Implantation of Mesenchymal Stem Cells Overexpressing Endothelial Nitric Oxide Synthase Improves Right Ventricular Impairments Caused by Pulmonary Hypertension

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Background—Pulmonary hypertension (PH) is a life-threatening disease. Bone marrow cell transplantation is reported to reduce the development of PH by increasing vascular beds in pulmonary circulation. However, adenoviral overexpression of endothelial nitric oxide synthase (eNOS) in the lung is also known to reduce PH. Because mesenchymal stem cells (MSCs) are potential cell sources for neovascularization, the implantation of MSCs overexpressing eNOS (MSCs/eNOS) may further improve the surgical results. We evaluated the efficacy of MSCs/eNOS implantation in monocrotaline (MCT)-induced PH rats.

Methods and Results—MSCs were isolated from rat bone marrow. PH was induced in rats by subcutaneous injection of MCT. One week after MCT administration, the rats received 3 different treatments: MSCs (MSC group), MSCs/eNOS (MSC/eNOS group), or nontreatment (PH group). As the negative control, rats received saline instead of MCT (control group). Right ventricular (RV) hypertrophy and the elevation of RV systolic pressure (RVSP) were evaluated 3 weeks after MCT administration. Moreover, the effects of MSCs/eNOS on survival were investigated in PH induced by MCT 3 weeks earlier. RVSP in both the MSC and MSC/eNOS groups was significantly lower than the PH group. RVSP in the MSC/eNOS group was significantly lower than the MSC group. The RV weight to body weight ratio was significantly lower in the MSC and MSC/eNOS groups than the PH group. The survival time of rats receiving MSCs/eNOS was significantly longer than the nontreatment rats.

Conclusion—Intravenous implantation of MSCs/eNOS may offer ameliorating effects on PH-related RV impairment and survival time. (Circulation. 2006;114[suppl I]:I-181–I-185.)

Key Words: adenovirus • endothelial nitric oxide synthase • mesenchymal stem cells • pulmonary hypertension

Pulmonary hypertension (PH) is a fatal and refractory disease. The refractoriness of PH is caused by a vicious cycle: PH damages the endothelial cells of pulmonary arteries; the damage attenuates production of adrenomedullin, endothelial nitric oxide synthase (eNOS), and other substances from the endothelium; and the attenuated production leads to the constriction of pulmonary arteries and increases their resistance resulting in PH. Numerous approaches have been performed to conquer this refractory disease. Although intravenous drug administration (ie, prostaglandin I2, endothelin receptor antagonist) or the inhalation of nitric oxide (NO) has shown to temporarily reduce PH, these effects have not been persistent.

Recently, the regenerative method and gene therapy have been introduced to break the vicious cycle of PH. To regenerate healthy normal endothelium, the transplantation of bone marrow cells, such as endothelial progenitor cells, endothelial progenitor-like cells, and mesenchymal stem cells (MSCs) has been documented.1,2 The gene transfer of adrenomedullin or eNOS exerts remedial effects on the damaged endothelium of the pulmonary arteries.2–5 Both therapies have achieved better results than conventional therapies. However, their therapeutic action cannot cure PH practically.

In the present study, MSCs gene-transduced with eNOS were implanted intravenously into rats. The gene-transduced MSCs were expected not only to regenerate endothelial cells thereby increasing the vascular beds of pulmonary arteries but also to enhance the secretion of NO from the endothelial cells. These processes are thought to decrease the constriction of pulmonary arteries and their resistance, resulting in a reduction in PH. The purpose of this study was to evaluate the efficacy of the eNOS gene-transduced MSCs implantation in monocrotaline (MCT)-induced PH rats.
Materials and Methods

Animals
Male Sprague-Dawley 5-week-old rats (Japan SLC, Shizuoka, Japan) were used in this study. All animals were maintained in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Expansion of Rat MSCs
Preparation of MSCs was as previously described. Briefly, bone marrow cells were isolated by flushing the cavity of femurs and tibias and transferred to a Primaria tissue culture dish 90 mm in diameter (BD Biosciences, Franklin Lakes, NJ). The isolated bone marrow cells were then cultured in minimal essential medium (MEM) (Sigma, St Louis, Mo) supplemented with 10% fetal bovine serum (BD Biosciences Clontech, Palo Alto, Calif), 100 μg/mL of streptomycin and 100 U/mL of penicillin (Sigma). When adherent cells were confluent (defined as passage 0), they were continuously cultured as MSCs until passage 3 to 5 using 0.25% trypsin and 1 mmol/L EDTA (Sigma) for 5 minutes. All culture experiments in this study were performed at 37°C and in 5% humidified CO2.

