in the artery was created by passing an inflated balloon through the lumen five times [19]. After balloon injury, the ultra miniature pressure transducer catheter SPR-671 (Millar Instruments, Houston, TX, USA) was advanced into the left ventricle to examine the hemodynamic effects of CNP infusion in three rats.

*2.2.2. Hemodynamic effects of CNP after balloon injury*

In the three rats in which a left ventricular catheter was inserted after balloon injury, the left ventricular pressure was digitally recorded at baseline and during CNP infusion at the rate of 0.1 μg/kg/min and 1.0 μg/kg/min for 5 min each.

*2.2.3. Treatment of rats*

The remaining 33 rats were divided into five groups with various post-injury short-term treatments as follows: (1) control, balloon injury only ($n$=6); (2) high-dose CNP, infusion of CNP at 1.0 μg/kg/min for 24 h ($n$=6); (3) low-dose CNP, infusion of CNP at 0.1 μg/kg/min for 24 h ($n$=6); (4) low-dose CNP+US/MBs, infusion of CNP at 0.1 μg/kg/min for 24 h begun with US/MBs treatment ($n$=9); and (5) US/MBs, US/MBs treatment only ($n$=6).

The treatment protocol is shown in Fig. 1. Infusion of CNP was initiated immediately after balloon injury. For the US/MBs treatment, the S4 transducer was positioned 4 cm from and perpendicular to the injured carotid artery via an acoustic coupler. US with a transmission frequency of 1.8 MHz and mechanical index of 1.0 was applied at a frame rate of 30 Hz for 10 min, during which MBs (10% Optison®) was infused via the femoral vein at 0.2 ml/min ($4\times10^6$ microbubbles/min) for the first 5 min of the 10-min insonation. The echograms were recorded at baseline, during, and after MBs infusion to ensure the presence of MBs in the carotid artery. The US/MBs treatment was initiated 10 min after balloon injury and repeated 5 times at 3-min intervals. Thus the US/MBs treatment was completed within the first 80 min of the 24-h infusion of CNP following balloon injury.

*2.2.4. Morphometric analysis*

All the rats excluding three of the nine rats in the low-dose CNP+US/MBs group were euthanized with an overdose of sodium pentobarbital and the carotid arteries were dissected, fixed, and sectioned for analysis 14 days after the balloon injury. The remaining three rats in the low-dose CNP+US/MBs group were kept for an additional 14 days to assess the long-term effect of the treatment following balloon injury and were subjected to the same histopathological analyses at 28 days after treatment. Intimal and medial areas were measured in four consecutive sections in each vessel with a digital analysis system using the NIH Image version 1.62 (National Institutes of Health, Washington, DC, USA), and the intima/media (I/M) ratio was calculated [10,11]. Morphometric analysis was performed by a person (IK) who was blinded to the treatment condition of each rat.

*2.3. Statistical analysis*

Data are expressed as mean±S.D. For comparison between multiple groups, one-way ANOVA with Bonferroni's correction was used. For comparison of hemodynamic parameters at different infusion rates of CNP, one-way repeated measures of ANOVA was used. Differences were considered significant at $P<0.05$.

## 3. Results

*3.1. In vitro study*

The cGMP level in SMCs was rapidly increased and peaked at 10 min after incubation with CNP. Fig. 2 compares the peak cGMP levels among the various conditions. The peak cGMP level was 2-fold higher with CNP plus combined US/MBs than with CNP alone. Neither MBs nor US alone augmented the effect of CNP on cGMP production in SMCs. Treatment with MBs combined with US in the absence of CNP showed no effect on cGMP production. Interestingly, the addition of MBs alone to CNP without US even reduced cellular cGMP as compared with CNP alone. The concentration of free CNP in the medium after the removal of MBs fractions from the mixture was reduced to $0.37\times10^{-7}$ mol/l (37% of the initial concentration of $1.0\times10^{-7}$ mol/l), which may suggest that MBs may change the dis-

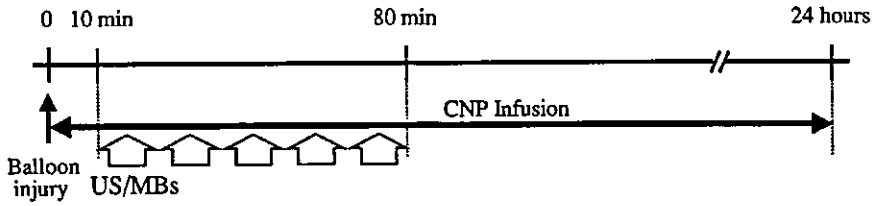


Fig. 1. Treatment protocol after balloon injury. Infusion of CNP was begun immediately after balloon injury and continued for 24 h. The US/MBs treatment shown as a white arrow was begun 10 min after balloon injury and repeated five times at 3-min intervals. The US/MBs treatment consisted of 10 min of US irradiation and MBs infusion for the first 5 min of the 10-min insonation. All US/MBs treatments were completed in the first 80 min of CNP infusion.

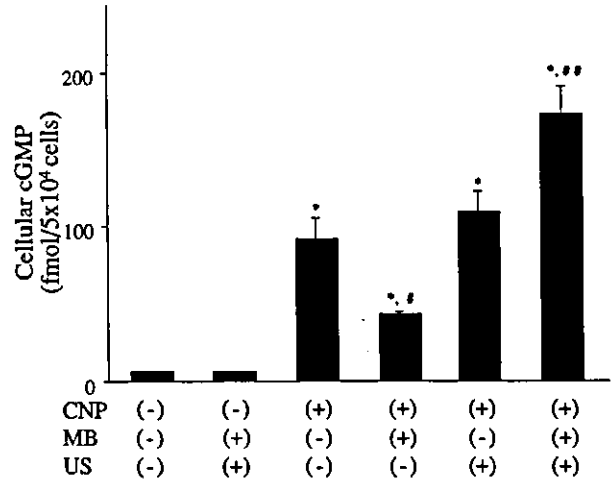


Fig. 2. Comparison of cellular cGMP concentrations in cultured smooth muscle cells in various conditions. A two-fold increase in the cellular cGMP level was exhibited with CNP(+)MB(+)US(+) compared with CNP(+)MB(−)US(−). Cellular cGMP concentration in CNP(+)MB(+)US(−) was about one-half that in CNP(+)MB(−)US(−). * $P<0.01$ vs. CNP(−)MB(−)US(−) and CNP(−)MB(+)US(+), # $P<0.05$ vs. CNP(+)MB(−)US(−), ## $P<0.001$ vs. CNP(+)MB(−)US(−).

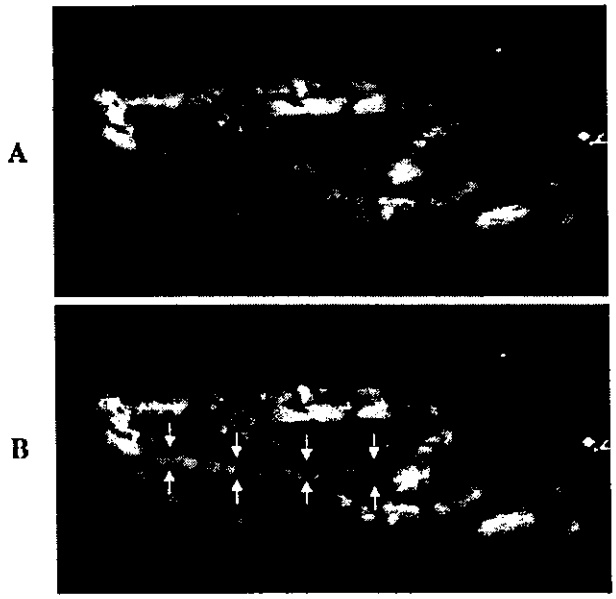


Fig. 3. Contrast echograms of carotid artery during US/MBs treatment. Although no appreciable structure for common carotid artery was demonstrated at baseline (A), a bright linear structure was visualized during MBs infusion as indicated by arrows (B).

tribution of CNP in the medium, presumably by trapping CNP on their surface.

### 3.2. In vivo study

#### 3.2.1. Hemodynamic effects of CNP after balloon injury

Table 1 summarizes the hemodynamic effects of low-dose and high-dose CNP obtained with high-fidelity recordings of the left ventricular pressure. Although no significant effects on left ventricular maximum $dP/dt$, left ventricular end-diastolic pressure, or heart rate were demonstrated, the peak systolic pressure was significantly decreased during high-dose CNP infusion.

