Transplantation of Circulating Endothelial Progenitor Cells Restores Endothelial Function of Denuded Rabbit Carotid Arteries

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Background and Purpose—Circulating endothelial progenitor cells (EPCs) play an important role in repair of injured vascular endothelium and neovascularization. The present study was designed to determine the effect of EPCs transplantation on the regeneration of endothelium and recovery of endothelial function in denuded carotid arteries.

Methods—Isolated mononuclear cells from rabbit peripheral blood were cultured in endothelial growth medium for 7 days, yielding EPCs. A rabbit model of common carotid artery denudation by passage of a deflated balloon catheter was used to evaluate the effects of EPCs on endothelial regeneration and vasomotor function. Immediately after denudation, autologous EPCs (10^5 cells in 200 μL saline) or 200 μL saline alone (control) were administered into the lumen of injured artery.

Results—Four weeks after transplantation, fluorescence-labeled colonies of EPCs were found in the vessel wall. Local transplantation of EPCs as compared with saline administration accelerated endothelialization and significantly improved endothelium-dependent relaxation when assessed 4 weeks after denudation (n=4 to 5, P<0.05). Transplantation of EPCs did not affect vasomotor function of arterial smooth muscle cells. Protein array analysis of conditioned media obtained from cultured EPCs demonstrated the ability of these cells to produce and release a number of proangiogenic cytokines.

Conclusions—We conclude that local delivery of cultured circulating EPCs into the lumen of denuded carotid arteries accelerates endothelialization and improves endothelial function. Paracrine effects of EPCs may contribute to regenerative properties of EPCs. (Stroke. 2004;35:2378-2384.)

Key Words: angiogenesis • carotid arteries • cell culture • cell transplantation • nitric oxide • stem cells

Circulating endothelial progenitor cells (EPCs) play important roles in angiogenesis and repair of injured endothelium.1–6 More recent findings suggest that transplantation of circulating EPCs or spleen-derived EPCs into the injured arterial wall has beneficial effects on vascular structure and function.7–9 Two types of EPCs (early EPCs and late EPCs) have been identified by culturing peripheral blood mononuclear cells.1,10,11 The early human EPCs are spindle-shaped in culture with peak growth at 2 to 3 weeks. Most of these cells have a life span of ≈4 weeks. A certain number of early EPCs can continue to grow into colonies of late EPCs, which emerge 2 to 3 weeks after start of mononuclear cell culture. They can be exponentially grown up to 12 weeks. The present study was designed to determine the effect of transplantation of early EPCs (derived from peripheral blood mononuclear cells cultured for 7 days) on regeneration of endothelium in denuded carotid arteries. Because previous studies have demonstrated that regenerated endothelium is dysfunctional,12 we hypothesized that transplantation of EPCs may help normalize endothelial control of vasomotor function. To determine whether this effect could be because of EPCs-induced alterations of smooth muscle cells reactivity, we also examined the effect of EPC transplantation on vasoconstrictor and vasodilator effects caused by direct activation of smooth muscle cells. Furthermore, existing evidence suggests that paracrine effect of EPCs and marrow-derived stromal cells due to secretion of growth factors may contribute to overall beneficial effect on endothelial repair.13,14 To address this possibility, we used a protein array approach to identify cytokines secreted by EPCs that may help explain their regenerative properties.

Materials and Methods

Peripheral Blood Mononuclear Cell Isolation, Characterization, and Labeling

Experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic (Rochester, Minn). Male New Zealand White rabbits (2.5 to 3 kg; Harlan Sprague-Dawley, Indianapolis, Ind) were used in all experiments.

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Animals were housed individually in a room with a 12-hour light/dark cycle. Rabbit peripheral blood (10 mL/kg body weight) was obtained from a central ear artery. Peripheral blood mononuclear cells were isolated by density gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences Corp). Mononuclear cells were then washed with MCDB 131 medium (GIBCO Industries, Inc) supplemented with hydrocortisone (Sigma-Aldrich), cAMP (EMD Biosciences, Inc), amphotericin B (Sigma-Aldrich), and 10 ng/mL vascular endothelial growth factor (VEGF, R&D Systems). Cells were plated at a density of \( \frac{5 \times 10^6}{500 \text{ wells}} \) cells/well on 6-well plates coated with human fibronectin (R & D Systems) in endothelial growth medium-2 (EGM-2, Cambrex Corp). Mononuclear cells were incubated at 37°C, 5% CO\(_2\), and fed daily with EGM-2. After 3 days, the nonadherent cells were removed. At day 7 of culture, the adherent cells (early EPCs, \( 11 \)) were harvested by trypsinization for transplantation.

