Human Endothelial Nitric Oxide Synthase Gene Delivery Promotes Angiogenesis in a Rat Model of Hindlimb Ischemia

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Objective—Endothelium-derived NO has been shown to mediate the mitogenic effect of vascular endothelial growth factor on cultured microvascular endothelium. To evaluate the role of endothelial NO synthase (eNOS) in angiogenesis in the ischemic hindlimb, we engineered an adenovirus containing human eNOS cDNA.

Methods and Results—After gene transfer, expression of eNOS in cultured cells was detected by increased intracellular cGMP and nitrate/nitrite levels and NO synthase activity. Adenovirus containing either the eNOS or luciferase gene was injected into the adductor muscle of rat hindlimbs immediately after femoral artery removal. Human eNOS protein was detected throughout the course of the experiment by immunostaining. Significant increases in blood perfusion were monitored by laser Doppler imaging from 2 to 4 weeks after gene delivery in the ischemic hindlimb of rats receiving eNOS compared with control rats receiving the reporter gene. An increase in regional blood flow was also detected after eNOS gene transfer by a fluorescent microsphere assay. eNOS gene delivery in the ischemic hindlimb resulted in significant increases in intracellular cGMP levels and in capillary density identified by anti–CD-31 immunostaining. Angiogenesis was further confirmed in mice after eNOS gene transfer by increased hemoglobin content in Matrigel implants.

Conclusions—Taken together, these results indicate that eNOS enhances angiogenesis and raises the potential of eNOS gene transfer for modulation of vascular insufficiency. (Arterioscler Thromb Vasc Biol. 2002;22:1279-1285.)

Key Words: endothelial NO synthase ■ angiogenesis ■ adenovirus ■ gene transfer ■ ischemia

Endothelial NO synthase (eNOS) has been shown to elicit multiple beneficial effects within the cardiovascular system.1–3 eNOS catalyzes the conversion of L-arginine to L-citrulline, producing NO. NO generated in endothelial tissues diffuses to surrounding smooth muscle cells (SMCs), where it activates soluble guanylate cyclase. Guanylate cyclase then converts GTP to cGMP, which mediates its vasorelaxation effect, in part, by causing a reduction in the intracellular calcium concentration. Some additional biological effects of NO in the vasculature include inhibition of platelet adhesion and vascular SMC proliferation and migration.4

NO plays a vital role in the regulation of vascular homeostasis. Our laboratory has previously demonstrated that delivery of eNOS via plasmid DNA results in a sustained decrease in blood pressure.5 Moreover, transgenic mice overexpressing eNOS exhibit a significant decrease in systolic blood pressure.6 In addition to its vasodilatory properties, NO has been implicated in the modulation of angiogenesis. The importance of NO in angiogenesis has been demonstrated in eNOS knockout mice, which have a significantly limited angiogenic potential.7 Increasing evidence exists to support the idea that eNOS works in conjunction with vascular endothelial growth factor (VEGF) to promote angiogenesis.8,9 VEGF is widely recognized for its ability to stimulate angiogenesis and has been used to restore blood flow in the ischemic rodent limb as well as in other animal models.10–12 Other studies have shown that VEGF increases the expression of eNOS in endothelial cells, suggesting that eNOS plays a role in the angiogenic response induced by VEGF.7,13,14 In addition, NO has been shown to be vital for the proliferation and migration of endothelial cells during angiogenesis,15–17 perhaps in part through the regulation of integrins in endothelial cells.18

To further clarify the role of eNOS in angiogenesis, we constructed a replication-deficient adenovirus harboring eNOS cDNA under the control of the cytomegalovirus (CMV) promoter. We then sought to test the potential role of eNOS in angiogenesis with an adenoviral vector in a rat model of operatively induced hindlimb ischemia. Our primary goal was to determine whether gene transfer of eNOS could promote angiogenesis in vivo. Because NO mediates the angiogenic signaling of VEGF, we determined whether an overexpression of eNOS (and hence, NO) would lead to increased angiogenesis. Our results showed that a continuous supply of NO via gene transfer was able to significantly augment angiogenesis in the rat.

