Nitric Oxide Synthase (nNOS) Gene Transfer Modifies Venous Bypass Graft Remodeling

Effects on Vascular Smooth Muscle Cell Differentiation and Superoxide Production

Nick E.J. West, MRCP; HuSheng Qian, MD, PhD; Tomasz J. Guzik, MD; Edward Black, FRCS; Shijie Cai, MD, PhD; Samuel E. George, MD; Keith M. Channon, MD, MRCP

Background—Pathological vascular remodeling in venous bypass grafts (VGs) results in smooth muscle cell (SMC) intimal hyperplasia and provides the substrate for progressive atherosclerosis, the principal cause of late VG failure. Nitric oxide (NO) bioactivity is reduced in VGs, in association with increased vascular superoxide production, but how these features relate to pathological VG remodeling remains unclear. We used gene transfer of the neuronal isoform of nitric oxide synthase (nNOS) to investigate how increased NO production modulates vascular remodeling in VGs and determined the effects on late VG phenotype.

Methods and Results—New Zealand White rabbits (n=60) underwent jugular-carotid interposition bypass graft surgery with intraoperative adenoviral gene transfer of nNOS or β-galactosidase. Vessels were analyzed after 3 days (early, to investigate acute injury/inflammation) or 28 days (late, to investigate SMC intimal hyperplasia). In early VGs, nNOS gene transfer significantly increased NOS activity and substantially reduced adhesion molecule expression and inflammatory cell infiltration. In late VGs, recombinant nNOS protein was no longer evident, but there were sustained effects on VG remodeling, resulting in a striking reduction in SMC intimal hyperplasia, a more differentiated intimal SMC phenotype, and reduced vascular superoxide production.

Conclusions—Intraoperative nNOS gene transfer has sustained favorable effects on VG remodeling and on the vascular phenotype of mature VGs. These findings suggest that early, transient modification of the response to vascular injury is a powerful approach to modulate VG biology and highlight the potential utility of NOS gene transfer as a therapeutic strategy in VGs. (Circulation. 2001;104:1526-1532.)

Key Words: grafting • superoxide • nitric oxide • vasculature • muscle, smooth

Venous bypass grafts (VGs) remain widely used for the surgical treatment of coronary artery disease but are prone to accelerated atherosclerosis and occlusion, occurring at rates of ≥50% at 10 years after implantation. Early VG injury, immediately after implantation into the arterial circulation, results in endothelial activation and inflammation, followed by vascular smooth muscle cell (SMC) migration and proliferation, resulting in VG intimal hyperplasia. Intimal SMCs in human and experimental VGs revert to an undifferentiated or synthetic phenotype after vascular injury, providing a substrate that facilitates the development of accelerated atherosclerosis.

Nitric oxide (NO), synthesized by nitric oxide synthases (NOS), plays key roles in maintaining vascular homeostasis by regulation of platelet adhesion and aggregation; vascular SMC migration, proliferation, and matrix synthesis; and inhibition of endothelial activation and inflammation. Deficient NO bioactivity is a characteristic feature of vascular disease states, and in VGs, NOS activity and NO production appear to be reduced early after bypass graft surgery, suggesting that loss of NO bioactivity may contribute to the injury response and to maladaptive VG remodeling.

Increasing evidence suggests that superoxide anion is an important contributor to reduced NO bioactivity in vascular disease states. Superoxide rapidly scavenges vascular NO and produces peroxynitrite, another pro-oxidant species. We have recently shown that superoxide production is enhanced in experimental VGs, predominantly because of increased NAD(P)H oxidase activity in dedifferentiated intimal SMCs. Superoxide production may play important roles in pathological VG remodeling, not only because of NO scavenging but also by direct effects on SMC mitogenesis, redox-sensitive gene expression, and regulation of matrix metalloproteinases.
We hypothesized that early restoration of NO bioactivity in VGs, by use of NOS gene transfer, would limit the early response to injury and that this early intervention could potentially exert sustained effects on the subsequent remodeling process. Adenoviral gene transfer is an effective strategy to augment vascular NOS protein levels and NO bioactivity in vivo. Gene transfer is a particularly attractive approach in VGs, in which the operative procedure provides the opportunity for direct local delivery of recombinant viral vectors without the need for delivery devices and minimizes risk of systemic exposure.

