Improvement of Relaxation in an Atherosclerotic Artery by Gene Transfer of Endothelial Nitric Oxide Synthase

Hiroaki Ooboshi, Kazunori Toyoda, Frank M. Faraci, Markus G. Lang, Donald D. Heistad

Abstract—Gene transfer with replication-deficient adenovirus is a useful tool to study vascular biology. We have reported that overexpression of endothelial nitric oxide (NO) in carotid arteries from normal rabbits augments vasorelaxation mediated by NO. In this study, we tested the hypothesis that adenovirus-mediated gene transfer of endothelial nitric oxide synthase (eNOS) improves impaired relaxation of atherosclerotic vessels. We used 2 replication-deficient adenoviruses: AdeNOS, which carries cDNA for eNOS, and Adβgal, which expresses β-galactosidase. Common carotid arteries from 10 New Zealand White (NZW; plasma cholesterol, 79±13 mg/dL) and 10 Watanabe heritable hyperlipidemic (WHHL; plasma cholesterol, 452±39 mg/dL) rabbits were incubated in organ culture with AdeNOS, Adβgal, or vehicle alone. Carotid arteries from WHHL rabbits had mild to moderate atherosclerotic lesions. Histochemical staining for β-galactosidase and immunohistochemistry for eNOS indicated transgene expression in the endothelium and adventitia in both NZW and WHHL rabbits. Expression of eNOS determined with Western blot analysis after incubation with AdeNOS tended to be higher in vessels from WHHL rabbits than NZW rabbits. Effects of transgene expression on vascular function were examined by recording isometric tension 1 day after transduction. After precontraction with phenylephrine, acetylcholine produced significantly less relaxation in vessels from WHHL rabbits than in vessels from NZW rabbits. Relaxation in response to acetylcholine was greater in carotid arteries from normal rabbits augments vasorelaxation mediated by NO. In this study, we tested the hypothesis that adenovirus-mediated gene transfer of endothelial nitric oxide (NO) in carotid arteries from normal rabbits augments vasorelaxation mediated by NO. In this study, we tested the hypothesis that adenovirus-mediated gene transfer of endothelial nitric oxide synthase (eNOS) improves impaired relaxation of atherosclerotic vessels. We used 2 replication-deficient adenoviruses: AdeNOS, which carries cDNA for eNOS, and Adβgal, which expresses β-galactosidase. Common carotid arteries from 10 New Zealand White (NZW; plasma cholesterol, 79±13 mg/dL) and 10 Watanabe heritable hyperlipidemic (WHHL; plasma cholesterol, 452±39 mg/dL) rabbits were incubated in organ culture with AdeNOS, Adβgal, or vehicle alone. Carotid arteries from WHHL rabbits had mild to moderate atherosclerotic lesions. Histochemical staining for β-galactosidase and immunohistochemistry for eNOS indicated transgene expression in the endothelium and adventitia in both NZW and WHHL rabbits. Expression of eNOS determined with Western blot analysis after incubation with AdeNOS tended to be higher in vessels from WHHL rabbits than NZW rabbits. Effects of transgene expression on vascular function were examined by recording isometric tension 1 day after transduction. After precontraction with phenylephrine, acetylcholine produced significantly less relaxation in vessels from WHHL rabbits than in vessels from NZW rabbits. Relaxation in response to acetylcholine was greater in carotid arteries from normal rabbits than in vessels treated with vehicle or Adβgal. Vasorelaxation in response to acetylcholine was inhibited by Nω-nitro-L-arginine. Responses to sodium nitroprusside were similar after treatment with vehicle alone, Adβgal, or AdeNOS in both groups of rabbits. Thus, overexpression of eNOS with an adenoviral vector improves impaired NO-mediated relaxation in atherosclerotic arteries. (Arterioscler Thromb Vasc Biol. 1998;18:1752-1758.)

Key Words: adenovirus • atherosclerosis • gene transfer • nitric oxide synthase • vasorelaxation

Replcation-deficient recombinant adenovirus is a promising vector for gene transfer to blood vessels.1–3 Adenoviral vectors can infect a broad range of cells, accommodate a cDNA insert up to ~8 kb, and provide relatively high efficiency for gene transduction,4–5 thus serving as useful tools for studies of vascular biology and potentially for gene therapy.

