eNOS Gene Transfer Inhibits Smooth Muscle Cell Migration and MMP-2 and MMP-9 Activity

Milind V. Gurjar, Ram V. Sharma, Ramesh C. Bhalla

Abstract—Vascular smooth muscle cell (SMC) migration is a critical step in the development of neointima after angioplasty. Matrix metalloproteinases (MMPs) degrade the basement membrane and the extracellular matrix, facilitating SMC migration. Transfer of the endothelial nitric oxide synthase (eNOS) gene to the injury site inhibits neointima formation. Neither the signaling pathways leading to NO-mediated inhibition of SMC migration and proliferation nor the alterations in these pathways have been characterized. We hypothesize that NO inhibits SMC migration in part by regulating MMP activity. To test this hypothesis, we transfected cultured rat aortic SMCs with replication-deficient adenovirus containing bovine eNOS gene and analyzed the conditioned medium for MMP activity. We observed that eNOS gene transfer significantly \((P<0.05)\) inhibited SMC migration and significantly \((P<0.05)\) decreased MMP-2 and MMP-9 activities in the conditioned medium. Similarly, addition of the NO donor DETA NONOate and 8-bromo-cGMP to the culture medium significantly decreased MMP-2 and MMP-9 activities in the conditioned medium collected 24 hours after treatment. Furthermore, Western blot analysis of the conditioned medium collected from eNOS gene–transfected SMCs showed a significant increase in tissue inhibitor of metalloproteinases-2 (TIMP-2) levels. Our data suggest that NO decreases MMP-2 and MMP-9 activities and increases TIMP-2 secretion, and this shifts the balance of MMP activity, which may favor the inhibition of cell migration because of inhibition of extracellular matrix degradation. (Arterioscler Thromb Vasc Biol. 1999;19:2871-2877.)

Key Words: endothelial nitric oxide synthase ■ gene transfer ■ matrix metalloproteinases ■ tissue inhibitor of metalloproteinases ■ cell migration ■ smooth muscle cells

Atherosclerosis and restenosis after angioplasty are characterized by formation of neointima, which contributes to occlusion of the artery lumen.1,2 Smooth muscle cells (SMCs) from the media proliferate and migrate toward the intima to give rise to the neointima. Migration of SMCs from media to intima critically depends on degradation of extracellular matrix (ECM) proteins by matrix metalloproteinases (MMPs). Therefore, efforts are being directed toward developing therapies to inhibit migration and proliferation of SMCs to prevent restenosis.3 In vivo, net matrix degradation is regulated through a balance between MMPs and their endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs).4 Remodeling of ECM as a result of increased secretion of MMPs has been implicated in many pathological conditions, including vascular proliferative responses in atherosclerosis and restenosis after angioplasty.5 The gelatinases MMP-2 and MMP-9 have been of particular interest in investigating the role of MMPs in basement membrane degradation.6,7 It has been shown that cytokines activate MMP-2 and MMP-9,8 and the concentration of these enzymes is increased after balloon injury.9 MMP inhibitors have been shown to inhibit SMC migration in vitro and neointima formation in vivo.10,11 Taken together, these findings suggest that modulation of MMP activity can be used as a possible therapeutic target to inhibit neointima formation after angioplasty.

The arterial endothelium is damaged in atherosclerosis and after balloon angioplasty. NO derived from endothelial cells has been shown to inhibit SMC migration and proliferation.12,13 NO donors14,15 and transfer of the endothelial nitric oxide synthase (eNOS) gene to SMCs have been shown to inhibit proliferation in vitro16 and neointima formation in vivo.17,18 However, the precise mechanism by which NO mediates its effects on the inhibition of SMC migration and proliferation and neointima formation is not known. Because MMPs play an important role in cell migration and NO inhibits SMC migration, we tested the hypothesis that NO inhibits SMC migration in part by inhibiting MMP-2 and MMP-9 activities. We used replication-deficient adenovirus to transfer the eNOS gene into rat aortic SMCs. We demonstrate that eNOS gene transfer inhibits SMC migration and MMP-2 and MMP-9 activities in SMCs. Treatment of SMCs with DETA NONOate and cGMP also resulted in a decrease in MMP-2 and MMP-9 activities. Also, there was an increase in TIMP-2 secretion after eNOS gene transfer. These results suggest that a decrease in the MMP-2 and MMP-9 activities and an increase in TIMP-2 secretion by NO may play an important role in inhibiting SMC migration by altering the ratio of MMPs to TIMPs.