Construction of MSCs with eNOS/Adeno-X Tet-Off Viruses
Polymerase chain reaction was performed using the oligonucleotides 5'-cttagaattgcgaacttggaag-3' (XbaI sense primer) and 5'-cttaaggattgtcaggaacca-3' (AflII antisense primer) with rat heart cDNA as a template to amplify a fragment consisting of the XbaI/AflII sites. The polymerase chain reaction product was subcloned into the XbaI/AflII sites of pShuttle (BD Biosciences Clontech) and sequenced by the dyeoxydeoxynucleotide chain termination method using the DYEnamic ET terminator cycle sequence kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions. The full-length eNOS cDNA was inserted into the multicloning site of pDNR-CMV in Adeno-X Tet-Off Expression System 2 (BD Biosciences Clontech) to construct eNOS/pDNR-CMV. The eNOS/Adeno-X Tet-Off vector was then generated from eNOS/pDNR-CMV and pLP-Adeno-X-TRE (BD Biosciences Clontech) by the Cre-loxP site-specific recombination method, and was packaged into infectious adenoviruses by transduction of HEK293 cells. The eNOS/Adeno-X Tet-Off virus particles were purified and concentrated with Virakit (Virapur, Carlsbad, Calif). The titer of the eNOS/Adeno-X Tet-Off virus was determined by cytopathic changes in infection of HEK293 cells by the serial dilution method. The cultured MSCs were exposed to the eNOS/Adeno-X Tet-Off viruses and the regulatory viruses, which had a tetracycline-controlled activator, at a multiplicity of infection of 2000 in 2 mL of fresh culture medium containing 2% fetal bovine serum for 1 hour. Next, the MSCs that had been exposed to the eNOS/Adeno-X Tet-Off viruses were cultured for 48 hours in 5% of complete culture medium, resulting in MSCs/eNOS. These MSCs were then harvested with phosphate-buffered saline containing 0.25% trypsin and 1 mmol/L EDTA immediately before use. In the case of MSCs/eNOS, the cells were harvested using rubber scrapers, because they were too vulnerable to be harvested by the trypsin method. These cells were washed and then resuspended in culture medium.

Measurement of eNOS Activity
NOS activity was measured from the conversion rate of L-[3H] arginine to L-[3H] citrulline in homogenates of MSCs. The MSCs were detached from tissue culture dishes using rubber scrapers and the cell suspension in phosphate-buffered saline was centrifuged at 15 000 rpm at 4°C for 5 minutes. The pellet was suspended in homogenization buffer containing 25 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, and 1 mmol/L EGTA. The reaction mixture contained 10 mmol/L NADPH, 0.6 mmol/L CaCl2, 1 μmol/L FAD, 1 μmol/L FMN, 5 μmol/L tetrahydrobiopterin, 0.1 μmol/L Calmodulin, 25 mmol/L Tris-HCl, pH 7.4, and L-[3H]arginine. The reaction was initiated by the addition of the homogenates and performed for 8 minutes at 37°C, after which the reaction was quenched with 0.4 mL of ice-cold stop buffer containing 50 mmol/L HEPES, pH 5.5, and 5 mmol/L EDTA. Some experiments were also performed in the presence of Nω-Nitro-l-arginine methyl ester, hydrochloride (L-NAME) (1 mmol/L) as an inhibitor of NOS. The amount of L-[3H] citrulline content was determined by liquid scintillation counting after separation from the reaction mixture by passage through a column containing the cation exchange resin Dowex 50WX-8 (100 μL of the Na+ form). As the negative control, MSCs were exposed to the eNOS/Adeno-X Tet-Off viruses and the regulatory viruses in tetracycline containing medium, resulting in the inhibition of eNOS gene transduction.

MCT-Induced PH Model
To examine the effects of MSCs/eNOS on both prevention and reversal of PH, the rats were randomly assigned to 2 experimental groups, followed by subcutaneous injection of 60 mg/kg MCT.