Nitric oxide (NO), produced by NO synthase (NOS), mediates endothelium-dependent relaxation6 and plays a major role in vascular function, including regulation of vascular tone and inhibition of platelet aggregation and leukocyte adhesion.7–9 The main source of NO in normal vessels is type III NOS (or endothelial NOS; eNOS).10 Recent studies that used viral vectors carrying eNOS have shown that overexpression of eNOS in normal or mechanically injured vessels produces improvement of vascular function.11–13 Endothelium-dependent relaxation is impaired in atherosclerotic or hypercholesterolemic vessels in humans and animals.14–17 Administration of L-arginine, the substrate of NOS, improves impaired relaxation in atherosclerotic vessels.18–20 Thus, one might anticipate that gene transfer of eNOS would be beneficial for atherosclerotic vessels. Production of oxygen-derived radicals is increased in atherosclerotic vessels, however.21,22 Superoxide anion reacts with NO, and superoxide dismutase improves NO-mediated responses in atherosclerotic arteries.23 Therefore, it is difficult to predict whether overexpression of eNOS via gene transfer would improve NO-dependent relaxation in atherosclerotic arteries in the presence of continued production of superoxide anion.

The goal of this study was to determine whether impaired endothelium-dependent relaxation is improved by overexpression of eNOS in atherosclerotic vessels. Thus, we transfected arteries from normal and atherosclerotic animals with a replication-deficient adenovirus that carries the cDNA for eNOS and examined vascular function.

Methods

Adenoviral Vectors

We used 2 replication-deficient recombinant adenoviruses encoding nuclear-targeted β-galactosidase (Adβgal) and eNOS (AdeNOS),...
both driven by a cytomegalovirus promoter. AdenOS was constructed by using cDNA for bovine eNOS (a generous gift from Dr D.G. Harrison, Emory University, Atlanta, Ga) according to our previous methods. AdGal was used as a control virus. Recombinant adenoviruses were triple plaque–purified to assure that viral suspensions were free of wild-type virus, and titers were determined by plaque assay on 293 cells. Purified viruses were suspended in PBS containing 3% sucrose and kept at −80°C until used.

**Transfection of Cell Culture**

To examine characteristics of eNOS, we transfected COS 1 cells with AdeNOS [1, 10, or 100 plaque-forming units (pfu)/cell] and measured the conversion of L-[15N]arginine to L-[15N]citrulline with cell homogenate 2 days later, as described in detail previously. To characterize the overexpressed enzyme, we tested calcium dependence by depletion of calcium and addition of 2.5 mmol/L EGTA in the reaction mixture. We also examined the specificity of NOS by addition of 10 mmol/L N-nitro-l-arginine methyl ester. Specificity of converted l-citrulline was confirmed by using thin-layer chromatography.

**Transduction of Arteries**

We studied 10 New Zealand White (NZW; 3.2 to 5.1 kg, 5 males and 5 females; plasma cholesterol, 79±13 mg/dL) and 10 homozygous Watanabe heritable hyperlipidemic (WHHL; 2.1 to 4.6 kg, 5 males and 5 females; plasma cholesterol, 452±39 mg/dL) rabbits. The rabbits in this study were different from those used in a previous study. Rabbits were euthanized by an overdose of pentobarbital (50 mg/kg), and common carotid arteries were removed. Vessels were placed in Krebs’ bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, KH2PO4 1.2, MgSO4·7H2O 1.2, d-glucose 11.1, NaHCO3 25.0, and CaCl2·H2O 2.54. Loose connective tissue was removed gently, without disruption of adherent adventitia. We cut each artery into 6 to 8 rings, each 3 mm long. Each ring was incubated with 100 μL of viral suspension that contained 3×108 pfu of virus (3×107 pfu/mL), either AdGal or AdeNOS. Other rings were incubated in vehicle (3% sucrose in PBS) as a control for transfection. Two hours later, the viral suspension or vehicle was removed. Rings were rinsed with PBS and incubated with Eagle’s modified essential medium with 100 U/mL penicillin, 100 μg/mL streptomycin, and 5% PBS. One day later, the rings were used for Western blot analysis, isometric tension examination, or histochemistry.

