

eNOS-Overexpressing Endothelial Cells Inhibit Platelet Aggregation and Smooth Muscle Cell Proliferation *in Vitro*

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ABSTRACT

Endothelial cell seeding of synthetic small diameter vascular grafts (SSDVG) has been shown to diminish thrombosis and intimal hyperplasia, resulting in improved graft patency. However, endothelial cell retention on seeded grafts when exposed to physiological shearing conditions remains poor. We report that the genetic engineering of endothelial cells to overexpress endothelial nitric oxide synthase (eNOS), may create improved anti-thrombotic and anti-hyperplastic endothelial cell phenotypes for SSDVG seeding. eNOS-overexpressing endothelial cells may potentially overcome the biochemical loss due to shear induced reduction in endothelial cell coverage on SSDVG. Bovine aortic endothelial cells (BAEC) were transfected with the human eNOS gene, and co-incubated with either human whole blood or bovine aortic smooth muscle cells (BASMC) *in vitro*. eNOS-transfected BAEC significantly overexpressed eNOS compared to control β -Gal-transfected and untransfected BAEC up to 120 h post transfection. In co-incubation and co-culture assays, human platelet aggregation decreased by 46% and BASMC proliferation decreased by 67.2% when compared to incubation with untransfected BAEC.

INTRODUCTION

THE DEVELOPMENT OF A SYNTHETIC small-diameter vascular graft (SSDVG) for coronary and peripheral bypass has been hindered by a number of biochemical processes inherent in the damaged or diseased small diameter vessel. Presently, blockages in the coronary vessels are corrected by autologous vein or artery grafts.¹ Although these natural replacements exhibit short- and mid-term success, they are not satisfactory permanent replacements and often require re-operation.²

SSDVG have failed in achieving widespread clinical use. In the design of SSDVG, synthetic biomateri-

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als have addressed the structural requirements of natural vessels but have not met the biochemical requirements. Failure occurs due to thrombosis in the acute phase and anastomotic hyperplasia in the chronic phase³ due to the proliferation of medial smooth muscle cells into the lumen of the graft or pannus ingrowth at the anastomoses. As the processes that cause hyperplastic response peak at 96 h post-implant,⁴ interruption of these processes may be sufficient to ensure vascular graft patency.

Because the endothelium is the natural modulator of homeostasis in the blood vessel,⁵ attempts at seeding a confluent luminal monolayer have been pursued.⁶⁻⁹ The natural ability of endothelial monolayers to counteract thrombosis and smooth muscle cell (SMC) proliferation after injury and thus increase graft patency has been established in animal⁹ and human¹⁰⁻¹² models. Although confluent monolayers can be grown on graft materials *in vitro*, much of this layer is lost when exposed to shearing stresses associated with normal blood flow.¹³ Subconfluent endothelial cell monolayers exhibit a pro-proliferative influence on smooth muscle cells.¹⁴⁻¹⁶ Biomaterial scaffold surface modifications aimed at preventing the loss of endothelial cells due to shear have not been successful.¹⁷⁻¹⁹ The design of a patent vascular graft utilizing endothelial cell seeding must take into account the loss of endothelium due to exposure to shear and the resultant subconfluent endothelial layer.

We hypothesize that nitric oxide (NO) over production by endothelial cells may overcome the pro-proliferative state produced when a part of the endothelial cell layer is inevitably lost due to shear. Nitric oxide is produced at basal levels in endothelial cells by endothelial nitric oxide synthase (eNOS)²⁰⁻²² at 50 nM per hour.²³ NO is a potent vasodilator, inhibitor of platelet²⁴ and leukocyte²⁵ adhesion, and inhibitor of SMC proliferation²⁶ and is largely responsible for vessel homeostasis. eNOS transfection of vessel wall after balloon injury has been reported to cause a 70% reduction of intimal hyperplasia 14 days after injury.²⁷

In this study, we describe human eNOS gene transfection of bovine aortic endothelial cells (BAEC) such that these engineered endothelial cells may be effective in preventing thrombosis and intimal hyperplasia even in subconfluent cultures.

MATERIALS AND METHODS

Experiments were designed to (1) characterize eNOS transfection and overexpression in BAEC, and (2) elucidate the effect the exposure of eNOS-overexpressing BAEC on human platelet (HP) aggregation and bovine aortic smooth muscle cell (BASMC) proliferation. We have concentrated most of our experimentation during the period of time between 72 and 120 h, as it is most significant to the vascular graft application.^{4,28} The time line of experiments conducted is shown in Fig. 1. Significance was established using the two-tailed Student *t*-test assuming equal variance with a *p* value of less than 0.05 considered statistically significant.