To examine the preventative effects of MSCs/eNOS, MCT-treated rats were divided into 3 groups 1 week after MCT injection: rats that received no treatment (PH group, n = 10); rats that received 1 × 106 MSCs (MSC group, n = 10); and rats that received 5 × 105 MSCs/eNOS (MSC/eNOS group, n = 10). For the negative control, rats received a subcutaneous injection of 0.9% saline instead of MCT (control group, n = 10). For cell implantation, rats were anesthetized with an intraperitoneal injection of pentobarbital following an inhalation of ether, and the right femoral vein then exposed to inject the cell suspension. Three weeks after MCT injection, after reanesthetization, rats were injected with a 3-Fr Miller catheter via the right jugular vein into the right ventricle to obtain measurements of right ventricular systolic pressure (RVSP). The rats were then euthanized and the hearts were harvested. The ratio of right ventricular free wall to body weight (RV/body weight ratio) was determined.

To examine reversal of PH, a separate set of MCT-treated rats were divided into 2 groups 3 weeks after MCT injection: MCT-treated rats that received 1 × 106 MSCs (PH-MSC group, n = 10); and MCT-treated rats that received 5 × 105 MSCs/eNOS (PH-MSC/eNOS group, n = 10). The survival time after MCT injection was compared among the PH-MSC group, the PH-MSC/eNOS group, and the PH group.

Statistical Analysis
All results are expressed as mean±standard error. Comparisons among groups were made with the ANOVA Kruskal-Wallis test. If ANOVA reveals a difference, Fisher protected least significant difference post-hoc test was used to determine significant differences. Significant changes were considered present when probability values were less than 0.05.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

Characters of MSCs
Most cells were spindle-like in shape and adhered to the plastic tissue culture dishes. The rest were small and round in shape, and attached to the spindle-like cells. Most of the gene-transduced MSCs appeared to continuously adhere to the tissue culture dishes when infected at high multiplicity of infection (Figure 1). The efficiency of gene transduction by the adenovirus vector was 90%, which was determined using lacZ gene-transduction.

The MSCs/eNOS induced NOS activity, and this activity was inhibited by the addition of L-NAME. MSCs without eNOS gene-transduction produced the same amount of NOS activity as the MSCs with eNOS gene-transduction and L-NAME (Figure 2).
Reduction of RV Impairment Induced by MCT

RVSP data are shown in Figure 3. RVSP was significantly elevated in the PH group compared with the control group (58.70 ± 1.86 versus 29.60 ± 1.44 mm Hg, \(P < 0.0001\)). However, RVSP in both the MSC and MSC/eNOS groups was significantly lower than in the PH group (58.70 ± 1.86 versus 42.30 ± 1.10 mm Hg, 58.70 ± 1.86 versus 32.60 ± 3.17 mm Hg; \(P < 0.0001\)). The RVSP of the MSCs/eNOS group was significantly lower than that of the MSC group (32.60 ± 3.17 versus 42.30 ± 1.10 mm Hg; \(P < 0.0001\)).

The RV/body weight ratio was significantly lower in both the MSC (0.462 ± 0.029 g/kg) and MSCs/eNOS groups (0.435 ± 0.042 g/kg) than in the PH group (0.590 ± 0.084 g/kg, \(P < 0.05\)) (Figure 4). Survival time in the MSC and MSC/eNOS groups was considerably longer than in the PH group (data not shown).

Reversal Effects

Survival time (Figure 5) in the PH-MSC/eNOS group was significantly longer than in the PH group (19.67 ± 3.11 versus 10.89 ± 3.63 days; \(P < 0.05\)). Although survival time of the PH-MSC group was also longer than that of the PH group, no significant differences were observed (15.6 ± 4.32 days). Similarly, although survival time of the PH-MSC/eNOS group was longer than that of the PH-MSC group, no significant differences were observed.

Discussion

In the current study, we have shown that the intravenous implantation of eNOS gene-transduced MSCs improves the progression of RV impairment caused by MCT-induced PH. The MCT-induced PH model is known to represent similar pathology to that of primary pulmonary hypertension, which is injured pulmonary arterial endothelium and induced media thickness. This conclusion is based on the following results: (1) both the MSC and MSC/eNOS groups significantly inhibited RVSP progression compared with the nontreated PH group; (2) both the MSC and MSC/eNOS groups significantly reduced the RV/body weight ratio compared with the nontreated PH group; and (3) the MSC/eNOS group significantly prolonged the survival time compared with the nontreated PH group.

