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Gene therapy inhibiting neointimal vascular lesion: *In vivo* transfer of endothelial cell nitric oxide synthase gene

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ABSTRACT **It is postulated that vascular disease involves a disturbance in the homeostatic balance of factors regulating vascular tone and structure. Recent developments in gene transfer techniques have emerged as an exciting therapeutic option to treat vascular disease. Several studies have established the feasibility of direct *in vivo* gene transfer into the vasculature by using reporter genes such as β-galactosidase or luciferase. To date no study has documented therapeutic effects with *in vivo* gene transfer of a cDNA encoding a functional enzyme. This study tests the hypothesis that endothelium-derived nitric oxide is an endogenous inhibitor of vascular lesion formation. After denudation by balloon injury of the endothelium of rat carotid arteries, we restored endothelial cell nitric oxide synthase (ec-NOS) expression in the vessel wall by using the highly efficient Sendai virus/liposome *in vivo* gene transfer technique. ec-NOS gene transfection not only restored NO production to levels seen in normal untreated vessels but also increased vascular reactivity of the injured vessel. Neointima formation at day 14 after balloon injury was inhibited by 70%. These findings provide direct evidence that NO is an endogenous inhibitor of vascular lesion formation *in vivo* (by inhibiting smooth muscle cell proliferation and migration) and suggest the possibility of ec-NOS transfection as a potential therapeutic approach to treat neointimal hyperplasia.**

The process of intimal hyperplasia is common to various forms of vascular diseases such as atherosclerosis, transplant vasculopathy, and restenosis following balloon angioplasty. The use of *in vivo* gene therapy for the treatment of vascular disorders has been speculated for several years. Recently, our laboratory and others have shown that neointima formation after balloon injury can be inhibited by antisense oligonucleotide transfection (1, 2). However, there has been no report of a "therapeutic" transfection of a functional gene whose product inhibits neointima formation. This study demonstrates the successful inhibition of neointimal hyperplasia by *in vivo* gene transfer by introducing the cDNA encoding the endothelial cell nitric oxide synthase (ec-NOS). Our study has two major implications: (*i*) that the prevention of neointimal hyperplasia by an *in vivo* gene transfer approach is feasible, and (*ii*) that NO is an effective *in vivo* inhibitor of vascular smooth muscle cell (VSMC) accumulation and that its enhanced expression by *in vivo* gene transfer may be a promising strategy for the treatment of vascular disease.

Injury to the endothelium plays an essential role in the "response to injury" hypothesis (3, 4). Experimental studies have shown that vascular injury induces local expression of mitogens and chemotactic factors mediating neointima formation. The lesion is characterized in part by the abnormal migration and proliferation of VSMCs in the intima. In addition, it is postulated that endothelial denudation may result in the loss of constitutively expressed endothelium-derived inhibitory factors capable of suppressing neointimal hyperplasia. Moreover, the pathogenesis of vascular disease is characterized by endothelial dysfunction in which endothelium-dependent vasorelaxation is impaired. Our laboratory and others have hypothesized that endothelium-derived NO may be an important endogenous inhibitor of vascular lesion formation, given its capacity to inhibit platelet aggregation (5), leukocyte adhesion (6), and VSMC proliferation and migration *in vitro* (7, 8). Indeed, recent studies in hypercholesterolemic rabbits showed that the supplementation with dietary L-arginine, the substrate for NOS, inhibited atherosclerotic lesion formation and improved endothelium-dependent vasorelaxation (9). Furthermore, systemic administration of NOS inhibiting arginine analogs accelerated atherosclerotic lesion formation and impaired vascular reactivity (10, 11). These studies suggest a direct role of NO in inhibiting vascular lesion development but cannot exclude the contribution of systemic and/or biochemical effects.

We chose to study the well-characterized rat carotid artery balloon injury model because it is a model of VSMC migration and proliferation with little initial regrowth of the endothelium after injury (12). We recognize that this model has limitations as a model for human restenosis. However, the rat carotid injury model is useful for studying the factors influencing VSMC migration and proliferation *in vivo*. We used this model as a means for the "proof of concept" that vascular-derived NO has an important role *in vivo* in response to vascular injury independent of systemic hemodynamic and/or biochemical factors. We tested the hypothesis that restoration of ec-NOS gene expression and local generation of NO by *in vivo* gene transfer can restore, at least in part, vascular function and inhibit neointima formation. This study provides direct evidence that local NO generation modulates vascular lesion formation *in vivo*.