Plasmid DNA

The eNOS gene (4077 bp), driven by the SV40 promoter and subcloned in a pSV-SPORT 1 plasmid (3160 bp, GIBCO BRL) was a generous gift from Dr. Kenneth D. Bloch (Massachusetts General Hospital). A β -Gal control vector (6821 bp, Promega) containing an SV40 promoter was used as a transfection control.

Cell culture

Both BAEC and BASMC were cultured in DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1 μ g/mL penicillin-streptomycin ("culture medium," GIBCO BRL) at 37°C with 5% CO₂ and 95% humidity. Human whole blood was freshly drawn from donors who had not taken any drug within the past 24 h. Blood was drawn with an 18-gauge needle so as to cause minimal platelet damage and 3.8% sodium citrate was added as an anti-coagulant.

BAEC transfection

BAEC were transfected with either pSV-SPORT-NOS (pSV-NOS, 7237 bp) DNA or β -Gal control vector DNA using Lipofectamine[®] (GIBCO BRL). Briefly, BAEC were isolated as previously described²⁹ and

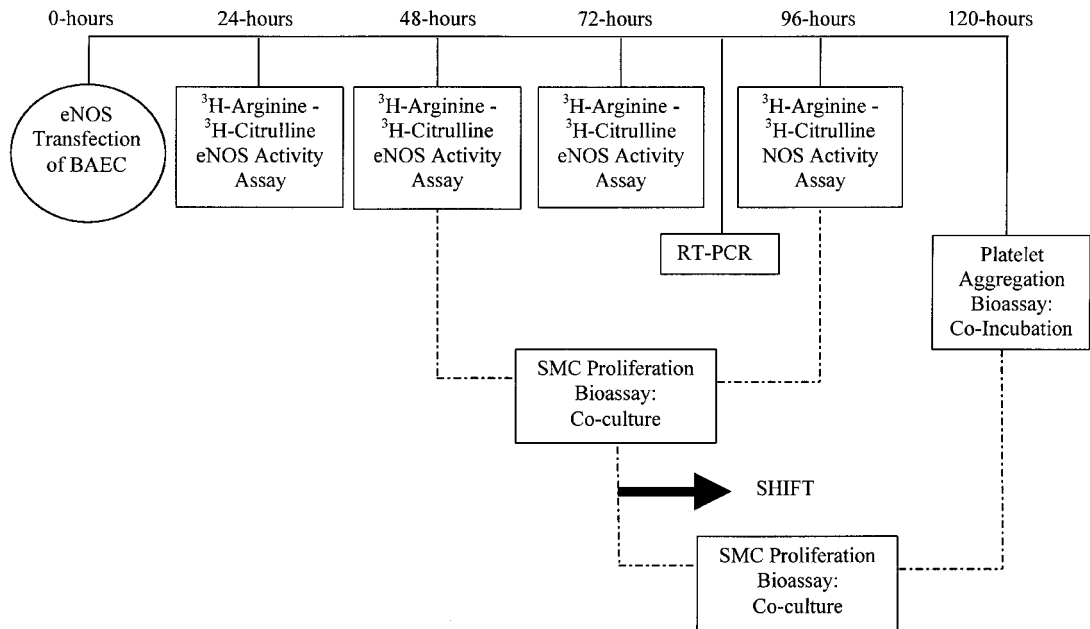


FIG. 1. Time line of various experiments performed. Arginine conversion assays were performed at 24, 48, 72, and 96 h post-transfection. The smooth muscle cell proliferation bioassay proceeded between 48 and 96 h and 72 and 120 h post-transfection (2 days). RT-PCR was performed on BAEC 84 h post-transfection. Platelet aggregation inhibition bioassay was conducted 120 h post-transfection.

used between passages six and twelve, long after endogenous eNOS expression is known to have ceased. BAEC were seeded into six-well plates (Costar) at 60,000 cells/well and were allowed to attach and grow to 60–80% confluence. Transfection was optimized to the use of 16 μ g of Lipofectamine[®] reagent to 1.5 μ g of plasmid DNA (CsCl₂ purified preparation). Transfections proceeded by standard techniques.

