Enhanced Therapeutic Angiogenesis by Cotransfection of Prostacyclin Synthase Gene or Optimization of Intramuscular Injection of Naked Plasmid DNA

Kazuya Hiraoka, MD; Hiromi Koike; Seiji Yamamoto, MD; Naruya Tomita, MD, PhD; Chieko Yokoyama, PhD; Tadashi Tanabe, PhD; Takashi Aikou, MD, PhD; Toshio Ogihara, MD, PhD; Yasufumi Kaneda, MD, PhD; Ryuichi Morishita, MD, PhD

Background—Although clinical trials of therapeutic angiogenesis by angiogenic growth factors with intramuscular injection of naked plasmid DNA have been successful, there are still unresolved problems such as low transfection efficiency. From this viewpoint, we performed the following modifications: (1) combination with vasodilation using prostacyclin and (2) changing the agents or volume of naked plasmid DNA in vivo.

Methods and Results—First, we examined cotransfection of the VEGF gene with the prostacyclin synthase gene in a mouse hindlimb ischemia model. Cotransfection of the VEGF gene with the prostacyclin synthase gene resulted in a further increase in blood flow and capillary density compared with single VEGF gene. Similar results were obtained with other angiogenic growth factors, such as hepatocyte growth factor (HGF). Alternatively, we changed the injection volume of the solution of plasmid DNA. Luciferase activity was increased in a volume-dependent manner. An increase in injection volume at 1 site rather than separate injections at multiple sites resulted in high transfection efficiency, which suggests that transfection of naked plasmid DNA is mediated by pressure. Interestingly, treatment with hyperbaric oxygen increased the transfection efficiency. Finally, we also examined the effects of different solutions. Saline and PBS, but not water, achieved high transfection efficiency. In addition, sucrose solution but not glucose solution resulted in high luciferase activity.

Conclusions—Overall, angiogenesis might be enhanced by cotransfection of prostacyclin synthase gene or an increase in injection volume and osmotic pressure. These data provide important information for the clinical application of therapeutic angiogenesis to treat peripheral arterial disease. (Circulation. 2003;108:2689-2696.)

Key Words: peripheral vascular disease ▪ vasodilation ▪ angiogenesis ▪ gene therapy

Critical limb ischemia is estimated to develop in 500 to 1000 individuals per million per year. In a large proportion of these patients, the anatomic extent and distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization. Because there is no optimal medical therapy for critical limb ischemia, novel therapeutic modalities are needed to treat these patients. Therapeutic angiogenesis may play a role in the treatment of patients with vascular disease for whom there is no other treatment option. Recently, the efficacy of therapeutic angiogenesis with vascular endothelial growth factor (VEGF) gene transfer, including VEGF165, VEGF121, and VEGF-2, has been reported in human patients with critical limb ischemia and myocardial ischemia. In addition to VEGF, we and others have reported the angiogenic properties of hepatocyte growth factor (HGF) in a rabbit ischemia model and human patients. Alternatively, a key transcription factor to stimulate VEGF, HIF-1, has also been used to stimulate angiogenesis in animal and human subjects. Interestingly, most successful clinical trials of the treatment of peripheral arterial disease (PAD) with angiogenic growth factors have been performed by intramuscular transfection of naked plasmid DNA, although gene transfer by direct injection of naked DNA into skeletal muscle is inefficient. Because it is apparent that a more efficient gene transfer method is required to achieve therapeutic effects, many investigators have focused on the adenoviral gene transfer method. Although the adenoviral vector is very efficient, there are some theoretical disadvantages, such as strong immunogenicity and safety. It might be assumed that the combination of different strategies to treat PAD, such as the vasodilator action of prostacyclin combined with therapeutic angiogenesis, would

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From the Division of Gene Therapy Science (K.H., S.Y., Y.K.), Department of Geriatric Medicine (K.H., T.O.), Division of Clinical Gene Therapy (H.K., N.T., R.M.), Graduate School of Medicine, Osaka University; Department of Pharmacology (C.Y., T.T.), National Cardiovascular Research Center, Osaka; and First Department of Surgery (T.A.), Faculty of Medicine, Kagoshima University, Sakuragaoka, Kagoshima, Japan.

