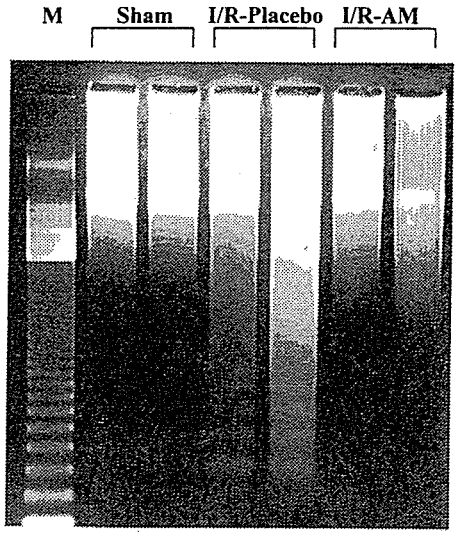

Figure 4. DNA ladder in sham, I/R-placebo, and I/R-AM groups. Although typical DNA ladder indicating fragmented DNA in cardiomyocytes was observed in I/R-placebo group, it was attenuated in I/R-AM group. M indicates molecular marker.

Discussion

In the present study, we demonstrated that short-term infusion of AM during the early phase of ischemia/reperfusion significantly reduced myocardial infarct size and inhibited myocyte apoptosis, and AM significantly decreased LVEDP and tended to improve LV dP/dt_{max} and dP/dt_{min}. We also demonstrated that AM enhanced Akt phosphorylation in cardiac tissue and that pretreatment with a PI3K inhibitor attenuated AM-induced cardioprotective effects against ischemia/reperfusion and inhibited AM-induced Akt phosphorylation.

Intravenous infusion of AM has beneficial hemodynamic and renal effects in patients with heart failure.[8] However, whether AM has direct cardioprotective effects in vivo remains unclear. In the present study, we demonstrated that short-term infusion of AM during the early phase of ischemia/reperfusion markedly reduced myocardial infarct size. Cardiomyocyte apoptosis is one of the major contributors to the development of myocardial infarcts,[15,16] which is related to the pathogenesis of heart failure. Thus, we examined whether AM has antiapoptotic effects in cardiomyocytes. Interestingly, short-term infusion of AM significantly reduced myocyte apoptosis after ischemia/reperfusion. This is the first study to demonstrate antiapoptotic effects of AM against myocardial ischemia/reperfusion injury, although AM has been shown to have antiapoptotic effects in vascular endothelial cells.[17,18] Given that cardiomyocyte apoptosis rather than necrosis contributes to myocyte death after ischemia/reperfusion, the antiapoptotic effects of AM may result in the reduced infarct size after ischemia/reperfusion.

In the present study, 60-minute infusion of AM improved cardiac function after ischemia/reperfusion, as indicated by a significant decrease in LVEDP and a tendency for an increase in LV dP/dt_{max} and a decrease in LV dP/dt_{min}. Previous studies have shown that the susceptibility to cardiac dysfunction

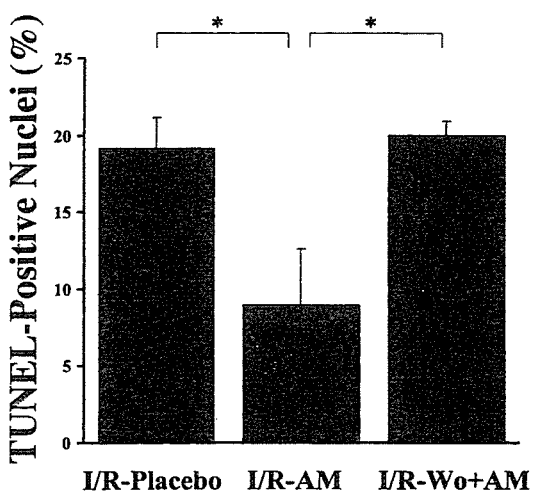

Figure 5. Quantitative analysis of TUNEL-positive nuclei in myocytes. Number of TUNEL-positive myocytes was lower in I/R-AM group than in I/R-placebo group. However, number of TUNEL-positive myocytes in I/R-Wo+AM group was as large as in I/R-placebo group. Data are mean±SEM. *$P<0.05$.

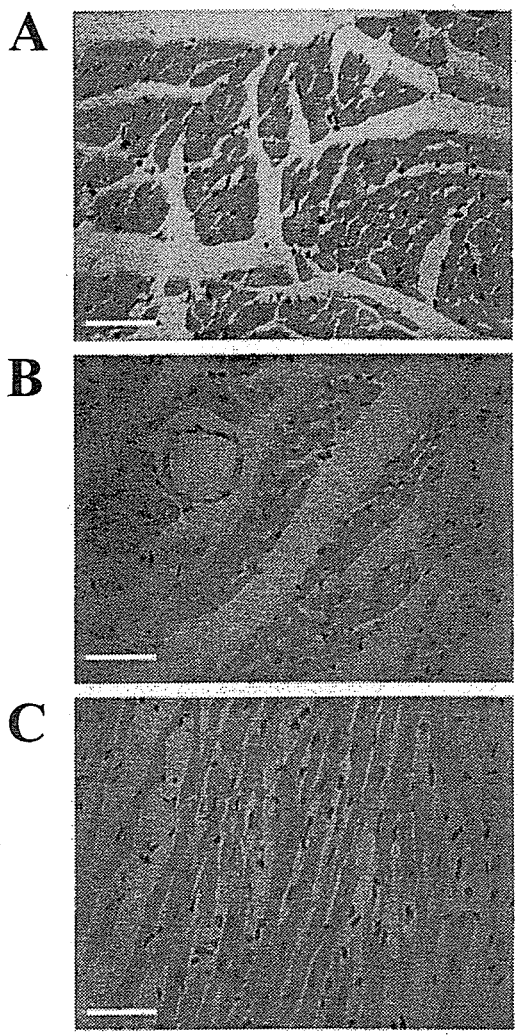

Figure 6. Immunohistochemistry for CRLR in rat cardiac tissue. Representative photomicrographs revealed that CRLR was localized in cardiomyocytes (A) and vascular endothelial cells (B). Negative control study (using mouse IgG) showed no positive staining in cardiac tissue (C). Original magnification ×400. Bar=20 μm.

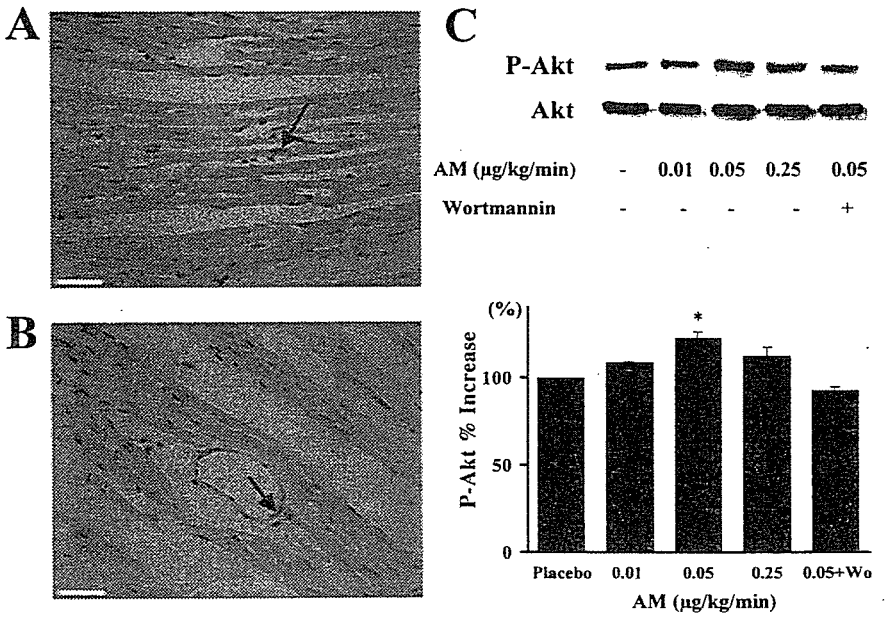

Figure 7. A and B, Immunohistochemistry for Akt phosphorylation in rat cardiac tissue. Infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) phosphorylated Akt predominantly in nuclei of cardiomyocytes (A, B) and vascular endothelial cells (B). Arrow indicates nuclei of cardiomyocytes with positive staining for P-Akt antibody. Arrowhead indicates nuclei of endothelium with positive staining for P-Akt antibody. Original magnification ×400. Bar=20 μm. C, Western blot analysis of AM-induced Akt phosphorylation in cardiac tissues. Infusion of AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) activated Akt in myocardial tissues exposed to ischemia/reperfusion. Pretreatment with wortmannin significantly inhibited AM-induced Akt phosphorylation. P-Akt indicates phosphorylated Akt; Wo, wortmannin. Data are mean±SEM. *$P<0.05$ vs placebo.

depends on the degree of myocyte apoptosis within 24 hours after ischemia/reperfusion.[19] Thus, the early prevention of myocyte apoptosis and the resultant reduced infarct size by AM may contribute to the hemodynamic improvement after ischemia/reperfusion. AM infusion reduced right ventricular systolic pressure, which may be attributable not only to the potent vasodilatory effects of AM but also to improvement in cardiac function.

Recently, Akt activation has been shown to reduce myocyte apoptosis and thereby prevent myocardial injury after transient ischemia.[10] Akt is the downstream effector molecule for signal transduction initiated by cardioprotective hormones such as insulin-like growth factor I.[20] Thus, Akt is considered to be a powerful survival signal in myocytes.[21] More recently, AM has been shown to activate the PI3K/Akt-pathway in vascular endothelial cells.[9] However, localization of AM-specific receptors in cardiac tissue had been unknown. The present study demonstrated that CRLR was present in rat cardiomyocytes and vascular endothelial cells and that AM infusion accelerated Akt phosphorylation in nuclei of cardiomyocytes and vascular endothelial cells. Furthermore, Western blot analyses demonstrated that AM 0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$ significantly increased phosphorylated Akt in cardiac tissue compared with placebo treatment and that pretreatment with wortmannin significantly inhibited Akt phosphorylation. Interestingly, pretreatment with wortmannin attenuated the AM-induced beneficial effects, such as reduction of infarct size, hemodynamic improvements, and inhibition of apoptosis. These findings suggest that AM infusion directly induces cardioprotective effects through the PI3K/Akt-dependent pathway.

In the present study, plasma AM level during infusion was much higher than baseline plasma level in rats, plasma level in normal human subjects (≈10 fmol/mL),[8] and plasma level in patients with acute myocardial infarction (≈14 fmol/mL).[22] These findings suggest that exogenously administered AM functions at pharmacological levels.