The early EPCs were characterized by endocytosis of acetylated low-density lipoprotein (acLDL), isolectin binding, and expression of endothelial marker Flk1. Cells were incubated with acLDL (Molecular Probes) at a concentration of 10 \( \mu \text{g/mL} \) for 4 hours. After washing twice with EGM-2, the cells were incubated with 5 \( \mu \text{g/mL} \) isolectin (Molecular Probes) for 30 minutes. Cells were washed with PBS, and examined using fluorescence confocal microscopy with absorption wavelengths at 555 nm (acLDL) and 495 nm (isolectin). Indirect immunofluorescence was applied to detect Flk1. \( 1,10,15 \) Cells were permeabilized by incubation with 0.1% Triton X-100 for 2 minutes before staining. Primary monoclonal antibody against human Flk-1 (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) and an isotype-matched mouse IgG (Santa Cruz Biotechnology, Inc) were used at working concentration of 1 \( \mu \text{g/mL} \). After labeling with a 1:500 dilution of Texas Red-conjugated goat anti-mouse IgG (Molecular Probes), cells were incubated with 10 \( \mu \text{mol/L} \) Hoescht 33342 (Sigma-Aldrich) for 5 minutes to counterstain the nuclei. Cells were viewed using fluorescence confocal microscopy.

To track the homing of EPCs in the carotid artery, a fluorescent carbocyanine dye, CM-Dil (Molecular Probes) was used to label early EPCs from 3 rabbits. Early EPCs were trypsinized and resuspended at 1 \( \times 10^6 \) cells/mL in EGM-2 containing CM-Dil (5 \( \mu \text{L/mL} \)) for 10 minutes at 37°C, and then for another 10 minutes on ice. Labeled cells were washed 3 times with PBS, and resuspended in 200 \( \mu \text{L} \) saline for subsequent administration.

**Conditioned Media Collection and Analysis**

Isolated mononuclear cells were plated on 6-well plates coated with human fibronectin at a density of 20 \( \times 10^6 \) cells/well, and cultured in EGM-2. Media were changed daily. At day 6 of culture, attached cells were washed with endothelial basal medium-2 without growth factors and serum (EBM-2, Cambrex Corp) and incubated to reach confluence. Media were collected and filtered to remove cell debris. The collected media were stored at \(-80°C\) until analysis.

**Figure 1.** Phenotype of EPCs. A through C, Fluorescence confocal microscopy illustrates that EPCs of day 7 were positive for uptake of Dil acLDL (A) and for staining of Alexa Fluor 488-conjugated isolectin (B). Overlaying A and B results in panel C (40× magnification). D and E, Immunofluorescence confocal microscopy of EPCs labeled with Flk-1 (D) and normal mouse IgG (E, as a control, 100× magnification). Cell nuclei were counterstained with Hoechst stain. F, Phase contrast micrograph showed that confluent EPCs, out-grown from day 7 EPCs, grew into monolayer with cobblestone appearance (10× magnification).
Corporation) once and incubated with EBM-2 (2 mL/well) for 24 hours (condition medium). The conditioned media (4 mL from 2 wells per sample) were collected for analysis of proteins using Human Cytokine Array V (RayBiotech, Inc). This detection system is designed for qualitative analysis of proteins present in conditioned media. Proteins specifically bound to spots of a membrane and were detected by a cocktail of biotin-conjugated antibodies (for 72 different cytokines). After incubation of the membrane with horseradish peroxidase–conjugated streptavidin and detection solution, the membrane was exposed to Biomax MR film for 10 minutes. Three independent experiments were performed. The cytokine spots present on the membrane in all 3 experiments were considered positive.

**Rabbit Carotid Artery Denudation and EPC Transplantation**

Animals were anesthetized with an intramuscular injection of ketamine (35 mg/kg), xylazine (5 mg/kg), and acepromazine (2.3 mg/kg). Both common carotid arteries were exposed through a midline incision to the neck. Heparin (300 U/kg) was injected intravenously through an ear vein. An arteriotomy was made in the right common carotid artery (RCA) after this vessel had been clamped, and a 3-French Fogarty balloon catheter (Baxter Healthcare Corp) was introduced into the lumen. Arteries underwent endothelial denudation by passage of a deflated balloon catheter (3 ×). Autologous early EPCs (10^5 cells in 200 μL) or 200 μL saline alone (control) were administered into the lumen of denuded arteries. After closing of arteriotomy and incubation of cells or saline for 20 minutes in injured vessel, the clamps were removed allowing resumption of blood flow. The left carotid arteries (LCA) that did not undergo arteriotomy served as internal controls.

