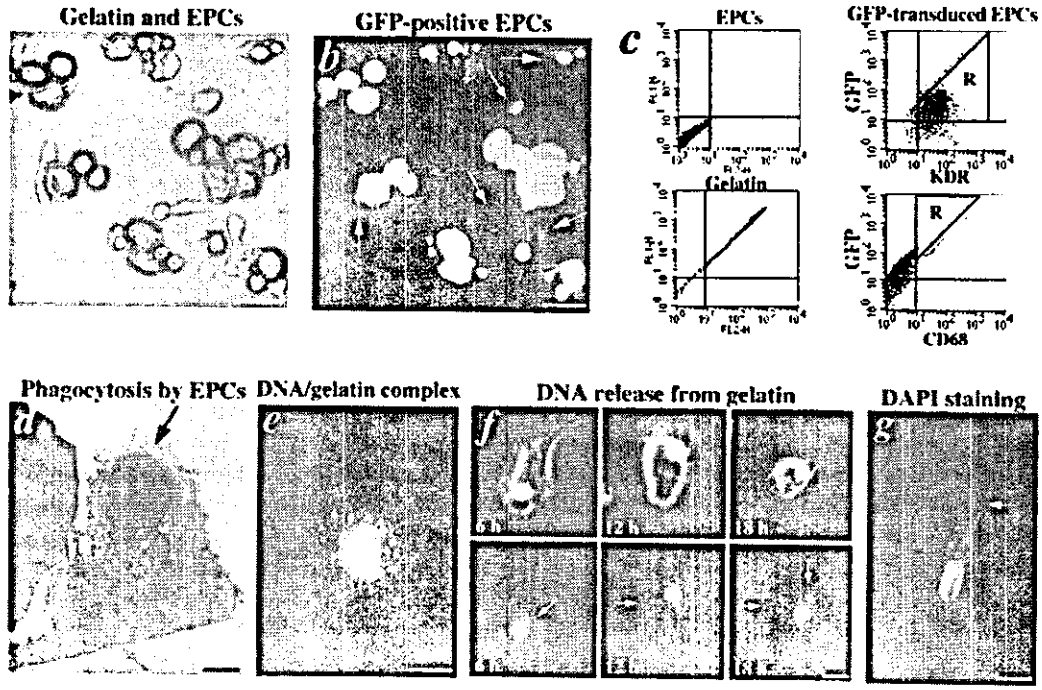

Figure 2. Ex vivo gene transfer into EPCs based on phagocytosing action. a, EPCs were cultured with ionically linked GFP DNA-gelatin complexes. b, GFP was highly expressed in EPCs (arrows) in same field as Figure 2a. c, Flow cytometric analyses of EPCs cultured with GFP DNA-gelatin complexes. Negative 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

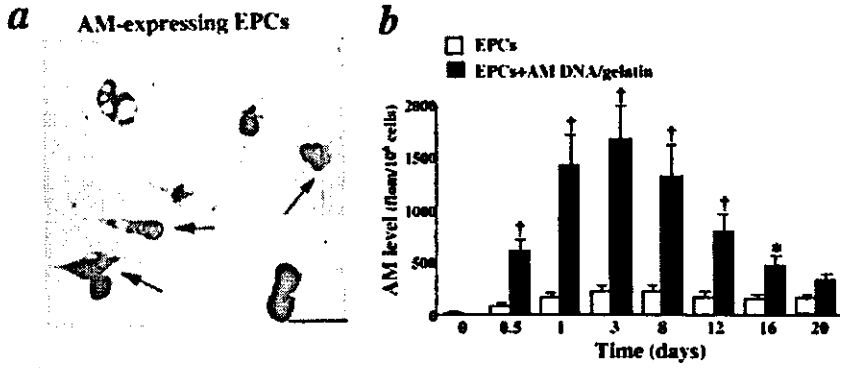

Figure 3. AM gene transfer into EPCs. a, Immunohistochemical analysis of AM in EPCs after gene transfer. Intense immunostaining for AM was observed in EPCs (arrows). Scale bar: 10 μm. b, Time course of AM secretion from EPCs during coculture with AM DNA-gelatin complexes. Data are mean±SEM. *$P<0.05$,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

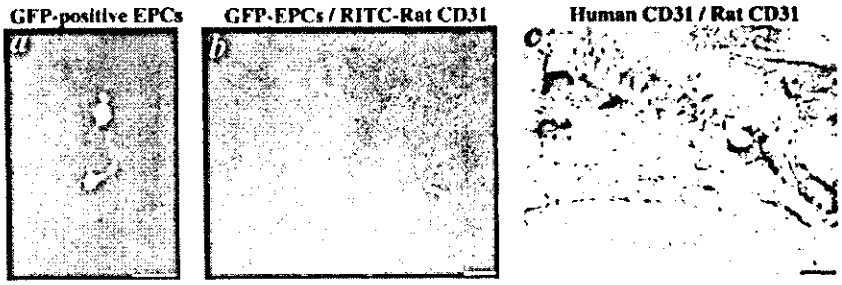

Figure 4. Distribution of EPCs in lungs of MCT rats. a, Intravenously administered GFP-expressing EPCs were incorporated into walls of pulmonary arterioles. b, Transplanted GFP-expressing EPCs were distributed on lung tissues. Pulmonary vasculature was detected by RITC-conjugated anti-rat CD31 (red). 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-

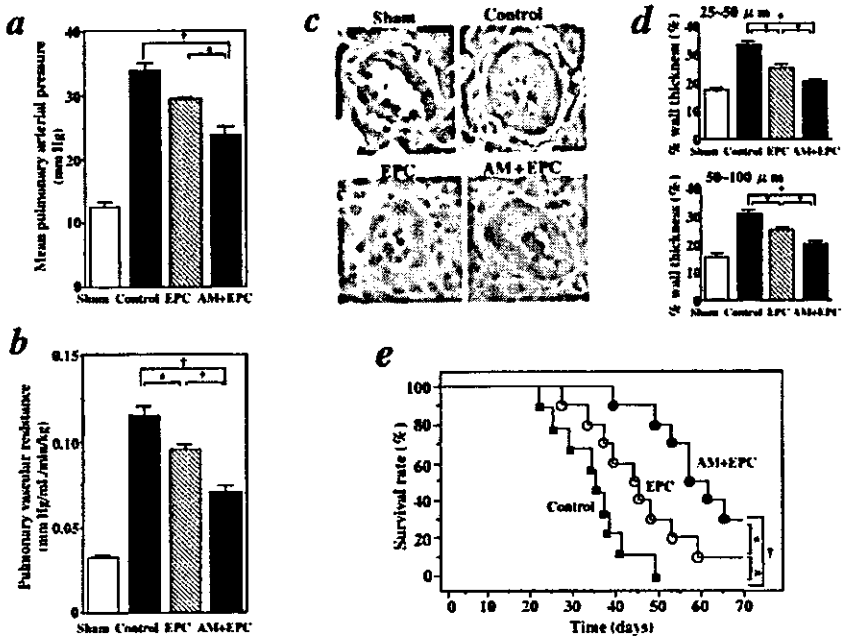

Figure 5. Effects of AM DNA-transduced EPC transplantation on mean pulmonary arterial pressure (a) and pulmonary vascular resistance (b) in MCT rats. c, Representative photomicrographs of peripheral pulmonary arteries in rats. Scale bars, 20 μm. d, Quantitative analysis of percent wall thickness of peripheral 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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References

1. Kitamura K, Kangawa K, Kawamoto M, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun.* 1993;192:553–560.
2. Archer S, Rich S. Primary pulmonary hypertension: a vascular biology and translational research "work in progress." *Circulation.* 2000;102:2781–2791.
3. Asahara T, Murohara T, Sullivan A, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Science.* 1997;275:965–967.

4. Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med.* 1999;5:434–438.
5. Gill M, Dias S, Hattori K, et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. *Circ Res.* 2001;88:167–174.
6. Kawamoto A, Gwon HC, Iwaguro H, et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation.* 2001;103:634–637.
7. Tabata Y, Nagano A, Ikada Y. Biodegradation of hydrogel carrier incorporating fibroblast growth factor. *Tissue Eng.* 1999;5:127–138.
8. Fukunaka Y, Iwanaga K, Morimoto K, et al. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. *J Control Release.* 2002;80:333–343.
9. Tabata Y, Ikada Y. Macrophage activation through phagocytosis of muramyl dipeptide encapsulated in gelatin microspheres. *J Pharm Pharmacol.* 1987;39:698–704.
10. Owji AA, Smith DM, Coppock HA, et al. An abundant and specific binding site for the novel vasodilator adrenomedullin in the rat. *Endocrinology.* 1995;136:2127–2134.
11. Yoshibayashi M, Kamiya T, Kitamura K, et al. Plasma levels of adrenomedullin in primary and secondary pulmonary hypertension in patients <20 years of age. *Am J Cardiol.* 1997;79:1556–1558.
12. Nagaya N, Satoh T, Nishikimi T, et al. Hemodynamic, renal and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. *Circulation.* 2000;101:498–503.
13. Nagaya N, Nishikimi T, Uematsu M, et al. Haemodynamic and hormonal effects of adrenomedullin in patients with pulmonary hypertension. *Heart.* 2000;84:653–658.
14. Murohara T, Ikeda H, Duan J, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *J Clin Invest.* 2000;105:1527–1536.
15. Kalka C, Masuda H, Takahashi T, et al. Vascular endothelial growth factor (165) gene transfer augments circulating endothelial progenitor cells in human subjects. *Circ Res.* 2000;86:1198–1202.
16. Dimmeler S, Aicher A, Vasa M, et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest.* 2001;108:391–397.
17. Kalka C, Masuda H, Takahashi T, et al. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci USA.* 2000;97:3422–3427.
18. Priller J, Flugel A, Wehner T, et al. Targeting gene-modified hematopoietic cells to the central nervous system: use of green fluorescent protein uncovers microglial engraftment. *Nat Med.* 2001;7:1356–1361.
19. Rosenberg H, Rabinovitch M. Endothelial injury and vascular reactivity in monocrotaline pulmonary hypertension. *Am J Physiol.* 1988;255:H1484–H1491.
20. Horio T, Kohno M, Kano H, et al. Adrenomedullin as a novel antimigration factor of vascular smooth muscle cells. *Circ Res.* 1995;77:660–664.

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10.1152/ajpheart.00548.2002.

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

Noritoshi Nagaya,[1] Hiroyuki Okumura,[2] Masaaki Uematsu,[3] Wataru Shimizu,[1] Fumiaki Ono,[1] Mikiyasu Shirai,[4] Hidezo Mori,[4] Kunio Miyatake,[1] and Kenji Kangawa[2]

[1]*Department of Internal Medicine, National Cardiovascular Center, Osaka 565-8565;*
[2]*Department of Biochemistry, National Cardiovascular Center Research Institute, Osaka 565-8565;*
[3]*Cardiovascular Division, Kansai Rosai Hospital, Hyogo 660-0064, Japan; and* [4]*Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Osaka, Japan 565-8565*

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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

Address for reprint requests and other correspondence: N. Nagaya, Dept. of Internal Medicine, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan (E-mail: nagayann@hsp.ncvc.go.jp).

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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 Scheffe'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.

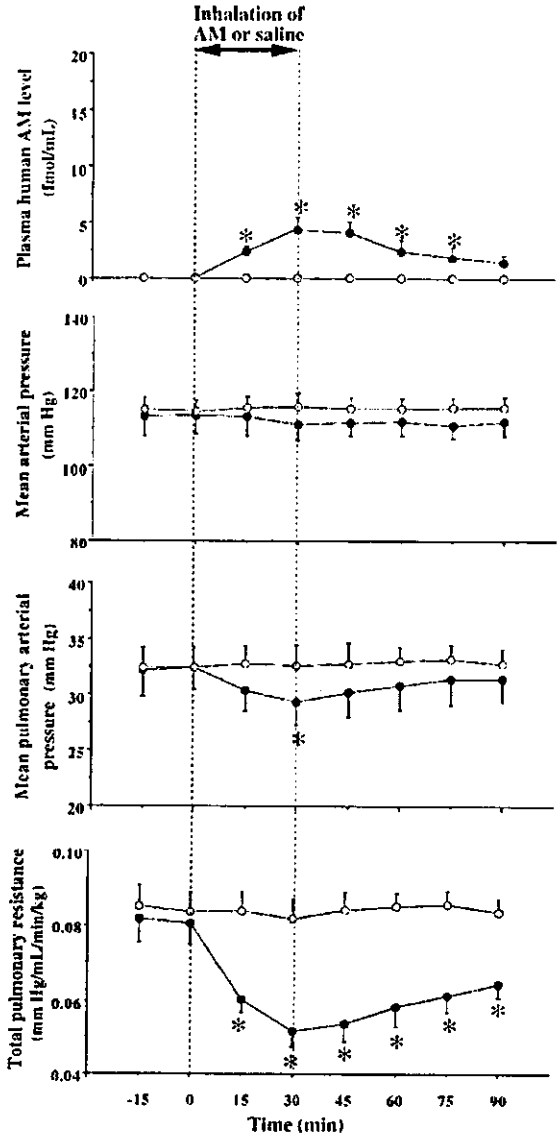

Fig. 1. Acute hemodynamic and hormonal responses to inhaled adrenomedullin (AM; ●) or saline (○) in monocrotaline (MCT)-treated rats (MCT rats). Data are means ± SE. *$P < 0.05$ vs. *time 0*.

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·min^{-1}·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·ml^{-1}·min^{-1}·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·min^{-1}·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·ml^{-1}·min^{-1}·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.

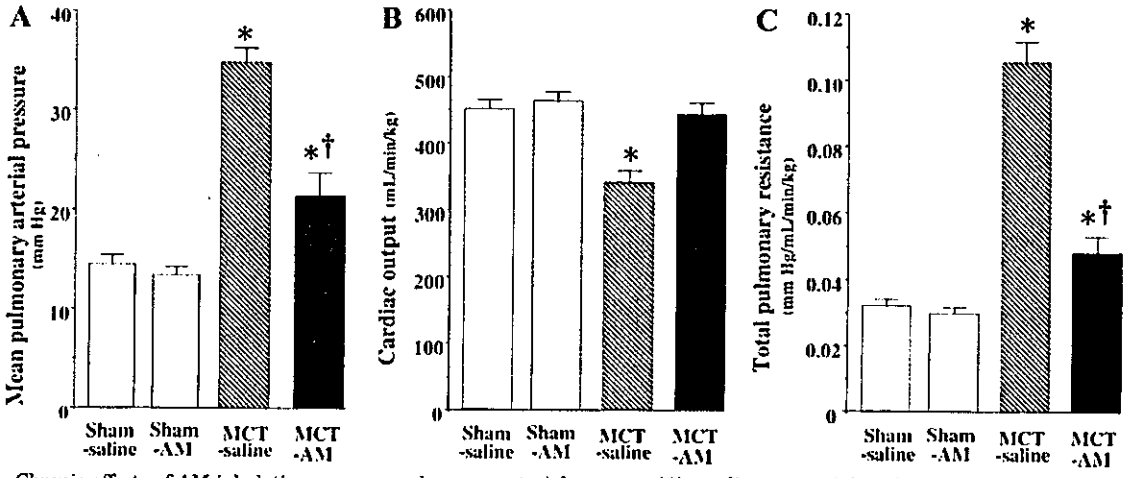

Fig. 2. Chronic effects of AM inhalation on mean pulmonary arterial pressure (*A*), cardiac output (*B*), and total pulmonary resistance (*C*). Sham-saline, sham rats given aerosolized AM; MCT-saline, MCT rats given aerosolized saline; sham-AM, sham rats given aerosolized AM; MCT-AM, MCT rats given aerosolized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

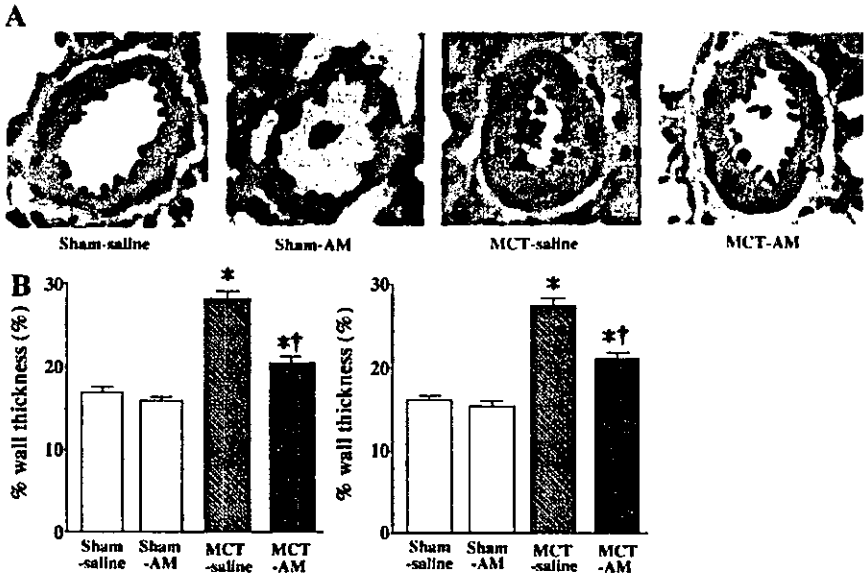

Fig. 3. *A*: representative photomicrographs of peripheral pulmonary arteries in the four groups. AM inhalation markedly inhibited hypertrophy of the vessel wall in MCT rats. Magnification, ×400. *B*: quantitative analyses of peripheral pulmonary arteries with an external diameter of 25–50 μm (*left*) 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; $\dagger P < 0.05$ 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.