Dr Morishita is a board member of and stockholder in AnGes MG, Inc.

Correspondence to Ryuichi Morishita, MD, PhD, Professor, Division of Clinical Gene Therapy, Osaka University, Graduate School of Medicine, 2-2 Yamada-oka, Suita 565-0871, Japan. E-mail morishit@cgt.med.osaka-u.ac.jp

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be useful to stimulate collateral formation, because prostacyclins are widely used for the treatment of PAD. An alternative idea is to achieve the optimal conditions for plasmid DNA transfer into skeletal muscle as follows: (1) increase the injection volume of plasmid DNA, (2) change the solution in which plasmid DNA is dissolved, and (3) apply hyperbaric oxygen (HBO) therapy with plasmid DNA.

Methods

Experiment 1: Cotransfection of VEGF or HGF With Prostacyclin Synthase Gene in Mouse Hindlimb Ischemia Model

Construction of Plasmids

To produce expression vectors, human VEGF cDNA, human HGF cDNA (2.2 kb), or human prostacyclin synthase cDNA (1.5 kb) was inserted into a simple eucaryotic expression plasmid that utilizes the cytomegalovirus promoter/enhancer. The vector used as a control was the cytomegalovirus expression vector plasmid, which does not contain VEGF, HGF, or prostacyclin synthase cDNA.

In Vivo Gene Transfer Via Direct Injection Approach

An ischemia model was created according to previous reports. “Naked” plasmid DNA encoding human VEGF, human HGF, human prostacyclin synthase, or control vector (200 μg/100 μL per body) was carefully injected directly into the mouse ischemic limb with a 27-gauge needle (Terumo) 10 days after surgery (day 10). Four separate injections of vector locally (intramuscularly into the ischemic limb, near both the proximal and distal arterial stumps) were performed. All protocols were approved by the Osaka University Institutional Animal Care and Use Committee. In all experiments, investigators performing the follow-up examinations were blinded to the identity of the treatment administered.

Concentrations of human VEGF and HGF in the hindlimb were determined by enzyme immunoassay with anti-human VEGF and HGF antibody 4 days after transfection. The antibody against human VEGF reacts only with human VEGF and not with mouse VEGF. The antibody against human HGF reacts only with human HGF and not with mouse HGF. Alternatively, we used a kit (Cayman Chemical Co) to examine production of 6-keto-prostaglandin F1α 4 days after transfection.

Measurement of Blood Flow by Laser Doppler Imaging and Capillary Density Measurement

Measurement of blood flow with laser Doppler imaging has been described previously. Because laser Doppler flow velocity correlates well with capillary density, we measured cardiac blood flow by means of laser Doppler imaging (Moor Instruments). Consecutive measurements were obtained over the same regions of interest (leg and foot). Low or no perfusion was displayed as dark blue, whereas the highest perfusion interval was displayed as white. Alkaline phosphatase staining was used as a specific marker of endothelial cells. To analyze the number of vessels in the ischemic hindlimb transfected with HGF vector or control vector, mice were killed and the muscle was removed. Three individual sections from the middle of the transfected muscle were analyzed. The number of vessels was counted under a light microscope (magnification, ×100) in a blinded manner.

Experiment 2: Optimization of Plasmid DNA Transfection In Vivo

In Vivo Gene Transfer Via Direct Intramuscular Injection Approach

Naked luciferase gene or control vector (500 μg/body) was carefully injected directly into the center of the pretilial muscle of the right hindlimb of Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) with a 27-gauge needle (Terumo). Luciferase gene expression vectors driven by the SV 40 promoter were obtained from a commercial source (Promega Corporation). Firefly luciferase activity was measured with a luciferase assay system (PicaGene; Toyo-Inki). Rats were killed 2 days after transfection of luciferase gene by direct injection of naked plasmid into the hindlimb.