#### 3.2.2. Contrast echography of carotid artery

Fig. 3 displays echograms obtained during US/MBs treatment in one rat. Although no appreciable structure for the common carotid artery was demonstrated before MBs infusion (Fig. 3A) because of the low carrier frequency of the ultrasound, a bright linear structure corresponding to the common carotid artery (arrows) was visualized in all rats that received MBs infusion during US/MBs treatment (Fig. 3B).

Table 1
Hemodynamics data

| | Baseline | Low-dose | High-dose |
|---|---|---|---|
| HR (bpm) | 249±3 | 267±30 | 259±16 |
| LVPSP (mmHg) | 102±4 | 102±3 | 82±6* |
| LVEDP (mmHg) | 5.9±0.9 | 6.5±0.5 | 4.1±0.1 |
| Max $dP/dt$ (mmHg/s) | 6233±208 | 6166±208 | 5300±100 |

Data are expressed as mean±standard deviation. HR, heart rate; LVPSP, left ventricular peak systolic pressure; LVEDP, left ventricular end-diastolic pressure; Max $dP/dt$, maximum $dP/dt$. * $P<0.05$ vs. baseline.

#### 3.2.3. Effects on neointimal formation

Fig. 4 displays representative histological sections of the carotid artery at the injured site at 14 days (Fig. 4A–E) and at 28 days (Fig. 4F) after treatment. Neointimal hyperplasia at the injured site was almost completely inhibited by high-dose CNP (Fig. 4B) compared with the control (Fig. 4A). Although low-dose CNP alone failed to prevent neointimal formation (Fig. 4C), combined US/MBs plus low-dose CNP successfully inhibited intimal thickening (Fig. 4D). The inhibition of intimal thickening persisted until 28 days after treatment in this group (Fig. 4F). US/MBs in the absence of CNP showed no effect on neointimal formation (Fig. 4E).

Fig. 5 compares the I/M ratios on day 14 among the groups. Although high-dose CNP reduced the I/M ratio by 82% compared to control, low-dose CNP produced only a non-significant 17% reduction in the I/M ratio. However, the US/MBs treatment exhibited a 4-fold augmentation of the effect of low-dose CNP, which resulted in an I/M ratio similar to that obtained with high-dose CNP. US/MBs alone resulted in no difference in the I/M ratio compared with control. The I/M ratio of the rats in the low-dose CNP+US/MBs group at 28 days after the short-term treatment was 0.16±0.04, which was similar to the ratio at 14 days obtained in the same treatment group.

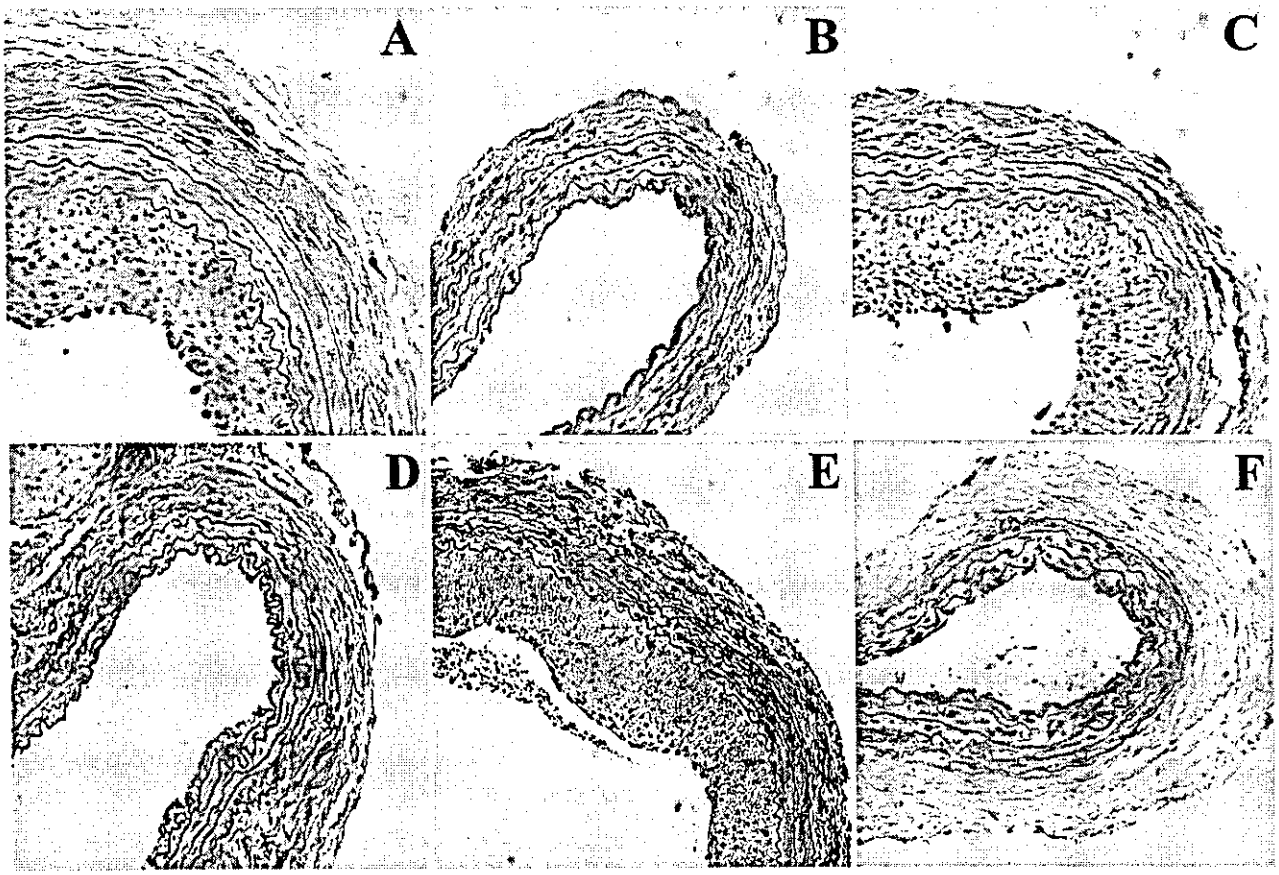


Fig. 4. Photomicrographs (hematoxylin–eosin staining, 100×) of the histological sections of carotid arteries 14 days (A–E) and 28 days (F) after balloon injury. Control (A) and those treated with high-dose CNP (B), low-dose CNP (C), low-dose CNP combined with US/MBs (D), and US/MBs alone (E) are shown. Neointimal formation was almost completely inhibited in high-dose CNP (B) and low-dose CNP+US/MBs (D,F). This effect in low-dose CNP+US/MBs persisted until 28 days (F).

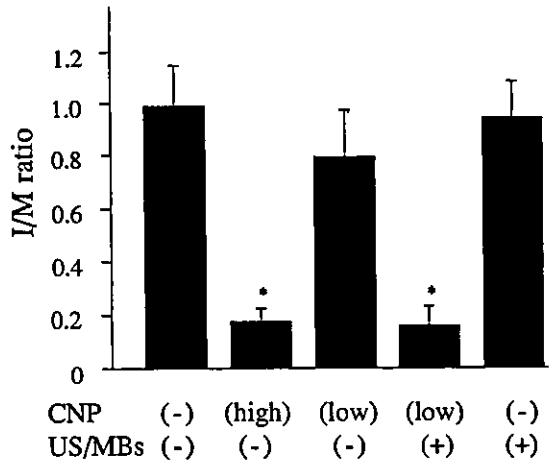


Fig. 5. Comparison of the I/M ratios among the groups. US/MBs, combined application of ultrasound and microbubbles; high, 1.0 μg/kg/min; low, 0.1 μg/kg/min. I/M ratios were similarly lower in high-dose CNP and low-dose CNP +US/MBs than balloon injury only (CNP(−)US/MBs(−)). * $P<0.0001$ vs. CNP(−)US/MBs(−).

## 4. Discussion

The present study demonstrated that a relatively short-term (24 h) treatment with high-dose CNP (1.0 μg/kg/min) immediately after balloon injury inhibited neointimal hyperplasia for 14 days in the rats, the extent of which was comparable to that achieved in previous studies employing a long-term (5-day [10] or 14-day [10,11]) continuous infusion of the same dose. Moreover, the combination of US/MBs treatment in the first 80 min of the 24-h CNP infusion augmented this effect, which persisted beyond 14 days at least until 28 days after the treatment. Thus, this study suggests a new treatment approach that employs combined US/MBs treatment and CNP infusion for a clinically feasible time period to prevent restenosis following intravascular interventions.