MATERIALS AND METHODS

Plasmid Construction. The full-length bovine ec-NOS cDNA (3.7 kb; kindly provided by T. Michel, Harvard Medical School) was isolated from a Bluescript II SK+ vector (13) by restriction enzyme digest with *Eco*RI and was then ligated into the *Eco*RI cloning site of the pUC-CAGGS expression plasmid (kindly provided by J. Miyazaki, Tokyo). The pUC-CAGGS vector was engineered by introducing the CAG promoter [cytomegalovirus immediate-early enhancer sequence connected to a modified (AG) chicken β-actin promoter], rabbit β-globin gene sequences including a polyadenylylation signal, and a simian virus 40 *ori* into the pUC13 vector [4.8 kb (14)]. To verify that the pUC/ec-NOS construct (8.5 kb) encodes a

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Abbreviations: NOS, nitric oxide synthase; ec-NOS, endothelial cell nitric oxide synthase; HVJ, hemagglutinating virus of Japan; VSMC, vascular smooth muscle cell.

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biologically active protein, pUC/ec-NOS was transfected into COS-7 cells. NOS activity was determined by the conversion of [^{14}C]arginine to [^{14}C]citrulline that was inhibitable by an arginine analogue (nitro-L-arginine methyl ester) (15), as well as by positive NADPH diaphorase staining (16) (data not shown). The pUC-CAGGS vector lacking the ec-NOS insert served as control vector.

Hemagglutinating Virus of Japan (HVJ) Liposome-Mediated Gene Transfer. pUC/ec-NOS was mixed with nuclear protein [high mobility group (HMG)-1] and incubated at 20°C for 1 h (17). HVJ liposomes were prepared by mixing dried lipids with the DNA/HMG-1 solution and subsequently with UV light-inactivated HVJ virus (purified Z strain; 20,000 hemagglutinating units/ml) (2). Rats (Sprague–Dawley; 400–450 g; Charles River Breeding Laboratories) were anesthetized (ketamine, 75–95 mg/kg; xylazine, 4–8 mg/kg i.p.), and a cannula was introduced into the left common carotid artery via the external carotid artery. After balloon injury of the left common carotid artery (ref. 18; 2F Fogarty catheter), the distal injured vessel segment was isolated by temporary ligatures. HVJ liposomes containing 30 μg of encapsulated ec-NOS or control vector plasmid per ml were infused into the distal injured segment and incubated for 10 min at room temperature under a distending pressure of 0.5 atm (1 atm = 101.3 kPa). After incubation, the infusion cannula was removed and blood flow to the common carotid artery was restored. The proximal injured segment of the vessel served as an untransfected control. All experiments involving animals were done in accordance with the institutional guidelines at Stanford University.

Western Blotting. For determination of ec-NOS protein expression after transfection, Western blotting was performed as described (19) with some modifications. Rats were sacrificed on day 4 after transfection; vessels were harvested and quick-frozen in liquid nitrogen. Crude enzyme preparations solubilized with 2% SDS/8 M urea were separated on denaturing SDS/7.5% polyacrylamide gels (80 μg per lane). Proteins were then blotted onto nitrocellulose (Hybond-ECL; Amersham) by wet electroblotting for 12 h. Blots were blocked overnight at 4°C with 5% nonfat dry milk in TBS-T (20 mM Tris·HCl/137 mM NaCl/0.1% Tween 20). Western blot analysis was performed with the highly specific anti-human as well as anti-bovine ec-NOS monoclonal antibody H32 (ref. 19; kindly provided by J. Pollack, Abbott). Specific proteins (135 kDa) were detected by enhanced chemiluminescence (ECL; Amersham) according to the manufacturer's instructions. Blots were incubated with the first antibody (1:10,000) for 1 h at room temperature and, after washing, with the second antibody (horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin antibody, 1:3000; Amersham) for 1 h. Prestained protein markers (Sigma) were used for molecular mass determinations. Positive controls were run with homogenates of bovine aortic endothelial cells.

NADPH Diaphorase Histochemistry. On day 4 after transfection, carotid arteries were perfused with phosphate-buffered saline; vessel segments were harvested and then frozen fixed in O.C.T. compound (Miles)/liquid nitrogen. Frozen sections (5 μm thick) were stained by an NADPH diaphorase reaction by incubating sections with 1 mM NADPH/0.2 mM nitroblue tetrazolium/0.1 M Tris·HCl, pH 7.2/0.2% Triton X-100 for 30 min at 37°C (16, 20).