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2871
Methods

Materials

Chemicals and materials were obtained from the following sources: TRIZol, total RNA isolation reagent, and fibronectin (human plasma), GIBCO BRL; platelet-derived growth factor (PDGF), R&D Systems Inc; DETA NONOate, Cayman Chemicals; Dowex AG50W-X8 (200-mesh), Ready-gels, Bio-Rad; [H]arginine (35 to 70 Ci/mmol) and [3H]UTP (3000 Ci/mmol), Amersham; random-primed DNA labeling kit, Boehringer Mannheim; 10% gelatin zymography precast gels, rebuffering buffer, developing buffer, and Seablu molecular weight marker, NOVEX; Transwell gelation assay dishes, Costar Inc; rabbit anti-eNOS polyclonal antibody, Santa Cruz Biotechnology; rabbit anti–TIMP-2 polyclonal antibody, Chemicon; chemiluminescence detection kit, Transduction laboratories; and goat anti–rabbit IgG horseradish peroxidase (HRP) conjugate, NADPH, FAD, tetrahydrobiopterin, calmodulin, nitro-L-arginine (LNA), 8-bromo-cGMP, and other chemicals and cell culture additives not listed were of the highest grade available from Sigma.

Cell Culture

Rats and guinea pigs used in the study were maintained and used in compliance with the principles set forth in the Guide for the Care and Use of Laboratory Animals and approved by the University of Iowa Animal Care and Use Committee. Aortic SMCs were cultured from Wistar male rats (8 to 10 weeks old; 200 to 300 g body weight). Aorta was removed from rats anesthetized with ether and kept in Ham’s F12 culture medium. Guinea pig coronary artery cells were obtained from 4- to 6-week-old guinea pigs, 600 to 900 g body weight. Hearts were removed from guinea pigs anesthetized with xylazine (1 mg/kg) and ketamine (80 mg/kg IP) and kept in Ham’s F12 culture medium. SMCs were cultured according to procedures established in our laboratory.19,20 The purity of SMCs was confirmed by immunohistochemical localization of smooth muscle–specific actin by use of monoclonal antibody.19,20 Cells were subcultured weekly in DMEM high-glucose medium supplemented with 10% FBS and antibiotics (100 μg/mL streptomycin, 100 U/mL penicillin, and 2.5 μg/mL Fungizone) and were used between the third and seventh passages.

Adenovirus-Mediated Gene Transfer

We used replication-deficient recombinant adenovirus, Ad5/RSVeNOS, containing bovine aortic endothelial cell NOS cDNA24 or a reporter gene (green fluorescent protein, GFP).16 Adenoviral vectors were prepared by The Vector Core, The University of Iowa College of Medicine, as described elsewhere.16,18,22–24 The adenovirus vector has had the sequence in the E1A, E1B, and E3 regions deleted, conferring the ability of the virus construct containing eNOS and GFP to replicate in nonpermissive cells. We have shown earlier that infected SMCs with adenovirus vectors carrying reporter or eNOS gene increased eNOS mRNA in a dose-dependent manner.16,23 In addition, our previous studies demonstrated that 50 to 100 pfu of viral vector per cell (50 to 100 multiplicity of infection, MOI) produces optimal gene expression of both reporter and eNOS genes.16,18,22,23; therefore, in this study we used 50 MOI of virus to transfer the eNOS gene. Confluent SMCs in 100-mm dishes were transfected by incubation with Ad5/RSVeNOS virus at 50 MOI (50 pfu/cell) in 2 mL of serum-free DMEM supplemented with 0.1% BSA and antibiotics for 3 hours. Then, 3 mL of serum-free DMEM/0.1% BSA was added to each dish of cultured cells and incubated for 24 hours. After 24 hours, virus-containing medium was removed, 4 mL of fresh serum-free DMEM/0.1% BSA was added, and cells were incubated for 24 hours. GFP gene–carrying control vector (Ad5/RSVGFPP)-transfected cells were used as control. Expression of eNOS gene was confirmed by Northern and Western blot analysis. eNOS activity was measured by citrulline assay on cell homogenates according to established protocols.16,18 Nitrite levels were measured in conditioned medium with Griess reagent as described before.25 The transfection efficiency of GFP protein was examined under a fluorescent microscope with a fluorescein filter, and eNOS protein was examined by immunohistochemistry followed by confocal microscopy as described earlier.16,18,22