Accordingly, we used intraoperative NOS gene transfer to locally increase NO production in experimental VGs to determine the effects on VG endothelial activation, inflammation, and vascular remodeling. Furthermore, we sought to investigate how early NO augmentation would modify SMC phenotype and superoxide production in late VGs.

**Methods**

**Animals, VG Surgery, and Gene Transfer**

Replication-deficient adenoviral vectors encoding recombinant β-galactosidase (Ad.βGal) or neuronal NOS (nNOS) (Ad.nNOS) were generated and purified as previously described. Ad.nNOS includes a rat nNOS cDNA driven by a CMV immediate/early promoter.

Male normocholesterolemic New Zealand White rabbits (2 to 2.5 kg; n=60) underwent interposition bypass grafting of the jugular vein to the ipsilateral carotid artery, as previously described, with intraoperative delivery of adenoviral vectors. Vein segments were gently flushed to remove residual blood, and virus solution (either Ad.nNOS [n=21] or Ad.βGal [n=10], 5×10⁹ pfu/mL, or sham infection with buffer alone [n=5]) was instilled under gentle distending pressure for 20 minutes at 37°C.

**Vessel Harvesting and Analysis**

Experimental VGs were harvested 3 (n=32) or 28 (n=28) days after surgery. Vessel weight and length were measured, then segments were immediately washed in ice-cold Krebs-HEPES buffer (composition in mmol/L: NaCl 99, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1, CaCl₂ 1.9, NaHCO₃ 25, glucose 11.1, and Na-HEPES 20) for superoxide assays or snap-frozen in liquid nitrogen for homogenization and Western blotting. The middle 4-mm segment of each VG was snap-frozen in OCT medium for cryosections after equilibration in PBS/30% sucrose.

**NADPH-Diaphorase and X-Gal Staining**

VG cryosections (6 µm) were fixed and evaluated for β-galactosidase or NOS activity by X-Gal or NADPH-diaphorase staining, respectively, as previously described. The area of vessel stained (µm² per high-power field with 20× objective) was assessed at 6 points around the vessel circumference; image analysis was performed by video capture and Leica Q500 MC Qwin software.

**Western Immunoblotting**

Portions of VG homogenate, equalized for protein content, were separated by SDS-PAGE and transferred to polyvinylidine difluoride membranes. nNOS protein was detected by use of a mouse monoclonal antibody (Transduction Laboratories), as described.

**Determination of Vascular NOS Activity**

NOS activity was determined by the conversion of ['H]arginine to ['H]citrulline, as previously described. Briefly, vessels were incubated in Krebs-Henseleit buffer containing ['H]arginine and 1 µmol/L A23187. After 4 hours, ['H]citrulline was separated from ['H]arginine and quantified by liquid scintillation counting. NOS activity was expressed as pmol citrulline formed · mg vessel wt⁻¹ · h⁻¹.

**Immunohistochemistry and Image Analysis**

Immunohistochemistry was performed with mouse monoclonal antibodies on VG cryosections (6 µm), as described. To investigate indices of inflammation in early VGs, antibodies against vascular cell adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1 (a kind gift of Dr M. Cybulsky, University of Toronto), macrophages (RAM 11, Dako), and polymorphs (CD18, Serotec) were used. Antibodies against smooth muscle α-actin (Sigma) and smoothelin (Chemicon) were used to study vascular SMC phenotype and differentiation. Immune complexes were visualized by avidin-biotin complex formation and Vector Red staining (Vector Laboratories). Intimal/medial thickness (µm) and cell counts (n/µm²) were assessed at 4 to 6 points distributed equally around the circumference of sections in ≥2 sections per vessel. Image analysis was performed as described above.

