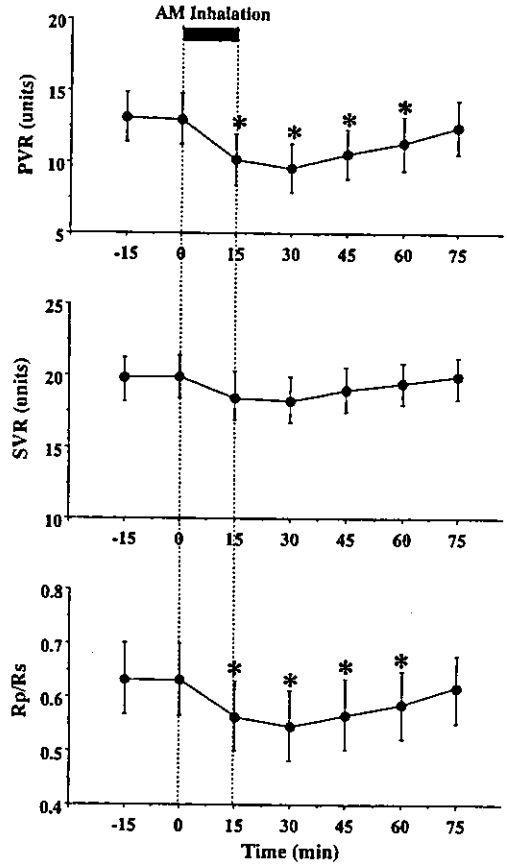

Figure 3. Changes in pulmonary vascular resistance (PVR), systemic vascular resistance (SVR), and ratio of pulmonary vascular resistance to systemic vascular resistance (Rp/Rs) by inhalation of aerosolized AM in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *P<0.05 vs value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

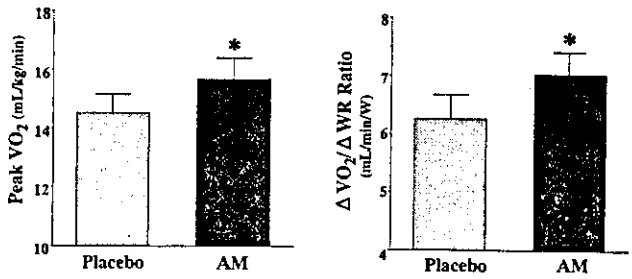

Figure 4. Changes in peak oxygen consumption (peak $\dot{V}_{O_2}$) and ratio of change in oxygen uptake to that in work rate ($\Delta\dot{V}_{O_2}/\Delta W$ ratio) by inhalation of aerosolized AM or placebo in patients with idiopathic pulmonary arterial hypertension. Data are mean±SEM. *P<0.05 vs placebo.

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, crossover 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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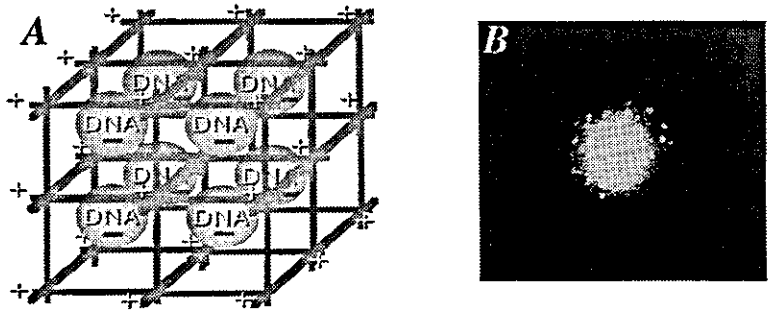

Figure 1. A, Schema of DNA-gelatin complex. Biodegradable gelatin can hold negatively charged plasmid DNA in its positively charged lattice structure. B, RITC-labeled AM DNA particles were incorporated into gelatin.

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.