To increase the pressure, the manchette of a phymgomonometer was wrapped around the muscle transfected with naked luciferase plasmid DNA immediately after transfection. To add pressure at the transfected sites, additional intramuscular injection of PBS solution without plasmid DNA was performed into the muscle transfected with naked luciferase plasmid DNA 0.5 or 5 hours after transfection. For HBO treatment (O2 exposure), on the morning of exposure, animals were placed in a hyperbaric chamber. The chamber was flushed with 100% O2 for 1.5 minutes to raise the O2 level to >99%. The animals were exposed to 100% O2 at 2 atm for 1 hour, immediately after transfection.

Statistical Analysis

All values are expressed as mean±SEM. ANOVA with subsequent Duncan test was used to determine the significance of differences in multiple comparisons. Differences with a probability value less than 0.05 were considered significant.

Results

Angiogenesis Induced by Intramuscular Injection of VEGF, HGF, or Prostacyclin Synthase Plasmid in Mouse Model

Initially, we measured human VEGF and HGF concentrations in the ischemic hindlimb after transfection. Expectedly, human VEGF or HGF was readily detected in the hindlimb transfected with human VEGF or HGF vector, whereas no human VEGF and HGF could be detected in muscles transfected with control vector at 4 days after transfection (Figures 1a and 1b, P<0.01). In addition, human 6-keto-prostaglandin...
F1a could be detected in the ischemic hindlimb transfected with human prostacyclin synthase but not in control vector (control: not detected; prostacyclin synthase: 3.26 ± 0.82 pg/g tissue; P < 0.01). After an increase in human VEGF concentration, injection of human VEGF vector into the ischemic hindlimb resulted in a significant increase in blood flow at 4 weeks after transfection (P < 0.01; Figures 2a and 2b). Moreover, transfection of human VEGF vector significantly increased capillary density in the mouse ischemic hindlimb around the injection site compared with control vector at 4 weeks after transfection (Figures 3a and 3b; P < 0.01). In contrast, injection of human prostacyclin synthase vector resulted in a weak but significant increase in capillary density at 4 weeks after transfection, as shown in Figure 3. Cotransfection of VEGF and prostacyclin synthase genes resulted in an additional increase in blood flow compared with single-gene transfection of VEGF alone at 4 weeks after transfection (Figure 2; P < 0.01). Similarly, capillary density was most markedly increased in mice transfected with VEGF and prostacyclin synthase genes compared with VEGF vector alone (Figure 3; P < 0.01). Then, we examined the enhanced effects of prostacyclin synthase gene with HGF, because the clinical trial with HGF is currently ongoing. Cotransfection of the prostacyclin gene and HGF gene resulted in a significant increase in blood flow and capillary density compared with the HGF gene alone (Figure 3; P < 0.01).

Comparison of Transfection Efficiency Into Rat Muscle In Vivo
To modify non–virus-mediated plasmid DNA transfection with high transfection efficiency, we examined the effects of the injection volume on transfection efficiency. Expectedly, luciferase activity was increased in a plasmid DNA dose-dependent manner (Figure 4; P < 0.01). Interestingly, as shown in Figure 4a, transfection of naked plasmid DNA was increased according to the increase in injection volume of solution (PBS; P < 0.01). Importantly, an increase in injection volume (100 µL at 1 site) rather than separate injections (25 µL at 4 sites or 12.5 µL at 8 sites) resulted in higher transfection efficiency (Figure 4b; P < 0.01). Therefore, we thought that the transfection efficiency of naked plasmid DNA might be related to the osmotic pressure. To clarify this point, we used the manchette of a sphygmomanometer on the hindlimb after transfection to increase the pressure from outside. Unexpectedly, neither manchette-mediated pressure of 150 or 300 mm Hg increased transfection efficiency (Figure 5). When repeated pressure by the manchette was used, transfection efficiency was not affected (Figure 5). In contrast, to increase the pressure inside, intramuscular injection of PBS without plasmid DNA was performed into the same site as plasmid DNA transfection. As shown in Figure 6, additional injection of PBS at 30 minutes after the first injection of plasmid DNA resulted in an increase in luciferase activity (P < 0.01). Nevertheless, similar additional injection...
of PBS alone at 5 hours after the initial transfection did not increase luciferase activity. In contrast, a change in injection speed did not affect the transfection efficiency (data not shown).