### *4.1. CNP to prevent vascular remodeling*

CNP secreted by endothelial cells has multiple effects, including vasodilation and inhibition of vascular SMCs

migration and proliferation through the cGMP cascade [7–9]. In atherosclerotic lesions, endothelial CNP production is decreased [20], whereas the CNP-specific receptor NPR-B of vascular SMCs is overexpressed [6]. Arterial interventions cause endothelial injury resulting in the loss of endothelium-derived anti-proliferative factors including CNP and nitric oxide, both of which regulate the intracellular cGMP levels in the arterial wall [21,22]. Therefore, compensation of CNP at the injured site has been attempted to prevent restenosis.

In this regard, a systemic high-dose administration that potentially affects systemic hemodynamics [23] was performed to yield a sufficient concentration of CNP at the target site for long periods [10,11]. Other authors used an infiltrator angioplasty balloon catheter to perform adenovirus-mediated transfer of the CNP gene [24]. However, after gene transfer, the reduced local CNP concentration may not be compensated until the expression of CNP in the target site becomes sufficient, which may explain the lack of a difference in the degree of neointimal hyperplasia between the sites with control gene transfection and those with CNP gene transfection in their study [24]. By contrast, in our study, a high-dose CNP infusion for only 24 h immediately after the injury was as effective in preventing neointimal formation as the 2-week continuous infusion at the same rate employed in previous studies [10,11]. Moreover, combined US/MBs treatment in the first 80 min of the 24-h CNP infusion at a much lower infusion rate (0.1 μg/kg/min), one-tenth the previously reported rate [10,11], significantly augmented the anti-proliferative effect of CNP to a level similar to that obtained with high-dose CNP. Furthermore, neointimal formation remained inhibited until 28 days after the treatment, after which the I/M ratio is known to barely increase [25,26]. This suggests that the effect of CNP in preventing neointimal proliferation may be related to the early events that occur in response to vascular injury.

CNP has been shown to inhibit migration of stimulated SMCs [8], which has been thought to lead to their proliferation and resulting in neointimal hyperplasia [27]. More recently, it has been reported that bone marrow-derived smooth muscle progenitor cells mobilized to the injured site can differentiate into SMCs to contribute to subsequent neointimal development [3]. CNP may inhibit the migration of SMCs or their progenitor cells, both of which occur early after vascular injury.

Alternative explanations for the favorable effect of relatively short-term CNP administration may be related to their possible anti-inflammatory actions. Balloon inflation induces an infiltration of inflammatory cells into the vessel wall, the greatest increase being seen at 24 h after injury [28]. Significant accumulation of neutrophils and macrophages occurs in the adventitia in the first 24 h after balloon injury [29], which precedes the onset of cell proliferation beginning between 48 and 72 h [30]. Growth factors or their inhibitors placed in the adventitia can significantly affect intimal development after balloon injury [31,32]. Therefore, the accumulation of inflammatory cells in the adventitia might be an important source for growth factors and cytokines stimulating intimal thickening after angioplasty. In addition, rapid accumulation of adhesion molecules on the luminal surface and neutrophil recruitment occur after endothelial denudation [33]. Thus, regardless of adventitial and luminal sides, infiltration of leukocytes plays a role in restenosis development. In this regard, sirolimus, which has recently been shown to prevent in-stent restenosis, blocks inflammation at the angioplasty site [34]. A monoclonal antibody to ICAM-1 significantly suppresses intimal hyperplasia in the rat model of restenosis [35]. The blockade of the selectin family by an analogue of sialyl-Lewis$^x$ [36] and the knock-out of P-selectin [37] can also reduce neointimal hyperplasia. Moreover, leukocyte depletion by either mustine or an antibody against the leukocyte common antigen has been shown to inhibit neointimal hyperplasia after balloon angioplasty in a rabbit model [38].

CNP derived from the CNP gene plasmid transfected to the site of balloon angioplasty prevented the adventitial inflammatory/proliferative response [24]. As was expected by the authors who reported the production and secretion of CNP by inflammatory cells [39], our preliminary data have shown that CNP has anti-inflammatory actions including the inhibition of the expression of adhesion molecules on neutrophils, which diminishes reperfusion injury in a renal ischemia/reperfusion model [40]. These data lead us to the hypothesis that CNP may exert anti-leukocyte actions when administered immediately after vascular injury, which may be a mechanism of inhibition of the neointimal proliferation at the early phase of the response to the vascular injury.

### 4.2. Mechanisms of enhancement of CNP by US/MB

Previous investigators successfully utilized ultrasound-mediated MBs destruction to induce 'cavitation' for 'sonoporation' [12] of the cell membrane to deliver genes or plasmid DNA into cells and tissues [13,14]. The 'cavitation' may allow a direct 'micro-injection' [41] of CNP into the arterial wall from the lumen side or a microvascular disruption [42] at the level of the vasa vasorum, both of which may increase CNP concentration in the arterial wall. In addition, the occurrence of a 'microstream' near the cell surface may increase the opportunities for ligand–receptor contacts. In fact, US/MBs augmented cGMP production in cultured SMCs that were not in the tissue but were exposed in the medium. Moreover, our in vitro experiments demonstrated that CNP concentration in the medium was reduced after removal of MBs fractions from the mixture and that production of cGMP in SMCs combined with both CNP and MBs in the absence of US was less compared with CNP alone. These results may

suggest another mechanism by which MBs trap CNP molecules on their surface to limit the action of CNP until the MBs are disrupted by exposure to US. This mechanism may be beneficial in in vivo settings, because diffusion of CNP or its degradation by neutral endopeptidase [43] can be minimized in the systemic circulation and local release of CNP only at the insonated site may be provided.

### 4.3. Clinical implications

Although the precise mechanisms remain unknown, the local enhancement of the bioeffects of CNP shown in this study has several advantages. The use of CNP, an endogenous molecule, eliminates possible hazards arising from the use of viral vectors or the gene itself in gene delivery. Both the MBs and US systems employed in the present study are approved for clinical use and safety. Moreover, when US and MBs were combined, one-tenth of the effective dosage of intravenous CNP achieved nearly complete prevention of neointimal formation. In contrast to the reported high-dose of CNP that decreased systolic blood pressure, low-dose CNP infusion exerted almost no significant hemodynamic effects in this study. This may be an advantage for the application of this proposed treatment in patients undergoing intravascular interventions, in whom an unfavorable blood pressure decline should be avoided. Of importance, a relatively short-term treatment with CNP and US/MBs early after the vessel injury has been found to be effective. This not only suggests that the effect of CNP may be related to the early events in response to the vascular injury, but also implies its future clinical application as an immediate adjunct to intravascular interventions.

### 4.4. Study limitations

Several limitations exist in this study. First, the long-term effects of the proposed treatment beyond 28 days post-procedure remain unknown. However, previous studies employing the carotid artery injury model also evaluated the neointimal formation 2 weeks after injury [10,11,44–46]. It is known that the mitogenesis of the intimal smooth muscle cells reaches its maximum level within 7 days after the injury [26] and that the I/M ratio peaks by 4 weeks and plateaus thereafter at least until 16 weeks after the injury [25]. Therefore, we also evaluated the outcome of the treatment at 14 days post injury and confirmed the persistence of the effect of the proposed treatment up to 28 days after treatment. Second, the precise mechanisms for the effectiveness of the present treatment are unknown. Finally, future studies are necessary to determine the minimally required dose or duration of CNP administration, and optimal MBs concentration and US parameters specific to variable acoustic properties of the tissues surrounding the target vessels.

### 4.5. Conclusions

A relatively short-term intravenous infusion of CNP immediately after vascular injury can inhibit neointimal formation. Short-term ultrasound irradiation with microbubble infusion at the beginning of the CNP infusion potentiates the effect. Thus, CNP infusion combined with ultrasound and microbubbles may provide a practical and safe approach for the prevention of restenosis following intravascular interventions.

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# 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-

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cytic 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 phagocytose 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.

### Fluorescent Staining for EPCs

Adherent cells on day 8 of culture were stained by acetylated LDL labeled with DiI (DiI-acLDL, Biomedical Technologies) and fluorescein isothiocyanate (FITC)-labeled lectin from ulex europaeus (Sigma). Double-positive cells for DiI-acLDL and FITC-labeled lectin were identified as EPCs, as reported previously.[15,16]

### Flow Cytometry

Adherent cells on day 8 of culture and green fluorescent protein (GFP) gene-transduced cells were analyzed by fluorescence-activated cell sorting (FACS; FACS SCAN flow cytometer, Becton Dickinson). Cells were incubated for 30 minutes at 4°C with phycoerythrin-conjugated mouse monoclonal antibodies against human CD14 (clone M5E2), CD31 (clone L133.1), CD68 (clone Y1/82A), and CD83 (clone HB15e; all from Becton Dickinson) and mouse monoclonal antibodies against human KDR (clone KDR-1, Sigma) and VE-cadherin (clone BV6, Chemicon). 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.[7] 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 ($5\times10^5$) 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.[8] 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 intrinsic 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, $1\times10^6$ EPCs, $1\times10^6$ 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 $1\times10^6$ 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=8), 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 $1\times10^6$ EPCs (EPC group, n=10), $1\times10^6$ 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.