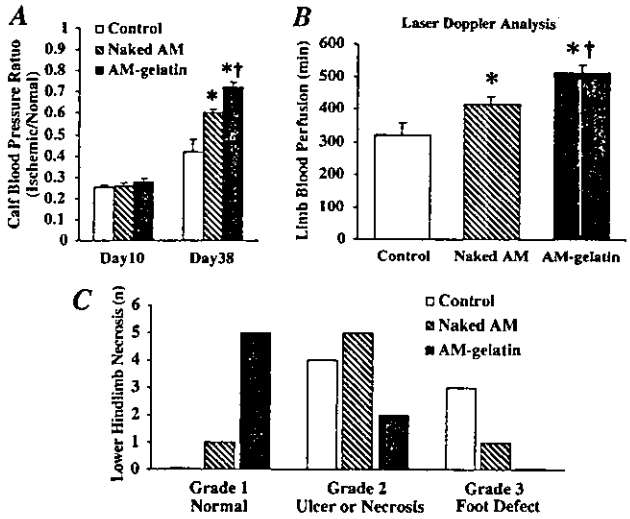

Figure 2. A, Calf blood pressure ratio (ischemic/normal hind limb) before (on day 10) and after (on day 38) gene transfer. B, Measurement of laser Doppler flow on day 38. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs naked AM group. C, Number of cases of each grade of lower hind limb necrosis 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.

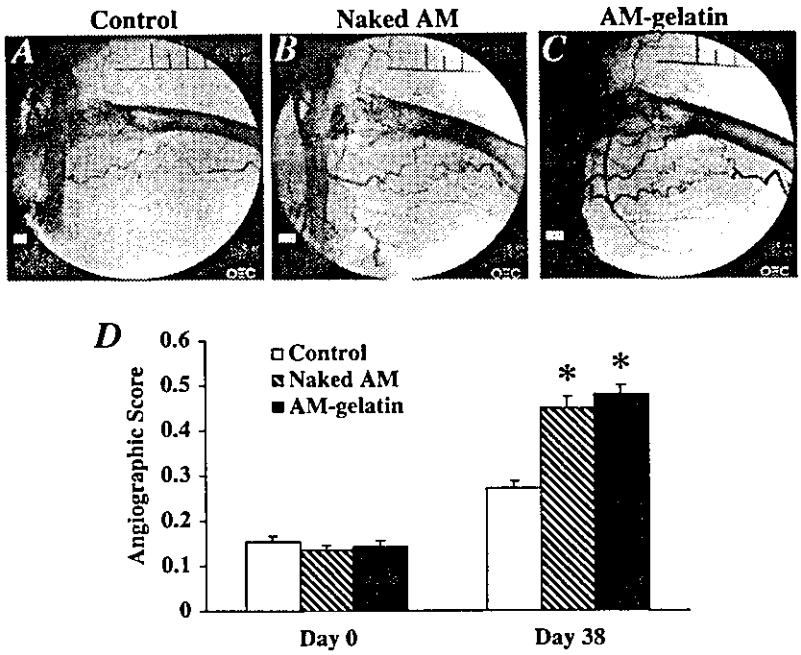

Figure 3. Representative angiograms of control group (A), naked AM group (B), and AM-gelatin group (C) on day 38. Collateral arteries were well developed in the naked AM and AM-gelatin groups. D, Angiographic score on days 0 and 38 in each group. Angiographic score on day 38 was significantly higher 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-

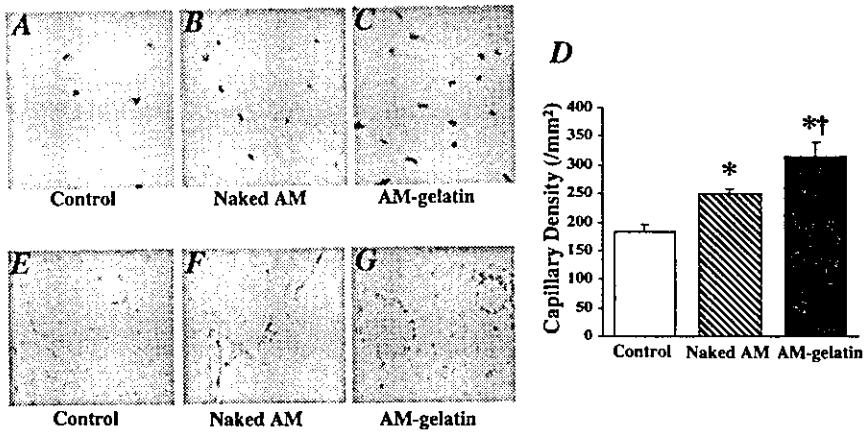

Figure 4. A through C, Representative examples of alkaline phosphatase staining in ischemic hind limb muscles. Magnification ×200. D, Quantitative analysis of capillary density in ischemic hind limb muscles. Data are mean±SEM. *$P<0.05$ vs control group; †$P<0.05$ vs naked AM group. E through G, Immunohistochemical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