**Assessment of Endothelialization**

Four weeks after carotid artery injury, animals were euthanized (by injection of a cocktail of 65 mg/kg ketamine +13 mg/kg xylazine + 22 μg/kg acepromazine intramuscularly, and 3 mL Sleepaway intravenously) 30 minutes after intravenous injection of 5 mL of 0.5% Evans blue (Sigma). Harvested arteries were immediately incised longitudinally to expose the luminal surface and rinsed with cold Krebs solution (4°C). Photos of en face carotid artery were taken. The areas with endothelialized (non-Evans blue stained area) and without endothelium (Evans blue stained area) were analyzed using Image Pro Plus (Media Cybernetics).

**Analyses of Vasomotor Function**

Both RCA and LCA were dissected. Rings (4 mm in length, the middle portion of each carotid artery was used in all experiments) were suspended for isometric force recording in organ chambers filled with 25 mL of gassed (94% O2 and 6% CO2) modified Krebs-Ringer bicarbonate solution.16 The rings were gradually stretched to 3.0 g of force and contracted twice by 40 mmol/L potassium chloride. After an hour of equilibration, contractions to phenylephrine (10^-6 to 10^-5 mol/L) Sigma Chemical) were obtained. Concentration-dependent response curves to acetylcholine (Sigma-Aldrich), and diethylammonium (Z)-1-(N,N-diethylamino)diazene-1-IM1,2-diolate (DEA-NONOate, Cayman Chemical) were cumulatively obtained during submaximal contractions to phenylephrine. Papaverine (3 × 10^-4 mol/L Sigma Chemical) was used to induce maximal relaxation of the vessels. Relaxations were presented as percentage of the maximal relaxation induced by papaverine.

**Statistical Analysis**

Data are presented as mean ± SEM. Concentration-response curves of different groups were compared by ANOVA for repeated measurements with Bonferroni correction (SigmaStat 2.03 for Windows). Comparison between 2 groups was made using Student t test. P<0.05 was considered statistically significant.

**Results**

**Characterization of EPCs**

Seven days after isolation, incorporation of acLDL and binding of isolecitin were detected in early EPCs (Figure 1A through 1C), which also expressed endothelial marker Flk1 (Figure 1D). Further culture (~2 to 3 weeks after isolation) gave rise to colonies of out-growth cells (late EPCs), which exhibited “cobblestone” morphology and monolayer growth pattern typical of endothelial cells at confluence (10, 11; Figure 1F). Only early EPCs were used in transplantation experiments.

**Cytokines Secreted by EPCs**

A sensitive antibody-based multiple-cytokine detection system17 was used to detect cytokines in conditioned media from early EPCs. As shown in Table, after culturing in EBM-2 (serum- and growth factor–free) for 24 hours, EPCs secreted predominantly angiogenic cytokines. We also detected few antiangiogenic, neurotrophic, and neuroregulatory cytokines. Angiogenic/arteriogenic cytokines including granulocyte colony-stimulating factor, granulocyte macrophage-colony-stimulating factor, platelet-derived growth factor-BB, and insulin-like growth factor were undetectable in all 3 samples.

**Transplantation of EPCs Enhanced Endothelialization**

Four weeks after carotid injury and local cell delivery, multiple colonies of DiI-labeled EPCs were observed in the wall of carotid arteries (Figure 2A). These colonies were
found in the non-Evans blue-stained area but not in the Evans blue-stained area (data not shown). Dil-labeled EPCs were not found in the uninjured LCA of the rabbits that underwent right carotid injury and local cell delivery (data not shown). Luminal staining for Evans blue showed that the surgical injury completely denuded carotid artery (Figure 2B). Transplantation of early EPCs enhanced endothelialization 4 weeks after injury (Figure 2B and 2C).

Effects of Transplantation of EPCs on Vasomotor Function
Four weeks after surgical procedures, neither injury alone nor injury plus local cell delivery affected contraction to phenylephrine, as compared with control carotid arteries without injury (Figure 3A). Endothelium-dependent relaxations to acetylcholine were significantly impaired in injured arteries. Local cell delivery normalized endothelium-dependent relaxation to acetylcholine (Figure 3B). On the other hand, endothelium-independent relaxations to DEA-NONOate were not affected by injury or injury plus cell therapy (Figure 3C).