Preclinical studies have demonstrated that a variety of antioxidative or antiapoptotic agents reduce myocardial infarct size after ischemia/reperfusion.[23,24] However, few agents are clinically available for patients with coronary artery disease. In contrast, the safety and hemodynamic benefits of short-term treatment with intravenous AM (0.05 $\mu g \cdot kg^{-1} \cdot min^{-1}$) have been demonstrated in patients with heart failure[8] and patients with myocardial infarction.[25] Given the results of the present study, a prospective, randomized, placebo-controlled clinical trial should be planned.

Conclusions

Short-term infusion of AM significantly attenuated myocardial ischemia/reperfusion injury. These cardioprotective effects were attributed mainly to the antiapoptotic effects of AM via a PI3K/Akt-dependent pathway.

Acknowledgments

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Effects of Adrenomedullin Inhalation on Hemodynamics and Exercise Capacity in Patients With Idiopathic Pulmonary Arterial Hypertension

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Background—Adrenomedullin (AM) is a potent pulmonary vasodilator peptide. However, whether intratracheal delivery of aerosolized AM has beneficial effects in patients with idiopathic pulmonary arterial hypertension remains unknown. Accordingly, we investigated the effects of AM inhalation on pulmonary hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

Methods and Results—Acute hemodynamic responses to inhalation of aerosolized AM (10 μg/kg body wt) were examined in 11 patients with idiopathic pulmonary arterial hypertension during cardiac catheterization. Cardiopulmonary exercise testing was performed immediately after inhalation of aerosolized AM or placebo. The work rate was increased by 15 W/min until the symptom-limited maximum, with breath-by-breath gas analysis. Inhalation of AM produced a 13% decrease in mean pulmonary arterial pressure (54±3 to 47±3 mm Hg, $P<0.05$) and a 22% decrease in pulmonary vascular resistance (12.6±1.5 to 9.8±1.3 Wood units, $P<0.05$). However, neither systemic arterial pressure nor heart rate was altered. Inhalation of AM significantly increased peak oxygen consumption during exercise (peak $\dot{V}O_2$, 14.6±0.6 to 15.7±0.6 mL · kg^{-1} · min^{-1}, $P<0.05$) and the ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}O_2/\Delta W$ ratio, 6.3±0.4 to 7.0±0.5 mL · min^{-1} · W^{-1}, $P<0.05$). These parameters remained unchanged during placebo inhalation.

Conclusions—Inhalation of AM may have beneficial effects on pulmonary hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension. (***Circulation.* 2004;109:351-356.**)

Key Words: peptides ■ hypertension, pulmonary ■ respiration ■ exercise ■ hemodynamics

Idiopathic pulmonary arterial hypertension is a rare but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right heart failure and death.[1,2] Although a variety of vasodilators have been proposed as potential therapy for this disease over the past 30 years,[3–7] some patients ultimately require heart-lung or lung transplantation.[8,9] Thus, a novel therapeutic strategy is desirable.

Adrenomedullin (AM) is a potent, long-lasting vasodilator peptide that was originally isolated from human pheochromocytoma.[10] Immunoreactive AM has subsequently been detected in plasma and a variety of tissues, including blood vessels and lungs.[11,12] It has been reported that there are abundant binding sites for AM in the lungs.[13] We 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.[14,15] Interestingly, AM has been shown to inhibit the migration and proliferation of vascular smooth muscle cells.[16,17] These findings suggest that AM plays an important role in the regulation of pulmonary vascular tone and vascular remodeling. In fact, we have shown that short-term intravenous infusion of AM significantly decreases pulmonary vascular resistance in patients with congestive heart failure[18] or pulmonary arterial hypertension.[19] Unfortunately, however, intravenously administered AM induced systemic hypotension in such patients because of nonselective vasodilation in the pulmonary and systemic vascular beds.

More recently, inhalation of aerosolized prostacyclin and its analogue iloprost has been shown to cause pulmonary vasodilation without systemic hypotension in patients with idiopathic pulmonary arterial hypertension.[20,21] In addition, inhalant application of vasodilators does not impair gas exchange because the ventilation-matched deposition of drug

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TABLE 1. Baseline Characteristics of Patients With Idiopathic Pulmonary Arterial Hypertension

Demographics	
Age, y	39±3
Male/female, n	2/9
NYHA functional class, n	
III	10
IV	1
Baseline hemodynamics	
MPAP, mm Hg	54±3
CI, $L \cdot min^{-1} \cdot m^{-2}$	2.4±0.1
PVR, Wood units	12.6±1.5
RAP, mm Hg	7±1
PCWP, mm Hg	7±1
Pulmonary function	
Sao_2, %	94±3
Svo_2, %	63±4
FVC, % predicted	86±4
FEV_1, % predicted	75±1
6-Minute walk test, m	355±35
Medication use, n	
Anticoagulant agents	10
Diuretics	9
Digitalis	7
Oral prostacyclin analogue	6
Calcium antagonists	2

NYHA indicates New York Heart Association; MPAP, mean pulmonary arterial pressure; CI, cardiac index; PVR, pulmonary vascular resistance; RAP, mean right atrial pressure; PCWP, pulmonary capillary wedge pressure; Sao_2, arterial oxygen pressure; Svo_2, mixed venous oxygen saturation; FVC, forced vital capacity; and FEV_1, forced expiratory volume in 1 second. Data are mean±SEM.

in the alveoli causes pulmonary vasodilation matched to ventilated areas.[20] In clinical settings, inhalation therapy may be more simple, noninvasive, and comfortable than continuous intravenous infusion therapy. Thus, the purpose of the present study was to investigate the effects of AM inhalation on hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

Methods

Study Subjects

Eleven patients with idiopathic pulmonary arterial hypertension (9 women and 2 men; age, 39±3 years) were included in this study. Idiopathic pulmonary arterial hypertension was defined as pulmonary hypertension unexplained by any secondary cause, on the basis of the criteria of the National Institutes of Health registry.[1] Ten patients were classified as New York Heart Association (NYHA) functional class III and 1 as class IV (Table 1). Two of the 11 patients (18%) were acute responders who showed a significant decrease in mean pulmonary arterial pressure of ≥20% with a decrease in mean pulmonary arterial pressure to <35 mm Hg and no change or an increase in cardiac index during short-term infusion of epoprostenol. Long-term medication, including anticoagulant agents, digitalis, and diuretics, was kept constant. Vasodilator agents, such as oral prostacyclin analogue and calcium antagonists, were stopped ≥12 hours before the study procedure was begun. The ethics committee of the National Cardiovascular Center approved the study, and all patients gave written informed consent.

Preparation of Human AM

Human AM was dissolved in saline with 4% D-mannitol and sterilized by passage through a 0.22-μm filter (Millipore Co). At the time of dispensing, randomly selected vials were submitted for sterility and pyrogen testing. The chemical nature and content of the human AM in vials were verified by high-performance liquid chromatography and radioimmunoassay. All vials were stored frozen at −80°C from the time of dispensing until the time of preparation for administration.

Hemodynamic Studies

Acute hemodynamic responses to AM inhalation were assessed in all patients while they were in a stable condition during hospitalization. Hemodynamic variables, including pulmonary arterial pressure, right atrial pressure, pulmonary capillary wedge pressure, and cardiac output (in triplicate), were determined with a thermodilution catheter (TOO21H-7.5F, Baxter Co).[22] A 22-gauge cannula was inserted into a radial artery for hemodynamic measurements and blood sampling. After an equilibration period of 30 minutes, baseline hemodynamics were measured. Then, AM (10 μg/kg body wt) was inhaled as an aerosol with a jet nebulizer (Porta-Nebu, MEDIC-AID) for 15 minutes, which resulted in a cumulative dose of 400 to 600 μg AM. Hemodynamic parameters were measured at 15-minute intervals starting 15 minutes before AM inhalation until 60 minutes after inhalation. Blood samples for AM measurement were taken at 15-minute intervals from 15 minutes before inhalation until 60 minutes after the end of inhalation.

Cardiopulmonary Exercise Testing

The effects of AM inhalation on exercise capacity were examined in 10 of 11 patients; 1 patient with NYHA class IV underwent the 6-minute walk test according to decision of attending physicians. Cardiopulmonary exercise testing was performed immediately after inhalation of aerosolized AM (10 μg/kg body wt) or saline in a double-blind, randomized, crossover design. This study was performed on 2 separate days, 1 week apart. The first cardiopulmonary exercise testing was performed within 10 days after the cardiac catheterization. The patients performed exercise seated on a cycle ergometer. They first pedaled at 55 rpm without any added load for 1 minute. The work rate was then increased by 15 W/min up to the symptom-limited maximum. Breath-by-breath gas analysis was performed with an AE280 (Minato Medical Science) connected to a personal computer running analyzing software.[23] The ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}o_2/\Delta W$ ratio) was calculated as the slope of oxygen consumption per unit workload from 1 minute after the start of load addition until 85% maximal $\dot{V}o_2$. Exercise capacity was evaluated by peak oxygen consumption (peak $\dot{V}o_2$), which was defined as the value of averaged data during the final 15 seconds of exercise. Ventilatory efficiency during exercise was represented by the $\dot{V}E$-$\dot{V}CO_2$ slope, which was determined as the linear regression slope of $\dot{V}E$ and $\dot{V}CO_2$ from the start of exercise until the RC point (the time until which ventilation is stimulated by CO_2 output and end-tidal CO_2 tension begins to decrease).

Measurement of Plasma AM, cAMP, and cGMP

Blood samples were immediately transferred into chilled glass tubes containing disodium EDTA (1 mg/mL) and aprotinin (500 U/mL) and centrifuged immediately at 4°C, and the plasma was frozen and stored at −80°C until assayed. Plasma AM level was measured by a specific immunoradiometric assay kit (Shionogi Pharmaceutical Co Ltd).[24] Plasma cAMP and cGMP were determined with radioimmunoassay kits (cAMP assay kit, cGMP assay kit, Yamasa Shoyu).[18]

Statistical Analysis

All data were expressed as mean±SEM unless otherwise indicated. Changes in hemodynamic and hormonal parameters by AM inhalation were analyzed by 1-way ANOVA for repeated measures,

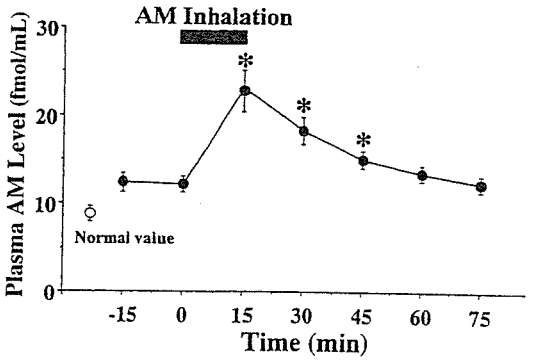

Figure 1. Changes in plasma AM level by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Normal value indicates plasma AM level derived from 15 age-matched healthy subjects. Data are mean±SEM. *P<0.05 vs value at time 0.

followed by Newman-Keuls test. Comparisons of exercise parameters between the 2 groups were analyzed with paired Student's t test. A probability value of P<0.05 was considered statistically significant.