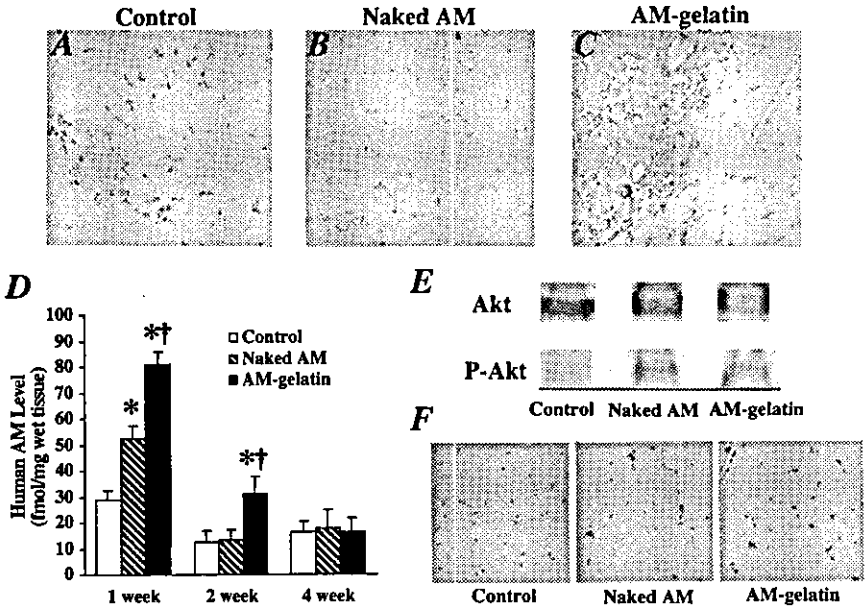

Figure 5. A through C, Immunohistochemistry for human AM 7 days after gene transfer. Intense immunostaining was observed surrounding gelatin in the AM-gelatin group. Magnification ×200. D, Time course of AM production in ischemic muscles after gene transfer. Data are mean±SEM. *$P<0.01$ vs control group; †$P<0.01$ 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.

Acknowledgments

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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.

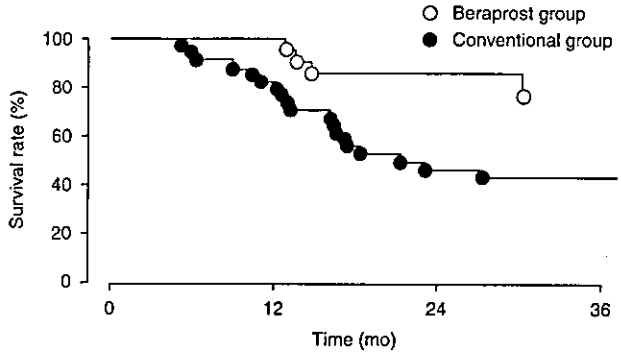

Fig. 1. Kaplan-Meier survival curves showing that outpatients treated with beraprost have a significantly higher survival rate than those treated with conventional therapy including calcium channel antagonists (log-rank test, $p < 0.001$).[20]

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

produces relatively selective pulmonary vasodilation.[55] Earlier studies have shown that sildenafil improves pulmonary hemodynamics particularly in attenuating rebound pulmonary hypertension after discontinuing inhaled NO.[56,57] In addition, sildenafil has been shown to augment the effect of inhaled NO for postoperative pulmonary hypertensive crises.[58] Thus, sildenafil may have synergistic effects with inhaled NO through inhibition of cGMP degradation. A single oral dose of sildenafil is as effective and selective a pulmonary vasodilator as inhaled NO.[59] A recent study has shown that oral sildenafil is a potent pulmonary vasodilator that acts synergistically with inhaled iloprost to cause strong pulmonary vasodilatation in severe pulmonary arterial hypertension.[60] These results suggest that phosphodiesterase inhibition by sildenafil may be a novel therapeutic strategy for the treatment of PPH. It is necessary to examine whether long-term treatment with sildenafil improves survival of patients with PPH. There is an ongoing placebo-controlled trial using sildenafil in pulmonary arterial hypertension.

