 4a-1, n), associated with frequent proliferation of satellite myoblasts (Figure 4a-1, arrows). Necrotizing muscle fibers were also evident in VEGF-treated mice (Figure 4b-1, n); however, the proliferation of satellite myoblasts was rare. In contrast, FGF-2-treated mice also had many proliferating myoblasts with a small number of necrotizing myofibers, suggesting early absorption of these necrotic materials (Figure 4c-1, arrows). In the muscle regenerating area, myoblasts surrounding necrotized muscle fibers (Figure 4a-2, n and arrows) were dominant, and massive regenerating muscles with central nuclei (Figure 4c-2, arrowheads) were prominent in muscles of FGF-2–treated mice. Even in this area, findings of muscle regeneration were ultimately rare in the VEGF group, and debris and necrotizing muscle fibers were dominant (Figure 4b-2). Generally, these areas were largely surrounded by necrotic muscles without any significant evidence of repair (Figure 4b-3, n), and only the lateral area showed viable and mature muscular tissue (Figure 4b-3). Computer-assisted quantification analysis revealed extensive necrotic or granulation tissue areas in VEGF165-treated muscles, as compared with luciferase- or FGF-2–treated muscles (Figure 4d).

VEGF165 Gene Transfer Increases Vessel Numbers With Less Blood Perfusion and Less SMA-Positive Cell Lining

To assess whether VEGF165 or FGF-2 gene transfer evokes functional neovessels, we performed another set of experiments not only for immunohistochemical analyses of vessel density in muscles at day 10, but also for a time-course blood perfusion analysis via a LDPI using the same animals. For immunostaining, we encountered only viable mature muscle areas for quantification in order to exclude angiogenesis involved in inflammatory response to granulation tissue.

In both cases of VEGF– or FGF-2–treated muscles (n=10, respectively), each muscular area had an increased number of vessels with PECAM-1–positive endothelial cells without significant difference between them (Figure 5; P<0.01 compared with findings in luciferase group). In contrast, the vessel number doubly positive for PECAM-1 and SMA was significantly higher in FGF-2–treated muscles, compared with data on VEGF-treated muscles (Figure 5; P<0.01). Control stain using an isotype-matched, nonimmunized antibody gave negative results (data not shown). These findings suggest that VEGF165 gene transfer increases immature capillaries more than mature ones.

In both mice treated with SeV-luciferase or SeV–FGF-2, physiological recovery of blood perfusion was apparently detected around the upper thigh on day 4 (data not shown), then a significant perfusion into the calf muscle was clearly seen in the FGF group on days 4, 7, and 10, compared with the limited perfusion in the thigh muscle in the luciferase group at these time points. Luciferase-injected mice had mildly atrophic limbs, whereas most of the FGF-2 group had undamaged limbs. In contrast, mice given VEGF165 had a sustained impairment of blood perfusion in the thigh muscle, and autoamputation followed. Quantification of LDPI indicated that mice given SeV–FGF-2 had a significantly higher blood perfusion than seen with that of SeV-luciferase with physiological recovery of limb circulation (Figure 5C). In contrast, blood flow in the thigh muscle treated with SeV–VEGF165 showed sustained lower perfusion and could not be sufficiently assessed at 7 days after surgery as a large number of mice had lost their limbs, at least at the knee level. These results suggest that the higher expression of VEGF165 in muscle was also likely to form capillaries without any significant blood perfusion.

In Vivo Blockade of Endogenous VEGF Abolishes Therapeutic Effects of FGF-2

Finally, to determine the exact role of upregulation of endogenous VEGF in FGF-2–treated mice, we assessed the effect of VEGF neutralization on the therapeutic effect of FGF-2, using the autoamputation model.

Although SeV–FGF-2 with nonimmune IgG showed reproducible results for improving limb prognosis (n=10), this therapeutic effect was significantly abolished by administration of VEGF-neutralizing antibody (n=5, P<0.0001) and reached a level similar to those seen in PBS- or luciferase-treated mice (n=8, respectively) (Figure 6). Thus, therapeutic effects of FGF-2 gene therapy are highly dependent on endogenous VEGF.

