Hybrid Cell–Gene Therapy for Pulmonary Hypertension Based on Phagocytosing Action of Endothelial Progenitor Cells

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Background—Circulating endothelial progenitor cells (EPCs) migrate to injured vascular endothelium and differentiate into mature endothelial cells. We investigated whether transplantation of vasodilator gene-transduced EPCs ameliorates monocrotaline (MCT)-induced pulmonary hypertension in rats.

Methods and Results—We obtained EPCs from cultured human umbilical cord blood mononuclear cells and constructed plasmid DNA of adrenomedullin (AM), a potent vasodilator peptide. We used cationic gelatin to produce ionically linked DNA-gelatin complexes. Interestingly, EPCs phagocytosed plasmid DNA-gelatin complexes, which allowed nonviral, highly efficient gene transfer into EPCs. Intravenously administered EPCs were incorporated into the pulmonary vasculature of immunodeficient nude rats given MCT. Transplantation of EPCs alone modestly attenuated MCT-induced pulmonary hypertension (16% decrease in pulmonary vascular resistance). Furthermore, transplantation of AM DNA-transduced EPCs markedly ameliorated pulmonary hypertension in MCT rats (39% decrease in pulmonary vascular resistance). MCT rats transplanted with AM-expressing EPCs had a significantly higher survival rate than those given culture medium or EPCs alone.

Conclusions—Umbilical cord blood–derived EPCs had a phagocytosing action that allowed nonviral, highly efficient gene transfer into EPCs. Transplantation of AM gene-transduced EPCs caused significantly greater improvement in pulmonary hypertension in MCT rats than transplantation of EPCs alone. Thus, a novel hybrid cell–gene therapy based on the phagocytosing action of EPCs may be a new therapeutic strategy for the treatment of pulmonary hypertension. ([Circulation. 2003;108:889-895.])

Key Words: pulmonary heart disease ■ natriuretic peptides ■ gene therapy ■ endothelium

The pulmonary endothelium plays an important role in the regulation of pulmonary vascular tone through the release of vasoactive substances such as nitric oxide, prostacyclin, and adrenomedullin (AM).1 Dysfunction of the endothelium may play a role in the pathogenesis of pulmonary hypertension, including primary pulmonary hypertension.2 Thus, pulmonary endothelial cells may be a therapeutic target for the treatment of pulmonary hypertension. Recently, endothelial progenitor cells (EPCs) have been discovered in adult peripheral blood.3 EPCs are mobilized from bone marrow into the peripheral blood in response to tissue ischemia or traumatic injury, migrate to sites of injured endothelium, and differentiate into mature endothelial cells in situ.4–6 These findings raise the possibility that transplanted EPCs may serve not only as a tissue-engineering tool to reconstruct the pulmonary vasculature but also as a vehicle for gene delivery to injured pulmonary endothelium.

We prepared biodegradable gelatin that could hold negatively charged protein or plasmid DNA in its positively charged lattice structure.7,8 We have shown that the gelatin is promptly phagocytosed and then gradually degraded by phagocytes, including macrophages.9 However, whether EPCs phagocytose ionically linked plasmid DNA-gelatin complexes remains unknown. If this is the case, the phago-
cycytic activity of EPCs would allow nonviral gene transfer into EPCs. Here we provide rationale of a novel hybrid cell–gene therapy for pulmonary hypertension.

AM is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.1 There are abundant binding sites for AM in the pulmonary vasculature.10 The plasma AM level increases in proportion to the severity of pulmonary hypertension, and circulating AM is partially metabolized in the lungs.11 Recently, we have shown that intravenous administration of AM significantly decreases pulmonary vascular resistance in patients with heart failure or primary pulmonary hypertension.12,13 These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone. Thus, we hypothesized that transplantation of AM DNA-transduced EPCs would improve monocrotaline (MCT)-induced pulmonary hypertension. To test this hypothesis, we investigated whether EPCs phagocyte DNA-gelatin complexes, which would allow nonviral gene transfer into EPCs; whether intravenously administered EPCs are incorporated into the pulmonary vasculature; and whether transplantation of AM DNA-transduced EPCs ameliorates MCT-induced pulmonary hypertension and improves survival in MCT rats.

Methods

Culture of EPCs

Human umbilical cord blood mononuclear cells were plated on fibronectin-coated dishes and cultured in Medium 199 supplemented with 20% FBS, bovine pituitary extract, vascular endothelial growth factor, basic fibroblast growth factor, heparin, and antibiotics, as reported previously.3,6,14 On days 4 and 8 of culture, nonadherent cells were removed, and medium was replaced. All mothers gave written informed consent, and the study was approved by the ethics committee.