NOS Assay. NO production on day 4 after ec-NOS transfection was measured by the chemiluminescence method (21). Vessel segments were incubated in PSS (2.2 mM Ca^{2++}) in the presence of A23187 (1 μM) and either L-arginine (100 μM) or *N*-monomethyl-L-arginine (100 μM) at 37°C for 2 h. NO and its one-electron oxidation products NO_2^- and NO_3^- (NO_x), respectively, can be measured by chemiluminescence detector after sample reduction in boiling acidic vanadium(III) and reaction with ozone at 98°C. Signals from the detector (model 2108; Dasibi) were analyzed by a computerized integrator and recorded as areas under the curve. Standard curves for NO_2^-/NO_3^- were linear over the range 100 pmol to 5 nmol of NO_x. All sample concentrations of NO_x fell within this range. NO_x was measured within 1 h after terminating the incubation experiment.

Measurement of DNA Synthesis. For bromodeoxyuridine (BrdUrd) staining, BrdUrd was injected into rats after vascular injury [100 mg/kg s.c. and 30 mg/kg i.p. at 18 h and 30 mg/kg i.p. at 12 h prior to sacrifice (22)]. Rats were sacrificed on day 4 after ec-NOS transfection. The carotid artery was removed after perfusion fixation (110 mmHg) with 4% (wt/vol) paraformaldehyde and processed for immunohistochemistry by using anti-BrdUrd antibodies (Amersham). The proportion of BrdUrd-positive cells was determined by cell counts under light microscopy in three or four different sections.

Measurement of Vascular Reactivity. At day 4 after ec-NOS transfection, vessels were carefully harvested and vascular rings were suspended from force transducers in organ chambers [filled with 25 ml of PSS (37°C; aerated with 95% O_2/5% CO_2) as described (9)] for continuous recording of isometric tension. Vascular rings were stretched to the optimal point of their length–tension relation (determined previously to be 0.8 g). Subsequently, all rings were contracted with KCl (50 mM) to establish the receptor-independent contractility of the vascular smooth muscle. After washing repeatedly with fresh PSS, vascular rings were exposed to increasing concentrations of L-phenylephrine (1 nM to 10 μM). After maximal tension was obtained, calcium ionophore A23187 (1 μM) was added to determine vascular relaxation. Finally, vessel rings were again contracted by adding nitro-L-arginine (300 μM). Data are expressed as percentage of the maximal response to L-phenylephrine.

Morphometric Analysis. At 2 weeks after ec-NOS transfection, rats were sacrificed and the carotid vessels were perfusion-fixed with 4% (wt/vol) paraformaldehyde, paraffin-embedded, sectioned (5 μm), and subsequently processed for light microscopy (hematoxylin/eosin). Medial and neointimal cross-sectional areas were determined by computerized morphometry by analyzing three or four individual sections of the middle portion of vessel segments (sections were coded to avoid biased analysis).

Statistical Analysis. All values are expressed as mean ± SEM. ANOVA with Tukey–Kramer multiple comparison posttests (INSTAT; Graphpad Software, San Diego) were used to determine significant differences in multiple comparisons. A P value of <0.05 was considered significant.

RESULTS

At 4 days after ec-NOS gene transfection, significant levels of protein expression were detected by Western blotting (Fig. 1). The antibody used specifically recognizes the ec-NOS isoform (135 kDa) and does not cross-react with the inducible forms of

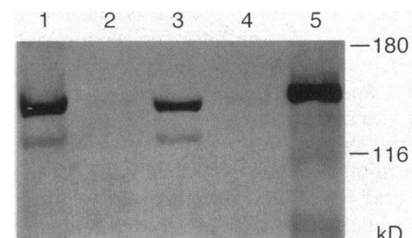

FIG. 1. Western blot demonstrating expression of ec-NOS protein after ec-NOS transfection into injured rat carotid arteries. Lanes: 1, ec-NOS transfected; 2, control vector transfected; 3, uninjured, untransfected; 4, injured, untransfected; 5, homogenate of cultured bovine endothelial cells.