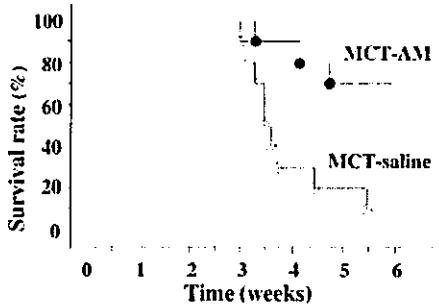

Fig. 4. Kaplan-Meier survival curves showing that MCT rats treated with aerosolized AM had a significantly higher survival rate than those given saline inhalation (log-rank test, $P < 0.01$).

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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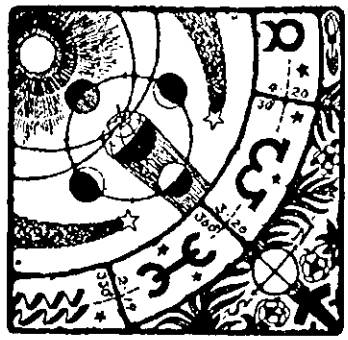
REFERENCES

1. **Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, Groves BM, Tapson VF, Bourge RC, Brundage BH, Koerner SK, Langleben D, Keller CA, Murali S, Uretsky BF, Clayton LM, Jobsis MM, Blackburn SD, Shortino D, and Crow JW.** A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. *N Engl J Med* 334: 296–301, 1996.
2. **Champion HC, Bivalacqua TJ, Toyoda K, Heistad DD, Hyman AL, and Kadowitz PJ.** In vivo gene transfer of prepro-calcitonin gene-related peptide to the lung attenuates chronic hypoxia-induced pulmonary hypertension in the mouse. *Circulation* 101: 923–930, 2000.
3. **Dschietzig T, Azad HA, Asswad L, Bohme C, Bartsch C, Baumann G, and Stangl K.** The adrenomedullin receptor acts as clearance receptor in pulmonary circulation. *Biochem Biophys Res Commun* 294: 315–318, 2002.
4. **Han ZQ, Coppock HA, Smith DM, Van Noorden S, Makgoba MW, Nicholl CG, and Legon S.** The interaction of CGRP and adrenomedullin with a receptor expressed in the rat pulmonary vascular endothelium. *J Mol Endocrinol* 18: 267–272, 1997.
5. **Heaton J, Lin B, Chang JK, Steinberg S, Hyman A, and Lippton H.** Pulmonary vasodilation to adrenomedullin: a novel peptide in humans. *Am J Physiol Heart Circ Physiol* 268: H2211–H2215, 1995.
6. **Higenbottam TW, Wheeldon D, Wells FC, and Wallwork J.** Long-term treatment of primary pulmonary hypertension with continuous intravenous epoprostenol (prostacyclin). *Lancet* 1: 1046–1047, 1984.
7. **Hoeper MM, Schwarze M, Ehlerding S, Adler-Schuermeyer A, Spiekerkoetter E, Niedermeyer J, Hamm M, and Fabel H.** Long-term treatment of primary pulmonary hypertension with aerosolized iloprost, a prostacyclin analogue. *N Engl J Med* 342: 1866–1870, 2000.
8. **Horio T, Kohno M, Kano H, Ikeda M, Yasunari K, Yokokawa K, Minami M, and Takeda T.** Adrenomedullin as a novel antimigration factor of vascular smooth muscle cells. *Circ Res* 77: 660–664, 1995.
9. **Ichiki Y, Kitamura K, Kangawa K, Kawamoto M, Matsuo H, and Eto T.** Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. *FEBS Lett* 338: 6–10, 1994.
10. **Ishizaka Y, Ishizaka Y, Tanaka M, Kitamura K, Kangawa K, Minamino N, Matsuo H, and Eto T.** Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. *Biochem Biophys Res Commun* 200: 642–646, 1994.
11. **Kakishita M, Nishikimi T, Okano Y, Satoh T, Kyotani S, Nagaya N, Fukushima K, Nakanishi N, Takishita S, Miyata A, Kangawa K, Matsuo H, and Kunieda T.** Increased plasma levels of adrenomedullin in patients with pulmonary hypertension. *Clin Sci (Lond)* 96: 33–39, 1999.
12. **Kano H, Kohno M, Yasunari K, Yokokawa K, Horio T, Ikeda M, Minami M, Hanehira T, Takeda T, and Yoshikawa J.** Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. *J Hypertens* 14: 209–213, 1996.
13. **Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, and Eto T.** Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem Biophys Res Commun* 192: 553–560, 1993.
14. **Lippton H, Chang JK, Hao Q, Summer W, and Hyman AL.** Adrenomedullin dilates the pulmonary vascular bed in vivo. *J Appl Physiol* 76: 2154–2156, 1994.
15. **McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, and Foord SM.** RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* 393: 333–339, 1998.
16. **McLaughlin VV, Genthner DE, Panella MM, and Rich S.** Reduction in pulmonary vascular resistance with long-term epoprostenol (prostacyclin) therapy in primary pulmonary hypertension. *N Engl J Med* 338: 273–277, 1998.
17. **Nagaya N, Nishikimi T, Uematsu M, Satoh T, Kyotani S, Sakamaki F, Kakishita M, Fukushima K, Okano Y, Nakanishi N, Miyatake K, and Kangawa K.** Plasma brain natriuretic peptide as a prognostic indicator in patients with primary pulmonary hypertension. *Circulation* 102: 865–870, 2000.
18. **Nagaya N, Nishikimi T, Uematsu M, Satoh T, Oya H, Kyotani S, Sakamaki F, Ueno K, Nakanishi N, Miyatake K, and Kangawa K.** Hemodynamic and hormonal effects of adrenomedullin in patients with pulmonary hypertension. *Heart* 84: 653–658, 2000.
19. **Nagaya N, Satoh T, Nishikimi T, Uematsu M, Furuichi S, Sakamaki F, Oya H, Kyotani S, Nakanishi N, Goto Y, Masuda Y, Miyatake K, and Kangawa K.** Hemodynamic, renal and hormonal effects of adrenomedullin infusion in patients with congestive heart failure. *Circulation* 101: 498–503, 2000.
20. **Nakamura M, Yoshida H, Makita S, Arakawa N, Niinuma H, and Hiramori K.** Potent and long-lasting vasodilatory effects of adrenomedullin in humans: comparisons between normal subjects and patients with chronic heart failure. *Circulation* 95: 1214–1221, 1997.
21. **Nootens M, Kaufmann E, Rector T, Toher C, Judd D, Francis GS, and Rich S.** Neurohormonal activation in patients with right ventricular failure from pulmonary hypertension: relation to hemodynamic variables and endothelin levels. *J Am Coll Cardiol* 26: 1581–1585, 1995.
22. **Nossaman BD, Feng CJ, Kaye AD, DeWitt B, Coy DH, Murphy WA, and Kadowitz PJ.** Pulmonary vasodilator responses to adrenomedullin are reduced by NOS inhibitors in rats but not in cats. *Am J Physiol Lung Cell Mol Physiol* 270: L782–L789, 1996.
23. **Ono S and Voelkel NF.** PAF antagonists inhibit monocrotaline-induced lung injury and pulmonary hypertension. *J Appl Physiol* 71: 2483–2492, 1991.
24. **Owji AA, Smith DM, Coppock HA, Morgan DG, Bhogal R, Ghatei MA, and Bloom SR.** An abundant and specific binding site for the novel vasodilator adrenomedullin in the rat. *Endocrinology* 136: 2127–2134, 1995.
25. **Rich S, Dantzker DR, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Koerner SK, Levy PC, Reid LM, Vreim CE, and Williams GW.** Primary pumonary hypertension: a national prospective study. *Ann Intern Med* 107: 216–223, 1987.
26. **Rubin LJ, Mendoza J, Hood M, McGoon M, Barst R, Williams WB, Diehl JH, Crow J, and Long W.** Treatment of primary pulmonary hypertension with continuous intravenous prostacyclin (epoprostenol): results of a randomized trial. *Ann Intern Med* 112: 485–491, 1990.
27. **Sakata J, Shimokubo T, Kitamura K, Nishizono M, Iehiki Y, Kangawa K, Matsuo H, and Eto T.** Distribution and characterization of immunoreactive rat adrenomedullin in tissue and plasma. *FEBS Lett* 352: 105–108, 1994.
28. **Walmrath D, Schneider T, Pilch J, Grimminger F, and Seeger W.** Aerosolized prostacyclin reduces pulmonary artery