**Western Blot Analysis for eNOS**

The relative levels of eNOS expressed in vessel rings from NZW and WHHL rabbits after gene transfer were determined by Western blot analysis. Twenty micrograms of protein from the homogenized vessel rings was resolved on 10% SDS polyacrylamide gels, together with lysate of COS 1 cells transduced with AdeNOS as a positive control, and prestained protein markers (Bio-Rad). Proteins were then blotted to a nitrocellulose membrane by using a semidry transfer apparatus (Bio-Rad). The membrane was blocked overnight at 4°C with 5% nonfat dry milk in PBS containing 0.1% Tween-20. Subsequently, the membrane was incubated with mouse monoclonal antibody to human eNOS (IgG1, used at 1:1000, Transduction Laboratories) for 1 hour at room temperature. The membrane was washed with PBS containing 0.1% Tween-20 and then incubated with horseradish peroxidase–conjugated goat anti-mouse IgG antibody (1:100 000, Sigma) for 30 minutes. After it was washed, the membrane was incubated with SuperSignal Ultra Chemiluminescent Substrate (Pierce) for 1 minute and then exposed to x-ray film (Biomax, Kodak) for 3 to 5 minutes. Bands corresponding to the size of eNOS (140 kDa) were scanned on a QCS3200 flatbed scanner (Imaprol) and analyzed by densitometry software (Volume Trace Motif, version 1.21, University of Iowa Image Analysis Facility). The ratio of band density of samples to the positive control was determined.

**Histochemical Analysis of Gene Expression for β-Galactosidase**

Vessel rings that were to be analyzed for transgene expression of β-galactosidase were rinsed twice with PBS and fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 10 minutes. After a thorough rinsing with PBS, the rings were incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Sigma) solution at room temperature. After incubation for 3 hours, the rings were rinsed in PBS and postfixed with 4% formaldehyde. Incubation with X-Gal was limited to 3 hours to prevent staining of endogenous β-galactosidase, which may be seen in the cytosol after longer (>4 hours) periods of incubation. The fixed tissue was processed for paraffin embedding, and sections (5 μm thick) were cut from the block with microtomes, placed on slides, and counterstained with nuclear fast red. Vessel sections were examined for positive staining of β-galactosidase (blue nuclei) by light microscopy.

**Immunohistochemistry for eNOS**

For immunohistochemical analysis for eNOS, serial 5-μm-thick frozen sections of the rings were adhered to poly-L-lysine–coated slides, allowed to dry in room air, and fixed in acetone and 1% paraformaldehyde at 4°C for 5 minutes. Goat serum (5%) and 0.2% BSA were used for blocking nonspecific binding of protein for 20 minutes. Mouse IgG1 monoclonal antibody to human eNOS (1:50, Transduction Laboratories) was then incubated for 30 minutes. After the slides were washed for 5 minutes in PBS, biotinylated goat anti-mouse IgG (Vector Laboratories) was applied for 30 minutes. After the slides were rinsed with PBS, avidin and biotinylated horseradish peroxidase complex (Vector Laboratories) were applied for 30 minutes. After a rinse with PBS, 0.05% diazenimobenzidine tetrahydrochloride and 0.01% HzO2 were applied for 5 minutes and washed with water. Vessel sections were counterstained with methyl green vector (Vector Laboratories) and examined for positive staining of eNOS (purple) by light microscopy.