Discussion

In the present study, using recombinant SeV to boost therapeutic genes in ischemic muscles, we characterized in vivo effects of angiogenic factors on critical limb ischemia. To our
Figure 4. a through c, Histological examination of the proximal portion of posterior thigh muscles in the limb salvage model, treated with SeV-luciferase (left series), SeV-VEGF165 (middle series), and SeV-FGF-2 (right series), 10 days after ischemic operation. High-powered view of subnumbered areas of top panels follows. The granulation tissue area treated with luciferase contained necrotic muscle fibers (a-1, n), associated with frequent proliferation of satellite myoblasts (a-1, arrows). Necrotizing muscle fibers were also consistent in VEGF-treated mice; however, the proliferation of satellite myoblasts was rare (b-1, n). Many proliferating myoblasts with a small number of necrotizing myofibers were noted in FGF-2-treated mice (c-1, arrows). Myoblasts surrounding necrotized muscle fibers (a-2, n and arrows) were dominant, and massive regenerating muscles with central nuclei (c-2, arrowheads) were prominent in muscles from FGF-2-treated mice. Even in this area with VEGF, debris and necrotizing muscle fibers were dominant (b-2). These were largely surrounded by necrotic muscles without any significant repair (b-4, n), and only a lateral area showed viable mature muscular tissue (b-3). Original magnifications: a through c, ×12; subnumbered areas, ×200, hematoxylin-eosin stain. d, Bar graph indicating quantitative squares of a histologically characterized area. Top posterior portions of thigh muscles were evaluated using a computer image analyser. Each value indicates mean of each percentage in total square of cross section of muscles.

Figure 5. Assessments of angiogenesis-related events after VEGF165 or FGF-2 gene transfer in a severely ischemic hind limb model. A, Typical immunohistochemical labeling of SMA-positive cells (left, blue-labeled perivascular cells) and PECAM-1-positive cells with circumference (right, orange-labeled endothelial cells) in serial sections, assessing maturation and quantitation of capillary vessels. Typical lesion of FGF-2–treated muscles with frequent double-positive vessels is demonstrated. Some PECAM-1-positive vessels are apparently lined by blue-labeled cells (arrows), suggesting mature capillaries. Some other PECAM-1-positive vessels are without blue cell lining (white arrows), suggesting immature vessels. A, Original magnification, ×400. B, Bar graph indicating quantifications of PECAM-1–positive vessels (solid black bars) and PECAM-1– and SMA-double positive vessels (hatched bars). Each group included 10 mice. *P<0.01. C, Computer-assisted quantitative analyses of hind limb blood flow in C57BL/6 limb salvage model demonstrated significantly enhanced ischemic (left)/untreated (right) limb blood perfusion ratios in mice injected with FGF-2 (n=8), compared with findings in luciferase (n=8) or VEGF165 (n=8). All mice in FGF-2 and mock groups had intact limbs (LSS=3 or 4). In the VEGF165 group, 12 mice were enrolled and 4 of them lost their limbs (LSS=0 or 1), thus their data were excluded and data on 8 animals were subjected to quantitative analyses. *P<0.01 vs other groups; #P<0.05 vs other groups; and ##P<0.05 vs SeV-luciferase–treated group.
knowledge, this is a first report demonstrating the clear limitation in use of VEGF165 in ischemic limbs.

We found that FGF-2 gene therapy was solely effective to treat critically ischemic limb and involves endogenous VEGF function in vivo. In contrast, VEGF165 gene transfer also strongly evoked endogenous VEGF in muscles, and inversely, led limbs to accelerated amputation associated with massive muscular edema, necrosis, and disturbed regeneration. In this process, significant increase in blood perfusion was not apparent, even though increased numbers of capillaries were present at levels similar to those seen with FGF-2. These results are of interest, because endogenous overexpression of VEGF was noted for both VEGF165 and FGF-2 treatments, whereas the recovery of blood perfusion and limb function in vivo probably relates to cellular damage of hind limb muscles, yet SeV-VEGF165 functions sufficiently to secrete a gene product similar to SeV–FGF-2, as shown in the in vitro study. Because histology and LDPI showed VEGF-induced muscle damage and sustained impairment of blood perfusion, respectively, it may be possible that cellular machineries of SeV-mediated transcription are disturbed. Another possible explanation regarding the mechanism for disturbed expression and deleterious action of VEGF might be induction of immunological response against human-derived protein. Because impaired expression was already noted on day 2, a possible immunological mechanism might be innate immunity, but not a major histocompatibility complex–restricted antigen-specific reaction, including induction of cytotoxic T cells and antigen-specific antibody production. However, this notion is not likely, because SeV-mediated intramuscular gene transfer of other human-derived proteins, including monocyte/macrophage chemoattractant protein-1, which is less homologous to murine counterpart than that of VEGF, showed comparable expression to that of FGF-2 (unpublished observation, 2001). We also demonstrated that neutralization of VEGF activity significantly attenuated the limb prognosis (Figure 3A). One more possibility regarding the lower immunoreactivity of VEGF may be ischemia-related disturbance of antibody recognition against recombinant protein. This also seems not likely, because we could detect significant elevation of human VEGF in severely ischemic limbs on day 1, as shown in Figure 2C, and disturbed expression was also seen in the nonischemic muscles similarly treated with SeV–VEGF165 (data not shown). These findings suggest that the deleterious effect of VEGF gene transfer may be the result of its biological activity, rather than of the nonspecific response to foreign protein.