Flow Cytometry

Adherent cells on day 8 of culture were stained by acetylated LDL staining for rat CD31 was performed on frozen sections with mouse anti-human CD31 (clone H9262, Immunotech), RITC-conjugated anti-mouse IgG antibody (DAKO). Isotype-identical antibodies served as controls.

Preparation of Biodegradable Gelatin and Plasmid DNA

We prepared biodegradable cationic gelatin, as a matrix to hold plasmid DNA, as reported previously.2 In brief, a gelatin sample with an isoelectric point of 9.0 was isolated from bovine bone collagen. Gelatin microspheres were prepared through the glutaraldehyde cross-linking of gelatin. The microspheres were washed with acetone and distilled water and then freeze-dried. We constructed the pcDNA1.1-CMV vector (Invitrogen) encoding human AM cDNA or GFP cDNA. The gelatin (5 to 30 μm in diameter, 2 mg) was added to plasmid DNA (200 μg/200 μL in PBS, pH 7.4). After 24-hour incubation at 4°C, DNA-gelatin complexes were obtained.

Ex Vivo Gene Transfer Into EPCs

EPCs (5×10^5) were cultured with ionically linked GFP or AM DNA-gelatin complexes (200 μg/2 mg) for 72 hours. To examine DNA localization, AM plasmid DNA was labeled by rhodamine B isothiocyanate (RITC), as reported previously.4 The nuclei of EPCs were stained by DAPI (Sigma). Immunocytochemistry for AM was performed with a mouse monoclonal antibody against human AM (46-52). Human AM level in culture medium (n=5) was measured by radioimmunoassay.

Assay for AM

The culture medium and lung tissues were acidified with acetic acid, boiled to inactivate intravascular proteases, and lyophilized. Human AM levels in culture medium, lung tissues, and plasma were measured with a radioimmunoassay kit (Shionogi).12

In Vivo Experimental Protocol

Male immunodeficient (F344/N rnu/rnu) nude rats weighing 100 to 120 g were randomly assigned to receive a subcutaneous injection of 60 mg/kg MCT or 0.9% saline. Seven days after MCT injection, 1×10^6 EPCs, 1×10^6 AM-expressing EPCs, or culture medium (500 μL each) was administered intravenously via the left jugular vein. Sham rats also received intravenous administration of 500 μL of culture medium. We used 1×10^6 cells per rat to obtain maximal effects of transplanted EPCs on the basis of dose-response experiments. This protocol resulted in the creation of 4 groups: MCT rats given EPCs (EPC group, n=9), MCT rats given AM-expressing EPCs (AM-EPC group, n=9), MCT rats given culture medium (control group, n=9), and sham rats given culture medium (sham group, n=8). Human mature pulmonary artery endothelial cells served as control cells.

Hemodynamic studies were performed 3 weeks after MCT injection. A polyethylene catheter was inserted into the right femoral artery. An umbilical vessel catheter was inserted through the right jugular vein into the pulmonary artery. Cardiac output was measured in triplicate by the thermodilution method. Pulmonary vascular resistance was calculated by dividing mean pulmonary arterial pressure by cardiac output.

Immunohistochemical and Immunofluorescence Staining

Immunohistochemistry was performed on paraformaldehyde-fixed, paraffin-embedded 5-μm sections of the lungs. To discern human endothelial cells from rat cells, we used mouse anti-human CD31 (DAKO) and mouse anti-rat CD31 (BD PharMingen) monoclonal antibodies. The sections were sequentially developed for the peroxidase and alkaline phosphatase substrates. Immunofluorescence staining for rat CD31 was performed on frozen sections with mouse anti-rat CD31 monoclonal antibody (BD PharMingen) and RITC-conjugated anti-mouse IgG antibody (DAKO).

Morphometric Analysis of Pulmonary Arteries

We analyzed the medial wall thickness of the pulmonary arteries in the middle region of the right lung (20 muscular arteries/rat, ranging in external diameter from 25 to 50 and from 51 to 100 μm). The medial wall thickness was expressed as follows: % wall thickness = [(medial thickness×2)/external diameter]×100.