pressure and improves gas exchange in the adult respiratory distress syndrome (ARDS). *Lancet* 342: 961–962, 1993.
29. **Wensel R, Opitz CF, Ewert R, Bruch L, and Kleber FX.** Effects of iloprost inhalation on exercise capacity and ventilatory efficiency in patients with primary pulmonary hypertension. *Circulation* 101: 2388–2392, 2000.
30. **Yoshibayashi M, Kamiya T, Kitamura K, Saito Y, Kangawa K, Nishikimi T, Matsuoka H, Eto T, and Matsuo H.** Plasma levels of adrenomedullin in primary and secondary pulmonary hypertension in patients < 20 years of age. *Am J Cardiol* 79: 1556–1558, 1997.
31. **Yoshihara F, Nishikimi T, Horio T, Yutani C, Takishita S, Matsuo H, Ohe T, and Kangawa K.** Chronic infusion of adrenomedullin reduces pulmonary hypertension and lessens right ventricular hypertrophy in rats administered monocrotaline. *Eur J Pharmacol* 355: 33–39, 1998.



3．PPH治療の今後の展望

再生医療

永谷 憲歳　国立循環器病センター研究所再生医療部室長　*Nagaya, Noritoshi*

論文のポイント

1. はじめに：
   肺高血圧症では肺血管内皮障害により血管作動性物質のバランスが破綻(収縮因子＞拡張因子)しており，正常な肺血管内皮細胞を再生させることが肺高血圧を軽減させる可能性がある．
2. 血管新生因子による肺高血圧治療：
   基礎的研究では血管新生因子を肺血管へ導入すると，肺血管内皮細胞が再生され，また血管内皮細胞のアポトーシスが抑制させて肺高血圧が軽減する．
3. 細胞移植による肺高血圧治療：
   血管内皮前駆細胞はモノクロタリン誘発肺高血圧ラットの肺細動脈と間質に付着し，血管内皮細胞となり肺高血圧をわずかに改善させた．
4. 遺伝子-細胞移植ハイブリッド治療：
   細胞移植と遺伝子治療を同時に行う遺伝子-細胞ハイブリッド治療法は，細胞による肺血管内皮の再生のみでなく，血管内皮前駆細胞をベクターとして肺組織選択的に遺伝子導入を可能にした．
5. 血管内皮前駆細胞への新たな遺伝子導入法の開発：
   血管内皮前駆細胞の貪食能を利用した細胞自身への非ウイルス性遺伝子導入に成功した．
6. アドレノメデユリン遺伝子と血管内皮前駆細胞を用いた肺高血圧治療：
   アドレノメデユリン遺伝子を導入した血管内皮前駆細胞の移植は，モノクロタリン誘発肺高血圧を有意に改善させた．

キーワード

肺高血圧／血管内皮前駆細胞／再生医療／アドレノメデユリン／血管新生因子

はじめに

原発性肺高血圧症(PPH)に対する治療としてプロスタサイクリン療法やエンドセリン(ET)受容体拮抗薬らが開発され，その有効性が報告されている．しかしなお治療抵抗性の症例が存在し，肺移植の適応とされながらもドナー不足により十分な治療が受けられないのが現状である．

PPH発症の原因の1つとして，肺血管内皮の機能障害が報告されている[1]．正常の肺血管内皮

細胞は様々な血管拡張因子を分泌して肺血管の低圧系を維持している．一方，肺高血圧症では肺血管内皮機能が障害されており，血管作動物質のバランスが破綻(収縮因子＞拡張因子)している．すなわちET-1，アンギオテンシンII，トロンボキサンA_2，セロトニンらの収縮因子が増加し，内因性血管拡張因子であるプロスタサイクリンや一酸化窒素の産生が相対的に低下している[1)-5)](図1)．

肺血管内皮機能障害の病態に着目した治療として，❶肺血管内皮細胞で産生される拡張因子の補充と収縮因子の抑制，❷正常な肺血管内皮細胞の再生促進という治療法が考えられる．後者の肺血管内皮再生のためには❶血管新生因子の蛋白投与または遺伝子導入，❷血管内皮前駆細胞，幹細胞を用いた細胞移植治療，❸血管新生因子と細胞移植の併用療法の3つの治療戦略が考えられる．本稿では肺血管の再生が肺高血圧の軽減に結びついた最近の基礎的研究を紹介する．

血管新生因子による肺高血圧治療

血管新生は様々な液性・細胞性因子によって密接にコントロールされており，血管内皮細胞増殖に対するプラスとマイナスの因子群のバランスによって恒常性が保たれている．現在，心血管領域では末梢動脈閉塞症や狭心症患者に対して血管新生因子(VEGF，FGF，HGF，アンジオポエチン-1)の蛋白や遺伝子を用いた血管新生療法の基礎的・臨床的研究が行われている[6)-8)]．これらの血管新生因子はPI 3 K-Akt経路を活性化し血管内皮細胞の生存，遊走，増殖，血管新生に関与し，血管新生を促すことが明らかとなった[9)]．

PPH患者の肺組織では，VEGFなどの血管新生因子の産生が亢進している[10)]．この現象が肺高血圧の原因か結果かは明らかでないが，PPHの病態に関与している可能性がある．基礎的研究においては，VEGF，FGF，HGF，アンジオポエチン-1の肺血管または肺実質への遺伝子導入で肺血管内皮細胞の再生・血管新生を促し，また血管内皮細胞のアポトーシスを抑制することで肺高血圧が軽減されることが示された[11)-13)]．肺高血圧実験モデルと実際のPPHでは，肺高血圧の発症機序が異なるが，血管新生因子の投与による肺血管再生治療が新たな肺高血圧治療となる可能性がある．

細胞移植による肺高血圧治療

従来成人個体における血管新生は既存の血管内

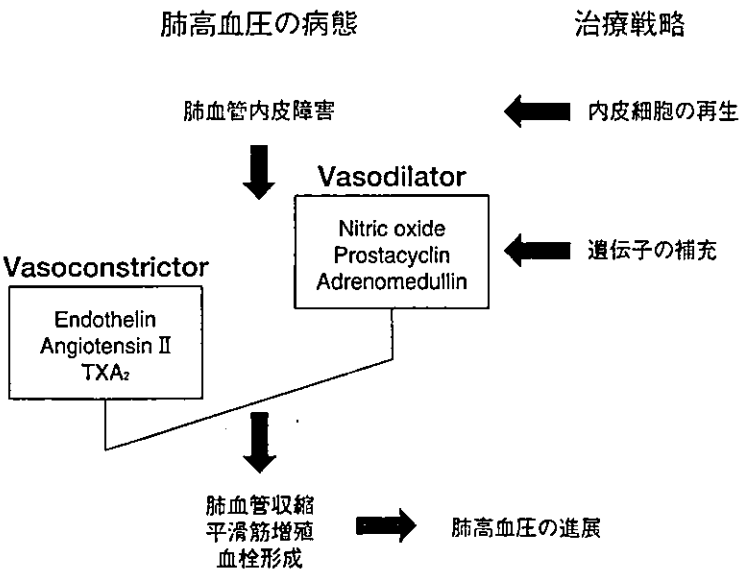


図1　肺高血圧症における血管作動物質のバランス破綻と病態に基づいた治療戦略

肺高血圧症では，肺血管内皮細胞から産生させる血管作動物質のバランスが破綻している(収縮因子＞拡張因子)．したがって血管拡張因子を補充すること，さらには血管新生因子や血管内皮前駆細胞を用いて正常な肺血管内皮細胞を再生させることが肺高血圧軽減につながる可能性がある．