**Functional Study of Carotid Rings**

Isometric tension was recorded to assess function of transfected vessels. Vascular rings that were treated with replication-deficient virus or vehicle were mounted on stainless steel hooks at optimal resting tension (3 g) in organ baths, bathed in Krebs’ bicarbonate solution at 37°C, and aerated with 95% O2–5% CO2. Tension was periodically adjusted to the desired level during a 45-minute equilibration period. The vascular rings were then contractured twice with 80 mmol/L KCl and rinsed 3 times after each contraction. Then, a concentration-response curve for phenylephrine (10−8 to 10−4 mol/L) was generated. Concentration-response curves for acetylcholine (10−9 to 10−3 mol/L) and sodium nitroprusside (10−8 to 10−4 mol/L) were also generated after precontraction of the vessels with an EC50 dose of phenylephrine. In separate experiments, concentration-response curves for acetylcholine were generated in the presence of N-nitro-L-arginine (100 μmol/L), which was applied 20 minutes before precontraction with phenylephrine. Acetylcholine chloride, L-phenylephrine hydrochloride, sodium nitroprusside, and N-nitro-L-arginine were obtained from Sigma Chemical Co and dissolved in normal saline. Contractile responses were expressed as percent contraction of the response to 80 mmol/L KCl, and relaxation was expressed as percent relaxation of the contraction produced by an EC50 dose of phenylephrine.

**Statistical Analysis**

Data are presented as mean±SEM. One-way ANOVA was used to test for the statistical difference among treatment groups, followed by Bonferroni’s corrected t test. An unpaired t test was used between NZW and WHHL rabbits. A value of P<0.05 was considered statistically significant.

**Results**

**Effect of Transduction of eNOS in Cell Culture**

There was minimal formation of l-citrulline (0.006±0.000 pmol·mg protein−1·min−1) from l-arginine in cells that

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were not treated with recombinant virus (control). Transfection of COS 1 cells with Adβgal did not change the activity of eNOS. In contrast, when COS 1 cells were transduced with AdeNOS, the activity of NOS increased by 26-fold and 186-fold (for 10 and 100 pfu/cell, respectively) (Figure 1). The increase in formation of L-citrulline after transduction with 10 pfu/cell of AdeNOS was inhibited in the absence of calcium or the presence of an inhibitor of NOS, Nω-nitro-L-arginine methyl ester.

**Western Blot Analysis for eNOS**

One day after incubation with AdeNOS, expression of eNOS was confirmed in carotid arteries treated with AdeNOS from both NZW and WHHL rabbits (Figure 2). The ratio of the amount of eNOS in AdeNOS-transduced arteries from WHHL rabbits to those from positive controls (0.79±0.32) tended to be greater (P>0.05) than those from NZW rabbits (0.44±0.25, n=4). The eNOS protein was not detectable in carotid arteries treated with vehicle or Adβgal from both NZW and WHHL rabbits after exposure of the band to the film for 3 to 5 minutes and was occasionally observed as a very thin band after exposure for a longer time.

**Histochemistry for β-Galactosidase and eNOS**

One day after incubation with Adβgal, positive staining for nuclear-targeted β-galactosidase was noted in the endothelium and adventitia in vessels from NZW and WHHL rabbits (Figure 3A and 3B). There was no detectable transgene expression of β-galactosidase in smooth muscle cells in either NZW or WHHL rabbits. Positive staining for β-galactosidase was not observed after AdeNOS or vehicle treatment. Mild to moderate atherosclerosis, characterized by patchy thickening of the intima-media, was observed in vessels from WHHL rabbits.

In vessels treated with Adβgal or vehicle, positive staining was observed in the endothelium, but not in the media or adventitia, in both NZW and WHHL rabbits (Figure 4A and 4B). In vessels treated with AdeNOS, positive staining for eNOS was observed in both endothelial and adventitial cells in both NZW and WHHL rabbits (Figure 4C, 4 days). The amount of eNOS expression in the endothelium appeared to be much greater in vessels treated with AdeNOS than in those
treated with Adβgal or vehicle in both NZW and WHHL rabbits.