Discussion
The results of the present study demonstrated that transplantation of autologous EPCs enhanced endothelialization and improved endothelial function of denuded carotid artery.
Transplantation of EPCs did not affect vasomotor function of smooth muscle cells, suggesting that detected effect was selective for endothelium. We provided evidence that cultured EPCs secreted a number of cytokines that could stimulate proliferation, migration, and survival of endothelial cells, therefore raising the possibility that paracrine function of EPCs may play an important role in endothelialization and recovery of endothelial function. Furthermore, we detected release of neurotrophic and neuroregulatory cytokines from the cultured EPCs, which may enable EPCs to exert an important trophic influence on neuronal tissue.

Both early and late EPCs have been isolated from the rabbit circulating blood and it appears that both cell types exert beneficial effects on endothelialization and neointimal formation in injured carotid artery. In the present study, we confirmed that early EPCs expressed endothelial markers and some of them outgrew into late EPCs, which had morphological features of vascular endothelium. We studied the effect of transplantation of early EPCs on endothelial injury and demonstrated that EPCs incorporated into injured vascular wall after transplantation of autologous early EPCs, suggesting that EPCs participate in endothelialization of denuded vessels. The patchy distribution of colonies of EPCs indicates that only a component of regenerated endothelium originates from transplanted EPCs. It appears that a major component of endothelial regeneration may be driven by the paracrine effects of EPCs on surrounding mature endothelium. Based on reported life span of early and late EPCs, it is likely that colonies of EPCs observed in carotid arteries represent late rather than early EPCs. However, further studies are needed to determine the nature of EPCs present in arterial wall 4 weeks after injury and transplantation of early EPCs. Interestingly, in the analysis of proteins secreted by cultured early EPCs, we detected a preponderance of proangiogenic cytokines (Table 1) that are known stimulators of proliferation, migration and survival of endothelial cells including VEGF, fibroblast growth factors, hepatocyte growth factor, and placenta growth factor. We also detected few antiangiogenic cytokines suggesting that EPCs can execute a complex regenerative program that involves orchestrated signaling with stimulatory and inhibitory molecules. According to the manufacturer, the detection range of the protein cytokine array is at least 100-fold greater compared with ELISA. However, the data obtained with the protein array are qualitative and do not allow any conclusion regarding relative contribution of different cytokines to overall regeneration of endothelium.

It has been reported that regenerated endothelium covered ~70% of denuded surface 4 weeks after injury of rabbit carotid arteries. In our study, endogenous mechanisms responsible for endothelial regeneration were even more efficient covering almost 85% to 90% of denuded surface. This difference could be explained by the fact that we did not impose severe injury on vascular wall by inserting deflated balloon catheter. Nevertheless, transplantation of EPCs improved endothelialization of de-
nuded arteries, suggesting that EPCs may have therapeutic value in treatment of vascular injury.

Studies of injured porcine coronary arteries demonstrated that endothelium-dependent relaxations to aggregating platelets and serotonin (mediated by NO) were impaired in arteries with regenerated endothelium.12–28 We report similar results in rabbit denuded carotid arteries. Despite 85% to 90% of endothelialization 4 weeks after denudation, the endothelium-dependent relaxations acetylcholine were significantly impaired. However, endothelialization by EPC transplantation remarkably recovered endothelium-dependent relaxation, suggesting that EPCs have beneficial effects not only on proliferation, and migration of endothelial cells, but can accelerate normalization of NO availability from regenerated endothelium. Indeed, VEGF and fibroblast growth factor, which have been reported to enhance expression and enzymatic activation of NO availability from regenerated endothelium,29 were found in the condition media of cultured EPCs (Table 1). Our data also demonstrated that vasoconstrictor effect of phenylephrine and endothelium-independent vasodilator effect of DEA NONOate were not affected by denudation of carotid artery or transplantation of EPCs, reinforcing our conclusion that the effect of EPCs was selective for endothelial cells. However, we cannot rule out the possibility that inhibition of neointimal formation by transplantation of EPCs may contribute to the beneficial effect of EPCs.7

It is important to note that EPCs have ability to produce and release neurotrophic proteins as shown in Table 1,30–32 This observation may have important implications for understanding the dynamic interaction between vascular endothelium and neuronal tissue, and may expand the concept of endothelial dysfunction to include impaired ability of endothelial cells to provide a trophic effect on brain parenchyma.

Findings reported in this study support the concept that transplantation of cultured EPCs has beneficial effect on endothelial regeneration and function after injury. Production and release of proangiogenic cytokines from EPCs may have an important role in enhanced endothelialization of injured carotid arteries. Identification of exact molecular mechanisms mediating beneficial effects of EPCs remains to be determined.

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