Survival Analysis

Seven days after MCT injection, 29 rats received intravenous injection of 1×10^6 EPCs (EPC group, n=10), 1×10^6 AM-expressing EPCs (AM-EPC group, n=10), or culture medium (control group, n=9). Survival was estimated from the date of MCT injection to the death of the rat or 10 weeks after transplantation.
Statistical Analysis
Data were expressed as mean±SEM. Comparisons of parameters among the 4 groups were made by 1-way ANOVA, followed by the Scheffe multiple comparison test. Comparisons of the time course of parameters between the 2 groups were made by 2-way ANOVA for repeated measures, followed by the Scheffe multiple comparison test. Survival curves were derived by the Kaplan-Meier method and compared with log-rank tests. A probability value <0.05 was considered statistically significant.

Results
EPCs From Human Umbilical Cord Blood
After 8-day culture of mononuclear cells, spindle-shaped or cobblestone-like adherent cells were observed (Figure 1a). Most of the adherent cells were double stained by DiI-acLDL and FITC-labeled lectin (Figure 1b, c, and d). These cells expressed endothelial cell–specific antigens (KDR, VE-cadherin, and CD31; Figure 1e). In contrast, the majority of adherent cells were negative for monocyte/macrophage marker CD68 and dendritic cell marker CD83. Although a small fraction of the adherent cells expressed monocyte marker CD14, this marker has been shown to also be expressed on activated endothelial cells and cultured EPCs.17 Thus, we confirmed that the major population of the adherent cells were EPCs.

Phagocytosis of DNA-Gelatin Complex by EPCs
EPCs were cultured with GFP DNA-gelatin complexes (Figure 2a). Interestingly, GFP was expressed in EPCs after 72-hour incubation (Figure 2b). Quantitative analyses by FACS confirmed a high incidence (76±3%, n=5) of GFP expression in adherent cells. KDR/GFP double-positive cells made up 70±2% of the adherent cells, whereas CD68/GFP double-positive cells accounted for 2±1% (Figure 2c). Transmission electron microscopy demonstrated that EPCs were phagocytosing DNA-gelatin complexes (Figure 2d). These results suggest that EPCs phagocytose DNA-gelatin complexes in coculture, which allows nonviral, highly efficient gene transfer into EPCs. Unlike gelatin, cationic liposome-mediated transfection efficiency was low (24±3%).

A number of DNA particles labeled by RITC were incorporated into gelatin (Figure 2e). RITC-labeled DNA particles were gradually released from gelatin within EPCs through gelatin degradation (Figure 2f). After 72-hour incubation, RITC-labeled DNA particles released from gelatin were distributed in the cytoplasm of EPCs (Figure 2g). These results suggest the ability of EPCs to take up DNA-gelatin complexes and dissolve the gelatin, freeing the DNA into EPCs. Unlike EPCs, human mature pulmonary artery endothelial cells did not phagocytose DNA-gelatin complexes.

When EPCs were cultured with AM DNA-gelatin complexes, intense immunostaining for AM was observed in EPCs impregnated with AM DNA-gelatin (Figure 3a). After 72-hour incubation, EPCs markedly secreted AM into the culture medium (10-fold increase compared with EPCs alone; Figure 3b). AM overproduction lasted for more than 16 days after gene transfer. AM secretion from EPCs was not influenced by the presence of gelatin (data not shown).

Incorporation of EPCs Into the Pulmonary Vasculature
GFP-expressing EPCs were administered intravenously 7 days after MCT injection. Three days after transplantation,
GFP-expressing EPCs were incorporated into the walls of pulmonary arterioles in MCT rats and composed pulmonary vasculature (Figure 4a). Transplanted GFP-expressing EPCs were distributed on lung tissues (Figure 4b). AM gene-transduced EPCs were similarly incorporated into the pulmonary vasculature (Figure 4c). Immunohistochemical analyses of rat and human CD31 demonstrated that the transplanted EPCs were of endothelial lineage and comprised a vessel structure similar to rat endothelial cells (Figure 4c). However, transplanted EPCs were rarely distributed to other tissues such as cardiac ventricles, kidneys, aorta, and brain (data not shown).