[³H]Arginine to [³H]citrulline conversion assay

The basal level eNOS enzyme expression in transfected and untransfected cells was quantified using a protocol modified from Bredt and Snyder.³⁰ Transfected BAEC were removed from their six-well plates by treatment with nonenzymatic dissociation medium (Sigma) for 7 min. The cells were counted. BAEC were lysed in a buffer solution containing 1% Triton X-100 (Sigma), 130 mmol/L NaCl, and 10 mmol/L sodium phosphate, in Tris-HCl (pH 7.5). Twenty-five microliters of the cell lysate was added to 100 μ L of a reaction mixture containing 50 mmol/L HEPES (GIBCO BRL), 1 mmol/L NADPH (Sigma), 1 mmol/L EDTA (Sigma), 1.25 mmol/L CaCl₂, 1 mmol/L dithiothreitol (Sigma), 10 μ g calmodulin (Sigma), and 3 μ L (9.25 MBq) of [L-2,3-³H]arginine (Dupont NEN). The mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 2 mL of a mixture of 20 mmol/L HEPES and 2 mmol/L EDTA. The resultant mixture was then passed over a Dowex AG W50-X8 resin (Na⁺ form, BioRad) which binds unreacted [³H]arginine and allows [³H]citrulline to pass. [³H]Citrulline was quantified by scintillation count (LKB 1209, Wallac Inc.) and normalized to 100,000 BAEC per well.

Reverse transcriptase-polymerase chain reaction to assay for eNOS mRNA

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay was conducted for quantitative analysis of eNOS mRNA in transfected BAEC. A 574-bp fragment of nonhomologous DNA (Clontech) was amplified with composite primers containing sequences complementary to the target DNA (5'-GCT-GGG-CCC-CCT-GCA-CTA-TGG-AGC-GCA-AGT-GAA-ATC-TCC-TCC-G-3') and the nonhomologous DNA (5'-CGC-AGC-GTG-AGC-CCG-AAA-ATG-TCT-TTG-AGT-CCA-TGG-GGA-GCT-TT-3'). An aliquot

of the above PCR-amplified DNA was amplified using target-specific primers (5'-GCT-GGG-CCC-CCT-GCA-CTA-TGG-AG-3' and 5'-CGC-AGC-GTG-AGC-CCG-AAA-ATG-TCT-3') and the product thus generated was quantified. Total RNA from eNOS-transfected BAEC was extracted at the desired time point and the first-strand cDNA was synthesized using the PCR Script system (GIBCO BRL). A fixed amount of the cDNA was mixed with known amounts of the competitive DNA and subjected to PCR amplification. The PCR products were separated electrophoretically and the intensities of bands were determined. With the amount of nonhomologous DNA in a given reaction known, the amount of target template, the mRNA levels were determined.

Platelet aggregation bioassay

The effect of β -Gal and pSV-NOS-transfected BAEC on the aggregation of human whole blood was probed using a modified whole-blood aggregometry assay.

Transfected BAEC were seeded onto the bottom of cylindrical cuvettes (Chrono-Log Corp) coated with Vitrogen (Collagen Corp) 4 days post-transfection. After overnight attachment, the culture medium was removed and replaced with 450 μ L of saline and 450 μ L of human whole blood. A mesh was placed over, but not contacting, the BAEC to allow for stirring of the cuvette contents at 1000 rpm without detaching the adherent BAEC. The HP within whole blood were activated with 1 μ g collagen (Type I, Chrono-Log Corp) and platelet aggregation was quantified by monitoring change in impedance over 10 min (Chrono-Log Corp). Impedance was normalized to 100,000 BAEC to factor out differences in cell densities.

To verify that the effects of eNOS-transfected BAEC on HP aggregation were mediated by NO, BAEC were tested at 3 days post-transfection. The culture medium on BAEC was removed and replaced with 1 mL of culture medium with and without 1mmol/L N^G -nitro-L-arginine methyl ester (L-NAME, Calbiochem) for 10 min. Four-hundred fifty microliters of this medium was combined with 450 μ L human whole blood and added to cylindrical cuvettes (Chrono-Log Corp). The HP within the blood-culture medium mixture were activated with 1 μ g collagen (Type I, Chrono-Log Corp) and platelet aggregation with and without L-NAME was quantified by monitoring change in impedance over 10 min (Chrono-Log Corp).

