Enhanced Inhibition of Neointimal Hyperplasia by Genetically Engineered Endothelial Progenitor Cells

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Background—Circulating endothelial progenitor cells (EPCs) have been reported previously. In this study, we examined the hypothesis that overexpression of vasculoprotective gene endothelial nitric oxide synthase (eNOS) and heme oxygenase-1 (HO-1) in EPCs enhances their ability to inhibit neointimal hyperplasia.

Methods and Results—EPCs were isolated from rabbit peripheral blood, expanded in culture, and transduced with pseudotyped retroviral vectors expressing human eNOS (eNOS-EPCs), HO-1 (HO-1-EPCs), or green fluorescent protein (GFP-EPCs). Transduction efficiency of EPCs ex vivo was >90%. Four groups of rabbits (n = 5 to 6 per group) were subjected to balloon angioplasty of the common carotid artery. Immediately after injury, ~5×10⁶ autologous EPCs or HO-1-EPCs were transplanted into the injured vessel. Control animals received an equivalent number of GFP-EPCs or Ringer’s saline. Two weeks after transplantation, eNOS and HO-1 transgene transcripts and proteins were detected in the transduced rabbit vessels. Endothelialization was enhanced in the EPC-transplanted vessels independently of gene transfer. Neointimal thickening was significantly reduced in the GFP-EPC–treated vessels relative to the saline control. Neointima size was further reduced in vessels treated with eNOS-EPCs. Surprisingly, no additional reduction was seen in vessels treated with HO-1-EPCs relative to GFP-EPCs. Thrombosis occurred in ~50% of the saline-treated vessels but was virtually absent in all EPC-transplanted vessels.

Conclusions—We conclude that transplantation of autologous EPCs overexpressing eNOS in injured vessels enhances the vasculoprotective properties of the reconstituted endothelium, leading to inhibition of neointimal hyperplasia. This cell-based gene therapy strategy may be useful in treatment of vascular disease. (Circulation. 2004;109:1769-1775.)

Key Words: endothelium ■ cells ■ hyperplasia ■ gene therapy

Endothelial progenitor cells (EPCs) originating from the bone marrow can be isolated from peripheral blood.¹² These cells are recruited to foci of injury,³ where they differentiate into endothelial cells that are capable of engrafting with the native tissue. This phenomenon suggests that EPC mobilization to sites of injury may participate in local tissue repair. The abundance of circulating EPCs, although low in basal conditions, increases severalfold after mobilization with cytokines and growth factors.⁴⁵ Recently, statin therapy was reported to enhance reendothelialization and to inhibit neointima formation in denuded arteries by increasing EPC mobilization from the bone marrow.⁶

Endothelial damage is a major cause of postangioplasty restenosis.⁷ The loss of endothelial cells during angioplasty decreases the availability of vasculoprotective molecules such as nitric oxide (NO) and prostacyclin as well as antioxidative systems such as heme oxygenase-1 (HO-1), with concomitant increases in the production of growth-promoting substances,⁷ which ultimately lead to the formation of neointima.⁸ Because endothelial dysfunction plays an essential role in vasculoproliferative disease, we postulate that transplantation of autologous EPCs overexpressing vasculoprotective genes such as endothelial NO synthase (eNOS) and HO-1 may have therapeutic potential as a strategy to enhance the biological properties of reconstituted endothelium. We have already shown that transplantation of autologous EPCs onto balloon-injured carotid artery leads to rapid reendothelialization of the denuded vessels,⁹ whereas others have reported that EPC transplantation onto decellularized artery reconstitutes a bioactive endothelial layer and confers sustained graft patency when these preparations are implanted as carotid interposition grafts.¹⁰ Thus, these studies demonstrate the feasibility of autologous EPC transplantation for repair of damaged vessels.

In the present study, we evaluated the efficacy of genetically modified autologous rabbit EPCs overexpressing eNOS...
or HO-1 in inhibiting neointimal hyperplasia and thrombosis in balloon-injured carotid arteries. We chose eNOS and HO-1 as therapeutic genes because the products of these genes exert antiproliferative, anti-inflammatory, and antithrombotic effects on the vessel wall.11–16 Our results indicate that EPC transplantation at the time of injury promotes reendothelialization and reduces neointimal hyperplasia of the injured vessels. In addition, overexpression of eNOS but not HO-1 further enhances the vasculoprotective properties of these cells. Considering the problems of thrombosis associated with drug-eluting stents, these findings suggest that transplantation of genetically engineered EPCs may be a novel alternative therapeutic strategy to prevent postintervention complications.