Results

All patients tolerated this study protocol. One patient developed a headache, and another patient had mild arterial hypoxemia during AM inhalation. None of them experienced other adverse effects, such as systemic hypotension, infection, or arrhythmia.

Plasma AM Level After Inhalation

Baseline plasma AM level in patients with idiopathic pulmonary arterial hypertension was significantly higher than the normal value, which was determined from pooled data of 15 age-matched healthy subjects (11.9±0.8 versus 9.3±0.1 fmol/mL, P<0.05). Inhalation of AM significantly increased the plasma AM level to 22.9±2.1 fmol/mL immediately after inhalation (Figure 1). The half-life of plasma AM after inhalation was approximately 20 minutes, and the elevation of AM lasted for >45 minutes. Plasma cAMP level increased significantly 30 minutes after the initiation of AM inhalation (10.8±0.7 to 12.0±0.6 pmol/mL, P<0.05), although plasma cGMP level was not significantly altered (6.5±1.0 to 6.8±1.0 pmol/mL, P=NS).

Hemodynamic Effects of AM Inhalation

Inhalation of AM significantly decreased mean pulmonary arterial pressure in patients with idiopathic pulmonary arterial hypertension (54±3 to 47±3 mm Hg, P<0.05) without a significant decrease in mean arterial pressure (85±4 to 83±4 mm Hg, P=NS) (Figure 2). AM inhalation slightly but significantly increased cardiac index by 12% (2.4±0.1 to 2.7±0.2 L · min^{-1} · m^{-2}, P<0.05). Thus, AM inhalation resulted in a 22% decrease in pulmonary vascular resistance (12.6±1.5 to 9.8±1.3 Wood units, P<0.05) (Figure 3). Inhaled AM did not significantly alter systemic vascular resistance. The ratio of pulmonary vascular resistance to systemic vascular resistance was decreased significantly at the end of inhalation (0.63±0.08 to 0.55±0.07, P<0.05). These hemodynamic effects of AM lasted for >45 minutes.

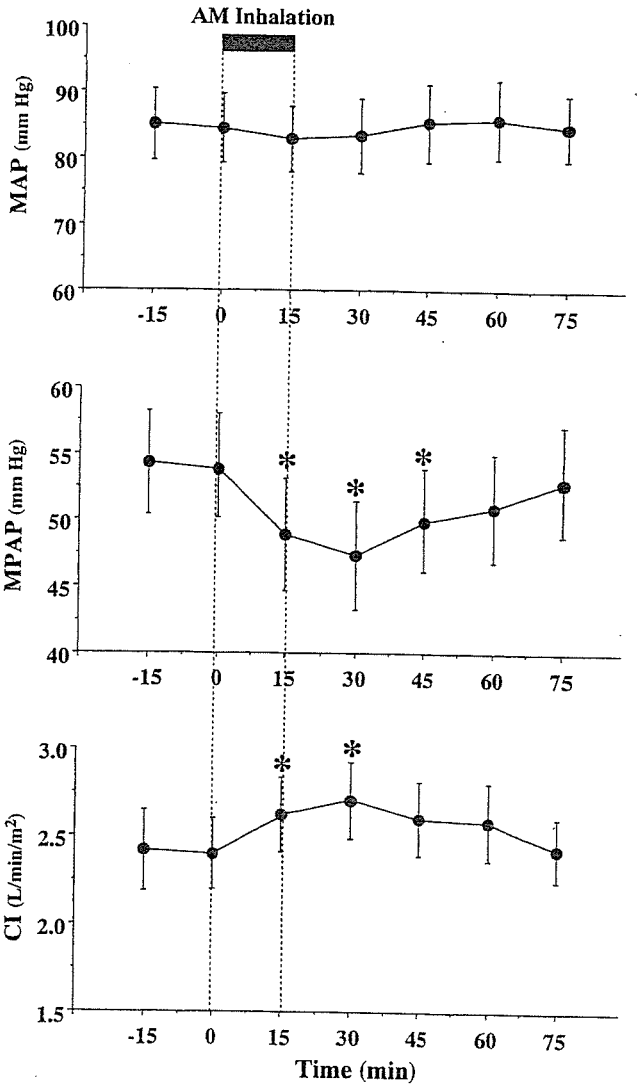

Figure 2. Changes in mean arterial pressure (MAP), mean pulmonary arterial pressure (MPAP), and cardiac index (CI) by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *P<0.05 vs value at time 0.

No significant change in heart rate, pulmonary capillary wedge pressure, or right atrial pressure was observed. There was no significant change in arterial oxygen saturation (94±3% to 93±3%).

Effects of AM Inhalation on Exercise Capacity and Ventilatory Efficiency

As the limiting symptom at the end of exercise, 6 patients reported muscle weakness and 4 reported dyspnea. There was no difference in these symptoms when exercise testing was performed with or without inhalation of AM. Inhalation of AM altered neither heart rate nor blood pressure either at rest or at peak exercise (Table 2). Inhalation of AM significantly increased peak workload (86±5 to 93±6 W, P<0.05) (Table 2). AM also significantly increased peak $\dot{V}O_2$ (14.6±0.6 to 15.7±0.6 mL · kg^{-1} · min^{-1}, P<0.05) (Figure 4). Inhalation of AM significantly increased $\Delta\dot{V}O_2/\Delta W$ ratio (6.3±0.4 to

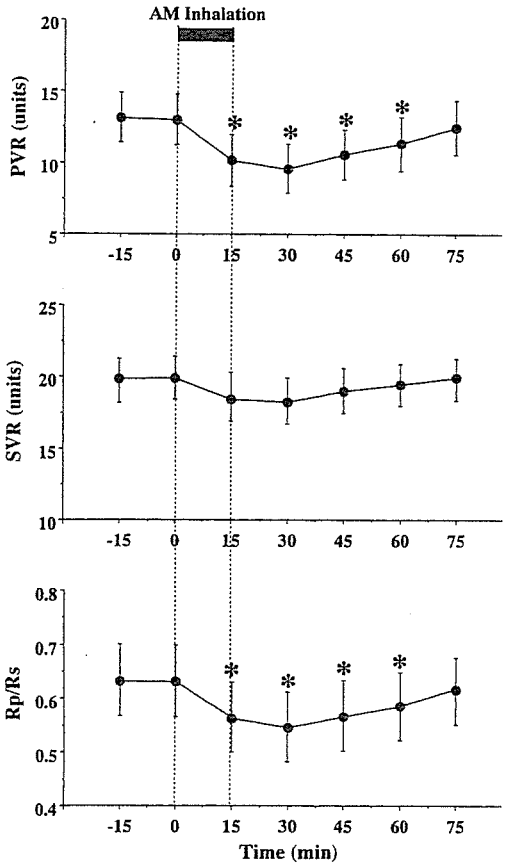

Figure 3. Changes in pulmonary vascular resistance (PVR), systemic vascular resistance (SVR), and ratio of pulmonary vascular resistance to systemic vascular resistance (Rp/Rs) by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *$P<0.05$ vs value at time 0.

7.0±0.5 mL · min^{-1} · W^{-1}, $P<0.05$). AM did not significantly alter the $\dot{V}_E$-$\dot{V}_{CO_2}$ slope (Table 2). No significant changes in arterial oxygen saturation were observed either at rest or at peak exercise. In 1 patient with NYHA class IV who did not undergo cardiopulmonary exercise testing, the distance walked in 6 minutes increased from 150 to 180 m by inhalation of AM.

Discussion

In the present study, we demonstrated that inhalation of AM improved hemodynamics with pulmonary selectivity and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

AM is one of the most potent endogenous vasodilators in the pulmonary vascular bed.[25–27] The vasodilatory effect is mediated by cAMP-dependent and nitric oxide–dependent mechanisms.[28,29] Endogenous AM production is enhanced in a variety of cardiovascular diseases through a compensatory mechanism.[14,30] Nonetheless, additional supplementation of AM has beneficial effects in these diseases.[18,19] These results suggest that endogenous AM level is not sufficient to improve deteriorated conditions despite the increased AM production. Interestingly, Champion et al[31] have shown that intratracheal gene transfer of calcitonin gene–related peptide, a member of the same peptide family as AM, to bronchial

TABLE 2. Changes in Exercise Parameters by Inhalation of AM or Placebo

Variables	Placebo	AM	*P*
Peak workload, W	86±5	93±6	<0.05
HR, bpm			
Rest	75±5	75±3	NS
Peak	144±6	148±6	NS
MAP, mm Hg			
Rest	85±3	87±5	NS
Peak	108±5	110±6	NS
Peak Borg score (D/L)	17/18	18/18	NS
Peak $\dot{V}_{O_2}$, mL · kg^{-1} · min^{-1}	14.6±0.6	15.7±0.6	<0.05
$\Delta\dot{V}_{O_2}/\Delta W$ ratio, mL · min^{-1} · W^{-1}	6.3±0.4	7.0±0.5	<0.05
$\dot{V}_E$-$\dot{V}_{CO_2}$ slope	37±2	36±2	NS
Sa_{O_2}, %			
Rest	97±1	97±1	NS
Peak	95±1	95±1	NS

HR indicates heart rate; MAP, mean arterial pressure; Peak Borg score (D/L), Borg score at peak exercise (dyspnea/leg fatigue); Peak $\dot{V}_{O_2}$, peak oxygen consumption; $\Delta\dot{V}_{O_2}/\Delta W$ ratio, $\dot{V}_{O_2}$ increase per unit workload; $\dot{V}_E$-$\dot{V}_{CO_2}$ slope, slope of regression line of relation between $\dot{V}_E$ and $\dot{V}_{CO_2}$; and Sa_{O_2}, arterial oxygen saturation. Data are mean±SEM.

epithelial cells attenuates chronic hypoxia-induced pulmonary hypertension in the mouse. These results raise the possibility that intratracheal delivery of a vasodilator peptide may be sufficient to alter pulmonary vascular function. In fact, in the present study, inhalation of AM significantly decreased pulmonary vascular resistance, whereas it did not alter systemic arterial pressure or systemic vascular resistance. The ratio of pulmonary vascular resistance to systemic vascular resistance was reduced significantly by AM inhalation. These results suggest that inhaled AM improves hemodynamics with pulmonary selectivity. This is consistent with earlier findings that inhaled prostacyclin or its analogue iloprost acts transepithelially with pulmonary selectivity and improves pulmonary hypertension.[20,21] Inhalation of AM slightly but significantly increased cardiac index in patients with idiopathic pulmonary arterial hypertension. Considering the strong vasodilator activity of AM in the pulmonary vasculature, the significant decrease in cardiac afterload may be responsible for increased cardiac index with

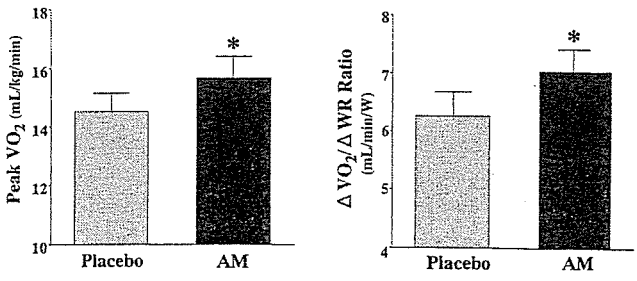

Figure 4. Changes in peak oxygen consumption (peak $\dot{V}_{O_2}$) and ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}_{O_2}/\Delta W$ ratio) by inhalation of aerosolized AM or placebo in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *$P<0.05$ vs placebo.