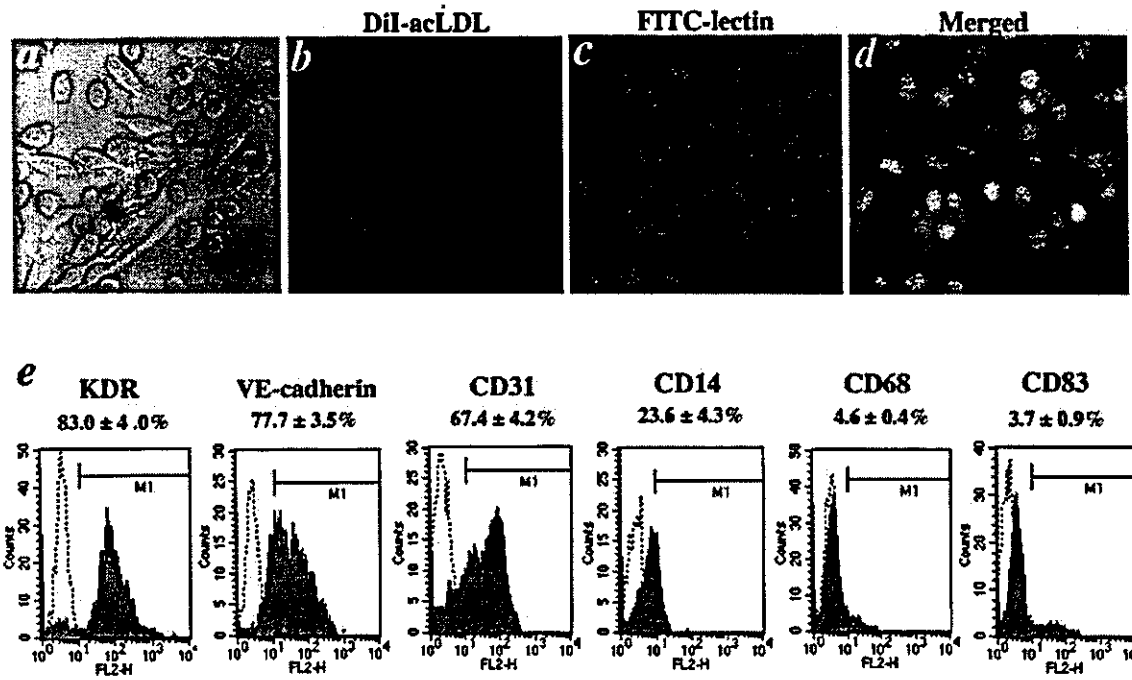


**Figure 1.** Characterization of EPCs derived from human umbilical cord blood. EPCs exhibited spindle-shaped or cobblestone-like morphology (a) and took up DiI-acLDL and FITC-labeled lectin in same field (b–d). e, Flow cytometric analysis of adherent cells on day 8. Most of adherent cells expressed endothelial lineage markers (KDR, VE-cadherin, and CD31), whereas they were negative for CD68 and CD83.

### 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,

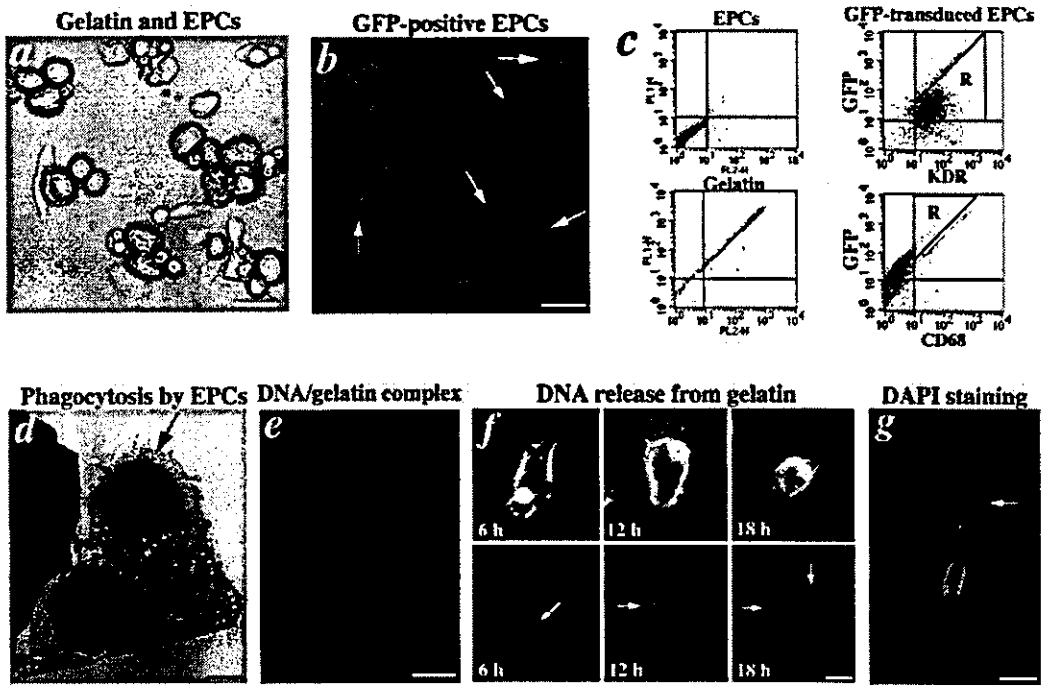


**Figure 2.** Ex vivo gene transfer into EPCs based on phagocytosing action. a, EPCs were cultured with ionically linked GFP DNA-gelatin complexes. b, GFP was highly expressed in EPCs (arrows) in same field as Figure 2a. c, Flow cytometric analyses of EPCs cultured with GFP DNA-gelatin complexes. Negative controls (EPC isocontrol and gelatin background) are shown in left panels. d, Transmission electron microscopy revealed that EPCs had phagocytosed GFP DNA-gelatin complexes (arrows). e, RITC-labeled DNA particles were incorporated into gelatin. f, RITC-labeled DNA particles (red, arrows) were released from gelatin through its degradation. g, RITC-labeled DNA particles released from gelatin (arrow) were distributed in cytoplasm of EPCs. Nuclei of EPCs were identified by DAPI staining. Scale bars: 10 μm (a and b); 2 μm (d and e); 5 μm (f and g).

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

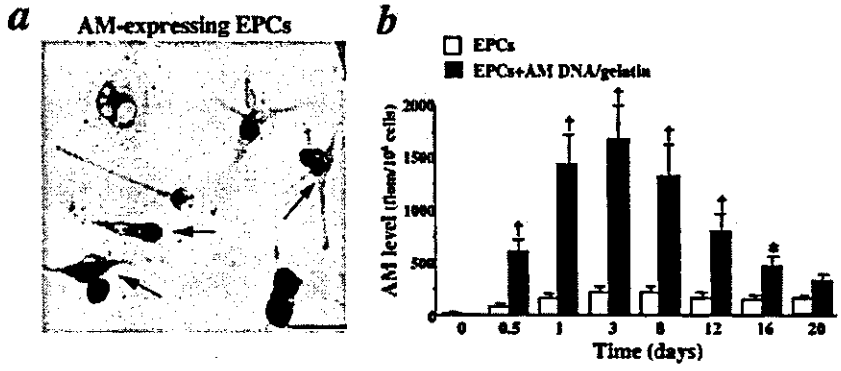


**Figure 3.** AM gene transfer into EPCs. a, Immunohistochemical analysis of AM in EPCs after gene transfer. Intense immunostaining for AM was observed in EPCs (arrows). Scale bar: 10 μm. b, Time course of AM secretion from EPCs during coculture with AM DNA-gelatin complexes. Data are mean±SEM. *$P<0.05$, †$P<0.001$ vs EPCs.

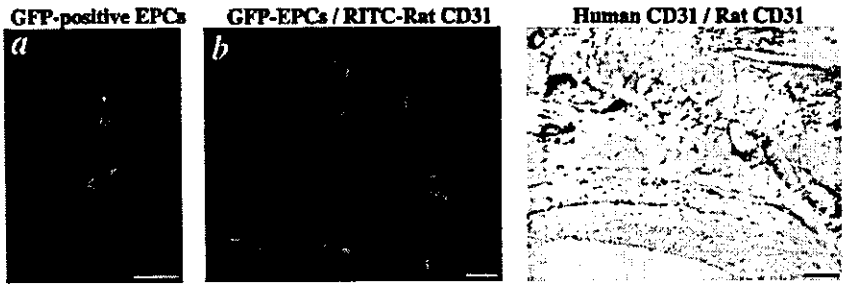


**Figure 4.** Distribution of EPCs in lungs of MCT rats. a, Intravenously administered GFP-expressing EPCs were incorporated into walls of pulmonary arterioles. b, Transplanted GFP-expressing EPCs were distributed on lung tissues. Pulmonary vasculature was detected by RITC-conjugated anti-rat CD31 (red). c, Immunohistochemistry for human CD31 (peroxidase, brown) and rat CD31 (alkaline phosphatase, pink). Scale bars: 50 μm.