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ischemic hindlimb, suggesting its potential efficacy in gene therapy for vascular deficiency.

Methods

Cell Culture

Human embryonic kidney (HEK 293) cells and human lung adenocarcinoma (A549) cells were supplied by American Type Tissue Collection. Both cell lines were maintained in DMEM (GIBCO-BRL) supplemented with 10% FBS (Sigma Chemical Co) and 1% antibiotic cocktail (GIBCO-BRL).

Construction of eNOS Adenovirus

Adenovirus was generated by using plasmids generously supplied by Dr. Mark Kay (Stanford University School of Medicine, Palo Alto, Calif) according to a modified method previously described.19 The plasmid DNA pShuttle was linearized by enzyme digestion at the SmaI site. A blunt-end CMV-eNOS fragment was obtained by excising the DNA from the pcDNA3.CMV-eNOS-pA plasmid with the use of NruI and EcoRV digestion. The CMV-eNOS fragment was then ligated to the shuttle vector. Next, pShuttle.CMV-eNOS was linearized by EcoRV digestion. A 4F2-pA fragment with XbaI cohesive ends was filled in by using the Klenow (large) fragment of DNA polymerase I. This fragment was then ligated to the shuttle plasmid to yield the final shuttle construct pShuttle.CMV-eNOS-4F2-pA. The integrity of the transgene cassette was tested by its ability to increase intracellular cGMP levels. The CMV-eNOS-4F2-pA transcription unit was released from the shuttle vector by I-Ceu-I and Pci-ScI-I sequentially. Meanwhile, the adenoviral gene-gnome backbone plasmid pAdHM4 was also cut with I-Ceu-I to release the complete linear adenoviral DNA. The DNA was transiently transfected into HEK 293 cells by using Effectene reagent (Qiagen) according to the manufacturer’s directions. The resulting virus was serially passaged 5 times. The final viral titer was then established by plaque assay. The virus was used to transduce the cell line A549 (human lung adenocarcinoma), which, unlike HEK 293, does not permit replication-deficient adenoviral growth. At a multiplicity of infection (MOI) of 10, there was no evidence of a cytopathic effect 72 hours after transduction.

CgMP Radioimmunoassay

Before adenovirus preparation, eNOS shuttle DNA or control green fluorescent protein (GFP) DNA (2 μg each) was used to transfect HEK 293 cells in 6-well plates according to the manufacturer’s protocol (Effectene reagent, Qiagen). HEK 293 cells were chosen because of their high transfection efficiency. Forty-eight hours after transfection, the medium was removed, and the cells were washed with PBS. Next, 250 μL of 0.1N HCl was added to each well, and the cells were gently rocked at 4°C for 2 hours. The incubation supernatant was then removed, and the cells were solubilized with 250 μL of 1N NaOH. cGMP levels in the supernatant were measured by radioimmunoassay as described.20–23 Total protein was estimated by the Lowry method, with BSA used as a standard. For the skeletal muscle samples, tissue was homogenized in 10 vol of 0.1N HCl and centrifuged at 20,800g for 15 minutes, and the supernatant was assayed as described above.

Nitrate and Nitrite Measurement

A549 cells were seeded in a 12-well plate and transduced 24 hours later with Ad.CMV-eNOS or Ad.CMV-GFP at an MOI of 50. We chose this cell line because of its high susceptibility to adenovirus transduction and because, unlike 293 cells, A549 cells will not lyse at the MOI used. After a 48-hour incubation, the medium was removed, and the cells were washed with PBS. Nitrate/nitrite levels were measured as previously described.24 Briefly, 500 μL of Krebs’ solution containing 0.1 mmol/L l-arginine and 1 μmol/L calcium ionophore was added. After 24 hours, the solution was assayed for nitrate/nitrite content by incubating the samples in the presence of nitrate reductase and NADPH. Diaminonaphthalene was then added, generating a fluorescent product on reaction with nitrate/nitrite. Product formation was measured by using a spectrophotometer set at an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

NOS Activity Assay

A549 cells were transduced with adenovirus carrying the eNOS or GFP gene, and 48 hours later, the cells were harvested by scraping in a solution of PBS. Cells were centrifuged for 10 minutes at 20 800g, and the pellet was used for the assay. Total NO synthase (NOS) activity was determined by the conversion of [1H]arginine to [1H]citrulline by using an NOS activity assay kit (Calbiochem) according to the manufacturer’s instructions.