Methods

Animals

Adult New Zealand White male rabbits (3 to 3.5 kg BW) were purchased from Millbrook Breeding Laboratory (Amherst, Mass). The animals were maintained on a 12/12-hour light/dark cycle in an ambient temperature of 24°C and 60% humidity. Food and water were provided ad libitum. The Harvard Medical Area Standing Committee on Animals approved all animal procedures.

Blood Collection

Animals were anesthetized with a mixture of ketamine (25 mg/kg) and xylazine (10 mg/kg) administered intravenously and supplemented as required. A left groin incision was made. The femoral vein was catheterized and 50 mL of blood were withdrawn into an equivalent volume of saline. The wound was closed and a protective collar was placed for 5 days to prevent disruption of the wound closure.

Cell Isolation and Culture Conditions

The isolation and culturing of EPCs was as described previously.9 First passage cells were used for all in vivo applications.

Cell Characterization

The immunohistochemical characterization of EPCs was performed as described previously.9 First passage cells were used for characterization. The cells were incubated with 1:50 dilution of monoclonal anti-human CD31 (PECAM-1, Dako, Carpenteria, Calif), or polyclonal goat anti-mouse VE-cadherin or anti-human eNOS (R&D Systems Inc, Minneapolis, Minn), or anti-human von Willebrand factor (vWF) (Dako). For detection of CD31 immunoreactivity, slides were incubated with 1:500 dilution of anti-mouse IgG biotin–alkaline phosphatase conjugate (Dako) followed by incubation in Fast Red (Vector Laboratories). For detection of VE-cadherin, eNOS, and vWF, the slides were incubated with 1:500 dilution of Alexa Fluor 546 rabbit anti-goat IgG conjugate (Molecular Probes). The eNOS and vWF slides were counterstained for 5 minutes with 10 μg of nuclear dye Hoechst 33342 (Sigma). Human umbilical vein endothelial cells (HUVECs) (Clonetics) were prepared and stained in the same manner and used as positive control.

Retroviral Production and Transduction

The vectors were constructed by ligating the full-length (4100-bp) human eNOS cDNA (a gift from Dr T. Michel, Harvard Medical School, Boston, Mass) or human HO-1 (987 bp) into the EcoRI or HindIII cloning sites, respectively, of pMSCV vector (BD Biosciences Clontech) downstream from the 5′ LTR promoter. The green fluorescent protein (GFP) gene (700 bp) together with the internal ribosomal entry site (IRES) were excised from pEGFP (Clontech) by XhoI/BluntHI digestion and cloned at the corresponding sites into the pMSCV vector. Generation and titering of VSV-G pseudotyped viral particles was performed by the Harvard Gene Therapy Initiative. The titers were ~0.5 to 1 × 10^7 IU/mL. First-passage EPCs at 30% to 40% confluence in 100-mm dishes were exposed to 100 μL of virus solution for 6 hours in the presence of 8 μg/mL polybrene (Aldrich Chemical). Cells were transplanted 4 days after transduction.

Balloon-Injury Model and Cell Transplantation

Balloon injury of the right common carotid artery was performed as described previously.9 Briefly, a 3F Fogarty balloon catheter (Baxter) was inserted through the external carotid artery, inflated with 300 μL of air, and passed 3 times along the length (3.5 to 4 cm) of the isolated segment. The injured segment was rinsed with saline, and ~5 × 10^7 autologous EPCs suspended in 300 μL of saline were instilled with a catheter fashioned from polyethylene tubing and incubated for 30 minutes. Unseeded vessel segments were incubated with an equivalent volume of saline.

Tissue Collection and Preparation

The injured segments were isolated 2 weeks after cell transplantation. The segments were excised and cut into 8 to 10 fragments, 4 of which were fixed overnight in 10% formalin and embedded in paraffin. The remaining fragments were snap-frozen in OCT compound (Miles) for preparation of frozen sections. For analysis of protein and RNA, vessels were snap-frozen in liquid nitrogen and stored at −80°C until extraction. Vessels with thrombosis were excluded from further analysis.