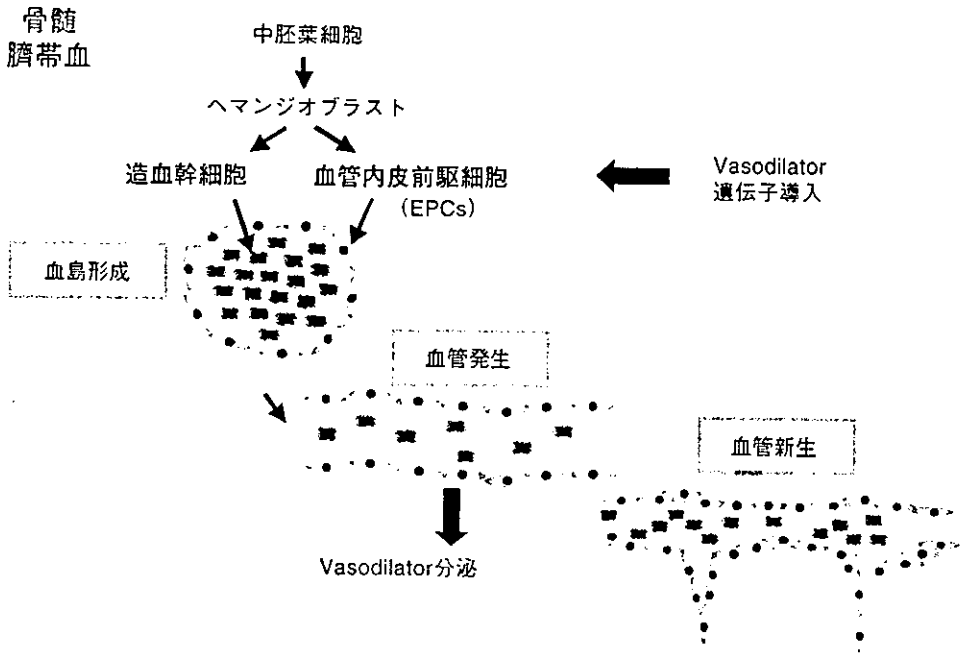


図2　血管の発生と遺伝子-細胞移植ハイブリット治療

ヘマンジオブラスト由来の造血幹細胞と血管内皮前駆細胞(EPCs)は血島を形成し，血管の発生に関与する．血管拡張因子の遺伝子を導入したEPCsを移植することで，細胞による血管再生と血管拡張因子による肺血管拡張が同時に可能となる(遺伝子-細胞移植ハイブリッド治療)．

皮細胞の発芽的増殖と遊走によるものと理解されていたが，1997年，Asaharaらは成人の末梢血中には内皮細胞に分化しうる血管内皮前駆細胞が存在することを明らかにした[14]．胎生初期の血管形成は，造血幹細胞が中心部に位置し血管内皮前駆細胞が辺縁部に位置する細胞群としていわゆる血島(blood island)から始まる(図2)．血島はその後互いに融合し合い，辺縁部に位置する血管内皮前駆細胞が血管内皮細胞となり血管壁を形成し，中心部に位置する造血幹細胞が血球系に分化して血液を形成する．CD 34は造血幹細胞のマーカーとして知られているが，血管内皮前駆細胞もまたCD 34陽性細胞の分画から得られる．これらの解剖学的または発生学的類似性から，造血幹細胞と血管内皮前駆細胞は共通の先祖細胞であるヘマンジオブラストから分化してくるものと考えられている．

血管内皮前駆細胞は血管障害部位への遊走能，血管再生能を持ち，生体内で虚血や血管内皮障害が起こったときに骨髄から強制動員され，血中を流れてその障害部位へ付着して血管内皮細胞に分化して血管を形成する[14]．この細胞の能力を利用して，急性心筋梗塞ラットに静脈から血管内皮前駆細胞を投与すると，血管新生さらには梗塞サイズが縮小され，心機能が改善することが報告された[15]．臨床の場においても血管内皮前駆細胞の移植は虚血性心疾患，閉塞性動脈硬化症の治療に有効であることが報告されている[16][17]．血管内皮前駆細胞の治療効果は❶血管内皮前駆細胞自身が血管内皮細胞に分化して血管形成に参加するため，❷血管内皮前駆細胞がVEGFらの血管新生因子を放出して局所の血管新生を促すためと考えられる．

われわれは血管内皮前駆細胞の移植による肺高血圧治療効果を動物実験で検討した[18]．ヒト臍帯血から単核球を分離し，VEGF下で培養することで血管内皮前駆細胞を得た．これらの細胞は内皮細胞に比較的特徴的な抗原(CD 31，VE-cadherin，KDR)を有していた．正常のラットに静脈から血管内皮前駆細胞を投与しても肺組織への付着は認

められなかったが，モノクロタリンで肺血管内皮細胞や間質に障害を与えた後に投与すると，血管内皮前駆細胞は肺細動脈と間質に付着し成熟した血管内皮細胞となった．また，血管内皮前駆細胞の移植は肺組織の血管数を増加させた．平均肺動脈圧に有意な変化は認められなかったが，肺血管抵抗のわずかな改善が認められた．その後，自家の血管内皮前駆細胞を移植すれば，モノクロタリン誘発肺高血圧を有意に抑制することが他施設から報告された[19]．しかし，血管内皮前駆細胞の移植のみでは肺高血圧治療効果に限界があると思われた．

遺伝子-細胞移植ハイブリッド治療

われわれは血管内皮前駆細胞の移植治療効果を高めるために遺伝子-細胞移植ハイブリッド治療を開発した．血管内皮細胞より産生される生体内で，最も強力な血管拡張ペプチドであるアドレノメデユリン[20]の遺伝子を用いた．アドレノメデユリンの特異的受容体は体血管よりむしろ肺血管に多数存在し[21]，血管平滑筋細胞の受容体に直接作用してcAMPを増加させまた血管内皮に働き一酸化窒素を介して血管拡張を来す[22][23]．われわれは，実際にアドレノメデユリンの経静脈的または経気道からの投与がPPH患者の肺血管抵抗を著明に低下させることを証明してきた[24][25]．近年，アドレノメデユリンはPI 3 K-Akt経路を活性化することで血管内皮細胞の生存，遊走，増殖，血管新生に関与することが明らかとなった．われわれは，血管内皮前駆細胞とアドレノメデユリンの相互作用に関する研究を行っており，アドレノメデユリン遺伝子導入によって血管内皮前駆細胞のアポトーシスが抑制されることや増殖が促進されることを明らかにした．またアドレノメデユリン遺伝子は血管内皮前駆細胞をベクターとして肺組織まで運ばれることで(Cell-based gene therapy)，アドレノメデユリンの発現による肺血管拡張作用が期待できる．こうして，細胞移植による治療効果と導入した遺伝子の発現による相乗効果で治療効果を増強させる，いわゆる遺伝子-細胞ハイブリッド治療法を生み出した．

血管内皮前駆細胞への新たな遺伝子導入法の開発

ブタの皮膚から得たゼラチンをグルタルアルデヒドの架橋反応により格子構造としエチレンジアミンを加えると正帯電ゼラチンが完成する[26]．この正帯電ゼラチンは蛋白のみでなく，一般に負に帯電したDNAと数時間接触することにより容易に電気的複合体を形成する(図3A，B)．このゼラチンは生体内で徐々に吸収されるため，ゼラチンに結合した蛋白やDNAの徐放，すなわち長時間の発現が可能である．われわれは，マクロファージなどの従来の貪食細胞と同様に，血管内皮前駆細胞がゼラチン/プラスミドDNA複合体を容易に貪食することを報告した(図3C)[18]．この血管内皮前駆細胞の貪食能を利用した*ex vivo*遺伝子導入は，ウイルスベクターを用いずに血管内皮前駆細胞自身への50～70％の高率の遺伝子導入を可能にした．またゼラチンはその分解とともにプラスミドDNAを徐放でき，長時間の遺伝子発現を可能にした．図4は，蛍光色素でラベルしたDNAが細胞内に貪食されたゼラチンから徐々に放出され核へ向かっている様子を可視化したものである．実際にこの方法でアドレノメデユリン遺伝子を血管内皮前駆細胞へ導入すると細胞質での蛋白の強発現が確認でき，その発現は2週間以上の長期間持続することがわかった(図5)．この遺伝子導入法は方法も比較的簡便であることから，画期的な遺伝子導入法として期待される．