1.2 Anticoagulation

The presence of endothelial injury in the pulmonary vascular bed develops pulmonary thrombi. Histopathologic studies on lung biopsy specimens show *in situ* arteriolar thrombosis in one-third to one half of the patients with PPH, and these microthrombotic lesions likely contribute to progression of the disease.[61] There is no prospective, randomized, placebo-controlled study showing a beneficial effects of anticoagulation therapy. However, Fuster et al.[62] reported in a retrospective study that survival was better in 78 patients who received oral anticoagulants than in 37 patients who were not anticoagulated. Rich et al.[13] showed that in patients who do not benefit from calcium antagonists, warfarin increased survival at 1 year from 62–91% and survival at 3 years from 31–47%. Based on these two studies, warfarin should be used in all patients with PPH unless there is an absolute contraindication. The current recommendation has been to target an international normalized ratio of 2- to 2.5-fold greater than control, a level that provides effective anticoagulation with a minimal risk of bleeding. Whether heparin would be more efficacious than warfarin, based on its inhibitory effects on smooth muscle proliferation remains unknown.

1.3 Inotropic Agents and Diuretics

Efficacy of chronic long-term inotropic therapy as a treatment modality remains controversial. However, Rich et al. reported that digoxin produced a modest increase in cardiac output in patients with pulmonary hypertension and RVr failure, as well as a significant reduction in circulating norepinephrine.[63] No detectable effects of digoxin on baroreceptor responsiveness were apparent. These results raise the possibility that digoxin has beneficial effects in patients with PPH, although long-term studies are necessary to confirm a therapeutic effect of digoxin.

Diuretics may be useful in reducing the increased intravascular volume and hepatic congestion in patients with right heart failure. Thus, diuretics generally afford symptom relief. However, the right ventricle is often highly dependent on preload, and care must be taken to avoid excessive diuresis, which may lead to a decrease in cardiac output in patients with PPH.

1.4 Oxygen Therapy

Oxygen therapy has been demonstrated to improve quality of life and decrease mortality in patients with pulmonary hypertension secondary to chronic long-term respiratory insufficiency.[64,65] In some patients with pulmonary arterial hypertension, significant ventilation-perfusion mismatching occurs that which results in hypoxic vasoconstriction exacerbating the underlying pulmonary hypertension. Thus, supplemental low-flow oxygen may alleviate the arterial hypoxemia and attenuate pulmonary vasoconstriction in some patients with PPH.

2. Noninvasive Assessment of Disease Severity

Accurate evaluation of both disease severity and the efficacy of vasodilator therapy is important in the management of patients with PPH. Mortality in patients with PPH is most closely associated with RV hemodynamic function and can be characterized by means of an equation using three variables: mean pulmonary artery pressure, mean right atrial pressure, and cardiac index.[66,67] However, risk stratification by a simple, noninvasive, and repeatedly available method is desirable. In order to assess current status of patients, we recommend repeated measurements of plasma brain natriuretic peptide (BNP),[68,69] serum uric acid (UA),[70,71] and the distance walked in 6 minutes.[72] Cardiopulmonary exercise testing[73,74] and echocardiography[75,76] can also reflect disease severity and predict poor outcome in patients with PPH. 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.