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 myocar-

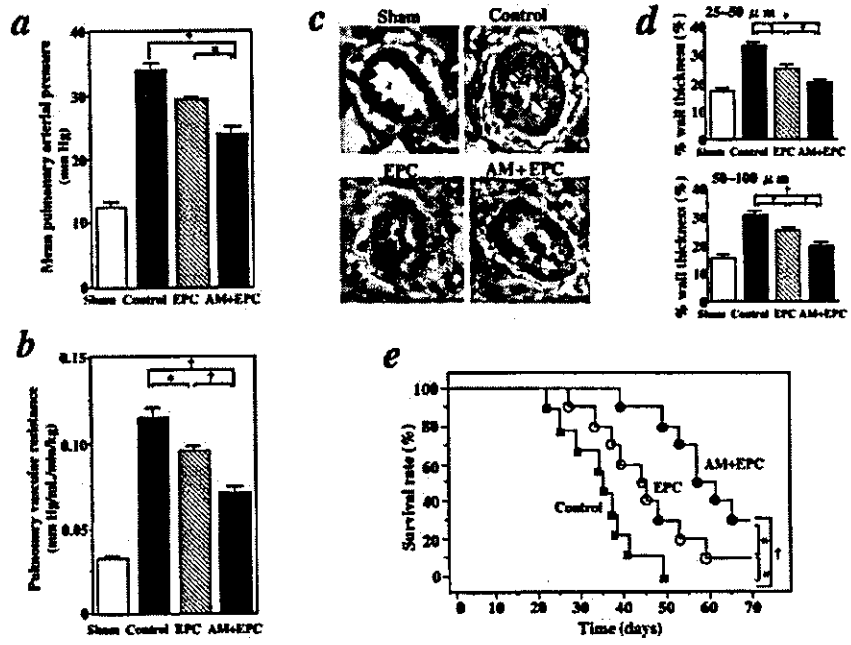


**Figure 5.** Effects of AM DNA-transduced EPC transplantation on mean pulmonary arterial pressure (a) and pulmonary vascular resistance (b) in MCT rats. c, Representative photomicrographs of peripheral pulmonary arteries in rats. Scale bars, 20 μm. d, Quantitative analysis of percent wall thickness of peripheral pulmonary arteries. e, Kaplan-Meier survival curves of MCT rats transplanted with AM-expressing EPCs (AM-EPC group, ●), EPCs alone (EPC group, ○), or culture medium (control group, □). Data are mean±SEM. *$P<0.05$; †$P<0.001$.

**Physiological Profiles of 4 Experimental Groups**

| | Sham (n=8) | Control (n=9) | EPC (n=8) | AM-EPC (n=9) |
|---|---|---|---|---|
| Body weight, g | 191±4 | 174±7 | 181±6 | 182±6 |
| RV weight, g/kg body weight | 0.59±0.02 | 1.04±0.05 | 0.91±0.03 | 0.77±0.04*† |
| Left ventricular weight, g/kg body weight | 2.42±0.03 | 2.49±0.05 | 2.46±0.04 | 2.44±0.09 |
| Heart rate, bpm | 398±10 | 390±11 | 398±15 | 387±11 |
| Mean arterial pressure, mm Hg | 112±4 | 100±5 | 104±3 | 98±4 |
| RV systolic pressure, mm Hg | 32±2 | 63±3 | 56±1* | 48±2*† |
| Plasma human AM, fmol/mL | 0 | 0 | 0.3±0.1* | 0.7±0.1*† |
| Lung human AM, fmol/g tissue | 0 | 0 | 11.9±0.6* | 23.0±2.3*† |

Control indicates MCT rats given culture medium; EPC, MCT rats given EPCs; AM-EPC, MCT rats given AM-expressing EPCs; and RV, right ventricular. Data are mean±SEM.

*$P<0.05$ vs control; †$P<0.05$ vs EPC.

dium after acute myocardial infarction.[6] These findings suggest that progenitor cells have the ability to sense injured tissues. In fact, in the present study, intravenously administered GFP-expressing EPCs were incorporated into pulmonary arterioles and capillaries in MCT rats and differentiated mature endothelial cells. MCT injures endothelial cells of small arteries and capillaries in the lungs, resulting in pulmonary hypertension.[19] 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.[14] 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 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.[1] Interestingly, EPCs cultured with AM DNA-gelatin complexes markedly secreted AM protein for more than 16 days. These results suggest relatively long-lasting AM secretion from EPCs. The consequence of this synthesis in MCT rats was a marked decrease in mean pulmonary arterial pressure and pulmonary vascular resistance. Histological examination revealed that transplantation of AM-expressing EPCs inhibited an increase in medial wall thickness of pulmonary arteries. Expectedly, transplantation of AM-expressing EPCs caused significantly greater improvement in pulmonary hypertension and vascular remodeling than transplantation of EPCs alone. Given the known potent vasoprotective effects of AM, such as vasodilation and inhibition of smooth muscle cell proliferation,[1,20] it is interesting to speculate that AM secreted from EPCs may act not only as a circulating factor but also as an autocrine/paracrine factor in the regulation of pulmonary vascular tone and vascular remodeling in MCT rats. Importantly, a single transplantation of AM-expressed 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.

## Acknowledgments

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10.1152/ajpheart.00548.2002.

# Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats

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**Nagaya, Noritoshi, Hiroyuki Okumura, Masaaki Uematsu, Wataru Shimizu, Fumiaki Ono, Mikiyasu Shirai, Hidezo Mori, Kunio Miyatake, and Kenji Kangawa.** Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats. *Am J Physiol Heart Circ Physiol* 285: H2125–H2131, 2003; 10.1152/ajpheart.00548.2002.—Adrenomedullin (AM) is a potent vasodilator peptide. We investigated whether inhalation of aerosolized AM ameliorates monocrotaline (MCT)-induced pulmonary hypertension in rats. Male Wistar rats given MCT (MCT rats) were assigned to receive repeated inhalation of AM ($n$ = 8) or 0.9% saline ($n$ = 8). AM (5 μg/kg) or saline was inhaled as an aerosol using an ultrasonic nebulizer for 30 min four times a day. After 3 wk of inhalation therapy, mean pulmonary arterial pressure and total pulmonary resistance were markedly lower in rats treated with AM than in those given saline [mean pulmonary arterial pressure: 22 ± 2 vs. 35 ± 1 mmHg (−37%); total pulmonary resistance: 0.048 ± 0.004 vs. 0.104 ± 0.006 $mmHg \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$ (−54%), both $P < 0.01$]. Neither systemic arterial pressure nor heart rate was altered. Inhalation of AM significantly attenuated the increase in medial wall thickness of peripheral pulmonary arteries in MCT rats. Kaplan-Meier survival curves demonstrated that MCT rats treated with aerosolized AM had a significantly higher survival rate than those given saline (70% vs. 10% 6-wk survival, log-rank test, $P < 0.01$). In conclusion, repeated inhalation of AM inhibited MCT-induced pulmonary hypertension without systemic hypotension and thereby improved survival in MCT rats.

vasodilator; hemodynamics; aerosol; survival

ADRENOMEDULLIN (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma (13). Immunoreactive AM has subsequently been detected in plasma and a variety of tissues, including blood vessels and the lungs (9, 27). It has been reported that there are abundant binding sites for AM in the lungs (24). We (11, 30) have shown that the plasma AM level increases in proportion to the severity of pulmonary hypertension and that circulating AM is partially metabolized in the lungs. Interestingly, AM has been shown to inhibit the migration and proliferation of vascular smooth muscle cells (8, 12). These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone and vascular remodeling.

In fact, experimental studies (5, 14, 22) have demonstrated that intralobar arterial infusion of AM induces pulmonary vasodilation in rats and cats. In humans, we have shown that short-term intravenous infusion of AM significantly decreases pulmonary vascular resistance in patients with congestive heart failure (19) or primary pulmonary hypertension (PPH) (18). Unfortunately, however, intravenously administered AM also decreases systemic arterial pressure in such patients because of its nonselective vasodilation in pulmonary and systemic vascular beds.