われわれが考案したこのゼラチンを用いた遺伝子-細胞移植ハイブリッド治療法は❶非ウイルス的に遺伝子導入を行うため安全性が高い，❷血管内皮前駆細胞が持つ虚血部・血管内皮障害部位へのhomingを利用して，静脈内投与による組織選択的治療が可能となる，❸機能遺伝子導入によって血管内皮前駆細胞の増殖を高め，一方アポトーシスを抑制することで，血管内皮前駆細胞自身の機能強化が可能である，❹遺伝子導入によって血管内皮前駆細胞から蛋白が過剰分泌され，パ

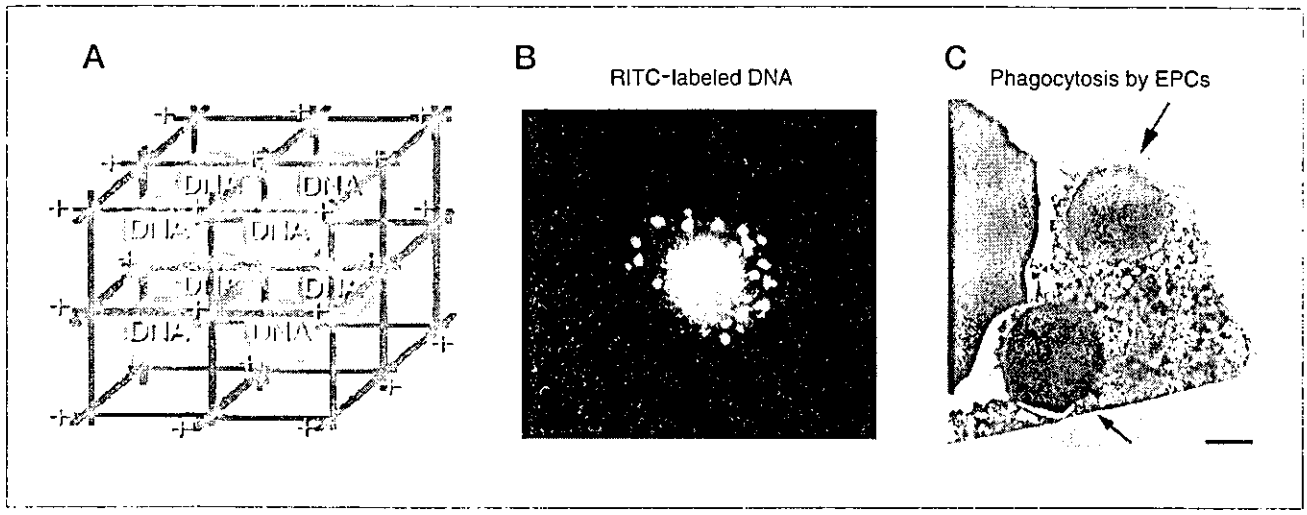


図3　DNA-ゼラチン複合体のシェーマ(A)とその実像(B)・電顕像(C)

DNA は赤色蛍光で表示(B)．(C)電顕像で血管内皮前駆細胞(EPCs)が DNA-ゼラチン複合体を貪食するのが確認された．

(文献 18 より改変，引用)

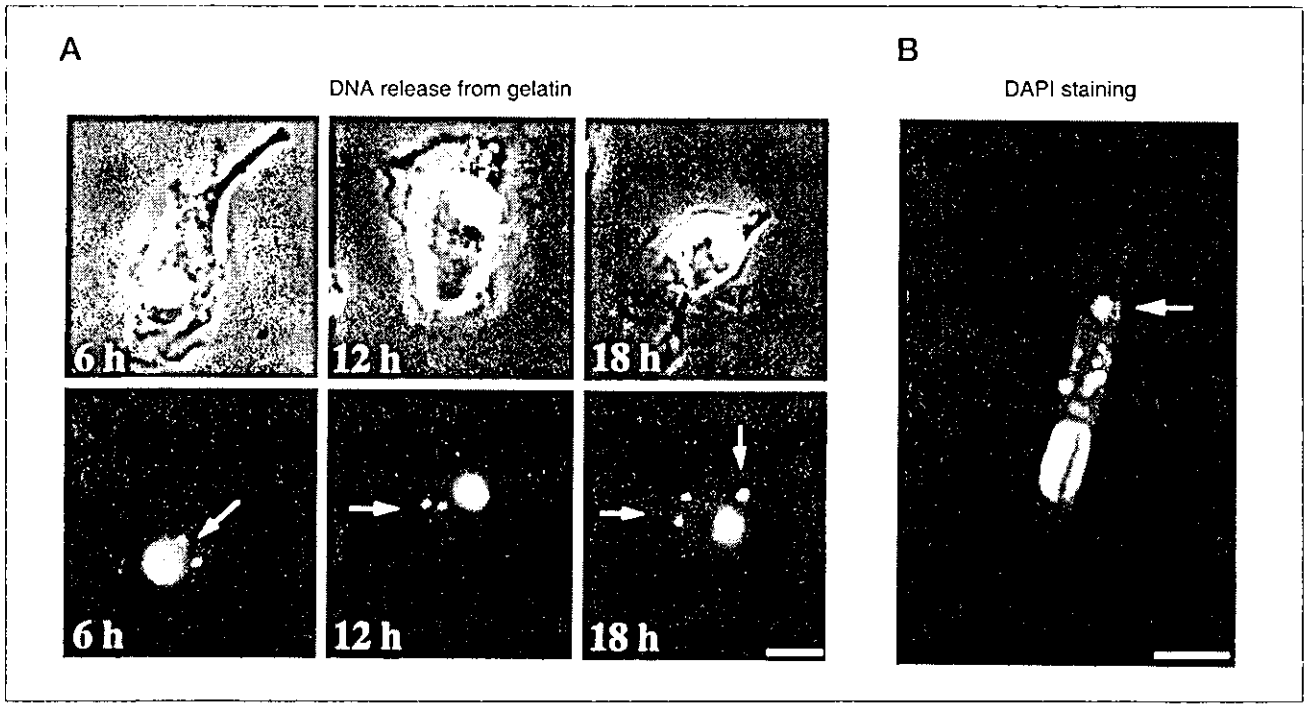


図4　血管内皮前駆細胞に貪食された DNA-ゼラチン複合体からの DNA 放出

(A)ゼラチンの細胞内での分解に伴い DNA は徐々に放出された．(B)ゼラチン(矢印)から DNA が核に向かって放出される像を示す．

ラクライン的に生理機能を発揮するらの利点がある．

アドレノメデユリン遺伝子と血管内皮前駆細胞を用いた肺高血圧治療

モノクロタリン肺高血圧ラットに，アドレノメデユリン遺伝子を導入した血管内皮前駆細胞を静脈内投与して，3 週間後の肺高血圧軽減効果を検討した[18]．血管内皮前駆細胞はゼラチン/DNA 複合体を貪食し，血管内皮前駆細胞自身への高率の遺伝子導入が可能であった．アドレノメデユリン遺伝子導入血管内皮前駆細胞は血管内皮前駆細胞単独の 10 倍のアドレノメデユリンを分泌し，2 週間以上発現が持続した(図 5)．経静脈的に投与した血管内皮前駆細胞は，肺細動脈内面と間質に