**Vascular Responses in Normal Arteries**

In carotid rings from NZW rabbits after transduction with Adβgal or AdeNOS, responses to phenylephrine were similar to those of control vessels that were not treated with virus (Figure 5A). After precontraction with phenylephrine, relaxation to acetylcholine was virtually identical between vessels treated with vehicle alone and those transfected with Adβgal (Figure 5B). Incubation of vessels with AdeNOS, however, resulted in enhanced relaxation in response to acetylcholine relative to vessels treated with vehicle or Adβgal. The EC₅₀ [log(mol/L)] for AdeNOS (−7.56±0.08) was significantly different from that for vehicle (−7.06±0.05, P<0.05) or Adβgal (−7.09±0.11, P<0.05). Relaxation in response to acetylcholine was inhibited after pretreatment of vessels with Nω-nitro-L-arginine in all vessels. Responses to sodium nitroprusside were not altered after transfection with Adβgal or AdeNOS (Figure 5C).

**Vascular Responses in Atherosclerotic Arteries**

In carotid rings from WHHL rabbits, phenylephrine produced a dose-dependent contraction, which was not altered by transfection with Adβgal or AdeNOS (Figure 6A). Maximum contraction in vessels from WHHL rabbits (149±4%) was similar to that of NZW rabbits (151±6%). In vehicle-treated rings, relaxation to acetylcholine was significantly smaller in WHHL (EC₅₀, −6.90±0.04) than NZW (−7.06±0.05, P<0.05) rabbits (Figure 7). Transduction of Adβgal did not alter the responses to acetylcholine (Figure 6B).

The major new finding of this study is that relaxation in response to low concentrations of acetylcholine was augmented after incubation of vessels from WHHL rabbits with AdeNOS, compared with incubation with vehicle alone or Adβgal (Figure 6B). The EC₅₀ for AdeNOS (−7.29±0.06)
was significantly different from that for vehicle (−6.90±0.04, P<0.05) or Adβgal (−7.02±0.07, P<0.05) in WHHL rabbits. The EC50 for AdeNOS in WHHL rabbits was lower than the EC50 for vehicle treatment in NZW rabbits and approached the value for AdeNOS in NZW rabbits. Relaxation to acetylcholine was inhibited markedly in all vessels after pretreatment of rings with Nω-nitro-L-arginine.

There were no differences in relaxation of vehicle-treated rings to acetylcholine in male and female NZW and WHHL rabbits (data not shown). Responses of arterial rings to acetylcholine were augmented similarly after incubation with AdeNOS, regardless of sex, in both NZW and WHHL rabbits. In vehicle-treated rings, responses of vessels to sodium nitroprusside were similar in NZW and WHHL rabbits. Transduction with Adβgal or AdeNOS did not alter the response to nitroprusside in vessels from WHHL rabbits (Figure 6C).

Discussion
The major new finding in this study is that adenovirus-mediated gene transfer of eNOS to carotid arteries from atherosclerotic rabbits restores NO-mediated responses to acetylcholine toward normal. This is, to our knowledge, the first demonstration of improvement of impaired endothelium-dependent relaxation in atherosclerotic vessels by gene transfer.

Endothelium-dependent relaxation is impaired in vessels from atherosclerotic and hypercholesterolemic humans or animals.14–17 Relaxation of the carotid artery in response to acetylcholine is impaired in WHHL rabbits, even without apparent lesions.27 In this study, we observed less relaxation of the carotid artery in response to acetylcholine in WHHL than NZW rabbits. Relaxation to sodium nitroprusside, an endothelium-independent vasorelaxant, was similar in normal and atherosclerotic animals, suggesting that impaired relaxation to acetylcholine in WHHL rabbits is not due to dysfunction of vascular smooth muscle.

We and others have accomplished transfer of eNOS cDNA to blood vessels.11–13 Although these studies demonstrated alteration in function after overexpression of eNOS in normal or mechanically injured vessels, functional effects of transfer of the eNOS gene to atherosclerotic vessels have not been examined. Because atherosclerotic arteries are possible candidates for gene therapy,29 it was important to determine whether overexpression of eNOS in atherosclerotic arteries produces functional changes. In this study, efficacy of the
transgene transduced with AdeNOS was confirmed by using the citrulline assay in cultured cells, and immunohistochemistry demonstrated expression of eNOS in the endothelium and adventitia of carotid arteries in both normal and atherosclerotic rabbits, which is consistent with our previous studies.13,29