Effects of Gene-Transduced EPC Transplantation on Pulmonary Hypertension
Pulmonary hypertension developed 3 weeks after MCT injection. Mean pulmonary arterial pressure was not strikingly decreased in the EPC group (−14%) but was significantly lower in the AM-EPC group (−29%) than in the control group (Figure 5a). Pulmonary vascular resistance was significantly lower in both the EPC group (−16%) and the AM-EPC group (−39%) than in the control group (Figure 5b). Importantly, the AM-EPC group showed significantly greater improvement in pulmonary vascular resistance than the EPC group. Right ventricular weight and right ventricular...
systolic pressure were significantly lower in the AM-EPC group than in the control and EPC groups (Table). AM levels in plasma and lung tissues were significantly higher in the AM-EPC group than in the other groups 2 weeks after transplantation. Unlike EPCs, transplantation of mature pulmonary artery endothelial cells did not significantly influence pulmonary hemodynamics in MCT rats.

Representative photomicrographs showed that hypertrophy of the pulmonary vessel wall after MCT injection was attenuated in both the EPC and AM-EPC groups (Figure 5c). Quantitative analysis also demonstrated a significant increase in percent wall thickness after MCT injection, but this change was markedly attenuated in the AM-EPC group (Figure 5d). Kaplan-Meier survival curves demonstrated that MCT rats transplanted with AM-expressing EPCs (AM-EPC group) had a significantly higher survival rate than those given culture medium (control group) or EPCs alone (EPC group; Figure 5e).

**Discussion**

In the present study, we present a new concept for cell-based gene delivery into the pulmonary vasculature that consists of 3 processes. First, cationic gelatin is readily complexed with plasmid DNA. Second, EPCs phagocytose ionically linked plasmid DNA-gelatin complexes in coculture, which allows nonviral gene transfer into EPCs with high efficiency. Third, transplanted gene-modified EPCs are incorporated into pulmonary vascular beds in MCT rats. This novel gene delivery system has great advantages over conventional gene therapy: nonviral, noninvasive, and highly efficient gene targeting into the pulmonary vasculature. These benefits may be achieved mainly by the ability of EPCs to phagocytose DNA-gelatin complexes and to migrate to sites of injured endothelium.

Tabata et al.7 and Fukunaka et al.8 demonstrated that gelatin can hold negatively charged protein or plasmid DNA in its positively charged lattice structure. In addition, Tabata et al.9 demonstrated that gelatin is promptly phagocytosed and gradually degraded by macrophages. The present study first demonstrated that EPCs phagocytosed ionically linked DNA-gelatin complexes, dissolved gelatin, and freed the DNA. Surprisingly, the transfection efficiency of this approach was markedly high. FACS analysis demonstrated that EPCs, not monocytes/macrophages, are the main contributors of GFP expression. These findings suggest that the phagocytosing action of EPCs allows nonviral, highly efficient gene transfer into EPCs themselves.

Recently, intravenously administered hematopoietic cells have been shown to be attracted to sites of cerebral injury.18 Intravenously injected EPCs accumulate in ischemic myocardial...
We also investigated whether transplantation of gene-modified EPCs causes additional improvement in pulmonary hemodynamics and survival in MCT rats. AM is one of the most potent vasodilators synthesized by vascular endothelial cells. MCT injures endothelial cells of small arteries and capillaries in the lungs, resulting in pulmonary hypertension. Taking these findings together, transplanted EPCs may circulate in the blood and attach to injured pulmonary endothelia in MCT rats. Thus, EPCs may serve not only as a vehicle for gene delivery to injured pulmonary endothelia but also as a tissue-engineering tool in restoring intact pulmonary endothelium. Transplantation of EPCs without gene modification slightly but significantly decreased pulmonary vascular resistance in MCT rats. EPCs have been shown to express endothelial nitric oxide synthase and produce nitric oxide. In the present study, we showed that EPCs produce AM even when its gene is not transduced. These results suggest that vasodilator substances secreted from EPCs contribute to improvement in pulmonary hypertension.

We also investigated whether transplantation of AM-expressing EPCs improved survival in MCT rats compared with administration of EPCs alone or culture medium. These results suggest that ex vivo gene transfer into EPCs greatly enhances the therapeutic effects of EPC transplantation. Additional studies are necessary to examine whether repeated administration of EPCs produces an even greater effect than single transplantation.

**Conclusions**

Human umbilical cord blood–derived EPCs have a phagocytosing action that allows nonviral, highly efficient gene transfer into EPCs. Transplantation of AM DNA-transduced EPCs causes significantly greater improvement in pulmonary hypertension and better survival in MCT rats than transplantation of EPCs alone. Thus, the novel hybrid cell–gene therapy based on the phagocytosing action of EPCs may be a new therapeutic strategy for the treatment of pulmonary hypertension.

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