To confirm these observations, we used HBO therapy, because HBO exposes an organism to an environment of pure oxygen under high pressure. Importantly, HBO treatment at 2 atm for 1 hour resulted in a significant increase in luciferase activity with an injection volume of both 100 and 300 µL (Figure 7; \( P < 0.01 \)). Because the combination of HBO increased the pressure onto cells, these results demonstrated that transfection efficiency with intramuscular injection of naked plasmid DNA was dependent on the pressure on the cell surface. Alternatively, an increase in osmotic pressure might affect transfection efficiency. Thus, we finally examined the effects of various solutions with plasmid DNA on transfection efficiency. In previous experiments, we used PBS solution as the injection vehicle. As shown in Figure 8a, saline as well as PBS demonstrated high transfection efficiency compared with other buffers. Unexpectedly, water diminished luciferase activity. To increase the osmotic pressure, we also examined the effects of glucose and sucrose solutions. Sucrose solution but not glucose solution significantly increased luciferase activity compared with water (Figure 8b; \( P < 0.01 \)), whereas both glucose and sucrose solutions increased luciferase expression. However, sucrose solution at 30% caused injury at the injected site in the muscle.

**Discussion**

In the present study, we introduce a new strategy, therapeutic angiogenesis with cotransfection of VEGF and prostacyclin synthase genes, as gene therapy for patients with critical limb ischemia. The reason we chose prostacyclin synthase was to consider the utility of vasodilator agents such as prostaglandins and phosphodiesterase type III inhibitors to treat human patients with PAD. A combination of angiogenesis induced by VEGF and vasodilation of newly generated blood vessels induced by prostacyclin would enhance blood flow recovery and maintain new vessel formation. The stimulatory effects of the prostacyclins on endothelial cell proliferation and vascular smooth muscle cell proliferation are both essential for angiogenesis.

**Figure 3.** Effect of transfection of human VEGF plasmid, HGF plasmid, or prostacyclin synthase plasmid (PGIS) on vascular formation at 4 weeks after transfection. a, Representative cross sections (×200). b and c, Effect of transfection of human VEGF plasmid (b), human HGF plasmid (c), or prostacyclin synthase plasmid (500 µg) on density of vessels. Abbreviations as in Figure 1. Each group contains 7 to 8 animals. \( * P < 0.01 \) vs control.