Femoral Artery Ligation and Adenovirus Administration

Male Wistar rats (~200 g, Harlan Inc, Indianapolis, Ind) were used for the present study. Animals were anesthetized intraperitoneally with 90 mg/kg ketamine and 10 mg/kg xylazine in PBS. An incision was made along the inner left hindlimb along the line of the femoral artery and vein. The proximal end of the femoral artery was tied with 4-0 silk suture (Ethicon) and electrocoagulated. The femoral artery was dissected free from the limb and its peripheral branches. The distal end was severed, and the artery was removed. A local injection of adenovirus carrying the eNOS cDNA (Ad.CMV-eNOS) or the luciferase gene (Ad.CMV-Luciferase) was delivered (1 × 109 pfu per rat in 100 μL of 10 mmol/L Tris-Cl, pH 8.0) after being divided among 4 to 5 injection sites in the adductor and surrounding muscles. All virus injections were administered immediately after surgery. A separate group received femoral artery ligation and injection of vehicle (100 μL of 10 mmol/L Tris-Cl) alone.

Immunostaining of Capillaries

Rat hindlimb skeletal muscle was excised 4 weeks after virus injection, fixed in zinc formalin, and embedded in paraffin. Sections (4 μm) were cut and stained with the use of hematoxylin and eosin. In addition, a second set of sections was immunostained (Vectastain ABC kit, Vector Labs) with antibody to platelet endothelial cell adhesion molecule (PECAM-CD-31, PharMingen). Capillaries were counted in 10 random fields of 0.25 mm2 for each condition.

eNOS Immunostaining

Three rats were euthanized per group at each time point after gene transfer (1, 2, 3, and 4 weeks), and rat adductor skeletal muscle was perfused with PBS, followed by 10% buffered formalin via cardiac injection. Paraffin-embedded sections (4 μm) were stained by using a monoclonal antibody specific for human eNOS (Transduction Laboratories) with the use of the DAKO Envision immunostaining kit according to the manufacturer’s instructions.

Laser Doppler Imaging

A laser Doppler blood flowmeter (Laser Doppler Perfusion Imager System, Liscia) was used to evaluate perfusion of left (ischemic) and right (nonischemic) murine hind limbs. The perfusion signal is subdivided into 6 different intervals, with each displayed as a separate color. Low or no perfusion is displayed as dark blue, whereas the highest perfusion interval is displayed as red. The stored perfusion values behind the color-coded pixels remain available for data analysis. Excess hair was removed from the hindlimbs with the use of a shaver. Before and during scanning, animals were placed on a heating plate at 37°C to minimize variations in temperature. After the laser Doppler images were recorded, the average perfusion values of the ischemic and nonischemic limbs were calculated on the basis of colored histogram pixels. To minimize variables, including ambient light and temperature, calculated perfusion was expressed as the ratio of left (ischemic) to right (nonischemic) hindlimb perfusion in each animal. Perfusion analyses were performed before and immediately after surgery and for 4 consecutive weeks thereafter.
Blood Flow Analysis by Microsphere Assay
The regional blood flow to skeletal muscle in both hindlimbs of rats was measured by using fluorescent microspheres 3 weeks after surgery. Rats were anesthetized intraperitoneally with ketamine (90 mg/kg) and xylazine (10 mg/kg). A PE-50 catheter was placed into the aortic arch via the carotid artery for microsphere injection. Both kidneys were ligated to increase the microsphere density in both hindlimbs. Microspheres (0.4 mL, $4 \times 10^{10}$ microsphere beads, 15-μm diameter, Molecular Probes) were injected at a rate of 0.4 mL/min and were flushed with 0.5 mL of 0.9% saline at a rate of 0.3 mL/min. Animals were euthanized, and the adductor and gastrocnemius muscles of both limbs were removed. Each muscle sample was weighed, cut into small pieces, and digested in 10 mL of 2 mol/L ethanol KOH containing 0.5% Tween 80 at 60°C for 48 hours with constant shaking. After complete digestion of the tissues, the microspheres were collected by centrifuging at 2000 g for 20 minutes and washing sequentially with 10 mL deionized water with and without 0.25% Tween 80. Finally, microspheres were dissolved in 3 mL of 2-ethoxyethylacetate, and the intensity of fluorescence was determined by using a fluorescence spectrometer. The regional blood flow in the ischemic limb was represented as the percentage of flow in the ischemic limb standardized with its tissue weight compared with that in the contralateral nonischemic limb standardized with its tissue weight.