AM. Interestingly, the hemodynamic effects of inhaled AM lasted for >45 minutes. A previous study demonstrated that intravenous injection of AM produces a long-lasting vasodilator response because of its long half-life (≈15 minutes).[32] The half-life of plasma AM after inhalation was longer (20 minutes). Thus, inhalation of AM may cause relatively long-lasting pulmonary vasodilator activity in patients with idiopathic pulmonary arterial hypertension. In the present study, plasma cAMP level increased after AM inhalation, suggesting that the hemodynamic effects of AM may be mediated by activation of cAMP.

Earlier studies have shown that peak $\dot{V}O_2$ during exercise is markedly lower in patients with idiopathic pulmonary arterial hypertension than in healthy subjects.[33,34] Peak $\dot{V}O_2$ is determined primarily by the maximal cardiac output during exercise and the potential for O_2 extraction by the exercising muscle.[35] Thus, the decreased peak $\dot{V}O_2$ may reflect insufficient oxygen delivery to the body during exercise, at least in part because of an inadequate increase in cardiac output under conditions of severe pulmonary hypertension. In the present study, inhalation of AM significantly increased peak $\dot{V}O_2$ in patients with pulmonary hypertension. AM also increased the $\Delta\dot{V}O_2/\Delta W$ ratio, which indicates oxygen transport per unit workload to the exercising legs. These results suggest that inhalation of AM improves exercise capacity in patients with idiopathic pulmonary arterial hypertension. It is possible that an increase in cardiac output during exercise may contribute to increases in peak $\dot{V}O_2$ and the $\Delta\dot{V}O_2/\Delta W$ ratio.

The major limitation of this pilot trial relates to the lack of a randomized, placebo-controlled group in acute hemodynamic studies, which was as result not only of invasive assessment of hemodynamics but also of the limited number of patients available. Nevertheless, cardiopulmonary exercise testing was performed in a double-blind, randomized, cross-over design. Thus, it is unlikely that the hemodynamic effects of inhaled AM are attributable to the placebo effect.

Inhalation therapy may be more simple, noninvasive, and comfortable than continuous intravenous infusion therapy. An experimental study demonstrated that repeated inhalation of AM (for 30 minutes, 4 times a day) inhibited monocrotaline-induced pulmonary hypertension and markedly improved survival in rats.[36] Recently, pulmonary delivery of a dry-powder insulin has been shown to improve glycemic control without adverse pulmonary effects.[37] Although further studies are necessary to maximize the efficiency and reproducibility of pulmonary AM delivery, combining AM inhalation therapy with other modalities that have a different mode of action may have beneficial effects in patients with idiopathic pulmonary arterial hypertension.

Conclusions

These preliminary results suggest that inhalation of AM may have beneficial effects on pulmonary hemodynamics and exercise capacity in patients with idiopathic pulmonary arterial hypertension.

Acknowledgments

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Adrenomedullin Gene Transfer Induces Therapeutic Angiogenesis in a Rabbit Model of Chronic Hind Limb Ischemia

Benefits of a Novel Nonviral Vector, Gelatin

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Background—Earlier studies have shown that adrenomedullin (AM), a potent vasodilator peptide, has a variety of cardiovascular effects. However, whether AM has angiogenic potential remains unknown. This study investigated whether AM gene transfer induces therapeutic angiogenesis in chronic hind limb ischemia.

Methods and Results—Ischemia was induced in the hind limb of 21 Japanese White rabbits. Positively charged biodegradable gelatin was used to produce ionically linked DNA-gelatin complexes that could delay DNA degradation. Human AM DNA (naked AM group), AM DNA-gelatin complex (AM-gelatin group), or gelatin alone (control group) was injected into the ischemic thigh muscles. Four weeks after gene transfer, significant improvements in collateral formation and hind limb perfusion were observed in the naked AM group and AM-gelatin group compared with the control group (calf blood pressure ratio: 0.60±0.02, 0.72±0.03, 0.42±0.06, respectively). Interestingly, hind limb perfusion and capillary density of ischemic muscles were highest in the AM-gelatin group, which revealed the highest content of AM in the muscles among the three groups. As a result, necrosis of lower hind limb and thigh muscles was minimal in the AM-gelatin group.

Conclusions—AM gene transfer induced therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia. Furthermore, the use of biodegradable gelatin as a nonviral vector augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of peripheral vascular diseases. (***Circulation.* 2004;109:526-531.**)

Key Words: peripheral vascular disease ■ angiogenesis ■ gene therapy ■ ischemia

Adrenomedullin (AM) is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.[1] AM and its receptor are expressed mainly in vascular endothelial cells and vascular smooth muscle cells.[2–4] AM not only induces vasorelaxation but also regulates growth and death of these vascular cells.[5–10] These findings suggest that AM plays an important role in maintaining vascular homeostasis in an autocrine and/or paracrine manner.

A recent study has shown that vascular abnormalities are present in homozygous AM knockout mice, suggesting that AM is indispensable for vascular morphogenesis.[11–13] More recently, AM has been shown to activate the PI3K/Akt-dependent pathway in vascular endothelial cells, which is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation.[7,14] These results raise the possibility that AM plays a role in modulating vasculogenesis and angiogenesis. However, whether AM induces therapeutic angiogenesis remains unknown.

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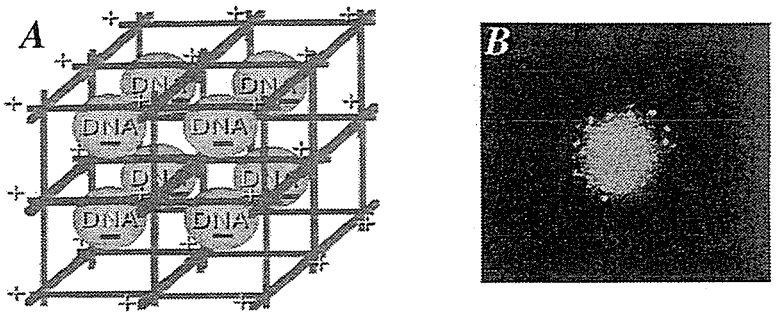

Figure 1. A, Schema of DNA-gelatin complex. Biodegradable gelatin can hold negatively charged plasmid DNA in its positively charged lattice structure. B, RITC-labeled AM DNA particles were incorporated into gelatin.

We prepared biodegradable gelatin that could hold negatively charged protein or plasmid DNA in its positively charged lattice structure.[15,16] Biodegradable gelatin has been widely used as a carrier of protein because of its capacity to delay protein degradation.[15] Similarly, ionically linked DNA-gelatin complexes can delay gene degradation.[16] These findings raise the possibility that gelatin may serve as a nonviral vector for gene therapy.

Thus, the purposes of this study were (1) to investigate whether AM gene transfer induces therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia and (2) to examine whether the use of biodegradable gelatin as a vector augments AM expression and thereby enhances the therapeutic effects of AM gene transfer.

Methods

Animal Model

All protocols were performed in accordance with the guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute. Twenty-one male Japanese White rabbits (body weight, 2.9±0.1 kg; Japan Animal Co, Osaka, Japan) were used for physiological and morphological assessment. In addition, 30 rabbits were used for radioimmunoassay, immunohistochemical examination, and Western blot analysis. After anesthetization with pentobarbital sodium (30 to 35 mg/kg), a longitudinal incision was made in the left thigh, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Hind limb ischemia was induced by ligation of the distal left external iliac artery and complete resection of the left femoral artery, as described previously.[17]

Construction of Plasmid DNA

To construct the expression vector for human AM, the *EcoRI/XhoI* fragment of the full-length human AM cDNA was ligated into the EcoRI/XhoI fragment of the pcDNA1.1-CMV expression plasmid (Invitrogen). To verify that the pcDNA1.1-CMV vector encoding AM cDNA produces a biologically active AM protein, the expression vector was transfected into 293 cells, and AM activity in the transfected cells was measured by high-performance liquid chromatography and radioimmunoassay. The pcDNA1.1-CMV vector encoding β-galactosidase (LacZ) cDNA was used as a control DNA.

Preparation of AM DNA-Gelatin Complex

Biodegradable gelatin was prepared from pig skin. The gelatin was characterized by a spheroid shape with a diameter of approximately 30 μm, water content of 95%, and an isoelectric point (pI) of 9 after swelling in water.[15,16] Gelatin can hold negatively charged protein or plasmid DNA in its positively charged lattice structure (Figure 1A). Dried gelatin (4 mg, pI 9) was added to human AM DNA solution (500 μg/100 μL in phosphate-buffered saline, pH 7.4). After mixture of DNA and gelatin, DNA-gelatin complexes were incubated at 37°C for 2 hours.

To visualize incorporation of DNA into gelatin, AM plasmid DNA was labeled with rhodamine B isothiocyanate (RITC), as reported previously.[16] In brief, the coupling reaction of RITC to plasmid DNA was carried out by mixing the two substances in 0.2 mol/L sodium carbonate-buffered solution (pH 9.7), followed by gel filtration with a PD 10 column (Amersham-Pharmacia). RITC-labeled AM DNA was incorporated into positively charged gelatin (Figure 1B).