**Vascular Superoxide Production**

Superoxide production was measured by lucigenin-enhanced chemiluminescence by previously described and validated methods. Briefly, intact VG segments were equilibrated in Krebs-HEPES buffer gassed with 95% O₂ /5% CO₂ for 30 minutes at 37°C. Chemiluminescence was measured in 2 mL Krebs-HEPES buffer at 37°C containing low-concentration lucigenin (5 µmol/L).

**Oxidative Fluorescent Microtopography**

In situ superoxide generation in VG sections was evaluated by use of the oxidative fluorescent dye dihydroethidium (DHE). Cryosections (30 µm) were incubated with DHE, and fluorescence images were obtained with a Bio Rad MRC 1024 scanning confocal microscope, as previously described. In each case, images were captured at identical microscope settings. The specificity of DHE staining for superoxide production was confirmed in preliminary experiments by a striking reduction in signal after preincubation with polyethylene glycol−conjugated superoxide dismutase.

**Statistical Analysis**

Data are expressed as mean±SEM. In all cases, n refers to number of animals. The statistical significance of differences was assessed by Student’s t tests or ANOVA, as appropriate; a value of P<0.05 was considered statistically significant.

**Results**

A total of 60 experimental VGs from 60 animals were studied. All animals survived to the intended time point, and all grafts were patent at the time of harvest.

**Efficient Adenoviral Gene Transfer to Experimental VGs**

Efficiency of adenoviral gene transfer, resulting in either nNOS or β-galactosidase expression, was assessed by NADPH-diaphorase or X-Gal staining, respectively (Table 1, Figure 2). The presence of functional NOS protein was determined by NOS activity assays and Western blotting (Figure 1) and localized by NADPH-diaphorase staining of vessel cryosections. Western blotting revealed recombinant nNOS protein in VGs 3 days after Ad.nNOS gene transfer, but not in control grafts, and NOS activity in these vessels was correspondingly increased. As expected from previous studies using first-generation adenoviral vectors in VGs, no
nNOS expression was evident by 28 days, suggesting that the changes in VGs harvested at this time are due to early effects on vessel remodeling, rather than direct effects of persistently increased NOS activity. Total NADPH-diaphorase staining was markedly increased in 3-day VGs after Ad.nNOS gene transfer compared with Ad.βGal-treated or sham-infected grafts and was localized predominantly to the endothelial surface (Figure 2). Numerous X-Gal–positive cells were observed in the endothelium of Ad.βGal-treated VGs, confirming the efficacy of gene transfer in these control vessels (Table 1).

Reduced Inflammation in Early VGs by nNOS Gene Transfer

To evaluate the acute endothelial activation and inflammation that are characteristic of the injury response in early experimental VGs, we carried out immunohistochemistry for the cellular adhesion molecules VCAM-1 and ICAM-1 and for infiltrating CD18-positive polymorphs and RAM 11–positive macrophages in VGs harvested 3 days after surgery (Figure 3). Ad.nNOS gene transfer significantly reduced both adhesion molecule expression and leukocyte infiltration in 3-day VGs (Figure 4). Early VG inflammation is associated with a 2- to 3-fold increase in vessel mass.17 In keeping with the immunohistochemistry findings, vessel wet weight-to-length ratio was significantly reduced in Ad.nNOS-treated grafts compared with β-galactosidase and sham controls (Table 1). By 28 days, inflammation had subsided in all grafts, with no differences between treatment groups (Table 2).