Morphometric Analysis

Assessment of endothelialization and neointima was performed as described previously.9 For histological analysis of endothelialization, 4 to 5 frozen sections from different segments of the vessel were incubated with 1:50 dilution of monoclonal anti-human CD31 antibody (Dako). Endothelialization was calculated as the ratio between the luminal surface covered by CD31-positive cells and the total luminal surface. For morphometric analysis of neointimal hyperplasia, paraffin–embedded sections were processed for elastin stains with Accustain elastic stain (Sigma). Neointimal and medial areas were measured in 6 to 8 individual sections taken from the middle portion of the segments by delineating the external elastic lamina, internal elastic lamina, and endothelium. Neointima/media ratios were used to compare neointima formation between the various groups.

Immunohistochemistry

Immunohistochemical detection of human eNOS or HO-1 in the vessel segments was performed by incubating 5-μm-thick frozen sections in 1:100 dilution of monoclonal anti-human eNOS or HO-1 antibodies (Transduction Laboratories). These antibodies do not cross-react with the endogenous antigens. Sections were then incubated in 1:500 dilution of biotinylated secondary antibody (Vector Laboratories). Sections were treated further with avidin-biotin conjugated to horseradish peroxidase, developed with DAB (Vector Laboratories), and counterstained with hematoxylin.

Western Blot

Immunoblotting detection of HO-1 and eNOS was performed essentially as previously described.11 Two hundred micrograms of protein was separated on SDS-PAGE under reducing and denaturing conditions and transferred to Hybond nitrocellulose membranes. The membranes were incubated in 1:500 dilution of monoclonal anti-human eNOS antibody (Transduction Laboratories) or 1:750 dilution of polyclonal anti-rat HO-1 (StressGen), followed by incubation with horseradish peroxidase–conjugated secondary antibody. Signal was detected by enzyme-linked chemiluminescence (Amersham).

Reverse Transcriptase–Polymerase Chain Reaction

For reverse transcriptase–polymerase chain reaction (RT-PCR) detection of human eNOS and HO-1, 200 ng of total RNA was used for first-strand cDNA synthesis and PCR amplification with the One-
Step Platinum Taq RT-PCR kit (Invitrogen). For eNOS, a 336-bp fragment was amplified for 40 cycles with the following primers: forward, 5'-AAGATCTCCGCTCGCTCA-3'; reverse, 5'-GCTTGTGAACGGATCTTTA-3'. For human HO-1, a 186-bp fragment was amplified with the following primers: forward, 5'-GCTCTTTGAGGAGTTGCAGG-3'; reverse, 5'-GTGTAAGGACCATCGGAGA-3'. The cycling conditions for both amplifications were 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds, followed by a terminal 10-minute elongation at 72°C.

Statistical Analysis
All results are presented as mean±SEM. Comparisons between groups were made by 1-way ANOVA. A probability value of ≤0.05 was considered to indicate statistical significance.

Results
Characterization of EPCs and Ex Vivo Gene Transfer
Under the culture conditions used in the present study, we found outgrowth colonies of adherent cells 6 to 8 days after plating. These colonies proliferated rapidly and exhibited cobblestone morphology (Figure 1A). Two weeks after plating, most colonies had reached near confluence. The cells could be passaged several times without discernible alterations in morphology or growth characteristics. After 10 days in culture, the majority of the cells expressed endothelial markers CD31 (Figure 1C), VE-cadherin (Figure 1E), eNOS (Figure 1G), and vWF (Figure 1I). Similar morphologies (Figure 1B) and pattern of CD31 (Figure 1D), VE-cadherin (Figure 1F), eNOS (Figure 1H), and vWF (Figure 1J) expression were observed in HUVECs.

Genetic Engineering of Blood-Derived EPCs
Figure 2 shows transduction of EPCs with retroviral vectors expressing GFP or the therapeutic genes eNOS and HO-1. Transduction efficiency with this vector was ≈80%, as determined by the number of GFP-positive cells (Figure 2, A and B). Human eNOS mRNA (Figure 2C) and protein (Figure 2D) were detected in lysates of cells transduced with MSCV-eNOS but not in cells transduced with MSCV-GFP. Similarly, we could detect human HO-1 mRNA (2E) and protein (2F) in HO-1-transduced cells. However we also detected low levels of HO-1 transcript and protein in untransduced and GFP-transduced cells, possibly because of sequence homology between human and rabbit HO-1 mRNA in the regions spanned by the primers and species cross-reactivity of the antibody used.