Study Protocol

Ten days after the induction of hind limb ischemia (day 10), AM DNA (naked AM group, n=7), AM DNA-gelatin complex (AM-gelatin group, n=7), or gelatin alone (control group, n=7) was administered intramuscularly into 3 different sites in the ischemic adductor muscle and 2 different sites in the semimembranous muscle. In addition, Lac Z DNA-gelatin complex served as a control DNA (Lac Z-gelatin group, n=5). The amount of plasmid was 500 μg (1 mL) and that of gelatin was 4 mg. Morphological and angiographic analyses and measurements of calf blood pressure and laser Doppler flow were performed 4 weeks after gene transfer (day 38). After completion of these measurements, the adductor, semimembranous, and gastrocnemius muscles were weighed in each hind limb.[18] The muscle weight ratio was calculated for each muscle as follows: muscle weight ratio=muscle weight in ischemic hind limb/muscle weight in nonischemic hind limb. Specimens of the adductor muscle of the ischemic hind limb were obtained for histological examination.

Measurement of Calf Blood Pressure

Calf blood pressure was measured on days 10 and 38 in both hind limbs with a Doppler flowmeter (Hayashi Denki Co, Ltd) and a 25-mm-wide cuff. The pulse of the posterior tibial artery was identified with the use of a Doppler probe, and the systolic blood pressure in both hind limbs was determined by standard techniques. The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic hind limb to that of the normal hind limb.[17]

Laser Doppler Blood Perfusion Analysis

Blood flow of the ischemic hind limb was measured with the use of a laser Doppler blood perfusion image system (moorLDI, Moor Instruments) on day 38.

Angiographic Analysis

Development of collateral arteries was evaluated by angiography on days 0 and 38. A 4F catheter was placed in the left internal iliac artery through the common carotid artery, and 3 mL contrast medium (Iopamiron 300, SCHERING) was injected with an automated angiography injector at a rate of 2.5 mL/s. Quantitative angiographic analysis of collateral vessel development in the ischemic hind limb was performed with the use of a 5-mm^2 grid overlay, as described previously.[17] The angiographic score was calculated for each film as the ratio of grid intersections crossed by opacified arteries divided by the total number of grid intersections in the ischemic medial thigh. The angiographic score was determined by 2 blinded observers.

Morphological and Histological Examination

The degree of lower hind limb necrosis and thigh muscle necrosis was macroscopically evaluated on graded morphological scales (grade 1 to 3) for peripheral tissue damage and muscle necrosis area of the adductor, semimembranous, and medial large muscles. Capillary density of the ischemic hind limb was evaluated by alkaline phosphatase staining, as reported previously.[17] A total of 10 different fields from three different sections were randomly selected, and the number of capillaries was counted under a ×40 objective. Capillary density was expressed as the mean number of capillaries per square millimeter. The number of myofibers in each field was also examined and the capillary/muscle fiber ratio calculated.

Radioimmunoassay for Human AM

Human AM production was examined 1, 2, and 4 weeks after gene transfer in the naked AM group, AM-gelatin group, and control group (n=5 each). The muscles were harvested for radioimmunoassay and immunohistochemical examination. Immunoreactive human AM level in rabbit muscles was determined by immunoradiometric assay with the use of a specific kit (Shionogi Co, Ltd).[19] Tissue content of vascular endothelial growth factor (VEGF) was examined by ELISA kit (R&D systems).

Immunohistochemistry for Human AM, Ki67 Antigen, and Phosphorylated Akt

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded 4-μm sections of ischemic thigh muscles 7 days after gene transfer. To elucidate AM expression after gene therapy, immunohistochemistry for human AM was performed with the use of a monoclonal antibody recognizing AM-(12–25) (1:100), as reported previously.[20] To evaluate the proliferative potential of AM, tissue sections were stained for Ki67, a marker for cell proliferation, with the use of monoclonal anti-Ki67 antibody (1:100) (DAKO). AM has recently been shown to promote proliferation of vascular endothelial cells at least in part through the PI3k/Akt pathway.[21] Thus, immunohistochemistry for phosphorylated Akt was performed with mouse monoclonal anti-phosphorylated Akt antibody (1:100) (Cell Signaling Technology).

Western Blot Analysis

To identify Akt phosphorylation in ischemic muscles after AM gene transfer, Western blotting was performed with the use of a commercially available kit (PhosphoPlus Akt [Ser473] Antibody Kit, Cell Signaling Technology). Ischemic muscles in the 3 groups were obtained 7 days after AM gene transfer. These samples were homogenized on ice in 0.1% Tween 20 homogenization buffer with a protease inhibitor (Complete, Roche). After centrifugation for 20 minutes at 4°C, the supernatant was used for Western blot analysis. The 50 μg of protein was transferred into sample buffer, loaded on 7.5% SDS-polyacrylamide gel, and blotted onto nitrocellulose membrane through the use of a wet blotting system. After blocking for 60 minutes, the membranes were incubated with primary antibodies (1:500) at 4°C overnight. The membranes were then incubated with secondary antibodies, which were conjugated with horseradish peroxidase (Cell Signaling Technology), at a final dilution of 1:2000. Signals were detected through the use of LumiGLO chemiluminescence reagents (Cell Signaling Technology).

Statistical Analysis

All results are expressed as mean±SEM. Statistical significance was evaluated by 1-way ANOVA followed by Fisher's analysis, Scheffe's F analysis, or Kruskal-Wallis test. A value of $P<0.05$ was considered statistically significant.

Results

Physiological and Morphological Assessment

Complete resection of the left femoral artery resulted in a similar decrease in calf blood pressure ratio among the 3 groups before the initiation of therapy (day 10) (Figure 2A). However, the calf blood pressure ratio on day 38 was highest in the AM-gelatin groups, followed by the naked AM group and subsequently the control group. The laser Doppler flow in hind limb was highest in the AM-gelatin group, followed by the naked AM group and the control group (Figure 2B). The calf blood pressure ratio and laser Doppler flow 4 weeks after gene transfer did not significantly differ between the control group and Lac Z-gelatin group. Lower hind limb necrosis was minimal in the AM-gelatin group, followed by the naked AM group and the control group (Figure 2C). Thigh muscle necrosis was also minimal in the AM-gelatin group. Similarly, the muscle weight ratio (ischemic/normal) on day 38 was highest in the AM-gelatin group (Table). Neither mean arterial pressure nor heart rate significantly differed among the 3 groups.

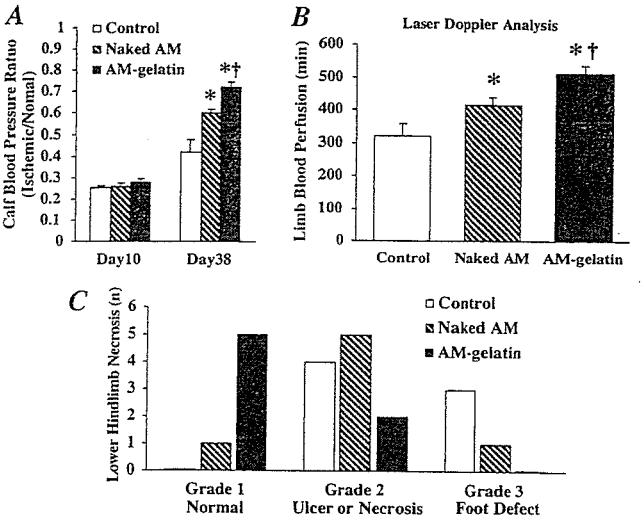

Figure 2. A, Calf blood pressure ratio (ischemic/normal hind limb) before (on day 10) and after (on day 38) gene transfer. B, Measurement of laser Doppler flow on day 38. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs naked AM group. C, Number of cases of each grade of lower hind limb necrosis on day 38. Lower hind limb necrosis was minimal in the AM-gelatin group. Number of necrosis or foot defect is statistically significant among the 3 groups ($P<0.05$ by Kruskal-Wallis test).

Angiographic Analysis

Angiograms 4 weeks after gene transfer (day 38) showed the development of collateral arteries in the naked AM and

Physiological Characteristics

	Control	Naked AM	AM-Gelatin
No. of rabbits	7	7	7
Body weight, kg	2.46±0.06	2.65±0.10	3.16±0.09
MAP, mm Hg	112±3	114±3	116±2
HR, beats/min	269±12	253±5	262±7
Muscle weight ratio	0.71±0.03	0.84±0.02*	0.95±0.02*†

MAP indicates mean arterial pressure; HR, heart rate; and muscle weight ratio, ratio of muscle weight in ischemic hind limb to that in nonischemic hind limb. Data are mean±SEM.

*$P<0.01$ vs control group; †$P<0.05$ vs naked AM group.

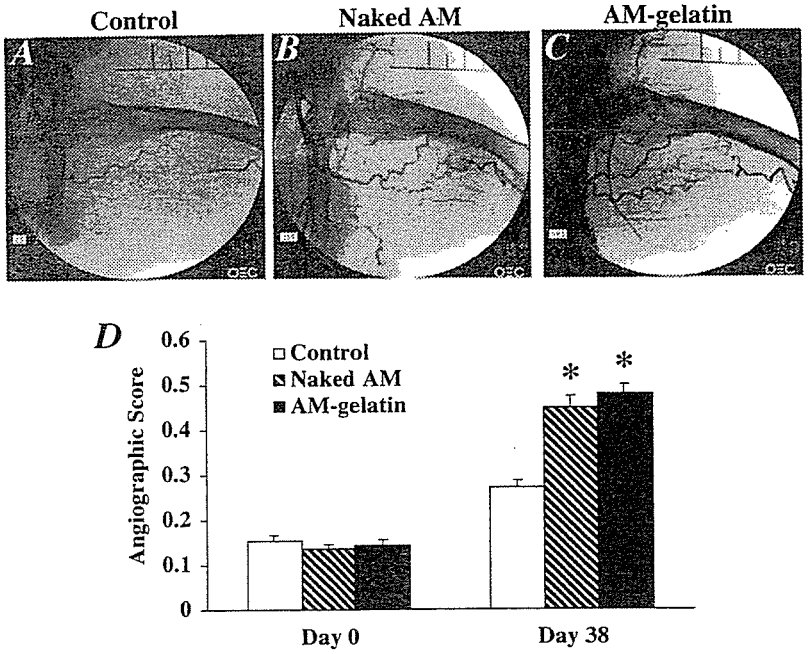

Figure 3. Representative angiograms of control group (A), naked AM group (B), and AM-gelatin group (C) on day 38. Collateral arteries were well developed in the naked AM and AM-gelatin groups. D, Angiographic score on days 0 and 38 in each group. Angiographic score on day 38 was significantly higher in the naked AM and AM-gelatin groups than in the control group. Data are mean±SEM. *$P<0.001$ versus control group.