Reduced Intimal Hyperplasia in Late VGs by nNOS Gene Transfer

To quantify SMC intimal hyperplasia in late VGs, we measured intimal and medial dimensions by image analysis of α-actin–immunostained cryosections from VGs harvested 28 days after surgery. All VGs had developed SMC intimal hyperplasia by 28 days. Ad.nNOS gene transfer greatly reduced VG intimal hyperplasia, however, as assessed by both weight-to-length ratio and quantitative morphometry (Table 2). Intimal thickness was almost halved in grafts treated with Ad.nNOS, with a corresponding reduction in intima-to-media ratio, because medial thickness was not different between groups (Table 2).

Modulation of Vascular SMC Phenotype by nNOS Gene Transfer

To evaluate the influence of NOS-induced VG remodeling on intimal SMC phenotype, we performed immunostaining for smoothelin, a cytoskeletal marker of fully differentiated (contractile) SMCs (Figure 5, Table 2). In sham- and Ad.βGal-treated VGs, the media and some SMCs in the deeper layers of the intima stained for smoothelin, whereas most α-actin–positive intimal SMCs did not stain for smoothelin, indicating a less differentiated (secretory) phenotype. In contrast, Ad.nNOS-treated VGs showed a striking increase in the proportion of smoothelin-positive intimal SMCs (Table 2), suggesting that NOS gene transfer not only reduces the magnitude of intimal hyperplasia in VGs but also results in modulation of intimal SMC phenotype toward a more differentiated phenotype.

### Table 1. Efficiency of Adenoviral Gene Transfer

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<th>Sham</th>
<th>Ad.βGal</th>
<th>Ad.nNOS</th>
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<tbody>
<tr>
<td>NADPH diaphorase</td>
<td>24.4±0.5</td>
<td>26.6±3.5</td>
<td>40.2±1.2*</td>
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<td>staining, μm²/HPF</td>
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<tr>
<td>X-Gal staining</td>
<td>0</td>
<td>38.2±1.3</td>
<td>0</td>
</tr>
<tr>
<td>, μm²/HPF</td>
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<td>Vessel weight:length</td>
<td>6.7±0.5</td>
<td>7.1±1.2</td>
<td>4.9±1.2*</td>
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<tr>
<td>ratio, mg/mm</td>
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HPF indicates high-power field. *P<0.05.

Figure 1. Evaluation of nNOS gene transfer in 3-day VGs. NOS activity was assessed by conversion of [H]arginine to [H]citrulline. Top, Western blots from sham- (left), Ad.βGal- (center), and Ad.nNOS-treated (right) VG homogenates. Left, Positive control for nNOS (rat pituitary lysate; +). *P<0.05 vs sham- and Ad.βGal-treated grafts.
Superoxide Generation From VGs
Because intimal SMCs are a major source of vascular superoxide generation in VGs, we next investigated the effect of reduced intimal hyperplasia and altered SMC phenotype on basal and NADH-stimulated superoxide production in VGs harvested at either 3 or 28 days after surgery (Figure 6). Early VGs had high levels of superoxide production, most likely reflecting the marked inflammatory cell infiltration. Ad.nNOS, however, reduced basal superoxide generation in 3-day VGs by one third, and by two thirds after maximal stimulation with NADH, compared with control VGs (β-galactosidase or sham gene transfer). In late VGs, basal superoxide production was lower in all groups, but Ad.nNOS treatment halved NADH-stimulated superoxide generation.

In situ oxidative fluorescent microtopography, using DHE staining in cryosections from VGs harvested after 28 days, revealed that superoxide production was localized predominantly to the SMCs in the hyperplastic VG intima (Figure 5). There was no difference in the pattern of DHE staining between sham- and Ad.βGal-treated VGs, but the reduction in intimal thickness in Ad.nNOS-treated VGs was associated with a substantial reduction in DHE staining when vessels were visualized by use of the same confocal scanning parameters.

Discussion
We have demonstrated that intraoperative NOS gene transfer markedly reduces endothelial activation and inflammation in early VGs and leads to a striking reduction in SMC intimal hyperplasia in mature VGs. Furthermore, NOS overexpression during the early phase of VG remodeling has important later effects on the biology of the mature VGs, as demonstrated by modulation of intimal SMCs toward a more differentiated phenotype and reduced SMC-related superoxide production.