In summary, we demonstrated that intramuscular overexpression of VEGF165 led to an accelerated limb amputation, whereas FGF-2 was safe and therapeutically effective for limb salvage in mouse models of critical limb ischemia. Further attention for the controversial effect of VEGF165 gene transfer in the ischemic limb should be paid, and the significant therapeutic effects and broader safety range of FGF-2 gene transfer warrant further investigations for possible clinical therapeutic angiogenesis.
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EXPANDED MATERIALS AND METHODS

Cells for In Vitro Gene Transfer and Expression Study
Bovine aortic vascular smooth muscle cells (BSMCS: passage 6-8) and human umbilical vein endothelial cells (HUVECs: passage 2-3) were isolated, as described (1-3). Rat cardiomyoblasts (H9C2) and African green monkey kidney cells (COS7) were purchased from American Type Culture Center. Two days after gene transfer, these cells were re-spread at \(10^5\) cells/well in 6-well plate. The next day, the medium was changed and incubated in the CO\(_2\) incubator, and then the medium was subjected to immunoassays exactly 24 hours later.

Luciferase Assay and Immunoassays
Luciferase assay was done using a luminometer (LB 9507, EG&G Berthold, Germany), and the data was expressed by relative light units (RLU)/mg protein (2, 4). Recombinant protein syntheses in tissue lysate were determined using commercially available Quantikine Immunoassay systems for human and murine VEGFs, and murine FGF2 (R&D Systems Inc. Minneapolis, MN) according to the manufacturer's instructions. Our pilot study indicated no significant cross-reactivity of immunoassays between human and murine VEGF, and the system for murine FGF2 completely cross-reacted with commercially available recombinant human FGF2 (data not shown). For value standardization, the wet weight (expressed as pg/g muscle) or total protein concentration was measured. For protein measurements, a commercially available protein assay system (Bio-Rad Laboratories, Hertfordshire, UK) was used (2, 4).

Laser Doppler Perfusion Image of the Blood Flow
Measurements of the ischemic (left)/normal (right) limb blood flow ratio, using a Laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, Devon, UK) were performed (5, 6). Before initiating laser scanning, mice were placed on a heating plate kept at 37 °C to minimize data variations due to body temperature. At a predetermined time point (before and on postoperative days 2, 4, 7 and 10), two consecutive scans were taken over the same region of interest (legs and feet) in each animal and essentially no difference was noted between the two scans. After scanning, the stored
images were subjected to computer-assisted quantification of blood flow, and the average flow of the ischemic and nonischemic feet was calculated. To minimize data variables due to ambient light and temperature, the LDPI index was expressed as the ratio of left (ischemic) to right (nonischemic) limb blood flow.

**Histological Analysis and Immunohistochemistry**

All thigh muscles of limb salvage model at 2 or 10 days after the surgical procedure were fixed with formalin, embedded in paraffin, and 5 μl-thick cross-sections stained with hematoxylin-eosin (H.E.) were used for histopathological examination. In case of VEGF-treated mice, more than 50% of them lost their limb, at least around knee level; thus the proximal half portion of medial and posterior thigh muscle group (including adductor magnus, biceps femoris, and semitendinosus) was used for all evaluations. Quantification of squares of the histological category, namely muscle necrosis without repair response, inflamed granulation tissue, muscle regeneration, and undamaged muscles, was done using MacScope software.