In the present study, we chose MSCs as a cell source because MSCs have favorable features for the genetically modified cell seeding technique.\(^{10,11}\) The separation from other subsets of bone marrow cells by the property of adhering to plastic dishes can be easily performed. Moreover, MSCs are known to be rapidly amplified and tough enough to survive even under low-serum conditions.\(^{8}\) Thus, some have suggested that MSCs have the pluripotent ability to become endothelial progenitor cells and other cell lineages.\(^{1,6,12,13}\)
Moreover, MSCs are stroma cells that feed other cells and produce various cytokines such as angiogenic factors, which are appropriate for each site. MSCs are reported to have the tendency to home in on injured locations. Although we have not confirmed the phenomena of lodging and homing to the pulmonary arteries, the fact that even the implantation of MSCs alone was able to prevent progression of RV impairment caused by PH may imply these phenomena. In fact, MSCs have been reported to lodge in the pulmonary circulation in PH models by intravenous administration.

Adenoviral gene transfer is one of the most reliable methods for introducing genes into mammalian cells. Infection by adenovirus is not cell-cycle dependent. The expression is transient, because adenoviral DNA normally remains episomal. Adenoviruses are capable of infecting various cells as well as organs. In the present study, the transduction efficiency into MSCs was high, as previously described.

Cell damage by eNOS overexpression was observed by many investigators. Kim et al also reported that NO is able to induce apoptosis. Hence, regulation of gene expression is essential to prevent cell death in the amplification of the recombinant adenovirus in HEK293 cells. In the current study, we succeeded in constructing a recombinant adenovirus with eNOS cDNA under the control of a tetracycline-regulated promoter.

The results of the present study imply the efficacy of MSCs implantation for PH-induced RV impairment. Although the underlying mechanism is not yet determined, several factors are expected to contribute. First, MSCs that lodged and homed to the lesion may have been regenerated, which may have increased the microvascular beds, eventually leading to a decrease in vascular resistance. Second, NO may have played a crucial role in vascular tone and regeneration. The eNOS gene-transduction would have induced the restoration of attenuated NOS production from the injured endothelium, resulting in vasodilation and regeneration. To determine the precise mechanism involved, morphological and/or histochemical examinations need to be conducted.

On the other hand, an overdose of MSCs is supposed to lead to pulmonary embolism. In a separate set of our experiments, $1 \times 10^6$ MSCs/eNOS caused animal death soon after administration (3 of 3 cases). Although the cause of death was not investigated, the amount of administered MSC appears critical and should perhaps be limited. In the present study, MSCs overexpressing eNOS offered therapeutic effects for RV impairment with half of the administered amount of MSCs. MSCs alone elicited the same effects with twice the amount. These results suggest that eNOS is able to reduce the amount of administered cells required and that the administration of MSCs overexpressing eNOS is preferable to MSCs alone in the treatment of RV impairment caused by PH.

In the clinical sphere, PH or RV impairment caused by PH is devastating diseases. For the treatment of PH, numerous approaches, including intravenous infusions of drugs or the inhalation of NO have been introduced, and have offered promising results. However, such effects have not been persistent. The genetically modified cell seeding technique used in the current study is expected to elicit therapeutic effects for a longer period. Furthermore, this seeding method of intravenous administration is less interventional and therefore, repeatable. In addition, this gene-transfer method is safer than other methods mediated by virus vectors, because it involves ex vivo gene transfer. Moreover, the pulmonary arterial pressure in patients with long-standing PH sometimes resists reducing, even after heart surgery. This method may be effective in combination with/or after surgical repair.

Although the results of the current study revealed that intravenous implantation of MSCs overexpressing eNOS may provide a new insight into overcoming the RV impairment caused by PH, several problems remain to be resolved. First, no histochemical analysis was performed in this study. Hence, whether regenerative responses including recruitment and homing of implanted MSCs occurred in the pulmonary microvasculature is unclear. Second, whether several factors other than pulmonary arterial pressure and RV hypertrophy contribute to the prolongation of survival time has not been established. The possibility exists that NO itself may have elicited cytoprotective effects through a certain mechanism independent of vascular regeneration and dilatation. Although several issues need to be addressed, to our knowledge, this is the first report of eNOS gene-transduced MSC seeding for the treatment of PH.

**Conclusion**

Intravenous administration of MSCs overexpressing eNOS reduced RV impairment caused by PH.

**Disclosures**

None.

**References**


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