AM-gelatin groups compared with that in the control group (Figure 3, A through C). Quantitative analysis of collateral vessels demonstrated that the angiographic score in both the naked AM and AM-gelatin groups was significantly higher than that in the control group (Figure 3D). Angiographic score did not significantly differ between the control group and Lac Z-gelatin group.

To examine the development of collateral vessels in an earlier stage, other rabbits (n=4 each) were examined 2 weeks after gene transfer (day 24). Angiograms showed significant collateral development in the naked AM and AM-gelatin groups compared with that in the control group.

Histological Examination

Alkaline phosphatase staining of ischemic hind limb muscle showed marked augmentation of neovascularization in both the naked AM and AM-gelatin groups compared with the control group (Figure 4, A through C). Quantitative analysis demonstrated that capillary density of the ischemic adductor muscle was highest in the AM-gelatin group (Figure 4D). Analysis of the capillary/muscle fiber ratio yielded similar results. Seven days after gene transfer, intense immunostaining for Ki67 was observed in vascular endothelial cells of the naked AM and the AM-gelatin groups (Figure 4, E through G).

AM Expression and Akt Phosphorylation After Gene Transfer

Seven days after gene transfer, modest immunostaining for human AM was observed in the naked AM group, whereas AM immunoreactivity was intense surrounding the gelatin in the AM-gelatin group (Figure 5, A through C). Tissue content of human AM was significantly increased both in the naked AM and the AM-gelatin groups 7 days after gene transfer (Figure 5D). The AM level in the AM-gelatin group was significantly higher that in the naked AM group. Two weeks after gene transfer, AM overexpression was observed only in the AM-gelatin group. The expression of endogenous VEGF and its receptors (Flt-1 and Flk-1) did not differ among the 3 groups (data not shown). Western blot analysis revealed that phosphorylated Akt in ischemic muscles was increased in both the naked AM and AM-gelatin groups 7 days after gene transfer (Figure 5E). Intense immunostaining for phosphory-

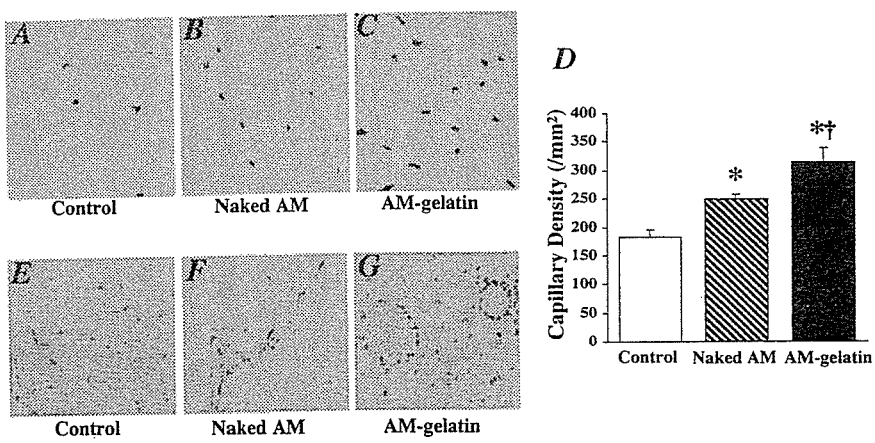

Figure 4. A through C, Representative examples of alkaline phosphatase staining in ischemic hind limb muscles. Magnification ×200. D, Quantitative analysis of capillary density in ischemic hind limb muscles. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs naked AM group. E through G, Immunohistochemical analysis of Ki67 antigen, a marker for cell proliferation. Magnification ×400.

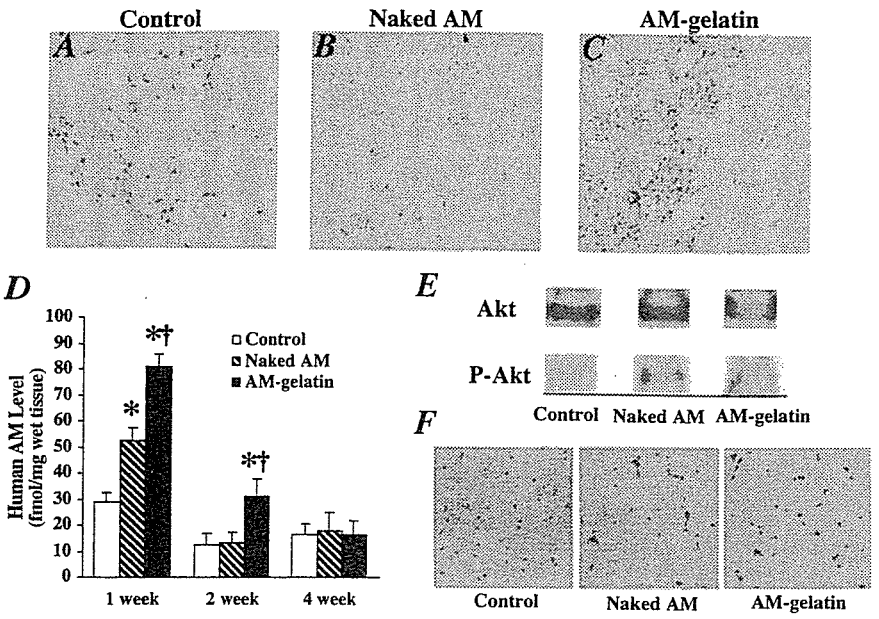

Figure 5. A through C, Immunohistochemistry for human AM 7 days after gene transfer. Intense immunostaining was observed surrounding gelatin in the AM-gelatin group. Magnification ×200. D, Time course of AM production in ischemic muscles after gene transfer. Data are mean±SEM. *$P<0.01$ vs control group; †$P<0.01$ vs naked AM group. E, Western blot analysis for Akt phosphorylation in muscles. F, Immunohistochemical staining for phosphorylated Akt 7 days after gene transfer. Phosphorylated Akt was distributed at least in endothelial cells. Magnification ×400.

lated Akt was observed at least in endothelial cells of the Naked AM and the AM-gelatin groups (Figure 5F).

Discussion

We demonstrated that (1) AM gene transfer induced hemodynamic and angiographic improvements in association with an increase in capillary density in a rabbit model of chronic hind limb ischemia. We also demonstrated that (2) administration of AM DNA-gelatin complexes markedly augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer.

AM has a variety of effects on the vasculature that include vasodilation,[1,5–7] inhibition of endothelial cell apoptosis,[8,9] and regulation of smooth muscle cell proliferation.[10] However, whether AM has angiogenic potential has remained unknown. In the present study, intramuscular administration of naked AM DNA augmented AM production in skeletal muscles, as indicated by increased tissue content and significant immunostaining of AM. As a result, AM gene transfer increased hind limb perfusion and ameliorated lower hind limb and thigh muscle necrosis in a rabbit model of hind limb ischemia. AM gene transfer may protect the ischemic hind limb partly by improving the blood flow in the ischemic hind limb because AM is originally identified as a potent vasodilating peptide.[1] Nevertheless, angiographic collateral development and high capillary density were observed in ischemic muscles after AM gene transfer. Ki67, a marker for cell proliferation, was detected in endothelial cells of microvessels after AM gene transfer. These results suggest that AM overproduction resulting from gene transfer may induce angiogenesis in a rabbit model of hind limb ischemia. Recent studies using AM gene knockout mice have shown that AM is essential for development of the vasculature during embryogenesis.[11–13] These studies support our results that AM may be an angiogenic factor. VEGF is known to induce angiogenesis and to regulate endothelial cell survival through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway.[22] Thus, the PI3K/Akt pathway is considered to regulate multiple critical steps in angiogenesis, including endothelial cell survival, proliferation, migration, and capillary-like structure formation.[14] A recent study has reported that AM promotes proliferation and migration of human umbilical vein endothelial cells at least in part through the PI3K/Akt pathway.[21] The present study demonstrated that phosphorylated Akt is increased at least in endothelial cells after AM gene transfer. AM gene transfer did not influence endogenous VEGF and its receptors. Taken together, it is interesting to speculate that AM may directly induce angiogenesis through the PI3K/Akt pathway.

In the present study, we used positively charged biodegradable gelatin as a nonviral vector. We have shown that basic fibroblast growth factor (bFGF) is ionically linked with gelatin, which enhances the angiogenic effects of bFGF by delaying protein degradation.[15] Thus, biodegradable gelatin has been used as a carrier of protein. However, little information is available regarding the therapeutic potential of gelatin as a nonviral vector for gene transfer. In the present study, we demonstrated that RITC-labeled AM DNA was incorporated into positively charged gelatin. In addition, intramuscular administration of AM DNA-gelatin complexes strongly enhanced AM production compared with that of naked AM DNA. These results suggest that biodegradable gelatin may serve as a vector for gene transfer. In fact, AM DNA-gelatin complexes induced more potent angiogenic effects in a rabbit model of hind limb ischemia than naked AM DNA, as evidenced by significant increases in histological capillary density, calf blood pressure ratio, laser Doppler flow, and muscle weight ratio and a decrease in necrosis of lower hind limb and thigh muscles. These results suggest that the use of biodegradable gelatin as a nonviral vector augments AM expression and enhances AM-induced angiogenic effects. The angiogenic effects of AM-gelatin complexes were comparable to those of bFGF-gelatin complexes (data not shown). AM DNA-gelatin complexes were distributed mainly in connective tissues. We have recently demonstrated that gelatin-DNA complex is readily phagocytosed by mac-

rophages, monocytes, endothelial progenitor cells, and so on, resulting in gene expression within these phagocytes.[23,24] These findings raise the possibility that AM secreted from these cells acts on muscles in a paracrine fashion. Unlike AM production in the naked AM group, AM overexpression in the AM-gelatin group lasted for longer than 2 weeks. Thus, it is interesting to speculate that delaying gene degradation by gelatin may be responsible for the highly efficient gene transfer.

Currently, a highly efficient and safe gene delivery system is needed for gene therapy in humans. The present study demonstrated that the use of gelatin, which is considered to be less biohazardous than viral vectors, enhanced the angiogenic potential of AM DNA. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of severe peripheral vascular diseases. However, the initial success of gelatin-mediated AM gene therapy reported here should be confirmed by long-term experiments, and extensive toxicity studies in animals are needed before clinical trials.