NOS (19). Expression of ec-NOS was present in the intact uninjured rat carotid artery but was almost undetectable in the injured vessel denuded of endothelium and transfected with the control vector. Slight differences in the size of the ec-NOS protein detected in tissue vs. cultured bovine aortic endothelial cells may be related to differences in posttranslational processing (glycosylation). The generation of NO by ec-NOS involves the cofactor NADPH; therefore, the NADPH diaphorase assay provides a useful *in situ* histochemical technique to qualitatively verify the functionality and localization of ec-NOS activity after transfection. As shown in Fig. 2, we observed intense staining within the medial layer of the ec-NOS-transfected vessel as compared to low background levels in the control vector-transfected vessel.

As a direct assessment of local generation of NO in ec-NOS gene transfected vessels, we determined the release of nitrogen oxides (NO_2^-/NO_3^-; NO_x) generated by enzymatic reaction and detected by chemiluminescence. The highest level of NO_x production (45.4 ± 12.9 pmol of NO_x per mg wet weight) was observed in the uninjured vessels with an intact endothelium (Fig. 3, CTRL). In contrast, minimal NO_x release was detected in injured untransfected (INJ; 4.6 ± 2.4 pmol per mg wet weight) and injured control vector-transfected vessels (INJ + CV; 4.6 ± 1.1 pmol per mg wet weight), respectively. However, *in vivo* gene transfer of the ec-NOS gene restored local NO_x generation (INJ + NOS; 34.7 ± 7.1 pmol per mg wet weight) to levels not significantly different from the uninjured vessel. The specificity of this assay of NO generation was confirmed by the abolition of NO_x generation by the arginine analogue *N*-monomethyl-L-arginine.

Previous studies in our laboratory and others have observed that a decrease in NO-mediated vasorelaxation is associated with vascular lesion formation (9). To address the biological effects of *in vivo* gene transfer of the ec-NOS gene on the vessel wall, we examined its influence on regulation of vascular tone. Specifically, we tested the hypothesis that transfection of the ec-NOS gene into the injured vessels with subsequent increased NO generation would depress vascular reactivity to vasoconstrictor stimulation. The maximal contraction to KCl (50 mM) was significantly reduced by 48% in the ec-NOS-transfected vessels compared to control vector-transfected vessel rings. Absolute values of contraction to KCl (50 mM) were as follows: CTRL, 0.41 ± 0.02 g; INJ, 0.29 ± 0.03 g; NOS, 0.13 ± 0.03 g; CV, 0.25 ± 0.04 g ($P < 0.05$ for CTRL vs. CV, NOS vs. CV; $P < 0.001$ for CTRL vs. NOS). These data indicated that the increased local generation of NO in the ec-NOS gene-transfected vessels depressed the contractile responsiveness. Indeed, blockade of NO generation with nitro-L-arginine (competitive inhibitor of NOS) restored the contractile response to levels comparable to the control vector-transfected vessels. Furthermore, in ec-NOS-transfected vessels precontracted with L-phenylephrine, activation of the Ca^{2+}/calmodulin-dependent ec-NOS enzyme with the calcium ionophore A23187 induced significant vasodilation (73.3% ± 10.1% of L-phenylephrine effect) compared to control vector-transfected vessels (11.7% ± 7.9%) (Fig. 4). These findings provided further confirmatory evidence that the ec-NOS-transfected vessels expressed increased generation of biologically active NO within the vessel wall.

On the basis of this documentation of *in vivo* transfer of the ec-NOS gene with demonstrable biological effects on the

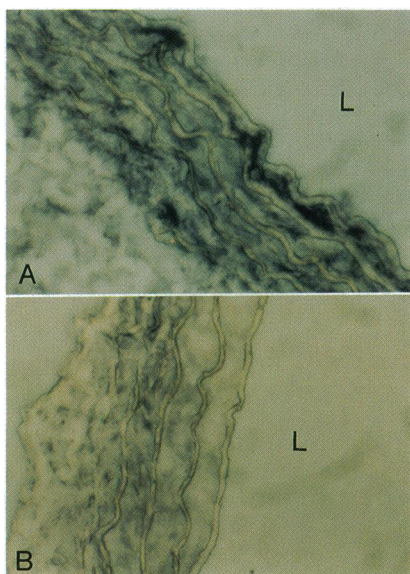

FIG. 2. Nitroblue tetrazolium reduction after ec-NOS transfection in ec-NOS-transfected (*A*) or control vector-transfected (*B*) vessel segments of injured rat carotid arteries. Cellular NOS activity can be detected by its reduction of nitroblue tetrazolium to form the dark-blue dye formazan (20). L, vessel lumen.