Recently, inhaled prostacyclin and its analog, iloprost, have been shown to cause pulmonary vasodilation without systemic hypotension in patients with PPH (7, 28, 29). In addition, the inhalant application of vasodilators does not induce negative side effects on gas exchange, because ventilation-matched deposition of the drugs in the alveoli causes pulmonary vasodilation matched to ventilated areas (28). In clinical settings, inhalation therapy may be more simple, noninvasive, and relatively comfortable than continuous intravenous infusion therapy. These findings raise the possibility that intratracheal delivery of aerosolized AM may have beneficial effects in patients with precapillary pulmonary hypertension.

Thus the purpose of the present study was to investigate whether inhalation of AM ameliorates monocrotaline (MCT)-induced pulmonary hypertension and thereby improves survival in MCT-treated rats.

## METHODS

*Animals.* Male Wistar rats weighing 80 to 100 g were used in this study. The rats were given a subcutaneous injection of 60 mg/kg MCT (MCT rats) and assigned to receive a single inhalation of AM ($n$ = 5) or 0.9% saline ($n$ = 5) or repeated inhalation of AM ($n$ = 8) or 0.9% saline ($n$ = 8). Sham rats not

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given a MCT injection also received repeated inhalation of AM ($n$ = 8) or 0.9% saline ($n$ = 8). An additional 20 rats were studied to evaluate the effects of inhaled AM on survival in MCT rats. Finally, rats that had developed pulmonary hypertension 3 wk after the MCT injection received repeated inhalation of AM ($n$ = 8) or 0.9% saline ($n$ = 8). All protocols were performed in accordance with guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute (Osaka, Japan).

*Preparation of AM.* Recombinant human AM was obtained from Shionogi (Osaka, Japan). The homogeneity of AM was confirmed by reverse-phase HPLC and amino acid analysis. AM was dissolved in 0.9% saline, and the solution was stored as 20-ml volumes containing 200 μg AM/tube at −80°C until the time of preparation for administration.

*Inhalation of AM.* We used an unrestrained, whole body aerosol exposure system. Each rat was placed in a plastic cage for aerosol delivery. AM or saline was aerosolized using an ultrasonic nebulizer (Soniclizer 305, Atom) connected to six cages. The 20 ml solution containing 200 μg AM was delivered as an aerosol into the six cages at a constant flow rate (0.6 ml solution/min) for 30 min. Inhalation of fluorescein isothiocyanate-dextran demonstrated that a single inhalation of AM delivered 0.5 μg AM to the lungs in each rat (5 μg/kg body wt).

To assess the acute effect of inhaled AM, hemodynamic studies were carried out at 3 wk after the MCT injection. Hemodynamics were measured at 15-min intervals before, during, and after a single inhalation of AM or saline. Blood was obtained from the carotid artery at the same time points for measurement of plasma AM.

To assess the chronic effect of inhaled AM, 30-min inhalation of AM (5 μg/kg body wt) or saline was repeated four times a day for 3 wk after the MCT injection. Finally, to investigate the effects of inhaled AM on developed pulmonary hypertension, aerosolized AM or saline was given for 1 wk to rats that had developed pulmonary hypertension 3 wk after the MCT injection. After completion of the inhalation therapy, hemodynamic studies were performed. Blood was then drawn from the carotid artery for measurement of plasma hormone levels. Finally, cardiac arrest was induced by the injection of 2 mmol KCl through the catheter. The ventricles and lungs were excised, dissected free, and weighed. The measurement of right ventricular weight excluded the interventricular septum. The ratio of right ventricular weight to body weight and the ratio of left ventricular weight to body weight were calculated as indexes of ventricular hypertrophy.

*Hemodynamic measurements.* Rats were anesthetized with intraperitoneal pentobarbital (30 mg/kg) and placed on a heating pad to maintain body temperature at 37–38°C throughout the study. A polyethylene catheter (PE-10) was inserted into the right femoral artery to measure heart rate and mean arterial pressure. An umbilical vessel catheter was inserted through the right jugular vein into the pulmonary artery for the measurement of right ventricular pressure and pulmonary arterial pressure. These hemodynamic variables were measured using a pressure transducer (model P23ID, Gould) connected to a polygraph and recorded with a thermal recorder (7758B system, Hewlett-Packard). A thermomicroprobe was advanced into the ascending aorta via the right carotid artery and connected to a cardiac output computer (Cardiotherm-500, Columbus Instruments). Cardiac output was measured in triplicate by the thermodilution method. Total pulmonary resistance was calculated by dividing the mean pulmonary arterial pressure by the cardiac output.

*Morphometric analysis of pulmonary arteries.* Paraffin sections 4 μm in thickness were obtained from the middle region of the right lung and stained with hematoxylin and eosin for examination by light microscopy. Analysis of the medial wall thickness of the pulmonary arteries was performed as described previously (23). In brief, the external diameter and the medial wall thickness were measured in 20 muscular arteries (ranging in external diameter from 25 to 50 and from 51 to 100 μm) per lung section. For each artery, the medial wall thickness was expressed as follows: percent wall thickness = [(medial thickness × 2)/external diameter] × 100. A lung section was obtained from individual rats for comparison among the four groups ($n$ = 5 each).

*Hormonal analysis.* The plasma AM level was measured by an immunoradiometric assay using a specific kit (Shionogi) (22). For the assessment of right ventricular function (17, 21), the plasma atrial natriuretic peptide (ANP) level was measured using an enzyme immunoassay kit (ANF Rat EIA kit; Peninsula, CA).

*Survival analysis.* To evaluate the effects of inhaled AM on survival in MCT rats, 20 rats received repeated inhalation of AM ($n$ = 10) or saline ($n$ = 10) four times a day from the date of the MCT injection until death. Survival was estimated from the date of the MCT injection to the death of the rat or 6 wk after the injection.

*Statistical analysis.* All data are expressed as means ± SE unless otherwise indicated. Comparisons of parameters among three groups were made by one-way ANOVA, followed by Scheffé's multiple-comparison test. Comparisons of the time course of parameters between two groups were made by two-way ANOVA for repeated measures, followed by Scheffé's multiple-comparison test. Survival curves according to the presence or absence of AM inhalation were derived using the Kaplan-Meier method and compared using a log-rank test. A $P$ value <0.05 was considered statistically significant.

## RESULTS

*Acute effect of single inhalation of AM.* Acute hemodynamic studies were carried out at 3 wk after the MCT injection. AM inhalation slightly increased the circulating level of human AM (from 0 to 3.6 ± 1.0 fmol/ml, $P < 0.05$). A 30-min inhalation of AM slightly but significantly decreased the mean pulmonary arterial pressure in MCT rats (from 32 ± 2 to 29 ± 2 mmHg, $P < 0.05$; Fig. 1) without a significant decrease in mean arterial pressure (from 113 ± 5 to 111 ± 4 mmHg, $P$ = not signficant). AM inhalation markedly increased cardiac output by 42% (from 405 ± 22 to 575 ± 34 ml·min$^{-1}$·kg$^{-1}$, $P < 0.05$) at the end of inhalation. Thus AM resulted in a 36% decrease in total pulmonary resistance (from 0.081 ± 0.006 to 0.052 ± 0.004 mmHg·ml$^{-1}$·min$^{-1}$·kg$^{-1}$, $P < 0.05$). The ratio of total pulmonary resistance to systemic vascular resistance was significantly decreased at the end of inhalation (from 0.29 ± 0.01 to 0.26 ± 0.01, $P <$ 0.05). Interestingly, these hemodynamic effects of AM lasted at least 60 min after the end of inhalation. Inhalation of saline did not alter any hemodynamic or hormonal parameter.

*Chronic effect of repeated inhalation of AM.* The physiological profiles of the four experimental groups are summarized in Table 1. Body weight was significantly lower in both MCT groups than in sham rats.