In addition to emphasizing the importance of NO in regulating VG intimal hyperplasia, our findings now indicate that early modulation of NO in VGs has sustained downstream effects that result in a substantial redirection of VG remodeling toward a phenotype characterized by reduced SMC proliferation, reduced intimal hyperplasia with increased SMC differentiation, and reduced vascular superoxide production. These late changes are not directly dependent on persistently increased NOS activity, because rabbit jugular VG transgene expression from first-generation adenoviral vectors typically persists for ≈14 days; by 28 days, there was no evidence of recombinant NOS activity. These observations suggest that important changes in VG biology may be achieved by targeting early events in the response to vascular injury and that long-term transgene expression or vector persistence may not be necessary to achieve biologically significant effects. In the injured rat carotid artery, early NOS augmentation modulates SMC expression of paxillin, a regulator of SMC differentiation, further supporting the hypothesis that the effects of NO on SMCs after vascular injury result in fundamental and sustained effects on SMC differentiation and phenotype.
Our study also highlights the importance of increased vascular superoxide production as both a marker of vascular injury and a potential mediator of SMC mitogenesis and phenotypic differentiation in vascular injury states.

In experimental VGs and in human bypass conduits, the predominant sources of vascular superoxide are NAD(P)H oxidases. In rabbit jugular VGs, intimal SMCs express NAD(P)H oxidase components and mediate the increase in vascular superoxide production in VGs. We now find that the modulation of VG remodeling by NOS gene transfer reduces both basal and NADH-stimulated vascular superoxide production. In early VGs, this is probably partly a result of a reduction in neutrophil infiltration, but in late VGs we found that superoxide production by intimal SMCs is reduced after NOS gene transfer, in association with the shift in SMC phenotype toward differentiation and quiescence. Downregulation of NAD(P)H oxidase–mediated superoxide production in SMCs may have important implications beyond a mere marker of phenotypic differentiation. Superoxide stimulates SMC mitogenesis in vitro, suggesting a role for NAD(P)H oxidase expression in regulating SMC proliferation in VG intimal hyperplasia and other vascular injury states. Interestingly, angiostatin II regulates expression of the NAD(P)H oxidase in vascular SMCs; previous studies have found a reduction in VG intimal hyperplasia after treatment with angiotensin II receptor antagonists. Thus, modulation of SMC phenotype leading to reduced superoxide production may be an important mechanism underlying the effects of NO in VG remodeling.

Gene transfer to modify VG pathobiology is an attractive strategy because veins are available for direct ex vivo gene delivery intraoperatively, reducing safety concerns over systemic delivery.

The following figures and tables illustrate the findings:

**Figure 4.** Quantification of inflammatory markers in 3- and 28-day VGs. Immunostaining for VCAM-1 and ICAM-1 (top) and leukocyte markers CD18 and RAM-11 (bottom) was quantified in 3- and 28-day VGs by image analysis. *P<0.05 vs sham- and Ad.βGal-treated grafts. †P<0.001 vs sham- and Ad.βGal-treated grafts. Bars show mean±SEM.

**Figure 5.** Immunohistochemical and DHE staining of 28-day VGs. Cryosections of VGs from each treatment group were stained for smooth muscle α-actin (top) or smoothelin (middle, a marker of SMC differentiation). Oxidative fluorescent microtopography using DHE was used to localize in situ superoxide generation in VGs (bottom). Top right, Bar=30 μm.

**Figure 6.** Superoxide generation in 3- and 28-day VGs. Superoxide production was determined from intact vessel rings from sham-, Ad.βGal-, and Ad.nNOS-treated VGs at 3 and 28 days by lucigenin-enhanced chemiluminescence (5 μmol/L lucigenin) (3 days: sham n=5, βGal n=5, nNOS n=6; 28 days: sham n=6, βGal n=5, nNOS n=6). *P<0.05 vs sham- and Ad.βGal-treated grafts. **P<0.005 vs sham- and Ad.βGal-treated grafts. Bars show mean±SEM.