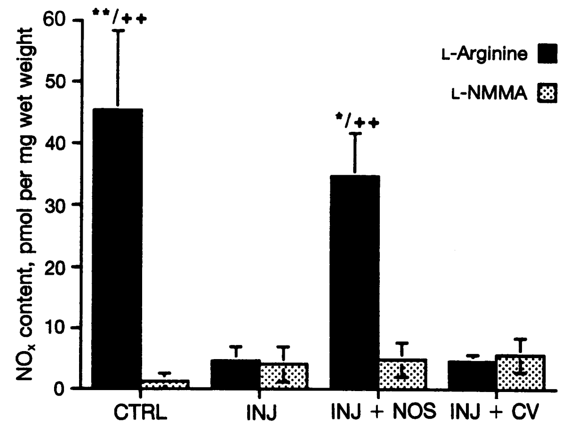

FIG. 3. NO production as measured by chemiluminescence (21) in injured, ec-NOS transfected (INJ + NOS); injured, control vector transfected (INJ + CV); injured, untransfected (INJ); and uninjured, untransfected (CTRL) rat carotid artery segments. NOS activity was stimulated by calcium ionophore (A23187; 1 μM) in the presence of L-arginine (100 μM) or *N*-monomethyl-L-arginine (L-NMMA) (100 μM). Mean ± SEM of four different experiments; * and **, $P < 0.05$ and $P < 0.01$ vs. INJ or INJ + CV; ++, $P < 0.01$ vs. L-arginine.

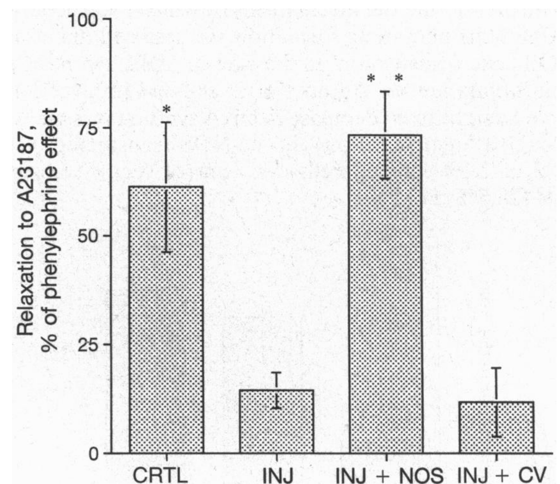

FIG. 4. Vascular reactivity of vessel rings obtained from uninjured (CTRL), injured untreated (INJ), injured and ec-NOS-transduced (NOS), and injured and control vector-treated (CV) rat carotid arteries (n = 3–7) to calcium ionophore (A23187; 1 μM). * and **, $P < 0.05$ and $P < 0.01$ vs. INJ or INJ + CV.