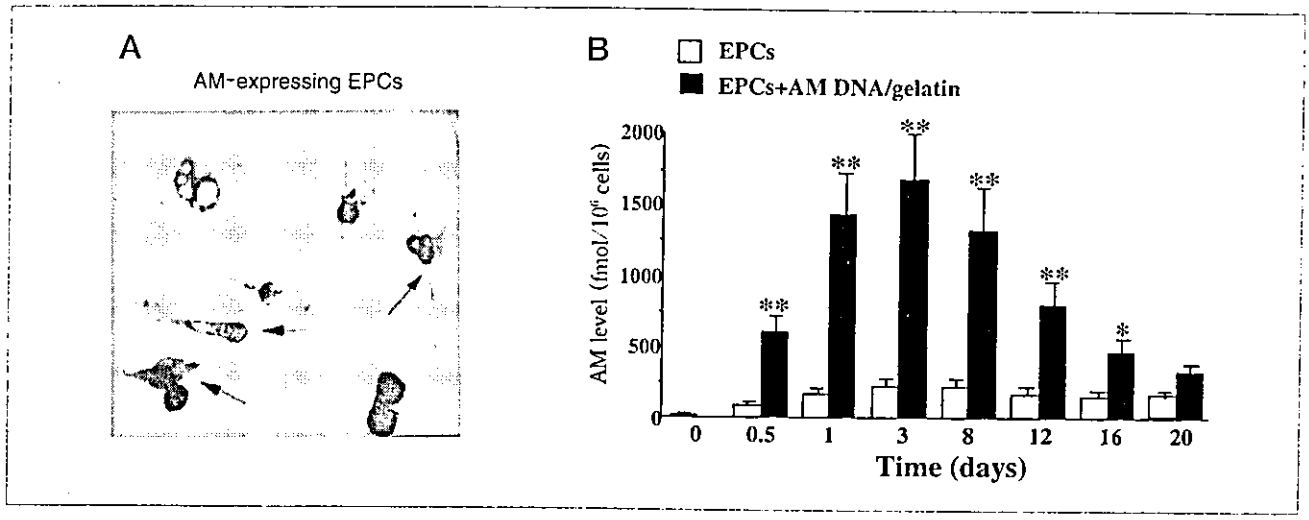


図5　ゼラチンによる遺伝子導入後の血管内皮前駆細胞(EPCs)でのアドレノメデユリン(AM)の発現
AMの免疫染色(A)と培養液中のAM濃度(B)

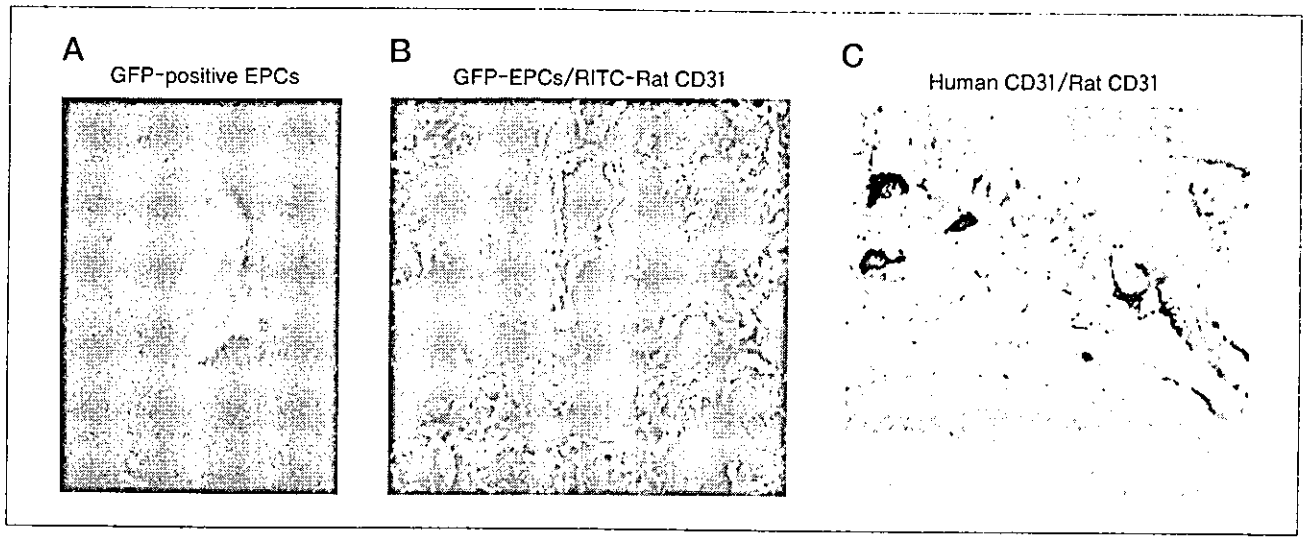


図6　遺伝子導入血管内皮前駆細胞(EPCs)による肺組織への付着と肺血管の形成
GFP遺伝子導入EPCsは肺細動脈の内面や肺組織の間質に付着し血管を形成した(A, B)．またヒトEPCsはラット肺組織内で血管内皮(CD 31陽性)を形成した(C)．

付着して血管を形成した(図6)．アドレノメデユリン遺伝子導入血管内皮前駆細胞の移植は，コントロール群に比べて平均肺動脈圧を有意に低下させ(24 ± 2 vs 34 ± 1 mmHg)，生存率を改善させた(図7)．これらの効果は血管内皮前駆細胞の単独投与より優っていた．既存の治療に抵抗性のPPH患者が少なからず存在することを考えると，この遺伝子－細胞移植ハイブリット治療法は重症肺高血圧症に対する新たな治療法となる可能性がある．

おわりに

肺高血圧症では，肺血管内皮の機能異常によって血管作動物質のバランスが破綻している．われわれは，血管拡張因子であるアドレノメデユリン遺伝子を導入した血管内皮前駆細胞を移植することで肺高血圧が軽減できることを証明した．細胞移植と遺伝子治療を同時に行う遺伝子-細胞移植ハイブリッド治療法は，細胞による肺血管内皮の再生のみでなく，血管内皮前駆細胞をベクターと

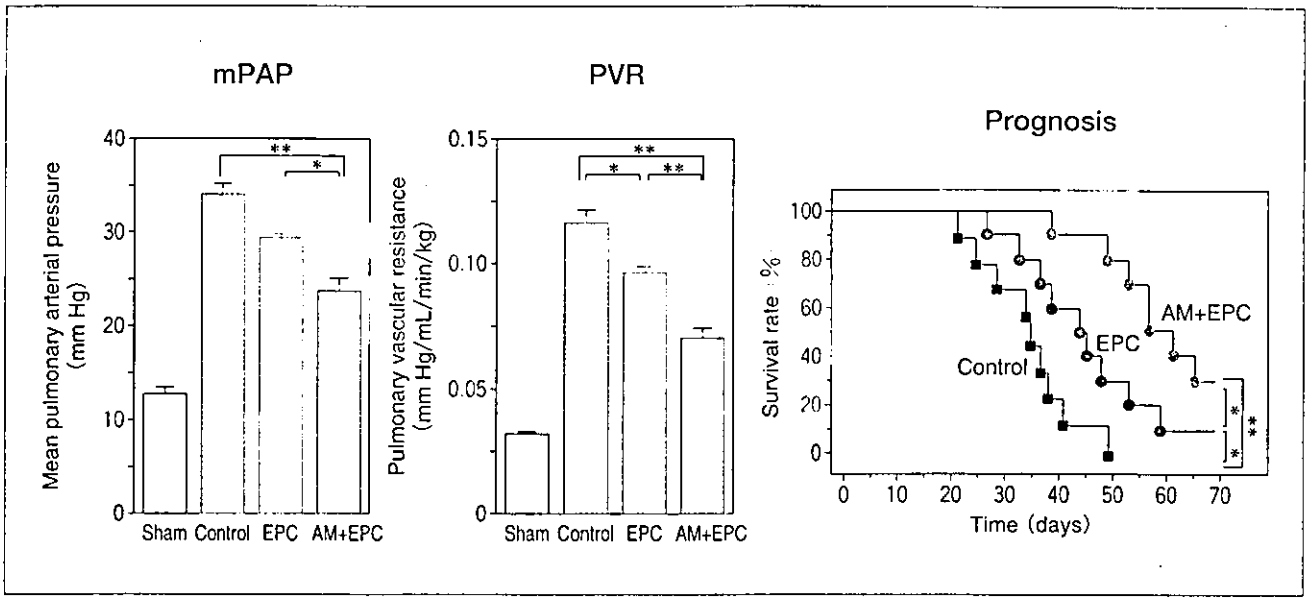


図7　アドレノメデュリン(AM)遺伝子導入EPCの移植による平均肺動脈圧(mPAP)，肺血管抵抗(PVR)，肺高血圧ラットの予後改善効果

Sham：正常ラット，Control：モノクロタリン肺高血圧ラットへvehicle投与，
EPC：肺高血圧ラットへEPC投与，AM＋EPC：肺高血圧ラットへAM遺伝子導入EPC投与
* $P < 0.05$，** $P < 0.001$