Hemoglobin Measurement
Male mice (weighing ~25 g) were subcutaneously injected with 0.5 mL growth factor–reduced Matrigel (Collaborative Biomedical Products) containing adenovirus (2 $\times 10^{10}$ pfu pmouse) in the abdomen, as previously described. After 10 days, the animals were euthanized, and the Matrigel plug was removed and separated from the skin. Hemoglobin content of the plugs was measured by using a hemoglobin assay kit (Sigma) according to the manufacturer’s instructions.

Statistical Analysis
Results are expressed as mean±SEM. Comparisons among groups were made by ANOVA with the Fisher protected least significant difference test. In experiments with only 2 groups, the Student t test was used. Differences were considered significant at P<0.05.

Results
Effects of eNOS Plasmid DNA on cGMP Production
To first determine the efficacy of the newly constructed eNOS plasmid, we measured cGMP levels (an indicator of NO) in cells transiently transfected with or without the construct. A 10-fold increase in cGMP levels was found in cells transfected with eNOS adenovirus plasmid DNA compared with GFP plasmid DNA (20.5±3.5 [eNOS] versus 1.75±0.15 [GFP] fmol/mg protein, P<0.001; n=2). HEK 293 cells were used because of their high transfection efficiency (>95% with GFP used as a reference). The cGMP levels in GFP-transfected cells were comparable to those of nontransfected cells (data not shown). These results demonstrate that the eNOS transgene was successfully introduced into the cells and that eNOS was active in the transfected cells.

Expression of eNOS in Cells Transduced With Adenovirus Containing eNOS cDNA
To further investigate the activity of the transgene once the virus was produced, we transfected cells (A549) with adenovirus and measured secreted nitrate/nitrite levels. Cells transduced with eNOS adenovirus, compared with cells transduced with adenovirus encoding GFP, showed an increase of ~3-fold (5.92±0.33 [eNOS] versus 1.92±0.07 [GFP] nmol/mL, P<0.01; n=3). NO generated from eNOS has a relatively short half-life and is rapidly converted to nitrate and nitrite. Therefore, measurement of nitrate/nitrite is a good indicator of eNOS activity. As an additional confirmation that eNOS gene transfer results in a functional eNOS gene product, we sought to measure the NO activity in A549 cells transfected with the virus. NO converts [H]arginine to [H]citrulline, which can be separated from another on the basis of charge. We found that cells transfected with eNOS virus exhibited a significant increase in levels of [H]citrulline versus cells transfected with GFP adenovirus, indicating that a functional enzyme was being produced (293 947±9974 [eNOS] versus 14 961±55 [GFP] cpm, P<0.0001; n=4 and 2, respectively). Furthermore, cells transfected with Ad.CMV-eNOS stained intensely for NADPH diaphorase activity, which is an indicator of NOS activity (data not shown). These data indicate that overexpression of eNOS results in a significant increase in NOS activity.