Study Limitation

First, histological capillary density, calf blood pressure ratio, and laser Doppler flow were significantly higher in the AM-gelatin group than in the naked AM group. However, the angiographic score did not significantly differ between the two. This discrepancy raises the possibility that conventional angiography may have insufficient resolution to fully visualize the angiogenic microvessels. Second, human AM level was slightly elevated in the control group. This implies that the anti-human AM antibody used in this radioimmunoassay had some cross-reactivity with endogenous rabbit AM. Nevertheless, human AM level in the muscles was highest in the AM-gelatin group within 2 weeks after gene transfer. These results suggest that AM DNA-gelatin complexes induces potent and long-lasting AM production.

Conclusions

Intramuscular administration of AM DNA induced therapeutic angiogenesis in a rabbit model of chronic hind limb ischemia. Furthermore, the use of biodegradable gelatin as a nonviral vector augmented AM expression and thereby enhanced the therapeutic effects of AM gene transfer. Thus, gelatin-mediated AM gene transfer may be a new therapeutic strategy for the treatment of peripheral vascular diseases.

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Drug Therapy of Primary Pulmonary Hypertension

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Contents

Abstract

Primary pulmonary hypertension (PPH) is a rare but life-threatening disease. Median survival, from the time of diagnosis, is considered to be 2.8 years . However, therapeutic medical advances over the past 2 decades have resulted in significant improvements in quality of life and survival in patients with PPH. Because pulmonary vasoconstriction, endothelial cell proliferation, smooth muscle cell proliferation, and *in situ* thrombosis contribute to the development of this disease, treatment with vasodilators, anti-proliferative agents, and anticoagulants is recommended.

Currently, oral administration of calcium channel antagonists and intravenous infusion of epoprostenol (prostacyclin) are established as treatment of PPH. Epoprostenol has vasoprotective effects including vasodilation, anti-platelet aggregation, and inhibition of smooth muscle cell proliferation. Interestingly, prostacyclin synthase deficiency in the lungs, and impaired prostacyclin production, have been linked to the development of pulmonary hypertension in this disease. As a result, continuous intravenous infusion of epoprostenol has become recognized as a therapeutic breakthrough that can improve hemodynamics and survival in patients with PPH.

The dramatic success of long-term intravenous prostacyclin is now leading to the development of epoprostenol analogs using newer drug delivery systems (oral beraprost, aerosolized iloprost, and subcutaneous treprostinil). In addition, promising drugs including endothelin antagonists and type V phosphodiesterase

inhibitors have recently been developed. Furthermore, gene therapy with endothelial nitric oxide synthase gene or prostacyclin synthase gene may hold great promise in the treatment of PPH.

Finally, accurate evaluation of disease severity and the efficacy of vasodilator therapy are important in the management of patients with PPH. In addition to invasive assessment by cardiac catheterization, we recommend repeated measurements of plasma brain natriuretic peptide, serum uric acid, and the distance walked in 6 minutes. These noninvasive parameters may be helpful as part of the evaluation of treatment in patients with PPH and, in particular, as a guide to the selection and timing for alternative therapies.

Primary pulmonary hypertension (PPH) is a rare but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right ventricular (RV) failure and death.[1,2] Because the presence of endothelial injury in the pulmonary vascular bed develops pulmonary vasoconstriction, smooth muscle cell proliferation, and *in situ* thrombosis,[3] a variety of vasodilators, anti-proliferative agents, and anticoagulants have been proposed as therapeutic agents of PPH. Despite an inability to cure PPH half a century after its initial description, therapeutic medical advances over the past 2 decades have resulted in significant improvements in quality of life and survival in patients with PPH. The most important advance is the continuous administration of prostacyclin through a permanently implanted central venous line.[4-8] In addition, new therapeutic strategy based on cellular and molecular mechanisms responsible for the pathogenesis of PPH have recently been proposed. This article will summarize current drug therapy of PPH and future perspectives in the treatment of PPH.

1. Current Medical Treatment

1.1 Vasodilators

The goal of vasodilator therapy for patients with PPH is to reduce pulmonary vascular resistance without producing systemic hypotension, and to improve quality of life and survival. Although a variety of vasodilators have been proposed as potential therapy for PPH over the past 30 years,[9-12] many of the agents used failed to achieve these goals without significant adverse effects. In 1992, however, Rich et al.[13] confirmed the beneficial effects of calcium-channel antagonists in selected patients.[13] A decrease in pulmonary vascular resistance in response to short-term vasodilator challenge occurs in about 20% of patients, and predicts a good response to chronic long-term therapy with oral calcium-channel antagonists. For patients unresponsive during acute short-term testing, continuous intravenous prostacyclin (epoprostenol) therapy has been undertaken. In 1996, Barst et al.[7] demonstrated that continuous intravenous administration of epoprostenol significantly improved survival of patients with PPH as compared with conventional therapy alone.[7] Based on these studies, both oral administration of calcium-channel blockers and intravenous infusion of prostacyclin have been established as treatment of PPH. Furthermore, promising drugs including prostacyclin analogs and endothelin antagonists have recently been developed (table I).

1.1.1 Calcium Channel Antagonists

Calcium channel antagonists are the oral drugs of choice for treatment of PPH. Earlier studies have demonstrated that oral administration of calcium channel antagonists decrease pulmonary arterial pressure and increase cardiac output, thereby decreasing pulmonary vascular resistance in some patients with PPH.[13-15] To predict which patients will have a therapeutic response to calcium channel antagonists, acute vasodilator challenge tests are generally performed, with inhaled nitric oxide (NO), adenosine, and in many institutions, prostacyclin.[16] Patients with PPH can be divided into two groups according to acute hemodynamic challenge tests: responders, with a 20% fall in pulmonary vascular resistance and nonresponders. In fact, the definition of a response varies from center to center, and a more appropriate definition is a substantial fall in pulmonary artery pressure rather than pulmonary vascular

Table I. Therapeutic options of vasodilators in the treatment of primary pulmonary hypertension (PPH)

Oral administration	
Nifedipine, diltiazem	Calcium channel antagonists
Beraprost	Prostacyclin analog
Bosentan	Endothelin antagonist
Sildenafil	Type V phosphodiesterase inhibitor
L-arginine	Precursor of nitric oxide
Intravenous administration	
Epoprostenol	Prostacyclin
Inhalant administration	
Iloprost	Prostacyclin analog
Nitric oxide	Nitric oxide
Subcutaneous administration	
Treprostinil	Prostacyclin analog

resistance. As a result, <10% of PPH patients may be true responders.

Because calcium channel antagonists dose-dependently decrease pulmonary vascular resistance, high doses of calcium channel antagonists are recommended to achieve the maximum beneficial effects in responders. Conversely, patients who are unresponsive to calcium channel antagonists seem to be unresponsive at any dose. Thus, calcium channel antagonists should be used only in responders.[13] The mechanism by which calcium channel antagonists provides benefit is primarily through vasodilatation. Because the agents do not have positive inotropic effects, the increase in cardiac output is attributable to a fall in pressure unloading of the right ventricle. For the clinician it is important to know that acute short-term withdrawal of the calcium channel antagonists can lead to fatal rebound effects.

Unfortunately, there are several factors limiting the widespread use of calcium channel antagonists in patients with PPH. First, it is estimated that responders to calcium channel antagonists represent <20% of all patients with PPH. Second, the much higher doses which are required to lower pulmonary vascular resistance are often associated with adverse effects such as systemic hypotension, tachycardia, and depressed myocardial contractility. Thus, long-term calcium channel blockade is contraindicated in patients with severe right heart failure. Third, these drugs have never been prospectively studied for their impact on survival or symptomatology in PPH. Even so, their relatively low cost and ease of administration make the calcium channel antagonists a therapeutic option for responders without severe right heart failure.

1.1.2 Prostacyclin Therapy

Prostacyclin, a metabolite of arachidonic acid, has vasoprotective effects including vasodilation, anti-platelet aggregation, and inhibition of smooth muscle cell proliferation.[17,18] Patients with PPH who are unresponsive to calcium channel antagonists are treated with intravenous epoprostenol.[4-8] Furthermore, the dramatic success of long-term intravenous epoprostenol is now leading to the development of prostacyclin analogs using newer drug delivery systems (oral beraprost, aerosolized iloprost, and subcutaneous treprostinil),[19-24] although beraprost and iloprost are not currently approved by the US FDA.

Epoprostenol

Epoprostenol, the synthetic form of prostacyclin, produces strong vasodilation and inhibition of platelet aggregation and vascular smooth muscle cell proliferation. Epoprostenol has a short half-life, and thus, long-term treatment requiring a continuous intravenous delivery system constituted essentially by a tunnelized central venous catheter and a portable infusion pump. Higenbottam et al.[4] was the first to use continuous intravenous epoprostenol in patients with PPH. Rubin and others have demonstrated that continuous intravenous administration of epoprostenol improves symptoms, hemodynamics, and long-term prognosis in patients with PPH.[5-7] In 1994, Barst et al.[6] reported that continuous intravenous epoprostenol improved survival in 17 patients who had failed conventional medical treatment. The 1-, 3-, and 5-year survival rates for the epoprostenol-treated patients were 87%, 63%, and 54%, respectively, compared with 77%, 41%, and 27% for the National Institute for Health (NIH) registry patients. In 1996, a 12-week prospective, randomized, multicenter, nonblind trial demonstrated that compared with conventional therapy, the continuous intravenous infusion of epoprostenol produced symptomatic and hemodynamic improvement, as well as improved survival in patients with severe primary pulmonary hypertension (New York Heart Association [NYHA] functional class III or IV).[7] Initially, epoprostenol treatment was considered as a bridge to transplantation in advanced cases of PPH, but recent experience has established this approach as a possible alternative to transplantation.

Epoprostenol therapy should be begun at a low dose (0.5–4 ng/kg/min) and thereafter, epoprostenol is gradually increased to the maximal tolerated doses. The dose of the medication is increased further if the adverse effect profile permits. Thus, the goal is to have patients receive as high a dose of epoprostenol as possible. Target dose for the first 2–4 weeks is usually approximately 5–10 ng/kg/min. Optimal dose is usually established by dose titration, although it varies among patients (0.5–200 ng/kg/min). Patients on epoprostenol require gradual upward dose titration to overcome tolerance to the medication. However, excessive epoprostenol in PPH can lead to a high cardiac output state, suggesting it has important positive inotropic effects. In this circumstance, reducing the dose can allow the cardiac output to return to normal without worsening the clinical state.[25] Nevertheless, it is important to know that acute short-term withdrawal of epoprostenol can lead to fatal rebound effects.