（文献18より改変，引用）

して肺組織選択的に遺伝子導入を可能にし，新たな肺高血圧治療法として期待される．

References

1) Archer S, Rich S : Primary pulmonary hypertension : a vascular biology and translational research "Work in progress". Circulation 102 : 2781-2791, 2000
2) Giaid A, Yanagisawa M, Langleben D, et al : Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. N Engl J Med 328 : 1732-1739, 1993
3) Tuder RM, Cool CD, Geraci MW, et al : Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension. Am J Respir Crit Care Med 159 : 1925-1932, 1999
4) Christman BW, McPherson CD, Newman JH, et al : An imbalance between the excretion of thromboxane and prostacyclin metabolites in pulmonary hypertension. N Engl J Med 327 : 70-75, 1992
5) Champion HC, Bivalacqua TJ, D'Souza FM, et al : Gene transfer of endothelial nitric oxide synthase to the lung of the mouse in vivo. Effect on agonist-induced and flow-mediated vascular responses. Circ Res 84 : 1422-1432, 1999
6) Isner JM, Pieczek A, Schainfeld R, et al : Clinical evidence of angiogenesis after arterial gene transfer of phVEGF 165 in patient with ischaemic limb. Lancet 348 : 370-374, 1996
7) Lederman RJ, Mendelsohn FO, Anderson RD, et al : Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study) : a randomised trial. Lancet 359 : 2053-2058, 2002
8) Aoki M, Morishita R, Taniyama Y, et al : Therapeutic angiogenesis induced by hepatocyte growth factor : potential gene therapy for ischemic diseases. J Atheroscler Thromb 7 : 71-76, 2000
9) Gerber HP, McMurtrey A, Kowalski J, et al : Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 273 : 30336-30343, 1998
10) Tuder RM, Chacon M, Alger L, et al : Expression of angiogenesis-related molecules in plexiform lesions in severe pulmonary hypertension : evidence for a process of disordered

angiogenesis. J Pathol 195 : 367-374, 2001
11) Partovian C, Adnot S, Raffestin B, et al : Adenovirus-mediated lung vascular endothelial growth factor overexpression protects against hypoxic pulmonary hypertension in rats. Am J Respir Cell Mol Biol 23 : 762-771, 2000
12) Zhao YD, Campbell AI, Robb M, et al : Protective role of angiopoietin-1 in experimental pulmonary hypertension. Circ Res 92 : 984-991, 2003
13) Ono M, Sawa Y, Matsumoto K, et al : In vivo gene transfection with hepatocyte growth factor via the pulmonary artery induces angiogenesis in the rat lung.Circulation 106 : 1264-1269, 2002
14) Asahara T, Murohara T, Sullivan A, et al : Isolation of putative progenitor endothelial cells for angiogenesis. Science 275 : 964-967, 1997
15) Kawamoto A, Gwon HC, Iwaguro H, et al : Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 103 : 634-637, 2001
16) Stamm C, Westphal B, Kleine HD, et al : Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 361 : 45-46, 2003
17) Tateishi-Yuyama E, Matsubara H, Murohara T, et al : Therapeutic Angiogenesis using Cell Transplantation (TACT) Study Investigators. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells : a pilot study and a randomised controlled trial. Lancet 360 : 427-435, 2002
18) Nagaya N, Kangawa K, Kanda M, et al : Hybrid cell-gene therapy for pulmonary hypertension based on phagocytosing action of endothelial progenitor cells. Circulation 108 : 889-895, 2003
19) Zhao YD, Deng Y, Zhang Q, et al : Regeneration of lung microvasculature by endothelial progenitor cells in experimental pulmonary arterial hypertension (PAH). Circulation Supplement Ⅳ-295, 2003 (abstract)
20) Kitamura K, Kangawa K, Kawamoto M, et al : Adrenomedullin : a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 192 : 553-560, 1993
21) Owji AA, Smith DM, Coppock HA, et al : An abundant and specific binding site for the novel vasodilator adrenomedullin in the rat. Endocrinology 136 : 2127-2134, 1995
22) Ishizaka Y, Ishizaka Y, Tanaka M, et al : Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. Biochem Biophys Res Commun 200 : 642-646, 1994
23) Nakamura M, Yoshida H, Makita S, et al : Potent and long-lasting vasodilatory effects of adrenomedullin in humans : comparisons between normal subjects and patients with chronic heart failure. Circulation 95 : 1214-1221, 1997
24) Nagaya N, Nishikimi T, Uematsu M, et al : Haemodynamic and hormonal effects of adrenomedullin in patients with pulmonary hypertension. Heart 84 : 653-658, 2000
25) Nagaya N, Kyotani S, Uematsu M, et al : Effects of adrenomedullin inhalation on hemodynamics and exercise capacity in patients with primary pulmonary hypertension. Circulation (in press)
26) Tabata Y, Nagano A, Ikada Y. Biodegradation of hydrogel carrier incorporating fibroblast growth factor. Tissue Engin 5 : 127-138, 1999

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Cell sheet engineering for myocardial tissue reconstruction

Tatsuya Shimizu, Masayuki Yamato, Akihiko Kikuchi, Teruo Okano*

Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

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Abstract

Myocardial tissue engineering has now emerged as one of the most promising treatments for the patients suffering from severe heart failure. Tissue engineering has currently been based on the technology using three-dimensional (3-D) biodegradable scaffolds as alternatives for extracellular matrix. According to this most popular technique, several types of 3-D myocardial tissues have been successfully engineered by seeding cardiomyocytes into poly(glycolic acid), gelatin, alginate or collagen scaffolds. However, insufficient cell migration into the scaffolds and inflammatory reaction due to scaffold biodegradation remain problems to be solved. In contrast to these technologies, we now propose novel tissue engineering methodology layering cell sheets to construct 3-D functional tissues without any artificial scaffolds. Confluent cells on temperature-responsive culture surfaces can be harvested as a viable contiguous cell sheet only by lowering temperature without any enzymatic digestions. Electrical communications are established between layered cardiomyocyte sheets, resulting in simultaneous beating 3-D myocardial tissues. Layered cardiomyocyte sheets in vivo present long survival, macroscopic pulsation and characteristic structures of native heart tissue. Cell sheet engineering should have enormous potential for fabricating clinically applicable myocardial tissues and should promote tissue engineering research fields.

Keywords: Myocardial tissue engineering; Cell sheet; Cardiac myocyte; Transplantation; Temperature-responsive culture surface

1. Introduction

Recently, alternative treatments for cardiac transplantation have been strongly requested to repair damaged heart tissue, because the utility of heart transplantation is limited by donor shortage. Cell therapy is now considered to be one of the most effective treatments for impaired heart tissue [1,2]. Direct transplantation of cell suspension has been researched since the early 1990s [3]. In these studies, survival of transplanted cells, integration of native and grafted cells, and improvement of host cardiac function have been reported. It is a critical point how to isolate and expand clinically transplantable myocardial cell source. Autologous myoblast transplantation has been performed clinically and the contraction and viability of grafted myoblasts have been confirmed [4]. Multipotent bone marrow cells or embryonic stem cells have been now aggressively investigated as possible candidates for human implantable myocardial cell source [5–8].

In direct injection of dissociated cells, it is difficult to control shape, size and location of the grafted cells. Additionally, isolated cell transplantation is not enough for replacing congenital defects. To overcome these problems, research on fabricating three-dimensional (3-D) cardiac grafts by tissue engineering technology has also now begun [9]. Tissue engineering has currently been based on the concepts that 3-D biodegradable scaffolds are useful as alternatives for extracellular matrix (ECM) and that seeded cells reform their native structure in according to scaffold biodegradation [10]. This context has been used for every type of tissue. In myocardial tissue engineering, poly(glycolic acid) (PGA), gelatin and alginate have been used as prefabricated biodegradable scaffolds. Papadaki et al. engineered 3-D cardiac constructs by using PGA scaffolds processed into porous meshes and rotating bioreactors [11]. Li et al. have demonstrated that transplantation of tissue-engineered cardiac grafts using biodegradable gelatin sponges replaced myocardial scar and right ventricular outflow track defect [12,13].

*Corresponding author. Tel.: +81-3-3353-8111x30234; fax: +81-3-3359-6046.
E-mail address: tokano@abmes.twmu.ac.jp (T. Okano).

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