SMC proliferation bioassay

The effect of β -Gal and pSV-NOS-transfected BAEC on BASMC proliferation was determined by [3 H]thymidine uptake. BASMC were exposed to transfected and untransfected BAEC from 48 to 96 h post-transfection. At least 42 h before transfection, BASMC were plated at a density of 24,000 cells/insert in 0.4-micron polycarbonate inserts (Millipore) coated with Vitrogen (Collagen Corp). The BASMC were allowed to adhere for 18 h in the presence of culture medium. The culture medium was then replaced with serum-free OptiMEM (GIBCO BRL) for 24 h to allow for cell quiescence. At 48 h post-transfection, the cells were introduced to co-culture with the transfected BAEC. At 72 h post-transfection, 1 μ Ci (9.25 MBq, Dupont NEN) [*methyl*- 3 H]thymidine was added to each well. At 96 h post-transfection, the BASMC were washed twice with phosphate-buffered saline (PBS) to remove unincorporated [3 H]thymidine, fixed in 100% methanol, and washed twice with 10% trichloroacetic acid (LabChem, Inc.). The plates were allowed to dry and 200 μ L of 0.2 N NaOH was added. The BASMC were incubated at 37°C for 30 min to solubilize the cells. Of the resulting solution, 20 μ L was added to 4 mL of scintillation fluid and proliferation was assayed by scintillation count (LKB 1209, Wallac Inc.). In addition to the BAEC-BASMC co-incubation during the 48–96 h time period, BASMC proliferation was assayed during co-incubation, 72–120 h post transfection (referred to as the “shift” assay).

RESULTS

Overexpression of eNOS in transfected BAEC

Changes in the basal level of eNOS expression were determined by [3 H]L-arginine to [3 H]L-citrulline conversion assay. The pSV-NOS-transfected BAEC were probed at 24, 48, 72, and 96 hr post-transfection. No increase in basal expression level was detected at the 24-h time point. After 48 h, an increase of eNOS

enzyme activity, 635% over control untransfected ($p < 0.001$) BAEC and 558% over β -Gal-transfected BAEC ($p < 0.05$) was detected. At 72 h, there was a 230% increase over basal levels ($p < 0.001$) and 210% increase over β -Gal-transfected BAEC ($p < 0.001$). By 96 h, there was a 133% increase over basal levels ($p < 0.001$) and 116% over β -Gal-transfected BAEC ($p < 0.001$), as shown in Fig. 2. At 48 h post-transfection, β -Gal-transfected BAEC also showed a significant increase in eNOS expression as compared to untransfected control BAEC that subsided by 72 h.

Quantification of eNOS mRNA in transfected BAEC

Increased expression of eNOS messenger was detected by RT-PCR in pSV-NOS-transfected BAEC and compared to expression in the β -Gal-transfected or untransfected control BAEC (Fig. 3). Messenger RNA concentrations were found to be at least 1:48 comparing between the controls (both β -Gal-transfected and untransfected BAEC) and the pSV-NOS-transfected BAEC at 84 h post-transfection.

Inhibition of platelet aggregation

The ability of eNOS-transfected BAEC to inhibit the aggregation of human platelets was assayed by modified whole blood aggregometry as described earlier. The pSV-NOS-transfected BAEC showed a significant inhibition of HP aggregation by 46% ($p < 0.01$) compared to both untransfected and β -Gal-transfected BAEC as shown in Fig. 4. The β -Gal-transfected controls did not inhibit platelet aggregation beyond that observed in the untransfected BAEC.

This inhibition of platelet aggregation was abolished using treatment with L-NAME. pSV-NOS-transfected BAEC inhibited platelet aggregation by 49% ($p < 0.01$), while these same pSV-NOS-transfected BAEC, when treated with L-NAME, caused no decrease in aggregation (Fig. 5).

Inhibition of BASMC proliferation

The effect of transfected BAEC on BASMC was determined. When BASMC were exposed to transfected and nontransfected BAEC from 48 to 96 h post-transfection, pSV-NOS-transfected BAEC inhibited BASMC