Time Course of Human eNOS Expression
To localize human eNOS delivered by adenovirus in our model, our immunohistochemical analysis used an antibody against eNOS that specifically recognizes the human form. Rats were euthanized (3 per group) at each time point of 1, 2, 3, and 4 weeks. Strong reactivity was seen in specimens 1 week after gene transfer, which steadily diminished to weaker levels by week 4 (although it was still detectable). Control tissue was used from uninjected contralateral limbs. Representative sections are shown from rats at each time point (n=3 per group) in Figure 1.

Laser Doppler Perfusion Imaging
We measured the ability of eNOS gene transfer to augment blood perfusion in the ischemic hindlimb of rats by using...
Figure 2. Time course of eNOS gene delivery on perfusion in the rat ischemic hindlimb. A, The left femoral artery was surgically removed, and either Ad.CMV-Luc or Ad.CMV-eNOS or vehicle alone was injected into left adductor and surrounding muscles (the animal’s left limb is shown on the right side of each image). Before and after surgery and weekly thereafter, perfusion was evaluated by laser Doppler perfusion imaging. Red areas indicate regions of relatively high perfusion, and blue areas indicate relatively low perfusion. B, Average perfusion was calculated and expressed as a ratio of ischemic limb/normoperfused limb. Bars represent mean±SEM (n=6 or 7 per group).
laser Doppler imaging (Figure 2A). This noninvasive technique allows for multiple time points of blood flow to be assessed. Animals were measured weekly, and body temperature was maintained on a heating pad before and throughout the course of the measurements. By week 2, quantitative analysis showed a significant increase in blood flow in rats receiving Ad.CMV-eNOS, which increased until week 4 (Figure 2B, week 4, 97.4 ± 3.0% [eNOS] versus 74.2 ± 3.1% [vehicle] and 74.0 ± 2.6% [luciferase], P<0.05; n=6 or 7).

Microsphere Determination of Regional Blood Flow After eNOS Gene Delivery

In addition to the laser Doppler perfusion analysis, we determined the blood flow in rats after gene delivery by using a fluorescent microsphere assay. The microspheres were injected into the left ventricle of anesthetized rats 3 weeks after the surgery and virus injection. Fluorescence in the adductor and gastrocnemius muscles was harvested, and fluorescence was measured. Results are expressed as fluorescence of ischemic limb/fluorescence of normoperfused limb standardized by weight of tissue. Bars represent mean±SEM (n=9 per group).

**Figure 3.** Blood flow assessment by injection of fluorescent microspheres. Microspheres were injected into the left ventricle of the heart 3 weeks after gene delivery. Adductor and gastrocnemius muscles were harvested, and fluorescence was measured. Results are expressed as fluorescence of ischemic limb/fluorescence of normoperfused limb standardized by weight of tissue. Bars represent mean±SEM (n=9 per group).

**Microsphere Determination of Regional Blood Flow After eNOS Gene Delivery**

To determine whether eNOS gene delivery increased the number of capillaries during ischemia, we measured the capillary density of skeletal muscle sections by immunostaining with antibody against CD-31. Representative sections are shown from rats injected with vehicle, Ad.CMV-Luc, and Ad.CMV-eNOS (Figure 4A). For quantification, the number of capillaries counted was adjusted per muscle fiber (Figure 4B, 1.42 ± 0.14 [eNOS] versus 0.82 ± 0.08 [vehicle] and 0.74 ± 0.03 [luciferase], P<0.01; n=5). These results indicate that delivery of eNOS can cause an increase in the number of capillaries formed in the ischemic hindlimb. A separate group of rats was used to determine whether an intramuscular injection of Ad.CMV-eNOS could increase cGMP levels, an indicator of NO formation. Three days after gene delivery, we observed a significant increase in the cGMP content of eNOS Gene Transfer Increases Capillary Density and Intracellular cGMP Content in Ischemic Hindlimb