Conditioning of Medium

After transfection, the cells were divided into unstimulated and stimulated groups. Two milliliters of serum-free DMEM/0.1% BSA was added to all cell culture dishes. Interleukin (IL)-1β was added to the stimulated group to make a final concentration of 10 ng/mL. For DETA NONOate and cGMP treatment, cells were serum-starved for 24 hours in 2 mL serum-free DMEM/0.1% BSA. Unstimulated cells were incubated with 2 mL serum-free DMEM/0.1% BSA. Cells were incubated for an additional 24 hours. After 24 hours, conditioned medium was collected, centrifuged to remove cell debris, and stored in aliquots at −70°C for future use.

Migration Assay

The chemotactic migration of eNOS gene– and GFP gene–transfected SMCs was measured with a transwell migration apparatus as described previously.1,12,26 PDGF-BB was diluted (10 ng/mL) in 0.6 mL DMEM/0.1% BSA and added to the lower wells of the chamber. The wells were covered with a PVP-free filter (Costar Inc) with 8-μm pores and coated on both sides with 6 μg/100 mm² fibronectin. Cells were trypsinized (0.01% trypsin/0.11 mmol/L EDTA), washed once in DMEM/0.1% BSA, and resuspended in DMEM/0.1% BSA at a density of ～10⁶ cells/mL. SMCs (75 000 to 100 000 cells in 0.1 mL) were added into the upper wells of the transwell chamber. A group of eNOS–gene–transfected cells were treated with 1 mmol/L of LNA 24 hours before and during the assay. The chambers were incubated for 6 hours at 37°C in an atmosphere of 95% air and 5% CO₂. At the end of incubation, cells were fixed and stained with hematoxylin. Nonmigrated cells on top of the filters were wiped off, filters were mounted, and migrated cells attached to the bottom of filter were counted in 6 random (×400 microscope) high-power fields.26 Cell migration was calculated as difference between the number of migrated cells in the eNOS-treated and control groups.

Northern Blot Analysis

Total RNA was extracted from eNOS gene– and control vector–transfected cultured SMCs as described previously with a commercial guanidinium isothiocyanate reagent (TRIZol, GIBCO BRL).19,27 RNA was quantified by spectrophotometry, and equal amounts of denatured RNA samples were separated by electrophoresis on 1.2% agarose-formaldehyde gels and transferred to Nitran membrane. Prehybridization was done at 42°C with herring sperm DNA. Hybridization was done with cDNA probes: eNOS (2-kbp EcoRI/Hinc II, obtained from Dr Thomas Michel, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA).28 Probes were radiolabeled by use of a random-primed DNA labeling kit. Blots were exposed to Kodak XAR-5 film with an intensifying screen for 24 to 48 hours at −70°C, developed, and photographed.

Zymography

Gelatinase activity in conditioned medium collected from cultures was measured by zymography.29 This procedure has been shown to quantitatively estimate both proenzyme and activated MMP enzyme activity. Equal amounts of conditioned medium (5 μL) were subjected to electrophoresis with Novex 10% zymography gels containing 0.1% gelatin. Gels were washed with renaturing buffer (Novex) for 30 minutes and incubated at 37°C for 20 hours in developing buffer (Novex). After 20 hours, gels were stained with Coomassie blue. All gels were calibrated with Seablu molecular-weight marker (Novex).