**Table 2.** VG Intimal Hyperplasia

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<th>Sham</th>
<th>Ad.βGal</th>
<th>Ad.nNOS</th>
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<tr>
<td>Vessel wet weight:length, mg/mm</td>
<td>14.4±4.8</td>
<td>12.7±1.4</td>
<td>8.2±0.6*</td>
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<td>Intimal thickness, μm</td>
<td>104±6.9</td>
<td>102±9.4</td>
<td>61.1±2.2*</td>
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<tr>
<td>Medial thickness, μm</td>
<td>35.0±3.4</td>
<td>40.1±2.4</td>
<td>38.4±4.0</td>
</tr>
<tr>
<td>Intima:media ratio</td>
<td>3.3±0.3</td>
<td>2.6±0.2</td>
<td>1.6±0.2*</td>
</tr>
<tr>
<td>Smoothelin staining, %</td>
<td>24±6</td>
<td>28±6</td>
<td>62±3†</td>
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*P<0.05; †P<0.001.
temic vector distribution. The ability to modify VG remodeling by early, transient expression of therapeutic transgenes, without the requirement for long-term gene expression, is also attractive. Indeed, “proof-of-concept” studies targeting SMC cell-cycle progression have demonstrated the feasibility of intraoperative VG gene therapy.26 Strategies aimed specifically at inhibiting SMC proliferation in VGs are effective in reducing intimal hyperplasia, but the pleiotropic actions of NO on endothelium, platelets, and smooth muscle suggest that NOS gene transfer may also be an attractive candidate for gene therapy in VGs.14 Furthermore, NOS gene transfer has significant effects on NO bioactivity in other vascular disease states associated with increased superoxide production and endothelial dysfunction.27,28

We elected to use nNOS for VG gene transfer because previous studies have shown that Ad.nNOS gene transfer increases or restores vascular NOS activity in vivo and can potentially reduce vascular inflammation.16,29 Other investigators have shown that both endothelial and inducible NOS (eNOS and iNOS) are effective in augmenting NO activity in ex vivo vein segments,30–32 suggesting that all of the NOS isoforms are potential candidates for VG gene therapy.14 Like eNOS, nNOS is calcium-calmodulin–regulated, but it has a higher Vmax, whereas iNOS has higher, constitutive activation. Matsumoto et al4 found that liposome-mediated eNOS gene transfer to canine VGs inhibited intimal hyperplasia resulting from reduced-flow conditions but did not assess early inflammation or the effects on VGs under normal flow conditions. Other studies have also shown that iNOS gene transfer is effective at reducing transplant arteriosclerosis,33 a pathology that shares similarities with VG intimal hyperplasia. It is also possible that adventitial NOS delivery, effective in augmenting NO bioactivity in arteries, may have similar effects in VGs.34 NOS activity depends on the availability of cofactors such as tetrahydrobiopterin; demonstration of increased NOS activity by arginine consumption suggests that tetrahydrobiopterin levels are sufficient to support increased NOS activity after gene transfer. Supplementation of tetrahydrobiopterin or other cofactors, however, may be a promising strategy to further augment NO activity.30

In summary, early overexpression of NOS by intraoperative gene transfer favorably affects VG remodeling by inhibiting early inflammatory changes and reducing late intimal hyperplasia. More importantly, intimal SMC differentiation is redirected toward a differentiated phenotype, associated with reduced superoxide production. These observations highlight the important role of NO in modulating both the early response to injury in VGs and the subsequent remodeling process and suggest that NOS gene transfer, or other strategies aimed at augmenting NO bioactivity in VGs, may have effects on long-term VG biology that could limit susceptibility to accelerated atherosclerosis.

Acknowledgments
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References

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