Gene Transfer and Expression In Vivo
Figure 3 shows human eNOS and HO-1 transgene expression in injured arteries 2 weeks after transplantation. Immunohistochemical staining of sections with the human-specific eNOS antibody showed intense positive staining in the endothelium of sections from the vessels transplanted with eNOS-EPCs (Figure 3A) but not in the sections from GFP-EPC–transplanted vessels (Figure 3B). We also detected intense human HO-1–specific staining in sections from HO-1-EPC–transplanted vessels (Figure 3C) but not in GFP-EPC–transplanted vessels (Figure 3D). Human eNOS mRNA (Figure 3E) and protein (Figure 3F) expression was detected in homogenates from the eNOS-EPC–transplanted vessels but not in the homogenates from GFP-EPC–transplanted vessels.

Human HO-1 mRNA (Figure 3G) and protein (Figure 3H) were found in the HO-1-EPC–treated vessels. Reduced amounts of HO-1–like immunoreactivity and message were also detected in GFP-treated vessels.

Reendothelialization of Denuded Carotid Artery
The reendothelialization of injured carotid arteries after EPC transplantation is shown in Figure 4. Immunohistochemical staining revealed ≈22% coverage of the luminal surface with CD31-positive cells 2 weeks after injury (Figure 4, A and E). Endothelialization of the denuded vessels was significantly enhanced in the GFP-EPC–treated (46%, Figure 4, B and E), eNOS-EPC–treated (53%, Figure 4, C and E) and HO-1-
EPC–treated (49%, Figure 4, D and E) vessels but did not differ between these groups.

**Inhibition of Neointimal Hyperplasia**

The effect of EPC transplantation on vascular lesion formation in carotid arteries is shown in Figure 5. No evidence of neointima was seen in uninjured vessels (data not shown). Balloon injury resulted in a prominent neointima in the arteries of saline-treated animals (Figure 5, A and B). Transplantation of GFP-EPCs reduced neointima development in the injured vessels (Figure 5, C and D). Morphometric analysis showed a decrease of 48.3% in neointima/media ratio in the GFP-EPC–transplanted vessels (0.294±0.051, n=6) compared with the saline control (0.569±0.116, n=5) (P<0.05) (Figure 5I). Transplantation of eNOS-EPCs led to 72.1% inhibition of neointima/media ratio (0.159±0.031, n=7) (Figure 5, E, F, and I) relative to saline control, and this reduction was significantly greater than that with GFP-EPC treatment (P<0.05). HO-1-EPCs also de-
creased neointimal/media ratio (0.235±0.085, n=6) to 58.7% of saline control but had no additional effect compared with GFP-EPCs (Figure 5, G, H, and I).

EPC transplantation dramatically reduced the incidence of thrombosis (Table). Approximately 50% of all unseeded vessels showed evidence of thrombosis at termination of the experiment. In contrast, EPC-treated vessels were virtually free of thrombosis (Table).

**Discussion**

Endothelial cell loss because of vascular injury is a major contributing factor to the local activation of pathophysiological events leading to the development of neointimal hyperplasia. NO, a key biological mediator released by the endothelial cells, and HO-1, a ubiquitous cytoprotective enzyme system has been show to inhibit the pathological processes leading to neointima formation. Accordingly, cell- and gene-based therapeutic strategies aimed at promoting early reendothelialization may be a treatment option for prevention of vascular diseases such as restenosis and graft failure. In the present study, we demonstrate that autologous EPC transplantation into balloon-injured carotid arteries leads to rapid reconstitution of the damaged endothelium and marked inhibition of neointima proliferation. Furthermore, the antiproliferative effect of EPCs is further enhanced by overexpression of eNOS, suggesting that transplantation of genetically modified EPCs may have potential as a therapeutic strategy for vasculoproliferative diseases.