Western Blot Analysis

Control and eNOS gene–transfected cells were serum-starved for 48 hours, then treated with IL-1β (final concentration of 10 ng/mL in DMEM/0.1% BSA) and incubated for 24 hours. After 24 hours, conditioned medium was collected, and cells were rinsed with ice-cold PBS and then lysed with 1 mL lysis buffer16,18,22 on a rocking platform for 30 minutes at 4°C. The lysates were centrifuged at 14 000 g for 10 minutes at 4°C. The supernatants were collected and total proteins quantified with BIO-RAD reagents. One hundred microliters of supernatant from each sample was mixed with 2× sample buffer and boiled for 5 minutes for immunoblot analysis. Equal amounts of total protein (20 μg) from each sample were run
cells (Figure 1, A and B). Calcium-dependent NOS activity in eNOS gene–transfected cells was \( \approx 225 \text{ pmol citrulline} \cdot \text{mg}^{-1} \cdot \text{min}^{-1} \), compared with no activity in GFP gene–transfected cells (Figure 1C). The NOS activity of eNOS gene–transfected cells was completely calcium-dependent and was inhibited by 1 mmol/L LNA. eNOS gene transfer increased PDGF-BB–stimulated NO production by 4- to 6-fold over GFP-transfected cells (Figure 1D). We have demonstrated earlier that adenoviral vectors produce >80% gene transfer of both reporter gene and eNOS gene in vascular cells. Similar transfection efficiency was observed in SMCs incubated with 50 MOI of viral constructs in this study (data not shown). These results demonstrate that transfected eNOS gene in rat aortic SMCs is functional and can be used to study the effect of NO on cell migration and regulation of MMP activity.

**Effect of eNOS Gene Transfer on SMC Migration**

PDGF released from restenotic or atherosclerotic lesions is a chemoattractant and proliferative agent for SMCs. It has also been observed that cultured SMCs express predominantly PDGF-BB receptors. Therefore, we used PDGF-BB (10 ng/mL) to test the effect of eNOS gene transfer on SMC migration in a modified Boyden chamber. eNOS gene transfer significantly (\( P<0.05, n=8 \)) inhibited PDGF-BB–induced chemotactic migration of rat aortic SMCs. There was a 30% decrease in migration in eNOS gene–transfected cells compared with GFP gene–transfected cells (Figure 2A). Treatment of eNOS gene–transfected SMCs with 1 mmol/L LNA, a specific inhibitor of eNOS, completely reversed this inhibitory effect, showing that inhibition of SMC migration is due to increased NO production in these cells. As in rat aortic SMCs, eNOS gene transfer significantly inhibited (40% to 50%) migration of guinea pig coronary artery SMCs (Figure 2B). Treatment of cells with control vector carrying the GFP gene in place of the eNOS gene had no effect on SMC migration (Figure 2, A and B), indicating that inhibition of cell migration by eNOS gene transfer is comparable to that produced by 500 \( \mu \text{mol/L DETA NONOate} \) treatment of coronary artery SMCs (Figure 2B). These results would suggest that eNOS gene transfer inhibits SMC migration by increasing local NO production in SMCs.

**Effect of eNOS Gene Transfer on MMP-2 and MMP-9 Activities**

MMP-2 and MMP-9 play an important role in the migration of cells and are increased after balloon injury. Therefore, using gelatin zymography, we examined whether local NO production by eNOS gene transfer to SMCs had any effect on MMP-2 and MMP-9 activities. eNOS gene transfer to SMCs significantly (\( P<0.05, n=8 \)) reduced the activated MMP-2 band compared with GFP gene transfection. There was a 40% decrease in the activated MMP-2 band in the eNOS gene–transfected SMCs compared with control cells transfected with GFP, whereas the pro-MMP-2 band was not altered by eNOS gene transfer (Figure 3A). This was not unexpected, because MMP-2 is constitutively expressed in SMCs and is regulated mainly at the posttranslational level.

**Data Analysis**

Zymograms and Northern and Western blots were scanned, and the relative intensity of bands was determined by densitometry. Statistical analysis was carried out by use of a commercially available program (STATVIEW, Cricket Software Inc), and differences were considered significant at \( P<0.05 \). The results are presented as mean±SEM, and \( n \) in parentheses represents the number of separate experiments.