The mechanisms responsible for impairment of endothelial function in atherosclerotic vessels are not completely clear. Initial hypotheses to explain the impairment related to increased diffusional barriers for NO,17,30 depletion of l-arginine,20 or altered receptor-coupling mechanisms.31 Expression of mRNA and protein of eNOS is greater in the atherosclerotic than the normal aorta, suggesting that the mechanism of impaired endothelial function is not due to a decrease in eNOS itself.32 Recent studies suggest that bioavailability of NO is reduced, at least in part, by the “quenching” of NO by superoxide anion in atherosclerotic vessels.21,22 Improvement of endothelium-dependent relaxation occurs after administration of l-arginine, a substrate of NOS, to atherosclerotic animals,18–20 although it has been proposed that the effect is not mediated by an increase in NO production but by a direct action of l-arginine.33,34 Because superoxide anion interacts with NO to produce peroxynitrite, it seemed possible that overexpression of NOS or overproduction of NO in atherosclerotic vessels might not lead to improvement of endothelium-dependent relaxation, because generation of additional NO might simply be inactivated by superoxide anion.

In our experiment, carotid arteries from WHHL rabbits that were transfected with AdeNOS showed enhanced relaxation to acetylcholine, compared with those transfected with Adβgal or vehicle. Therefore, overexpression of eNOS improves NO-dependent relaxation in atherosclerotic vessels. Bioavailability of NO may be a balance between production of NO and quenching by superoxide anion.35 Thus, it seems possible that an increase in production of NO might lead to increased bioavailability of NO. Peroxynitrite itself has weak vasodilator effects, which may be mediated by an increase in cGMP and activation of the ATP-sensitive potassium channel.36 We cannot exclude the possibility that peroxynitrite may contribute to improvement of vasorelaxation to acetylcholine after gene transfer of eNOS.

The amount of eNOS protein in AdeNOS-transduced vessels tended to be greater in WHHL than NZW rabbits, on the basis of Western blot analysis. Because endogenous rabbit eNOS was not detected in arteries that were treated with vehicle or Adβgal, eNOS of AdeNOS-treated arteries expressed by Western blotting corresponds to eNOS derived from AdeNOS. Thus, the finding suggests that gene transfer of eNOS to atherosclerotic vessels tended to be more efficient than that to normal arteries. Alternatively, the half-life of transduced eNOS may differ in normal and atherosclerotic arteries. We have observed that transgene expression after gene transfer of β-galactosidase was greater in the atherosclerotic than the normal aorta29 and in atherosclerotic than normal carotid and basilar arteries from rabbits (D.D. Lund et al, unpublished data, 1998). The current findings, with Western blot analysis of eNOS, are concordant with our previous findings.29

As we observed previously,31 vessels without endothelium do not respond to acetylcholine even after incubation with AdeNOS, despite expression of eNOS in the adventitia, probably because cholinergic receptors are not present in the adventitia. Thus, in the current study, overexpression of eNOS in the endothelium presumably accounted for the augmented vascular relaxation to acetylcholine. Immunohistochemical analysis also suggested that eNOS expression in the endothelium was greater in vessels treated with AdeNOS than in those treated with Adβgal or vehicle in both NZW and WHHL rabbits.

In the current study, maximal responses to acetylcholine in atherosclerotic arteries tended to be greater after transduction with AdeNOS than after vehicle treatment or transduction with Adβgal, but no differences achieved statistical significance. The variance was greater in atherosclerotic than in normal arteries, and this variance contributes to the absence of statistical significance. In addition, the maximal response of vehicle-treated arteries to acetylcholine was already >60%, which provides less potential for improvement after treatment with AdeNOS.

It will be important to determine whether long-term overexpression of eNOS alters vascular function in atherosclerotic arteries, because NO is reported to attenuate cell proliferation and interaction of platelets and leukocytes with the endothelium and may contribute to regression of atherosclerosis. We used adenoviral vectors that provide relatively short-term expression of transgene.1–5 However, recent advances with adenoviral and other vectors, including adenoassociated virus,37 may enable examination of long-term effects of overexpression of eNOS in atherosclerotic arteries.

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References
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