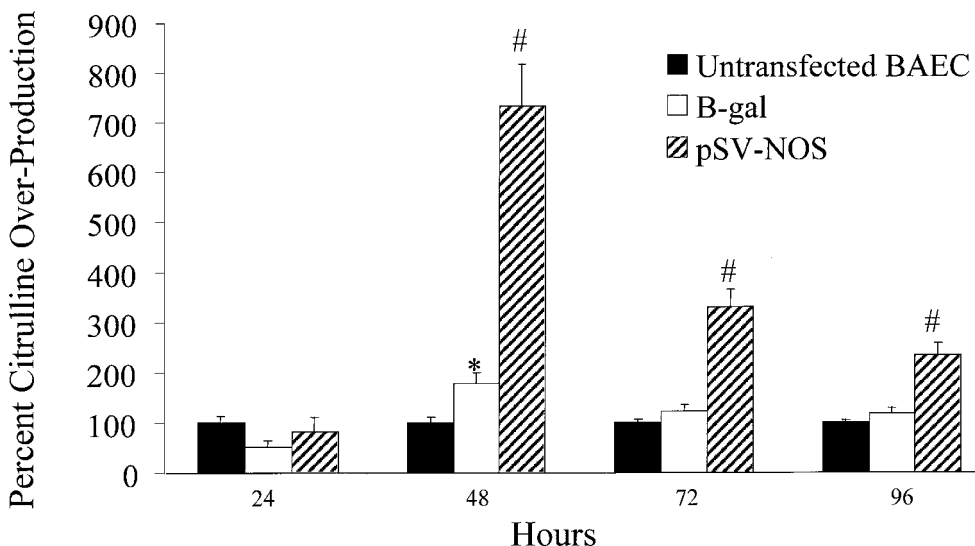


FIG. 2. [L-2,3-³H]Arginine to [L-2, 3-³H]citrulline conversion assay. The conversion of [³H]arginine to [³H]citrulline was measured at 24, 48, 72, and 96 h post-transfection. Data were normalized to, and are shown as, percent of non-transfected BAEC NO production. Percent increase NO production is graphed and shown as mean + SEM. $n = 12$ for each set at each time point. * $p < 0.05$ with respect to untransfected control BAEC; # $p < 0.05$ with respect to untransfected and β -Gal-transfected control BAEC.

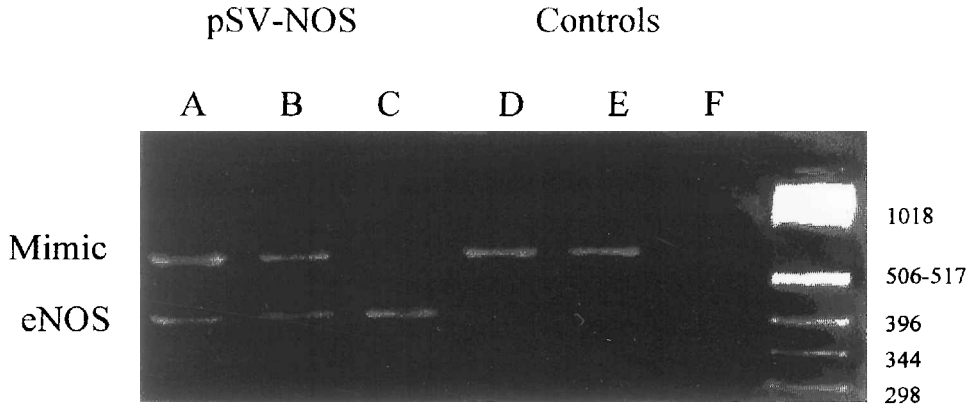


FIG. 3. Gel representation of RT-PCR results. BAEC at 84 h post-transfection were probed for the presence of eNOS mRNA. Lanes A, B, and C and similarly lanes D, E, and F denote decreasing concentrations of mimic cDNA (100 am/ μ L, 10 am/ μ L, and 1 am/ μ L), such that an intensity comparison between the standard and β -Gal-transfected and untransfected control cDNA can be made. Mimic cDNA was added to the PCR reaction in known concentrations as a standard. In this way, a direct comparison may be made regarding the concentration of eNOS mRNA initially present in untransfected and β -Gal-transfected cells.

proliferation by 82% with respect to untransfected BAEC ($p < 0.01$; Fig. 6). β -Gal transfected BAEC caused a 63% decrease in proliferation of BASMC compared to untransfected control BAEC ($p < 0.05$).

When the co-culture period was shifted to the 72- to 120-h time period, β -Gal-transfected BAEC inhibited BASMC proliferation by 7.4% when compared with untransfected controls (not statistically significant). pSV-NOS-transfected BAEC showed 67.8% inhibition of BASMC ($p < 0.05$, with respect to untransfected and β -Gal-transfected BAEC).