**Figure 4.** Effect of eNOS gene transfer on capillary density in the ischemic hindlimb of rats. Adenovirus carrying human eNOS or GFP was locally injected into the hindlimb of rats after femoral artery removal. Three weeks later, capillary density was assessed by immunostaining paraffin-embedded sections with an antibody against PECAM-1 (CD-31). A, Sections from rats injected with vehicle, control virus, and eNOS are shown. B, Results were quantified, and data are expressed as capillaries per muscle fiber (mean±SEM, n=5 per group).
An increasing amount of evidence that implicates NO as a modulator for angiogenesis has been reported recently. In the present study, we sought to determine the role of eNOS in angiogenesis by a gene transfer approach. We have successfully generated an adenovirus that expresses a functional human eNOS in vivo. This was demonstrated by increased levels of cGMP and immunoreactive human eNOS in rat skeletal muscle. On production of the adenovirus, we investigated the ability of eNOS to induce angiogenesis in animal models. We found that a single injection of adenovirus encoding human eNOS resulted in transgene expression that could be robustly identified 1 week after gene transfer and could still be weakly detected 4 weeks after injection.

Our results showed that overexpression of eNOS in the ischemic hindlimb can significantly improve capillary density and regional blood flow. Angiogenesis is an intricate process that involves a host of factors and signals. In a hypoxic state brought about by tissue ischemia, VEGF expression is upregulated, leading to a cascade of signaling to induce angiogenesis. Consequently, a certain amount of revascularization occurs in response to ischemic conditions. However, on the basis of our results, the reperfusion that results from the newly formed vessels causes a recovery of only 70% to 80% of the original perfusion. We found that overexpression of eNOS results in a significant increase in angiogenesis over the normal response to tissue ischemia. This was observed by using laser Doppler perfusion imaging as well as a fluorescent microsphere approach to determine regional blood flow in the hindlimb.

A central question in the present study was whether the increased blood flow observed after eNOS gene transfer is a result of the vasodilation property of eNOS or is due to an increase in blood vessel growth. Two lines of evidence support the latter. First, because expression of a transgene by adenovirus decreases rapidly in a matter of weeks, it is unlikely that the blood flow augmentation seen in rats receiving Ad.CMV-eNOS is due to vasodilation. Rather, it is more likely that the necessary signaling for blood vessel growth had already been accomplished by eNOS at this time point. Second, and more important, eNOS gene transfer has been shown by us to result in a significant increase in capillary density in the rat ischemic hindlimb as opposed to only dilation of existing vessels. Therefore, our data indicate that adenovirus-mediated eNOS gene delivery results in an increase in the spontaneous angiogenic response induced by tissue ischemia.

In addition to the ischemic hindlimb model, another simple approach to test the angiogenic potential of a substance in vivo is to use implanted Matrigel containing the test substance. Matrigel is a solution of basement membrane proteins that is liquid at 4°C and solid at 37°C. We used Ad.CMV-eNOS suspended in Matrigel to determine whether gene delivery of eNOS could result in angiogenesis. Adenovirus is capable of transducing tissue adjacent to the Matrigel implant, and this has been demonstrated by using 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside staining after injection of Matrigel containing adenovirus encoding lacZ. We injected the mice abdominally with the solution of Matrigel and adenovirus. Ten days later, the animals were euthanized, and the gel implants were removed. To evaluate the angiogenic response, we measured the hemoglobin content of the gel plugs introduced by the invading blood vessels. There was a marked increase in the amount of hemoglobin within the Matrigel containing Ad.CMV-eNOS compared with control with or without Ad.CMV-Luc, indicating an increase in functional blood vessels.

In conclusion, our data suggest that eNOS gene delivery may be a viable alternative in the treatment of peripheral vascular disease due to hypertension or other circulatory disorders in which augmentation of blood flow and capillary density would be advantageous.

**Acknowledgments**

This work was supported by National Institutes of Health grant HL-29397. We thank Dr James K. Liao at Harvard Medical School, Cambridge, Mass, for graciously providing the human CMV-eNOS construct used in this study for the preparation of adenovirus.

**References**


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Arterioscler Thromb Vasc Biol. 2002;22:1279-1285; originally published online June 20, 2002; doi: 10.1161/01.ATV.0000026613.18742.67

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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