Capillary densities of both ischemic and nonischemic skeletal muscle tissues at the end of limb rescue experiment (10 days after surgery) using limb salvage model were analyzed for the evidence of modified vascularity via intramuscular gene transfer of angiogenic factors. The hind limb skeletal muscles were fixed overnight in methanol, embedded in paraffin and 5-μm-thick sections were prepared. Anti-CD31 monoclonal antibody was used to identify ECs, and visualization was done by the EnVision system (Dakopatts, Santa Barbara, CA). Similar to histological examination, the proximal portion of medial and posterior thigh muscle group was used for evaluation. To exclude non-specific capillary formation due to granulation tissue or muscle regeneration, areas only with mature muscle fibers were used for capillary density quantification. Ten microscopic fields from three different sections from each animal were counted by 2-independent pathologists (I.M. and Y.Y.), and capillary density labeled with CD31 was expressed as the number of capillaries per square millimeter.
ADDITIONAL FIGURES AND SUPPORTING INFORMATIONS

Figure 1: Characterization of SeV-mediated Gene Transfer In Vivo

We first assessed the levels and time course of transgene expression using luciferase in the mild ischemia model. Mice given 100 μg/30g body weight of pCMV-luciferase (roughly estimated 50-times higher than clinical dose) (7, 8) showed significant luciferase activity (mean±S.D.=5.1±3.9 x 10^6 RLU/mg protein, n=6, same as follows), while SeV showed a dose-dependent increase at approximately 5-times at 10^7 plaque forming units (pfu) (2.4±1.8 x 10^7, n=12) and 120-times at 10^8 pfu (7.3±4.7 x 10^8, n=6) 2 days after gene transfer, compared to that of plasmid-based delivery. Further, the results of time course were as follows; the plasmid group showed a relatively high level of luciferase activity at 2 days after gene transfer (2.5±1.9 x 10^6, n=6), and followed by a decline (day 7: 1.4±2.3 x 10^4, n=6 and day 14: 2.7±2.0x 10^4, n=6). C57BL/6 mice received an intramuscular injection of 10^8 pfu of SeV-luciferase also showed an apparent decline in a time-dependent manner (day 2: 7.3±4.3 x 10^8, n=12, day 7: 3.4±4.7 x 10^7, n=12, and day 14: 2.6±1.2 x 10^4, n=12). In contrast, balb/c nu/nu mice retained the expression level fairly well (day 2: 9.4±3.7 x 10^8, n=12, day 7: 1.3±1.9 x 10^7, n=12, and day 14: 0.9±1.3 x 10^7, n=12).

Figure 2: SeV-mediated Expression of Angiogenic Factors In Vitro

We assessed secretion of angiogenic proteins in vitro, using various culture cells not only of muscle lineage (BSMCs and H9C2), but also HUVECs and COS7 cells. In these cells, SeV-mediated transgene expression was relatively stable, and lasting for over 1 month (10). We did this because the present FGF2 vector (SeV-FGF2) does not contain a classical signal sequences for secretion, although numerous studies including ours demonstrated that FGF2 without secreting sequences could be expressed extracellularly (18-20). As expected, the efficient secretion of FGF2 protein into the culture medium was at a similar level to that seen with VEGF165 (ex. MOI=10: VEGF165 vs FGF2=4,354.9±2,794.3 vs 3,682.9±1,063.1 in HUVECs, 275.2±58.9 vs 398.0±154.0 in BSMCs, 16,987.4±4,748.5 vs 5,976.2±381.2 in H9C2, and 3,864,884.6±491,383.1 vs 1,547,237.1±176,502.7 in COS7 cells, pg/10^5 cells/24 hours, n=3, respectively).

Figure 3: Schematic representation of possible effects of angiogenic factor gene
transfer in severely ischemic limbs.

a. Natural course of acute limb ischemia. Acute limb ischemia induces endogenous VEGF and FGF2, resulting in physiological restoration of blood flow.

b. Effect of VEGF165 gene transfer in ischemia limb. Local expression of exogenous VEGF165 in muscle, when it exceeds physiological levels, may induce muscular edema and accelerated limb ischemia, resulting in marked enhancement of endogenous VEGF expression and reduction of transgene expression. As a result, limb ischemia would be accelerated further, resulting in muscle necrosis and limb amputation.

c. Effect of FGF2 gene transfer. Transgene expression of FGF2 induces endogenous VEGF, resulting in effective angiogenesis and limb salvage.
REFERENCES


8. Isner JM, Baumgartner I, Rauh G, Schainfeld R, Blair R, Manor O, Razvi S, Symes JF. Treatment of thromboangiitis obliterans (Buerger's disease) by
Figure 1

[Diagram showing data points and trends over time for different conditions, with labels indicating sample sizes (n=6, n=12) and time points (2 days, 7 days, 14 days).]
Figure 2

Recombinant Protein Secretion (pg/ml x 10^6 cells/24 hour)

[Bar chart showing protein secretion levels for different cell lines: HUVEC, BSMC, H9C2, COS7. The chart includes bars for VEGF, FGF2, and basal release.]