**Figure 4.** a, Comparison of luciferase activity achieved by transfection of naked plasmid DNA at various injection volumes into skeletal muscle in vivo at 2 days after transfection. 100, 200, 300, and 400 µL indicate rats transfected with naked luciferase plasmid DNA (200 or 800 µg) with 100 to 400 µL of PBS solution, respectively. Each group contains 6 animals. \( ** P < 0.01 \) vs 100 µL. b, Comparison of luciferase activity achieved by transfection of naked plasmid DNA at various injection volumes and at different sites into skeletal muscle in vivo at 2 days after transfection. 200 µg/100X1 indicates transfected with luciferase plasmid DNA (200 µg) with 100 µL of PBS solution at 1 site; 200 µg/25X4, transfected with luciferase plasmid DNA (200 µg) with 25 µL of PBS solution at 4 sites (total 100 µL); 200 µg/12.5X4, transfected with luciferase plasmid DNA (200 µg) with 12.5 µL of PBS solution at 4 sites (total 100 µL); and 400 µg/25X4, transfected with luciferase plasmid DNA (400 µg) with 25 µL of PBS solution at 4 sites (total 100 µL). Each group contains 10 animals. \( ** P < 0.01 \) vs 200 µg/100X1. RLU indicates relative light units.
Figure 5. Comparison of luciferase activity by transfection of naked plasmid DNA with manchette wrapping of skeletal muscle at 2 days after transfection. Manchette of sphygmomanometer was wrapped around muscle transfected with naked luciferase plasmid DNA immediately after transfection. 200/100 indicates plasmid DNA (200 \mu g) with 100 \mu L of PBS solution without manchette wrapping; 10sX10(300), plasmid DNA (200 \mu g) with 200 \mu L of PBS solution with compression by manchette for 10 seconds 10 times at 300 mm Hg; 10sX30(300), plasmid DNA (200 \mu g) with 200 \mu L of PBS solution with compression by manchette for 30 times at 300 mm Hg; 1mX10(150), plasmid DNA (200 \mu g) with 200 \mu L of PBS solution with compression by manchette for 1 minute 10 times at 150 mm Hg; 1mX10(300), plasmid DNA (200 \mu g) with 200 \mu L of PBS solution with compression by cuff for 1 minute 10 times at 300 mm Hg; 5mX1(150), plasmid DNA (200 \mu g) with 200 \mu L of PBS solution with compression by manchette for 5 minutes once at 150 mm Hg; and 5mX1(300), plasmid DNA (200 \mu g) with 200 \mu L of PBS solution with compression by manchette for 5 minutes once at 300 mm Hg. Each group contains 4 animals. *P<0.05 vs 200/100. RLU indicates relative light units.

Figure 6. a, Comparison of luciferase activity achieved by transfection of naked plasmid DNA (200 \mu g) with additional injection of PBS solution into skeletal muscle at 2 days after transfection. Additional intramuscular injection of PBS solution without plasmid DNA was performed into muscle transfected with naked luciferase plasmid DNA 0.5 or 5 hours after transfection. 200/100 indicates plasmid DNA (200 \mu g) with 100 \mu L of PBS solution; 200 \mu g/100+300 (0.5h), plasmid DNA (200 \mu g) with 100 \mu L of PBS solution and injected with 300 \mu L of PBS solution alone at 30 minutes after first injection; 200 \mu g/100+300 (5h), plasmid DNA (200 \mu g) with 100 \mu L of PBS solution and injected with 300 \mu L of PBS solution alone at 5 hours after first injection; and 200 \mu g/400, plasmid DNA (200 \mu g) with 400 \mu L of PBS solution. Each group contains 4 animals. **P<0.01 vs 200 \mu g/100. b, Comparison of luciferase activity by naked plasmid DNA (800 \mu g) with additional injection of PBS solution into skeletal muscle at 2 days after transfection. 800 \mu g/100 indicates plasmid DNA (800 \mu g) with 100 \mu L of PBS solution; 800 \mu g/100+300 (0.5h), plasmid DNA (800 \mu g) with 100 \mu L of PBS solution and injected with 300 \mu L of PBS solution alone at 30 minutes after first injection; 800 \mu g/100+300 (5h), plasmid DNA (800 \mu g) with 100 \mu L of PBS solution and injected with 300 \mu L of PBS solution alone at 5 hours after first injection; and 800 \mu g/400, plasmid DNA (800 \mu g) with 400 \mu L of PBS solution. Each group contains 4 animals. **P<0.01 vs 800 \mu g/100. RLU indicates relative light units.
clin gene on angiogenesis were also observed in the case of cotransfection of HGF. These enhanced effects may be due to the synergistic effects of different mechanisms, including the participation of migrated blood-derived cells, because recent reports have documented an important contribution of blood cells to the pathogenesis of angiogenesis.28