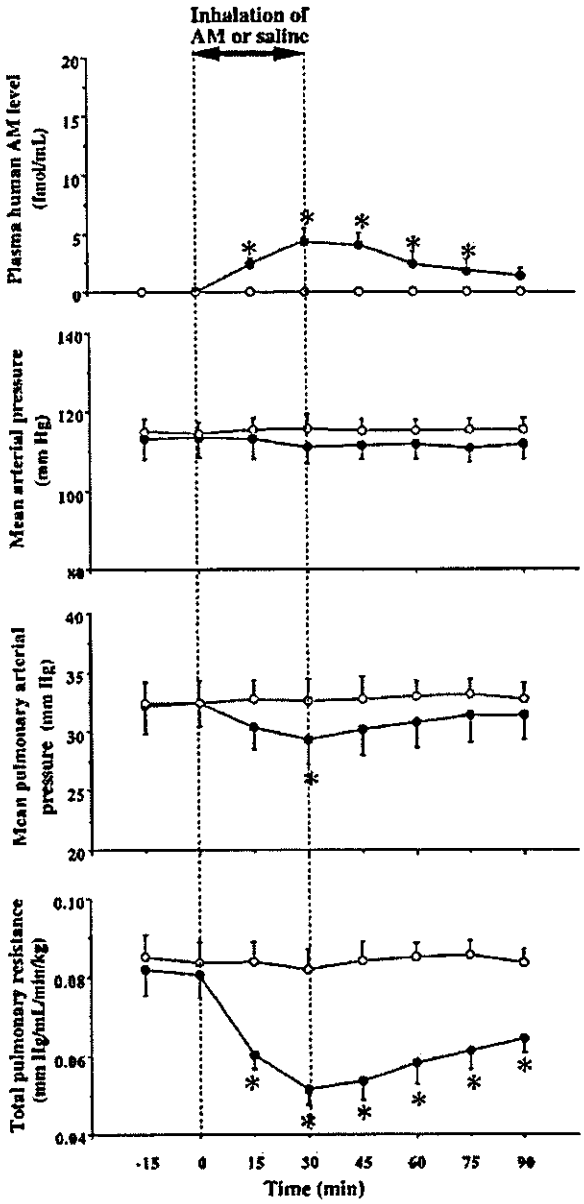


Fig. 1. Acute hemodynamic and hormonal responses to inhaled adrenomedullin (AM; ●) or saline (○) in monocrotaline (MCT)-treated rats (MCT rats). Data are means ± SE. *$P < 0.05$ vs. *time 0*.

Right ventricular weight was significantly lower in MCT rats receiving repeated inhalation of AM than in those given aerosolized saline. There was no significant difference in left ventricular weight among the four groups.

Three weeks after the MCT injection, pulmonary hypertension developed compared with findings in sham rats, but the rise in mean pulmonary arterial pressure was markedly attenuated in MCT rats treated with repeated inhalation of AM (by 37%) compared with that in MCT rats given aerosolized saline (22 ± 2 vs. 35 ± 1 mmHg, $P < 0.05$; Fig. 2). Cardiac output was significantly higher in MCT rats treated with AM (by 30%) compared with that in MCT rats given saline (444 ± 18 vs. 342 ± 18 $ml \cdot min^{-1} \cdot kg^{-1}$, $P < 0.05$). Therefore, total pulmonary resistance was markedly lower in MCT rats treated with AM (by 54%) compared with that in MCT rats given saline (0.048 ± 0.004 vs. 0.104 ± 0.006 $mmHg \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$, $P < 0.05$). Similarly, the increase in right ventricular systolic pressure was significantly attenuated by AM inhalation (Table 1). In contrast, neither mean arterial pressure nor heart rate differed among the four groups. The ratio of total pulmonary resistance to systemic vascular resistance was markedly lower in MCT rats treated with aerosolized AM (by 44%) compared with that in MCT rats given aerosolized saline (0.19 ± 0.01 vs. 0.34 ± 0.01, $P < 0.05$). Inhalation of AM did not significantly alter any hemodynamic parameters in sham rats.

Representative photomicrographs of pulmonary arteries showed that hypertrophy of the pulmonary vessel wall was inhibited in MCT rats treated with AM compared with that in MCT rats given saline (Fig. 3). Quantitative analysis of peripheral pulmonary arteries demonstrated that the percent wall thickness of pulmonary arteries was significantly lower in MCT rats treated with aerosolized AM than in those given aerosolized saline (20 ± 1% vs. 28 ± 1% in vasculature with an external diameter of 25–50 μm and 21 ± 1% vs. 27 ± 1% in vasculature with an external diameter of 51–100 μm, both $P < 0.05$; Fig. 3). Inhalation of AM did not significantly alter vascular morphology in sham rats.

*Effect of AM inhalation on long-term prognosis in MCT rats.* Kaplan-Meier survival curves demonstrated that MCT rats treated with aerosolized AM had a significantly higher survival rate than those given saline (70% vs. 10% in 6-wk survival, log-rank test, $P < 0.01$; Fig. 4). No definite adverse effects were detected after repeated inhalation of AM.

*Effect of AM inhalation on developed pulmonary hypertension.* AM or saline was inhaled by rats that had developed pulmonary hypertension 3 wk after the MCT injection. Mean pulmonary arterial pressure was significantly lower in MCT rats treated with AM (by 14%) compared with that in rats given saline (32 ± 1 vs. 37 ± 1 mmHg, $P < 0.05$). Cardiac output was also higher in MCT rats treated with AM (by 15%) compared with that in rats given saline (360 ± 11 vs. 313 ± 14 $ml \cdot min^{-1} \cdot kg^{-1}$, $P < 0.05$). Therefore, total pulmonary resistance was significantly lower in MCT rats treated with AM (by 24%) compared with that in rats given saline (0.091 ± 0.005 vs. 0.119 ± 0.008 $mmHg \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$, $P < 0.05$).

## DISCUSSION

In the present study, we demonstrated that *1*) a single inhalation of AM using an ultrasonic nebulizer induced relatively long-lasting pulmonary vasodilation without systemic hypotension, *2*) repeated inhalation

Table 1. *Physiological profiles of the four experimental groups*

| | Sham | | MCT | |
|---|---|---|---|---|
| | Sham-Saline | Sham-AM | MCT-Saline | MCT-AM |
| *n* | 8 | 8 | 8 | 8 |
| Body weight, g | 150 ± 3 | 154 ± 3 | 132 ± 2* | 146 ± 4† |
| RV/body wt, g/kg | 0.59 ± 0.02 | 0.58 ± 0.01 | 0.92 ± 0.06* | 0.66 ± 0.02† |
| LV/body wt, g/kg | 2.32 ± 0.04 | 2.27 ± 0.05 | 2.48 ± 0.05 | 2.33 ± 0.05 |
| Heart rate, beats/min | 409 ± 15 | 428 ± 20 | 424 ± 15 | 413 ± 14 |
| Mean arterial pressure, mmHg | 120 ± 3 | 117 ± 3 | 104 ± 3* | 115 ± 3† |
| RV systolic pressure, mmHg | 35 ± 1 | 34 ± 1 | 67 ± 2* | 45 ± 3*† |
| Right atrial pressure, mmHg | 2 ± 1 | 2 ± 1 | 7 ± 1* | 2 ± 1† |
| Plasma ANP level, pg/ml | 275 ± 40 | 238 ± 29 | 694 ± 61* | 346 ± 44† |

Values are means ± SE; *n*, number of rats. Sham-saline, sham rats given aerosolized saline; sham-AM, sham rats given aerosolized AM; MCT-saline, rats treated with monocrostaline (MCT) and given aerosolized saline; MCT-AM, rats treated with MCT and given aerosolized AM; RV, right ventricular LV, left ventricular; ANP, atrial natriuretic peptide. *$P < 0.05$ vs. sham-saline; †$P < 0.05$ vs. MCT-saline.

of AM ameliorated MCT-induced pulmonary hypertension and attenuated the development of pulmonary vascular remodeling, and *3*) inhalation of AM improved survival in MCT rats without definite adverse effects.

PPH is a rare but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right ventricular failure and death (25). Although intravenous administration of prostacyclin has become recognized as a therapeutic breakthrough (1, 6, 16, 26), some patients with PPH are refractory to this treatment. Thus a new therapeutic strategy for the treatment of PPH is desirable.

AM is one of the most potent endogenous vasodilators in the pulmonary vascular bed (5, 13, 14, 22). The vasodilating effect is mediated by a cAMP-dependent and/or nitric oxide-dependent mechanism (10, 20). Recently, we (19) have shown that intravenous administration of AM markedly decreases pulmonary vascular resistance in patients with PPH. Nevertheless, systemically administered AM decreases systemic arterial pressure, which may be harmful in treating patients with PPH. In the present study, inhalation of AM markedly decreased total pulmonary resistance, whereas it did not significantly decrease mean arterial pressure. The ratio of total pulmonary resistance to systemic vascular resistance was significantly reduced by AM inhalation. These results suggest that this novel route of AM administration causes relatively selective pulmonary vasodilation. Expectedly, inhalation of AM markedly increased the cardiac index in MCT rats, consistent with our previous results from intravenous delivery (18). Considering the strong vasodilator activity of AM in the pulmonary vasculature, the significant decrease in cardiac afterload may be responsible for the increased cardiac index with AM. Interestingly, the hemodynamic effects of AM lasted at least 60 min after a single inhalation of AM. Although a single inhalation of AM delivered 0.5 μg AM into the lungs in each rat, it induced only a slight increase in the plasma AM level (3.6 ± 1.0 fmol/ml). These results raise the possibility that inhaled AM is retained in lung tissue for a while and acts transepithelially on the pulmonary vasculature. Thus inhalation of AM may cause potent, long-lasting pulmonary vasodilator activity in MCT rats.