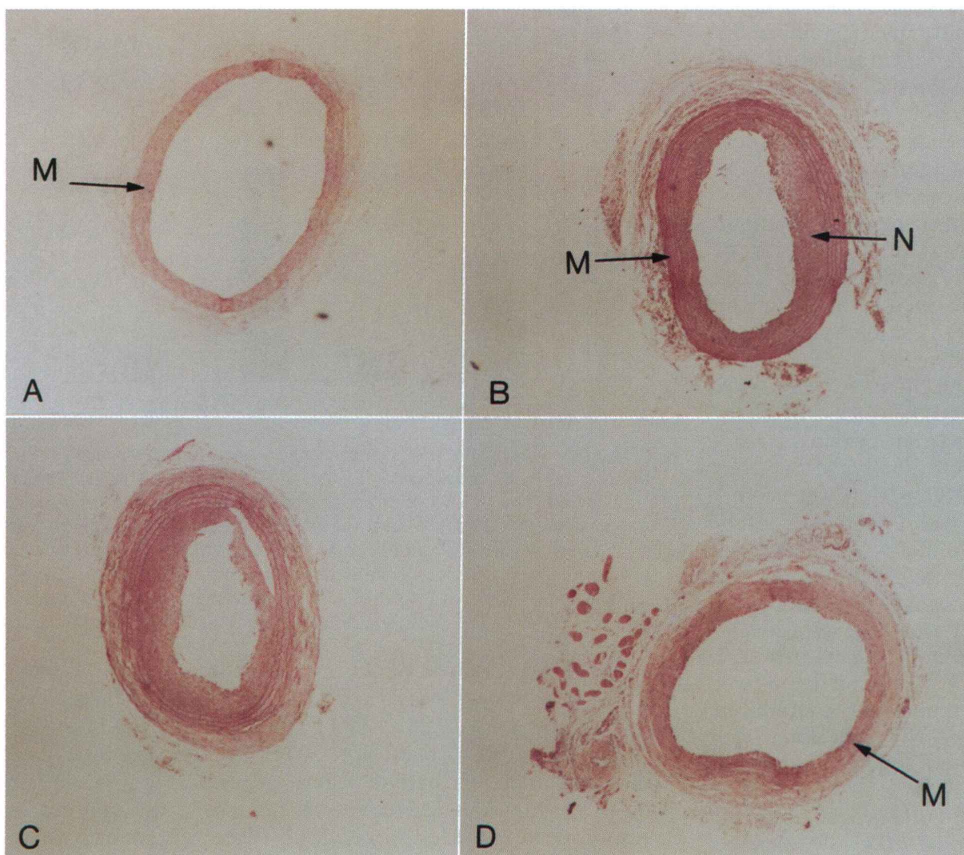

FIG. 5. Effect of ec-NOS (or control vector) transfection on neointima formation in balloon-injured rat carotid arteries. Representative cross-sections from rat carotid arteries are shown. (*A*) Uninjured. (*B*) Injured untreated. (*C*) Injured with control vector transfection. (*D*) Injured with ec-NOS transfection. M, media; N, neointima. (×25.)

regulation of vessel tone, we examined the effect of restored local NO generation on vascular lesion formation 2 weeks after injury (Figs. 5 and 6). Histological examination and morphometry revealed a 70% reduction in neointimal area (INJ + NOS, 0.059 ± 0.01 mm^2; INJ + CV, 0.20 ± 0.02 mm^2; INJ, 0.15 ± 0.02 mm^2; $P < 0.001$). Medial areas were 0.15 ± 0.01 mm^2 (INJ + NOS), 0.16 ± 0.02 mm^2 (INJ + CV), and 0.12 ± 0.01 mm^2 (INJ). The ratio of neointimal to medial area was 0.39 ± 0.06 (INJ + NOS) vs. 1.1 ± 0.08 (INJ + CV) or 1.3 ± 0.06 (INJ; Fig. 6). These results demonstrate that *in vivo* gene transfer of the ec-NOS transgene inhibits neointimal hyperplasia via increased local generation of NO within the vessel wall.

To further define the mechanisms by which local generation of NO inhibits neointima formation, we assessed the effect of ec-NOS gene transfection on the rate of DNA synthesis after vascular injury *in vivo*. At day 4 after ec-NOS transfection, we observed a significant decrease in DNA synthesis (as measured by BrdUrd incorporation) in ec-NOS-transfected vessels (15.3% ± 2.1% labeled cells) vs. control vector-transfected vessels (23.5% ± 3.2%).

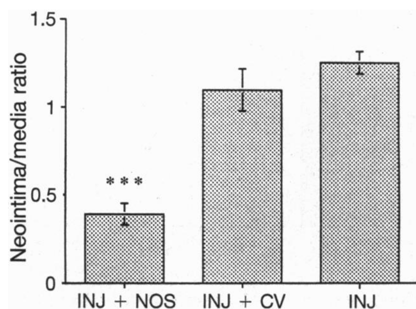

FIG. 6. Neointima/media ratio of injured ec-NOS-transfected (INJ + NOS), control vector (INJ + CV)-transfected, or injured untreated (INJ) rat carotid arteries. ***, $P < 0.001$ (n = 5–7).

DISCUSSION

Experimental studies have shown that vascular injury induces local expression of mitogens and chemotactic factors, which mediate neointimal lesion formation. This process is characterized in part by the abnormal migration and proliferation of VSMCs in the intima (4). In addition, the denudation of the endothelium after balloon injury may result in the loss of endothelium-derived inhibitory factors capable of suppressing neointimal hyperplasia. Endothelium-derived NO may be an important endogenous inhibitor of vascular lesion formation, given its multifactorial function in maintaining vascular homeostasis (4, 23). Accordingly, we tested in this study whether the restoration by *in vivo* gene transfer of ec-NOS gene expression and increased local generation of NO after vascular injury can inhibit neointima formation. To assess the biological response to transfection of the ec-NOS gene on vascular lesion formation *in vivo*, we chose the well-characterized rat carotid artery balloon injury model (24). This model is ideal for testing our hypothesis because there is no significant regrowth of the endothelium after injury (24, 25). We did not observe reendothelialization in histological examinations either at day 4 or at 2 weeks after ec-NOS transfection. Therefore, in the given time frame, ec-NOS gene expression can be restored only by successful *in vivo* gene transfer.