**Results**

**eNOS Gene Transfer**

Northern and Western analysis demonstrated expression of eNOS mRNA and protein in Ad5/RSVeNOS-transfected SMCs (Figure 1, A and B). Calcium-dependent NOS activity in eNOS gene–transfected cells was \( \approx 225 \text{ pmol citrulline} \cdot \text{mg}^{-1} \cdot \text{min}^{-1} \), compared with no activity in GFP gene–transfected cells (Figure 1C). The NOS activity of eNOS gene–transfected cells was completely calcium-dependent and was inhibited by 1 mmol/L LNA. eNOS gene transfer increased PDGF-BB–stimulated NO production by 4- to 6-fold over GFP-transfected cells (Figure 1D). We have demonstrated earlier that adenoviral vectors produce >80% gene transfer of both reporter gene and eNOS gene in vascular cells. Similar transfection efficiency was observed in SMCs incubated with 50 MOI of viral constructs in this study (data not shown). These results demonstrate that transfected eNOS gene in rat aortic SMCs is functional and can be used to study the effect of NO on cell migration and regulation of MMP activity.

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Because vessel injury results in release of cytokines, which activate MMP-2 and induce MMP-9, we tested whether NO had any effect on MMP-2 and MMP-9 activities in response to IL-1β stimulation. We stimulated SMCs with 10 ng/mL IL-1β for 24 hours after transfection by a modified Boyden chamber assay as described in Methods section. Multiple high-power (×400) fields were counted and data expressed as mean±SEM (n=8). The y axis represents cells migrated vs control (GFP-transfected cells). LNA (1 mmol/L) was added to a group of eNOS gene–transfected cells 24 hours before and during cell migration to demonstrate that the inhibition of cell migration by eNOS gene transfer was due to NO production. eNOS gene transfer inhibited SMC migration compared with GFP-transfected cells in rat aortic SMCs (A). Inhibition of migration due to eNOS gene transfer was reversed by addition of LNA to the transfected cells. Similarly, transfer of eNOS gene and treatment of cells with 500 μmol/L DETA NONOate inhibited cell migration in guinea pig coronary SMCs vs control cells (B). *Significant (P<0.05) decrease in cell migration by eNOS gene transfer was reversed by addition of LNA to the transfected cells. Similarly, transfer of eNOS gene and treatment of cells with 500 μmol/L DETA NONOate inhibited cell migration in guinea pig coronary SMCs vs control cells (B). *Significant (P<0.05) decrease in cell migration due to eNOS gene transfer compared with those transfected with GFP (Figure 3B).

Effect of DETA NONOate and cGMP on MMP-2 and MMP-9 Activities
To confirm that eNOS gene transfer–mediated effects on MMP activity is due to the NO/cGMP pathway, we tested the effect of the NO donor DETA NONOate (half-life 18 hours at 37°C) and 8-bromo-cGMP on MMP-2 and MMP-9 activities. Conditioned medium collected from cells treated for 24 hours with 500 μmol/L DETA NONOate and 100 μmol/L 8-bromo-cGMP showed a decrease in activated MMP-2 by 45% to 50% in unstimulated and IL-1β–stimulated groups but had no effect on pro-MMP-2 bands (Figure 4, A, B, and C). In addition, treatment of SMCs with DETA NONOate and 8-bromo-cGMP decreased MMP-9 activity by 80% to 90% in the IL-1β–stimulated group (Figure 4D). These results confirm our eNOS gene transfer experiments and demonstrate that NO/cGMP pathway activation leads to a decrease in activated MMP-2 and induction of MMP-9 by inflammatory cytokines.

Effect of eNOS Gene Transfer on TIMP-2 Expression
MMP activity is modulated in vivo by TIMPs. Overexpression of TIMPs inhibits SMC migration in vitro and neointima formation in vivo. Also, TIMP-2 has been shown to inhibit MMP-2 and MMP-9 activities and is involved in regulating MMP-2 activation. Therefore, using Western blot analysis...
of the conditioned medium, we investigated whether eNOS gene transfection altered TIMP-2 secretion. Densitometric analysis of Western blots (Figure 5A, representative blot) showed a significant increase (\(P\), 0.05, \(n\) = 8) in the levels of TIMP-2 in the conditioned medium collected from eNOS gene–transfected cells compared with control (Figure 5, A and B). eNOS gene transfer increased TIMP-2 secretion by almost 100% compared with GFP gene–transfected cells (Figure 5B). A significant difference (\(P\), 0.05, \(n\) = 6) was also observed in TIMP-2 levels in conditioned medium of eNOS and GFP gene–transfected cells in response to IL-1\(\beta\) stimulation (Figure 5, A and B).