Interestingly, unlike calcium channel antagonists, the clinical and hemodynamic improvements cannot always be predicted by the patients' acute response to initial vasodilator testing. McLaughlin et al.[8] demonstrated that in patients with PPH, long-term therapy with epoprostenol lowers pulmonary vascular resistance (−53%) beyond the level achieved in the short-term with intravenous adenosine. In addition to its role as a potent vasodilator, epoprostenol decreases platelet aggregation and inhibits smooth muscle cell proliferation. Therefore, it is possible that long-term treatment with epoprostenol inhibits vascular remodeling and vascular growth in patients with PPH. A recent study has shown that observed survival with epoprostenol therapy at 1, 2, and 3 years was 87.8%, 76.3%, and 62.8% and was significantly

greater than the expected survival of 58.9%, 46.3%, and 35.4% based on historical data.[26] Sitbon, et al.[27] have also shown that overall survival rates at 1, 2, 3, and 5 years were 85%, 70%, 63%, and 55%, respectively. Thus, epoprostenol has been demonstrated to improve long-term survival in patients with PPH.[26-28] Nevertheless, it should be noted that the long-term survival is far from perfect even with this treatment. Thus, lung transplantation should be considered in a subset of patients who remain in NYHA functional class III or IV or in those who cannot achieve a significant hemodynamic improvement after 3 months of epoprostenol therapy, or both.

Side effects with long-term epoprostenol treatment are common and include flushing, jaw pain, diarrhea, headache, leg pain, abdominal cramping, nausea, and hypotension.[4-8] Because the continuous infusion of epoprostenol requires the placement of a permanent in-dwelling venous catheter, the risk of potentially life-threatening infections exists. Furthermore, caution has been suggested regarding the use of epoprostenol in patients in whom a diagnosis of pulmonary veno-occlusive disease is suspected.[29,30] In such cases, a high incidence of pulmonary edema has been reported, presumably because of increased pulmonary perfusion in the presence of downstream vascular obstruction.

Despite some adverse effects, many studies have demonstrated that continuous intravenous epoprostenol induces dramatic improvements in symptoms, hemodynamics, and survival in patients with PPH. We therefore recommend consideration of continuous intravenous epoprostenol for patients with PPH in NYHA class III or IV who are unresponsive to oral vasodilator treatment. However, lung transplantation should be considered for patients who are refractory to epoprostenol therapy.

Beraprost

Beraprost is a newly developed prostacyclin analog with a stable structure because of its cyclopentabenzofuranyl skeleton.[31] Unlike epoprostenol, beraprost permits oral ingestion.[32] Like epoprostenol, beraprost produces strong vasodilation and inhibition of platelet aggregation.[33] We demonstrated that treatment with beraprost significantly decreased mean pulmonary arterial pressure and total pulmonary resistance by 13% and 25% during a mean follow-up period of 53 days.[20] Although retrospective, the Kaplan-Meier survival curves demonstrated that the 1-, 2-, and 3-year survival rates for the beraprost group were 96%, 86%, and 76%, respectively, compared with 77%, 47%, and 44%, respectively, in the conventional group (figure 1). Although this study did not include patients with the most severe forms of PPH, the oral administration of beraprost may have beneficial effects on the survival of the patients with milder forms of PPH. However, the survival data of beraprost was derived from a retrospective, preliminary study. A recent randomized, double-blind, placebo-controlled (Arterial Pulmonary Hypertension and Beraprost European [ALPHABET] study) has demonstrated that beraprost improves symptoms and exercise capacity in patients with pulmonary arterial hypertension.[34] Given the potential risks and high medical costs of the invasive method, orally active beraprost may be worth trying in such patients before the intravenous epoprostenol therapy is considered.

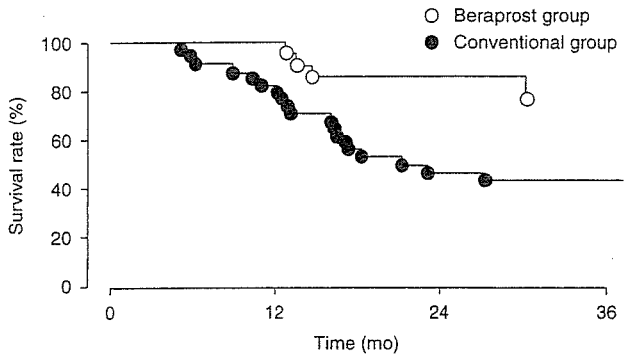

Fig. 1. Kaplan-Meier survival curves showing that outpatients treated with beraprost have a significantly higher survival rate than those treated with conventional therapy including calcium channel antagonists (log-rank test $p < 0.001$).[20]

Iloprost

Iloprost is a chemically stable prostacyclin analog. The inhalation of iloprost has been used to mitigate some of the problems associated with intravenous epoprostenol administration. This inhalational strategy can cause selective pulmonary vasodilation without systemic effects.[21,22,35] In addition, inhaled vasodilators do not induce negative adverse effects on gas exchange because ventilation-matched deposition of the drugs in the alveoli causes pulmonary vasodilation matched to ventilated areas.[36] In clinical settings, inhalation therapy may be more simple, noninvasive, and more comfortable than continuous intravenous infusion therapy. Two uncontrolled non-comparative trials have demonstrated acute short-term and long-term improvement in symptoms and hemodynamics in patients with pulmonary arterial hypertension including PPH.[21,22] Recently, a long-term, randomized, placebo-controlled study on the effect of inhaled iloprost in 203 patients with pulmonary arterial hypertension (NYHA functional class III or IV) was performed in Europe.[37] Repeated daily inhalations of 2.5 or 5μg of iloprost (six or nine times per day; median inhaled dose, 30 μg/day) were compared with inhalation of placebo. There were increases in the distance walked in 6 minutes of 36.4m in the iloprost group as a whole and of 58.8m in the subgroup of patients with PPH. Hemodynamic values were significantly improved at 12 weeks when measured after iloprost inhalation. Overall, 4.0% of patients in the iloprost group (including one who died) and 13.7%

percent of those in the placebo group (including four who died) did not complete the study ($p = 0.024$). These results suggest that inhaled iloprost is an effective therapy for patients with severe pulmonary hypertension. Further studies are necessary to examine a prognostic benefit of this treatment.

Treprostinil

Treprostinil is a tricyclic benzidine analog of epoprostenol that has more stability at room temperature and a longer half-life than epoprostenol. These characteristics allow the administration of the compound by intravenous and subcutaneous routes. Subcutaneous administration can be accomplished by an ambulatory insulin-pump delivery system. In this case, all the problems linked to a permanent, central venous line such as infections are avoided and the management of the system is much simpler. A preliminary study showed that subcutaneous treprostinil induced beneficial hemodynamic changes in patients with PPH.[23] A multicenter study demonstrated that chronic long-term subcutaneous treprostinil improved symptoms, functional capacity, and hemodynamics in patients with pulmonary arterial hypertension.[24] However, the most frequent adverse effect was pain and redness at the local infusion site which may prevent some patients from receiving adequate doses. Further studies are necessary to confirm benefits of subcutaneous treprostinil.

1.1.3 Endothelin Antagonists

Endothelin (ET)-1 is a potent vasoconstrictor and smooth muscle mitogen.[38] Plasma ET level is raised in patients with pulmonary hypertension.[39,40] Local production of ET-1 is also increased in pulmonary vascular endothelial cells in such patients.[41] Thus, raised levels of ET-1 may contribute to elevated pulmonary vascular resistance in patients with PPH. The effects of ET-1 are mediated through two receptor types: ET-A and ET-B. ET-A is present on vascular smooth muscle cells and mediates vasoconstriction and proliferation. Therefore, its blockade should be helpful in the treatment of PPH.

In a preliminary study,[42] the orally administered dual endothelin-receptor antagonist bosentan (125mg twice daily) decreased pulmonary vascular resistance and improved exercise capacity in patients with pulmonary arterial hypertension. Although most of endothelin-receptor antagonists have potential adverse effects, bosentan was well tolerated and free of these apart from a dose-dependent increase in liver enzyme levels. In a double-blind, placebo-controlled study,[43] 213 patients with pulmonary arterial hypertension (primary or associated with connective-tissue disease) were randomized to receive placebo or 62.5mg of bosentan twice daily for 4 weeks followed by either of two doses of bosentan (125 or 250mg twice daily) for a minimum of 12 weeks. Patients treated with bosentan had an improved 6-minute walking distance; the mean difference between the placebo group and the combined bosentan groups was 44m. Bosentan also improved the Borg dyspnea index and World Health Organization (WHO) functional class and increased the time to clinical worsening. These results suggest that endothelin-receptor antagonism with oral bosentan is an effective approach to therapy for pulmonary arterial hypertension.

1.1.4 Nitric Oxide

NO is a potent vasodilator that also inhibits platelet adhesion and smooth muscle cell proliferation.[44] Earlier studies have shown that inhaled NO ameliorates persistent pulmonary hypertension of the newborn or after cardiac surgery.[45,46] NO inhalation has been shown to improve hemodynamics with pulmonary selectivity and improve exercise capacity in patients with pulmonary hypertension.[47] In addition, NO has been widely used as an early test of vasodilator response in patients with PPH.[48] The acute short-term responsiveness to inhaled NO seems to predict the subset of patients who might be responsive to oral calcium channel antagonists. Furthermore, long-term continuous inhalation of NO had beneficial effects in some patients with PPH.[49] However, this treatment, requires a continuous inhalation device, hence proving uncomfortable and expensive. In addition, this treatment carries the risk of an acute rebound that occurs within minutes after interruption of therapy.

Because NO is synthesized from the amino acid L-arginine by NO synthase,[50] supplementation of L-arginine may have beneficial effects on cardiovascular diseases.[51,52] In fact, we demonstrated that oral supplementation of L-arginine significantly increased plasma L-citrulline, which indicated enhancement of NO production, and produced a 16% decrease in pulmonary vascular resistance without significant systemic hypotension in patients with pulmonary arterial hypertension.[53] One-week supplementation of L-arginine improved exercise capacity. These results suggest that oral supplementation of L-arginine may have beneficial effects on hemodynamics and exercise capacity in patients with PPH. There is an ongoing Pulmonary Hypertensional L-Arginine Supplemental Therapy (PHAST) trial that has been designed to confirm the therapeutic effect of L-arginine.

1.1.5 Phosphodiesterase Inhibitors

Sildenafil is a selective inhibitor of type V phosphodiesterase which breaks down cyclic guanosine monophosphate (cGMP) and limits cGMP-mediated NO vasodilation.[54] Sildenafil is well tolerated and available as an oral preparation. The effects of phosphodiesterase inhibition are best known in the penile vascular bed, hence its use in the treatment of erectile dysfunction. Interestingly, there are high concentrations of the type V enzyme in the pulmonary vasculature. As a result, orally administered sildenafil