Our laboratory has introduced a very efficient method for *in vivo* gene transfer into the vasculature that employs the viral coat of the Sendai virus (HVJ) in a DNA–nuclear protein–liposome complex (2, 17). Using this gene transfer approach, we have achieved high expression levels of ec-NOS in injured rat carotid arteries. Furthermore, we demonstrated therapeutic effects utilizing *in vivo* gene transfer of a cDNA encoding a functional enzyme. We used four complementary experimental methods to verify successful *in vivo* ec-NOS gene transfer into the vessel wall: (*i*) transgene protein expression was documented by Western blotting; (*ii*) localization of

enzyme expression in the vessel wall was verified by *in situ* histochemical staining by the NADPH diaphorase reaction; (*iii*) enzymatic activity of the transgene product was confirmed by measurement of increased NO generation from transfected vessel segments using the chemiluminescence method; and (*iv*) biological effectiveness of the transgene product was assessed by changes in vascular reactivity induced by the increased local generation of NO, thereby potentially counterbalancing vasospasm induced by vascular injury. Whether our transgene is still expressed as a functional gene 2 weeks after transfection was not tested in this study. However, expression of gene product and NOS activity were demonstrated on day 4 after injury, at which time VSMC DNA replication has occurred and cells are migrating out of the media (24). Given that these cellular processes are essential for lesion formation and are initiated during this critical time period, we focused our characterization of transgene expression during this time frame. It has been shown that interventions that inhibit cell proliferation and/or cell migration during this initial acute phase prevent neointima formation at least 2 weeks after injury (1, 2, 26).

Previous studies have established that the pathogenesis of neointima formation involves abnormal regulation of both cell migration and proliferation. It is interesting to note that the results of this study suggest that the inhibitory effect of NO on neointima lesion formation appears to be more profound than that observed on DNA synthesis. These findings are consistent with *in vitro* studies which indicate that NO may have not only antiproliferative but also antimigratory effects (8). Furthermore, it is also conceivable that the inhibitory effect of NO on cell migration involves an indirect effect on the expression of a chemotactic factor. NO is a potent inhibitor of platelet aggregation and activation (5) and therefore could impair gene expression and local release of platelet-derived growth factor BB (27). Given the critical role of platelets and their release of platelet-derived growth factor BB on cell migration and neointima formation in this model (26), we speculate that the inhibitory effect of NO on neointima formation may also be mediated by its capacity to inhibit platelet aggregation. Thus, the local generation of NO may inhibit lesion formation by inhibiting platelet aggregation, leukocyte adhesion (6), and/or VSMC migration and proliferation. Recently, it was shown that endothelial injury of the rat carotid artery induces the production of NO from L-arginine, probably by an inducible form of NOS (iNOS) present in VSMCs (28). This induction of iNOS in VSMCs appears to be transient due to downregulation by mitogens activated after vascular injury (29, 30), and blockade of iNOS by an NOS inhibitor after vascular injury in rat carotid artery had no effect on neointima formation (31). The transfection, in our study, of an ec-NOS construct lacking NOS-specific gene regulatory sites has the advantage that the activity of this transgene cannot be modulated transcriptionally by factors induced after vascular injury.

Although the rat carotid model may not be a good model of human disease, this study provides direct evidence that endothelium-derived NO is an important local inhibitor of neointimal hyperplasia *in vivo*. These results are consistent with the postulate that the regulation of vascular growth and migration *in vivo* involves a delicate balance of stimulatory vs. inhibitory factors. We speculate that the loss of endothelial-derived NO may play a fundamental role in the pathogenesis of vascular diseases such as atherosclerosis. Our data suggest that ec-NOS gene transfer may be useful for gene therapy of neointimal hyperplasia and associated local vasospasm. Further studies in other animal models of vascular injury must be carried out before one can extrapolate the usefulness of this approach to treating human restenosis.

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