### Discussion

Migration of vascular SMCs and remodeling of the ECM are important events in the development of restenosis after balloon angioplasty. \(^{35}\) Under physiological conditions, endothelial cells and SMCs exhibit a very low turnover rate. NO secreted by endothelial cells has been shown to inhibit SMC migration and proliferation and inhibit synthesis and secretion of ECM proteins.\(^{12,13}\) Vascular injury leads to loss of endothelium and stimulation of SMCs, which initially go through a proliferative phase, followed by a secretory phase, to form the neointima.\(^{36}\) Therefore, in this study we examined whether eNOS gene transfer into SMCs can decrease their migration by local NO production.

Transwell migration assay showed an inhibition of SMC migration in eNOS gene–transfected cells compared with the control group. Moreover, eNOS gene transfer produced inhibition of SMC migration comparable to that produced by 500 \(\mu\)mol/L DETA NONOate in coronary artery SMCs. Our results are in agreement with previously published results showing NO-mediated inhibition of SMC migration in vitro\(^{14,26}\) and in vivo.\(^{17,18}\) LNA, a specific inhibitor of eNOS, completely reversed this inhibitory effect, confirming the specificity of NO donor–mediated effects on SMC function. Our results demonstrate that transfection of eNOS gene into SMCs can effectively inhibit their migration in response to chemotactic agents like PDGF and provide a unique model system to investigate cellular and molecular mechanisms of NO-mediated inhibition of SMC migration.

MMPs are enzymes that are involved in breakdown of ECM and hence play a critical role in SMC proliferation and migration and remodeling of ECM.\(^{4,5}\) Of special interest are MMP-2 and MMP-9, whose activities are increased at the site of vascular injury.\(^{9}\) Inhibition of MMP-2 and MMP-9 by overexpression of TIMPs by gene transfer\(^{10}\) and synthetic peptide inhibitor\(^{11}\) has been shown to inhibit SMC migration in vitro and neointima formation in vivo. MMPs are secreted as zymogens and require activation after being secreted. Therefore, regulation of MMP activation provides an important mechanism in regulating ECM turnover. MMP-2 is secreted in its zymogen form, progelatinase A, and then activated to its active form by membrane-type MMPs.\(^{33,34}\) TIMP-2 has been shown to inhibit the activation of progelatinase A.\(^{37}\) Therefore, we examined whether eNOS gene transfer inhibits SMC migration by inhibiting MMP-2 and MMP-9 activities or by increasing TIMP-2 secretion.
and IL-1α showed a significant increase in TIMP-2 levels in both unstimulated (a)* and IL-1β-stimulated (b)* groups compared with conditioned medium collected from cells transfected with the control vector carrying genes. *Significant (P<0.05) increase in TIMP-2 level in eNOS gene–transfected cells vs GFP-transfected cells.

Figure 5. Effect of eNOS gene transfer on TIMP-2 secretion. SMCs were transfected with eNOS gene or control vector, and conditioned medium was collected from control and IL-1β cells as described in Figure 3. Western blot analysis of the conditioned medium was performed, and A shows a representative blot. Data are presented as mean±SEM (n=8). Conditioned medium collected from eNOS gene–transfected SMCs showed a significant increase in TIMP-2 levels in both unstimulated (a)* and IL-1β-stimulated (b)* groups compared with conditioned medium collected from cells transfected with the control vector carrying genes. *Significant (P<0.05) increase in TIMP-2 level in eNOS gene–transfected cells vs GFP-transfected cells.