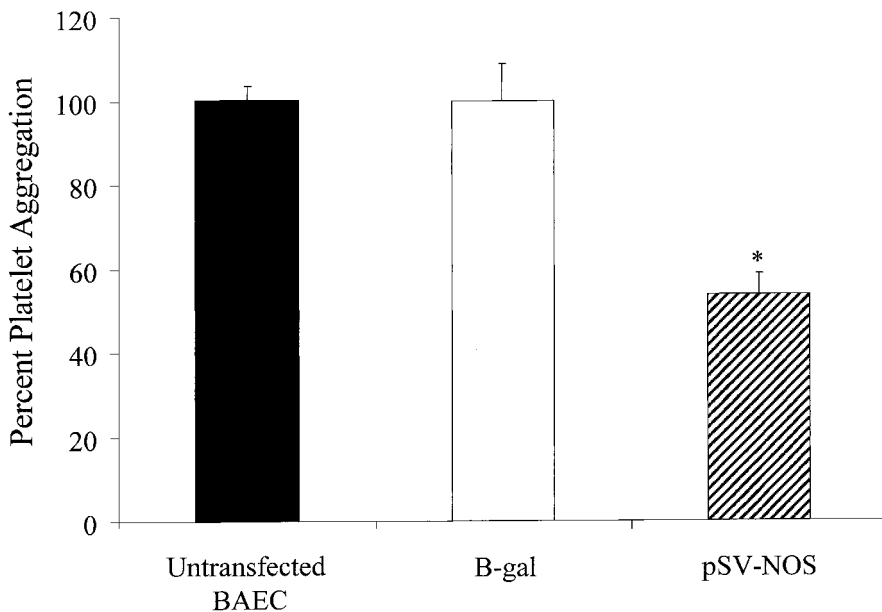


FIG. 4. Human platelet aggregation assay. BAEC at 120 h post-transfection were exposed to activated whole human blood. Percent aggregation with respect to untransfected BAEC is shown as mean + SEM. $n = 6$ for each set. # $p < 0.05$ with respect to both untransfected and β -Gal-transfected control BAEC.

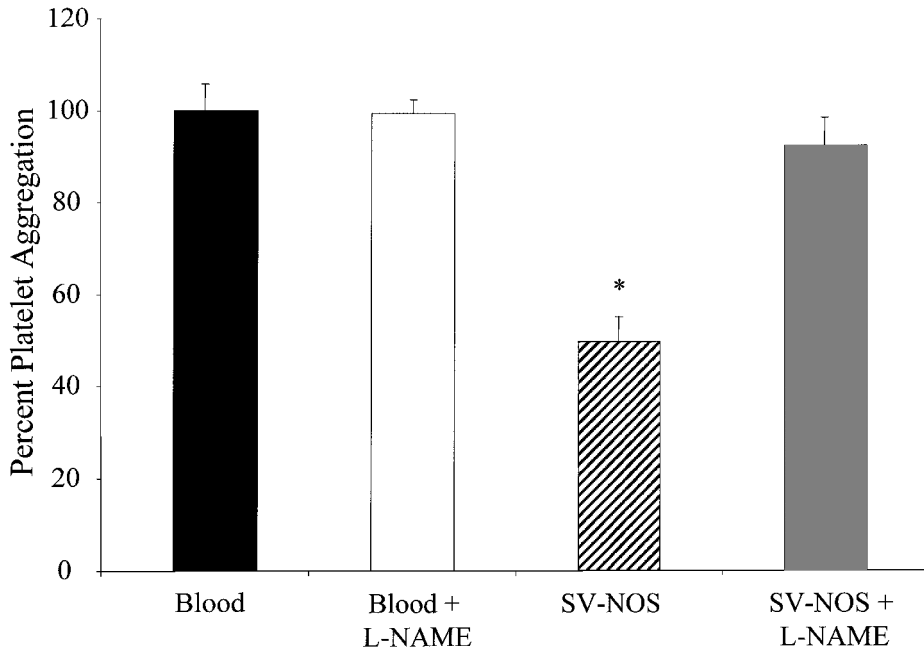


FIG. 5. Human platelet aggregation assay with L-NAME. BAEC at 72 h post-transfection were exposed to culture medium with and without L-NAME. The resultant medium was exposed to activated human whole blood. Percent aggregation with respect to untransfected BAEC is graphed as mean + SEM. $n = 3$; $\#p < 0.05$ with respect to whole blood, whole blood with L-NAME, and culture medium from BAEC treated with L-NAME.

DISCUSSION

In the vascular graft application, the inhibition of acute thrombosis and chronic intimal hyperplasia is critical to the success of the implant. The milieu associated with these responses, including early release of basic fibroblast growth factor and platelet-derived growth factor, peaks at 96 h post implant.⁴ Thus, our design was aimed at examining the effect of eNOS transfection at this critical time point. Further, our hypothesis is that transient overexpression of eNOS is sufficient to inhibit the majority of platelet aggregation and subsequent SMC proliferation.

The endothelium is a key modulator of hemostatic and homeostatic function within the blood vessel.⁵ The impressive arsenal of tools possessed by endothelial cells including thrombomodulin,³¹ anti-thrombin III,³² prostacyclin,³³ and NO,³⁴ maintains the surface properties of the intima.