The use of endothelial cells for seeding of denuded vessels has been reported previously. However, the cells used in
these earlier studies were cultured from veins or adipose tissue, neither of which is suitable for broad clinical application. EPCs present several advantages for vascular therapy. These cells are readily available from a nondepleting, self-renewing source such as peripheral blood1-2 and can be easily harvested, expanded, and selectively differentiated in culture conditions.4,9,10 Second, EPCs are amenable to ex vivo genetic engineering with viral vectors,9 making them ideal vehicles for delivery of therapeutic genes to sites of injury. Third, the transplanted EPCs are capable of proliferating and rapidly repopulating denuded vessels in vivo.9,10 More significantly, the use of autologous endothelial cells for transplantation circumvents the potential immunogenic problems associated with heterologous cell transplantation.

Our results suggest that the therapeutic effect of EPC transplantation in inhibition of neointima hyperplasia may be, at least in part, a result of accelerated reendothelialization of the denuded vessel. We have recently reported that EPC transplantation leads to >50% reendothelialization of balloon-injured rabbit carotid arteries as early as 4 days after transplantation.9 In the present study, we found sustained endothelial coverage of the denuded vessels 2 weeks after transplantation. This degree of reendothelialization is greater than what could be accounted for by endogenous repair alone. We postulate that the ability of the transplanted cells to rapidly restore endothelial integrity at the site of injury may act to restore endothelium-dependent bioactivity and suppress more effectively the activation of smooth muscle proliferation and migration from the underlying media, thereby resulting in attenuation of neointimal proliferation. Furthermore, the intact endothelium also forms an antithrombotic surface, which would minimize the occurrence of thrombosis.

The present study also evaluates the use of EPCs as vehicles for local delivery of vasculoprotective genes. We transplanted genetically modified autologous EPCs overexpressing eNOS or HO-1 into balloon-denuded carotid arteries to evaluate the feasibility and therapeutic potential of this cell-based gene therapy strategy for the inhibition of neointima hyperplasia and thrombosis. eNOS and HO-1 are, in many regards, ideal therapeutic genes for vascular disease. eNOS-derived NO, together with the byproducts of heme catabolism by HO-1, exert pleiotropic cytoprotective effects in the vessel wall, reducing oxidative stress and modulating vascular tone and platelet adhesion and inhibiting smooth muscle proliferation and migration and the development of atherosclerosis.11-16 Furthermore, several studies have demonstrated the therapeutic potential of eNOS and HO-1 gene transfer in stabilizing vessel wall function and limiting neointima proliferation after revascularization procedures.19-21 Our results clearly indicate that eNOS overexpression enhances the vasculoprotective effect of EPCs, suggesting that a combinatorial approach using EPCs for transfer of eNOS may be an effective strategy to enhance the function of reconstituted endothelium in damaged vessels. Unexpectedly, we did not observe any additional therapeutic effect with HO-1 overexpression by EPCs, despite levels of transgene expression that were comparable to eNOS. This is in contrast to previous studies that have reported significant inhibition of neointimal thickening after adenovirus-mediated delivery of HO-1 gene to the damaged vessel wall.21 Several reasons may account for the differences. First, HO-1 expression by adenovirus is more robust than the level of expression seen with our retroviral vector. Considering that CO has a lower affinity for soluble guanylate cyclase than NO,22 it is likely that a higher level of HO-1 expression would be necessary to confer comparable therapeutic effects. Second, the paracrine effects of CO emanating from the transplanted HO-1-EPCs may be limited by diffusion to the underlying smooth muscle cells. Thus, although our results do not discount the therapeutic value of HO-1 gene transfer, they do reveal that in the context of cell-based vascular gene therapy, NO synthase gene transfer, on a molar basis, provides more effective vascular protection than HO-1 gene transfer, possibly because of greater affinity of NO for soluble guanylate cyclase.

In conclusion, the present study shows that autologous EPC transplantation effectively prevents thrombosis and reduces neointimal hyperplasia in denuded carotid arteries by promoting reendothelialization and that this effect is further enhanced by genetic engineering with eNOS. Considering the problems of thrombosis and restenosis associated with endovascular intervention and stents, these findings suggest that transplantation of genetically modified EPCs expressing vasculoprotective genes such as NO may be a feasible therapeutic option that could be administered as an adjuvant to revascularization procedures to render the treated vessels resistant to thrombosis and restenosis.


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