Alternatively, it might be assumed that a higher transfection efficiency of angiogenic growth factors would increase the clinical utility of these treatments. Currently, researchers are using adenoviral gene transfer in clinical trials instead of naked plasmid DNA. However, the potential toxic effects of adenovirus, such as strong immunogenicity, are well known. Of particular importance, adverse side effects of adenovirus were reported in 1999.22 In deference to the safety issue, an ideal clinical therapy would use intramuscular injection of naked plasmid DNA despite its low transfection efficiency. From this viewpoint, we focused on the plasmid DNA-based gene transfer method, because this method appears to be safest. To increase transfection efficiency, we modified the plasmid DNA transfection method. We have previously reported high transfection efficiency of ultrasound-mediated plasmid DNA transfection with echo contrast microbubbles (Optison).29,30 When ultrasound exposure was used in the presence of microbubbles, an ~300-fold increment in transgene expression after transfection was reported in in vitro experiments.31 In addition, we confirmed the utility of ultrasound-mediated plasmid DNA transfection with Optison in rat skeletal muscle and rat carotid artery.29,30 The increase in transfection efficiency was due to the appearance of transient holes in the cell membrane caused by spreading of the bubbles. Given the efficient transfection of intramuscular plasmid DNA injection with ultrasound with Optison, we thought that instability of the cell membrane at high osmotic pressure might increase the transfection efficiency. The present data demonstrated that the transfection efficiency is determined by the amount of plasmid DNA and the injection volume in skeletal muscle. This phenomenon appears to be due to an increase in pressure on the cell surface. For human gene therapy, the present modification based on plasmid DNA might be superior, because electroporation or ultrasound would cause cell toxicity. A simple change in the solution or volume might be most feasible.

What is the clinical relevance of the second-generation therapeutic angiogenesis strategy based on plasmid DNA with intramuscular injection? First, it is possible to decrease the amount of plasmid DNA, thereby decreasing the potential cost. Second, it is possible to achieve efficient transfection without viral vectors. Avoidance of viral gene transfer methods, such as adenovirus, may increase the safety and open up a wide variety of targeted diseases for gene therapy. Third, further modification of gene-delivery instruments, such as combination with HBO therapy, may expand the utility of the present modification toward human clinical gene therapy with angiogenic growth factors. Fourth, expectedly, these...
modifications did not alter toxicity in the clinical setting. Fifth, although a single intramuscular injection of VEGF plasmid was sufficient to prevent necrosis, enhancement of therapeutic angiogenesis by increasing the dose or injection time or increasing transfection efficiency with viral and nonviral vector systems would be important. To treat a wider range of patients such as those with critical limb ischemia with diabetes or those undergoing hemodialysis, it is necessary to achieve higher efficiency. However, when increasing the dose or injection time, cost and toxicity issues might be a problem. From this viewpoint, the stimulation of collateral growth factors from a transgene requires a long period. Thus, considering the clinical setting, this second generation of therapeutic angiogenesis may be useful for treating PAD patients. In addition to prostacyclin synthase, other genes related to the vasodilative response, such nitric oxide synthase, might be useful. Alternatively, pharmaceutical drugs such as oral prostacyclin analogues or phosphodiester type III inhibitors combined with gene therapy with a single gene are more likely to be effective in the clinical setting. A recent study using VEGF121 reported that the vasodilator response to nitroglycerin of patients was significantly restored by transfection of the VEGF121 gene. These findings clearly indicate the potential enhancement of angiogenesis by a pharmacological approach. These experimental findings are thus encouraging for the treatment of PAD, although these areas clearly require further investigation and monitoring with regard to safety issues.

Overall, therapeutic angiogenesis might be enhanced by improvement of the transfection efficiency by an increase in injection volume and osmotic pressure or cotransfection of the prostacyclin synthase gene. A novel therapeutic strategy with plasmid DNA of angiogenic growth factors with HBO therapy or cotransfection of the prostacyclin synthase gene to stimulate angiogenesis may be useful to reduce clinical symptoms without the use of viral vectors in angiogenesis-dependent conditions such as wound healing, ischemic heart disease, myocardial infarction, and PAD.

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References
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