The ACCORD study²⁸ suggested that the administration of NO precursors for 2 days to patients after coronary balloon angioplasty resulted in a significant increase in luminal diameter that was maintained 6 months post-injury. However, this study also showed that end points such as incidence of myocardial infarction and death were not significantly reduced in the long term. The authors attributed this long-term effect to the fact that there was no difference in luminal loss.²⁸ The implication here is clear. Although NO was sufficient in alleviating the immediate effects of intimal denudation, this short-term gain was lost due to the fact that the intima was denuded. This is analogous to the vascular graft application in which the endothelium is missing from the intima.

Therefore, we believe that the use of other cells, which may be more abundant than endothelial cells such as mesothelial cells, fibroblasts, or smooth muscle cells, may be sufficient to increase patency in the short term but will not allow for the beneficial effects of the other endothelial factors. Simple administration of NO donors will similarly be insufficient in the long term.

Methods of cationic liposomal transduction may produce more stable expression. We acknowledge that retroviruses will produce more long-term expression than will liposomes. However, retroviral infection may

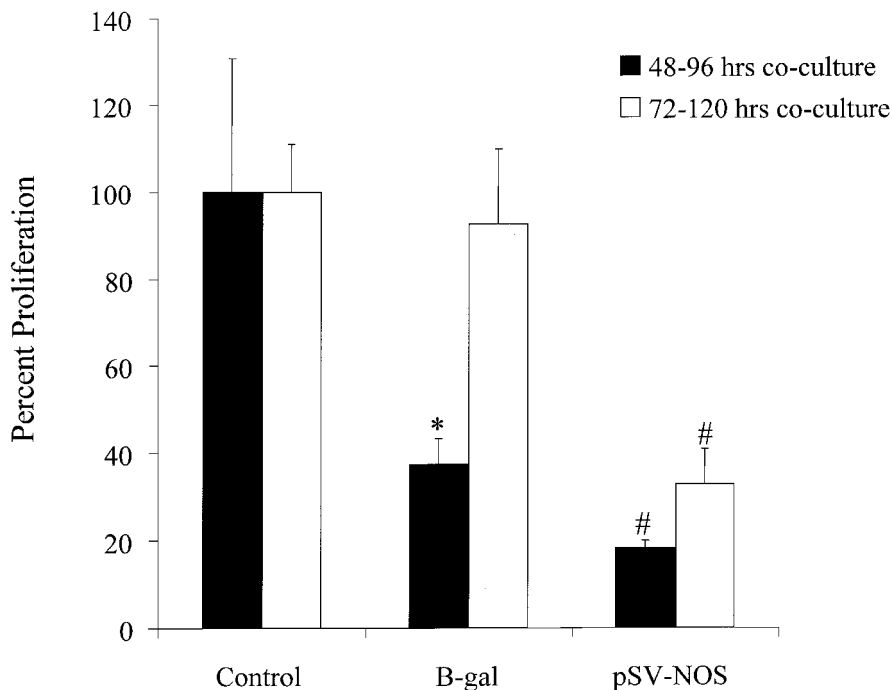


FIG. 6. Smooth muscle cell proliferation assay. BASMC were exposed to transfected BAEC for 2 days from 48 to 96 h post-transfection ($n = 6$) and from 72 to 120 h post-transfection ($n = 4$, except for β -Gal where $n = 3$). Percent [3 H]thymidine uptake with respect to untransfected BAEC is shown as mean + SEM. * $p < 0.05$ with respect to untransfected control BAEC; # $p < 0.05$ with respect to both untransfected and β -Gal-transfected control BAEC.

decrease the ability of endothelial cells to attach to a substrate^{35,36} and may produce insufficient expression³⁷ of eNOS.

Endothelial cell seeding of the luminal surface of a vascular graft for increased vascular graft patency requires that a fully confluent layer remain adherent or that compensation for flow-induced loss in endothelial coverage be designed. Because there is no surface modification that allows for perfect adhesion of an endothelial layer on a vascular graft in the presence of physiological shear, gene transfer may make compensate biochemically for cell losses due to flow. By seeding eNOS-transfected endothelial cells onto vascular grafts, the biochemical function of the lost endothelial cells may be compensated for by the increase in NO production by the remaining cells.³⁸

NO overproduction must allow for the inhibition of both platelet aggregation and smooth muscle cell proliferation, but cannot be at levels that are cytotoxic as is seen with inducible nitric oxide synthase (iNOS).³⁹ Cell death of the NO overproducing BAEC or of co-cultured BASMC was not observed in our cultures.