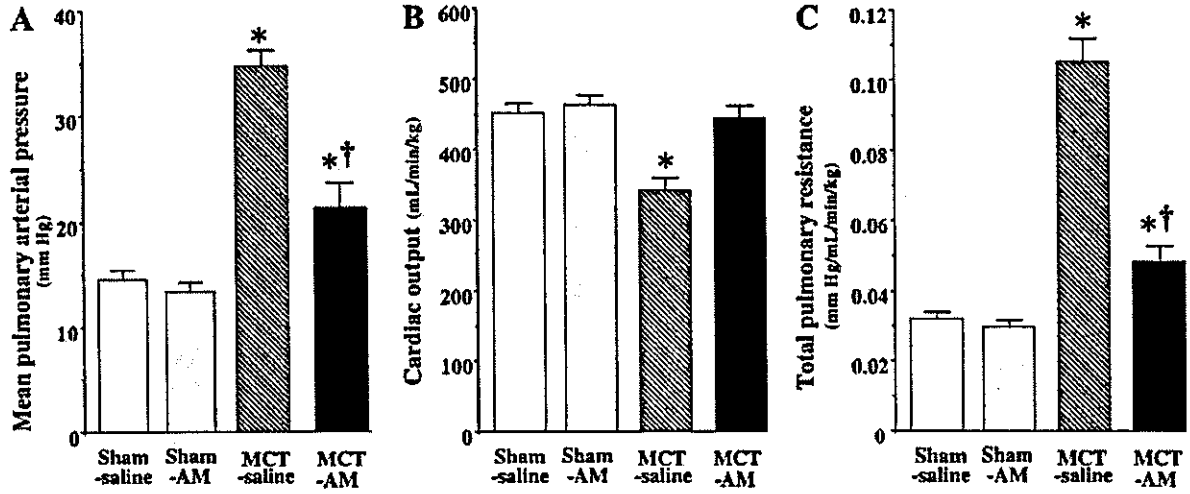


Fig. 2. Chronic effects of AM inhalation on mean pulmonary arterial pressure (*A*), cardiac output (*B*), and total pulmonary resistance (*C*). Sham-saline, sham rats given aerosolized AM; MCT-saline, MCT rats given aerosolized saline; sham-AM, sham rats given aerosolized AM; MCT-AM, MCT rats given aerosolized AM. Data are means ± SE. *$P < 0.05$ vs. sham-saline; †$P < 0.05$ vs. MCT-saline rats.

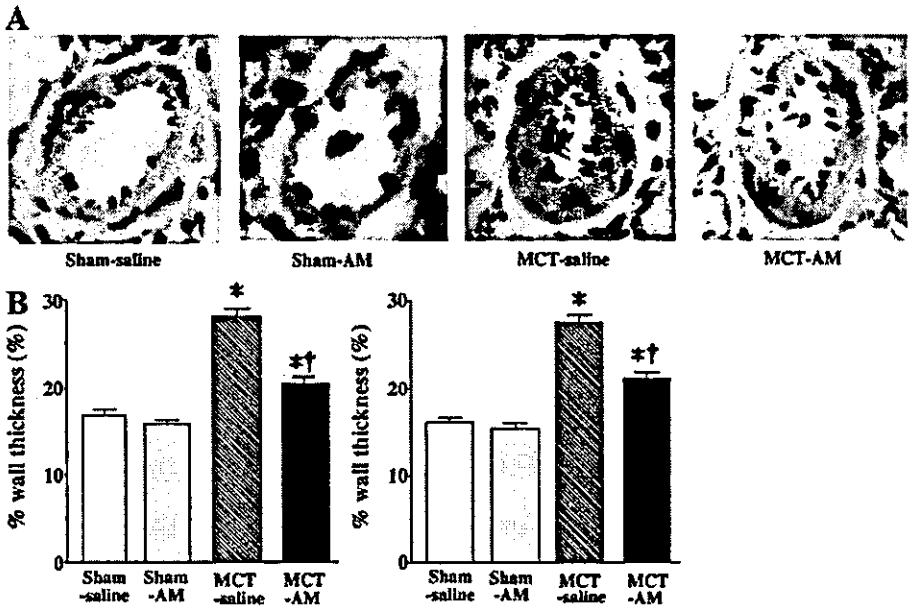


Fig. 3. *A*: representative photomicrographs of peripheral pulmonary arteries in the four groups. AM inhalation markedly inhibited hypertrophy of the vessel wall in MCT rats. Magnification, ×400. *B*: quantitative analyses of peripheral pulmonary arteries with an external diameter of 25–50 μm (*left*) or 51–100 μm (*right*). The percent wall thickness was calculated as [(medial thickness × 2)/external diameter] × 100. Abbreviations are as in Table 1 and Fig. 2. Data are means ± SE. *$P <$ 0.05 vs. sham-saline rats; †$P < 0.05$ vs. MCT-saline rats.

The present study also demonstrated that repeated inhalation of AM four times a day for 3 wk markedly decreased mean pulmonary arterial pressure and total pulmonary resistance in MCT rats without systemic hypotension. The potent, long-lasting pulmonary vasodilator effect of inhaled AM may contribute to the strong inhibition of the development of pulmonary hypertension. In addition, considering intermittent delivery of AM to the lungs, the chronic effects of inhaled AM appear to go beyond acute pulmonary vasodilation. In the present study, inhalation of AM inhibited an increase in the medial wall thickness of peripheral pulmonary arteries of MCT rats. Earlier studies (8, 12) have shown that AM inhibits the migration and proliferation of vascular smooth muscle cells. Given the known potent vasoprotective effects of AM, such as vasodilation and inhibition of smooth muscle cell migration and proliferation, it is interesting to speculate that AM trapped in the bronchial epithelium or alveoli leaks to the pulmonary arteries to maintain pulmonary vascular integrity in MCT rats. Inhalation of AM also decreased plasma ANP, a potential marker for right ventricular dysfunction (17, 21). It is possible that the decreased pulmonary vascular resistance by AM may ameliorate increased wall stress in the right ventricle and improve right ventricular dysfunction in MCT rats.

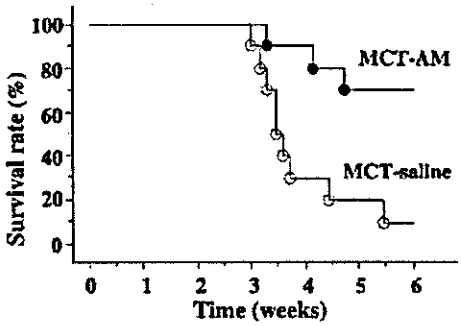


Fig. 4. Kaplan-Meier survival curves showing that MCT rats treated with aerosolized AM had a significantly higher survival rate than those given saline inhalation (log-rank test, $P < 0.01$).

Importantly, Kaplan-Meier analysis demonstrated that the 6-wk survival rate for MCT rats treated with aerosolized AM was significantly high (70%) compared with those given saline (10%). Thus treatment with aerosolized AM may be an alternative approach for severe pulmonary hypertension that is refractory to conventional therapy.

In the pulmonary circulation, the AM receptor acts not only as a functional receptor but also as a clearance receptor, the expression of which is stimulated by basal AM itself (3). Thus exogenously administered AM may have differing effects depending on the basal levels of AM.

Champion et al. (2) showed that intratracheal gene transfer of prepro-calcitonin gene-related peptide (CGRP) to the lung attenuates chronic hypoxia-induced pulmonary hypertension in mice. The gene for AM belongs to the CGRP family, and the receptors for CGRP and AM bind both peptides (15). In addition, the AM receptor is expressed at high levels in the pulmonary vascular endothelium, and there is an interaction of CGRP and AM with the receptor in the pulmonary endothelium (4). Thus it is not surprising that AM attenuates pulmonary hypertension in a similar manner as CGRP. In fact, we (31) have previously reported a beneficial effect of AM in a rat model of pulmonary hypertension. In our previous study, however, AM was administered subcutaneously. In contrast, in the present study, AM was inhaled to ameriolate pulmonary hypertension, which may have a pharmacological

and clinical implication of the treatment for this disorder.

In conclusion, repeated inhalation of AM inhibited MCT-induced pulmonary hypertension without systemic hypotension and thereby improved survival in MCT rats. Thus long-term treatment with aerosolized AM may be a new therapeutic strategy for the treatment of pulmonary hypertension.

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## DISCLOSURES

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