Analysis of MMP activity by zymography demonstrated that SMCs expressing the eNOS gene showed a decrease in activated MMP-2 activity under basal and IL-1β-stimulated conditions. Also, pro-MMP-9 activity was decreased in conditioned medium collected from eNOS gene–transfected cells stimulated with IL-1β. Inhibition of MMP-2 activation and decreased pro-MMP-9 activity were also observed in cells treated with 500 μmol/L DETA NONOate and 100 μmol/L 8-bromo-cGMP, demonstrating that eNOS gene transfer–mediated inhibition of MMP activity is due to NO and may involve the NO/cGMP pathway. Our results also show that inhibition of MMP-2 and MMP-9 by NO was associated with an increase in TIMP-2 secretion under basal and IL-1β-stimulated conditions. Decreased MMP-2 and MMP-9 activities and increased TIMP-2 levels in turn may contribute to the decreased cell migration observed in this study. In support of this contention, recent studies have demonstrated that in response to vascular injury, levels of MMP-2 protein and its activation are significantly increased, whereas TIMP-2 levels are significantly decreased, in balloon-injured rat carotid arteries. In addition, it has recently been shown that levels of the cardiovascular system–specific MMP inhibitor TIMP-4 are increased 2 weeks after vascular injury, which correlates temporally with cessation of SMC migration and onset of collagen deposition, suggesting an important role for MMPs in promoting SMC migration in response to vascular injury. All these studies taken together would indicate that MMPs are important for SMC migration in response to injury and atherosclerosis, and our in vitro observations may provide a biochemical explanation for the decrease in neointima formation in eNOS gene–transfected rat carotid artery.17,18

The role of NO in regulating MMP synthesis and secretion in vascular SMCs has not been investigated. Previously, Trachtman et al43 showed that iNOS induction by cytokines increased MMP activity in rat mesangial cells. Also, addition of the NO donor SNAP to the culture medium increased MMP-2 activity in a dose-dependent manner. In contrast, we observed that eNOS gene transfer or treatment of cells with DETA NONOate inhibited MMP activity in control as well as IL-1β-stimulated cells. These differences could be due to either different NO donors or the different cell types used in the 2 studies. Although mechanisms for eNOS gene transfer–mediated inhibition of MMP-9 secretion in IL-1β-stimulated SMCs are not clear at present, it is possible that increased production of NO by eNOS gene transfer may inhibit activation of the NF-κB signaling pathway. In support of this contention, a recent study demonstrated that NO donors inhibit iNOS induction in response to IL-1β stimulation in rat aortic SMCs by inhibiting NF-κB activation.41 NO could also directly regulate the enzymes involved in MMP activation or MMPs themselves. Direct modulation of MMP activity by NO donors via MMP peroxynitrite formation and nitrosylation has been demonstrated.42-43 Rajgopalan et al42 showed an increase in MMP-2 activity by peroxynitrite when cells were incubated with conditioned medium collected from rat pulmonary fibroblast. However, neither study demonstrated a direct effect of NO donors on MMP activity or synthesis/secretion in cell culture.

Our study demonstrates an inhibitory effect of eNOS gene transfer, NO donor DETA NONOate, and dibutyryl-cGMP on MMP and TIMP synthesis/secretion in vascular SMCs in culture. Although we cannot rule out peroxynitrite formation in SMCs treated with IL-1β or NO donors, it is unlikely that the effects of eNOS gene transfer on MMP synthesis/secretion seen in SMCs are due to peroxynitrite formation and direct modulation of MMP activity. First, eNOS gene transfer increases NO production to a limited extent only when cells are stimulated with hormones or growth factors (Figure 1D), and the levels of NO secreted into conditioned medium are 50 to 100 times lower than with iNOS induction by IL-1β. Second, dibutyryl cGMP also inhibits MMP-2 activation and MMP-9 secretion, suggesting that the effects of eNOS gene transfer are mediated by the NO/cGMP signaling pathway.

In summary, we have demonstrated that eNOS gene expression and NO donors inhibited SMC migration and decreased MMP-2 activation and MMP-9 secretion. These observations suggest that a decrease in MMP activity by NO may play an important role in inhibiting cell migration. Inhibition of MMP-2 activation and/or net activity and increased secretion of TIMP-2 might favor decreased proteolysis. These findings provide a possible mechanistic explanation for endothelial cell–mediated inhibition of SMC migration and proliferation. Although our results indicate that NO decreases MMP-9 synthesis/secretion while increasing TIMP-2 secretion, it would be interesting to investigate the effect of eNOS gene transfer on the transcriptional regulation of MMP-2, MMP-9, and TIMP-2 to demon-
strate that eNOS gene transfer decreases MMP gene transcription while increasing TIMP-2 gene expression.

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