Our data indicate that eNOS transfection in BAEC leads to an increase in eNOS expression by endothelial cells beginning at 48 h post-transfection and continuing until at least 96 h post-transfection. The increase of NOS activity in β -Gal-transfected cells was observed at 48 h and is thought to be due to transfection stresses on the BAEC. Endothelial cells have been shown previously to modulate NOS activity in response to both chemical^{40,41} and mechanical stresses.⁴² This effect was not observed in β -Gal-transfected BAEC beyond 48 h post-transfection. Although we do not fully understand the mechanism of the transient NOS upregulation in β -Gal-transfected endothelial cells, it is important to note that eNOS expression levels were always significantly higher in pSV-NOS-transfected BAEC compared to either β -Gal-transfected or untransfected controls, 48–96 h post transfection and that the β -Gal-transfected endothelial cells were exhibiting a transient, transfection-dependent eNOS expression increase. Currently, we are investigating the causes of the elevated NOS expression in β -Gal-transfected BAEC that we believe may be due to the toxicity of naked DNA, cationic liposomes, and their complexes (data not shown).

The increase in basal levels of eNOS was confirmed at 84 h post-transfection by quantitative RT-PCR, which showed that there is at least a 48-fold increase of eNOS messenger in the pSV-NOS-transfected BAEC as compared to the untransfected controls. The 84-h time point was chosen such that the increase in mRNA present would be correlated to the effects seen at 96 h. The significantly higher eNOS mRNA levels in pSV-NOS-transfected cells compared to untransfected basal levels at this time point suggests that eNOS overexpression continues for at least 84 h.

eNOS-transfected BAEC showed a greater ability to inhibit platelet aggregation than nontransfected controls in whole-blood aggregometry, which has been previously used as a predictor for vascular graft patency.⁴³ Human whole blood showed a 46% decrease in aggregation when exposed to pSV-NOS-transfected BAEC (120 h post-transfection) as compared to either β Gal-transfected or untransfected BAEC controls; this effect was abolished using short-term 1mmol/L L-NAME treatment of eNOS-transfected BAEC. Consistent with published reports,⁴⁴ experiments in our laboratory confirmed that 1mmol/L L-NAME is not toxic for at least a 2-h exposure (data not shown).

The effect on platelets at this time point suggests that eNOS overexpression continues until at least the fifth day post-transfection. This abolition of effects on platelet aggregation confirms that effects seen on platelet aggregation are mediated by NO. Our data suggest that, in our experimental model, 54 eNOS-transfected endothelial cells would produce the same inhibition of platelet aggregation as 100 untransfected cells. We suggest that this has implications for graft patency with shear induced loss of endothelial cell coverage.

The data regarding smooth muscle cell proliferation show the ability of pSV-NOS transfected BAEC to inhibit BASMC proliferation over a period of 2 days encompassing 2 to 4 days post-transfection by 82% and by 68% over the period 3 to 5 days post-transfection with respect to untransfected controls. The β -Gal-transfected BAEC induced a 63% reduction 48 to 96 h post-transfection and showed 7.4% reduction 72 to 120 h post-transfection in SMC proliferation rates with respect to untransfected BAEC controls. These observations are consistent with observed increases in eNOS activity at 48 h in β -Gal-transfected BAEC and in pSV-NOS. When the co-culture was shifted to the later period, the ability of β -Gal-transfected BAEC to affect BASMC proliferation was abolished. This correlates well with the fact that significant NOS activity in β -Gal-transfected BAEC ends in the second day after transfection. These data further suggest that shear-induced endothelial cell loss may be partially compensated by the transfection of endothelial cells with eNOS. Our data suggest that 25 transfected endothelial cells produce the same effect in inhibiting smooth muscle cell proliferation as 100 untransfected endothelial cells.

Shear effects on the expression of eNOS on endothelial cells has been well documented.^{45,46} It could be argued that when transfected endothelial cells are exposed to shearing conditions, that they will be indistinguishable from nontransfected endothelial cells in terms of NO production. While it is true that the sustained calcium independent upregulation^{45,46} of eNOS will contribute significantly to the total NO output, it is important to note that the shear-responsive elements in the inserted gene are intact. Therefore, the overexpression observed may increase in the face of shear and become more biologically relevant.

The enhanced ability of eNOS-transfected endothelial cells to inhibit platelet aggregation and smooth muscle cell proliferation through increased NO production may potentially compensate biochemically for flow-induced loss of endothelial cell coverage on SSDVG. Therefore, genetically engineered endothelial cells that overexpress eNOS may be better suited to seeding SSDVG compared to endothelial cells that are not engineered.

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