2.1 Plasma Brain Natriuretic Peptide Level

BNP is secreted predominantly from cardiac ventricles via a constitutive pathway.[77] BNP production is enhanced by the degree of myocardial stretch, damage, and ischemia in the ventricle.[77,78] Thus, plasma BNP level has been used as a noninvasive marker of left ventricular dysfunction and a prognostic indicator in a variety of patients with left-sided heart failure.[79,80] Recently, we

have shown that plasma BNP increases in proportion to the degree of RV dysfunction in pulmonary hypertension (figure 2).[68,69] Plasma BNP level correlated positively with total pulmonary resistance ($r = 0.79$, $p < 0.001$) and correlated negatively with RV ejection fraction ($r = -0.71$, $p < 0.001$). We have also shown that plasma BNP changes in association with chronic changes in hemodynamics, thereby serving as a potential indicator of the efficacy of vasodilator therapy in patients with PPH. More recently, we have demonstrated that high levels of plasma BNP, and in particular, a further increase in plasma BNP despite vasodilator therapy, may have a strong, independent association with increased mortality in patients with PPH.[69] Interestingly, patients with a supramedian level of BNP (> 180 ng/L) had a significantly lower survival rate than those with an inframedian level (figure 3). Thus, plasma BNP may serve as a noninvasive prognostic indicator of PPH, which may complement invasive standard prognostic markers.

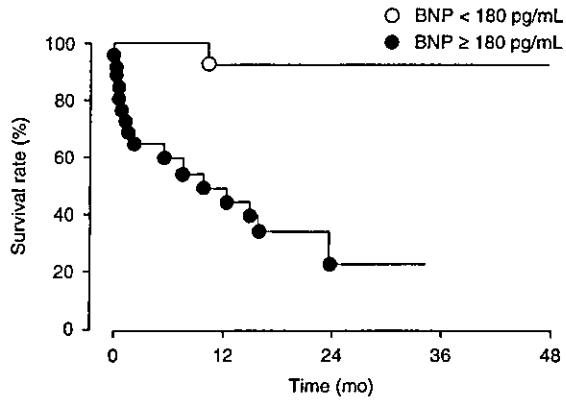

Fig. 3. Kaplan-Meier survival curves according to median value of plasma BNP levels in patients with PPH. **BNP** = brain natriuretic of peptide; **PPH** = primary pulmonary hypertension.[69]

2.2 Serum Uric Acid Level

Serum UA, the final product of purine degradation, has been shown to be increased in hypoxic states, such as chronic heart failure,[81,82] cyanotic congenital heart disease[83,84] and obstructive pulmonary disease.[85] Because tissue ischemia and hypoxia deplete adenosine triphosphate and promote degradation of adenine nucleotides to inosine, hypoxanthine, xanthine and UA,[86,87] increased serum UA levels may reflect impaired oxidative metabolism in such diseases. Recently, we and others[70,71] demonstrated that serum UA levels were significantly elevated in patients with PPH. Serum UA levels correlated negatively with cardiac output and correlated positively with total pulmonary resistance. Tissue hypoperfusion and hypoxia resulting from reduced cardiac output in severe PPH induce both overproduction of UA and impaired UA excretion, leading to increased serum levels in patients with PPH. Interestingly, serum UA level significantly decreased with vasodilator therapy associated with a reduction in total pulmonary resistance. Patients with high serum UA had a significantly higher mortality rate than those with low serum UA.[70] These results suggest that serum UA increases in proportion to the clinical severity of PPH and has independent association with long-term mortality of patients with PPH.

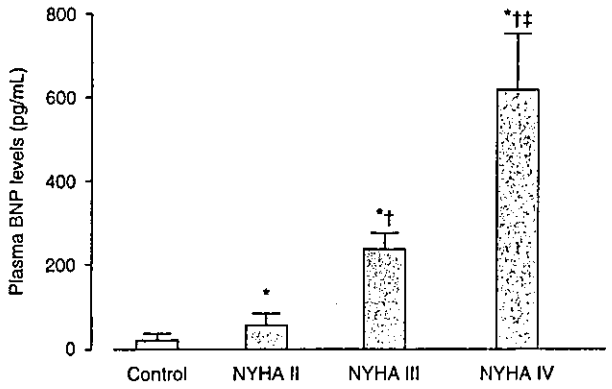

Fig. 2. Plasma BNP level in patients with PPH according to New York Heart Association (NYHA) functional class. **BNP** = brain natriuretic peptide; **PPH** = primary pulmonary hypertension. * indicates $p < 0.05$ versus control; † = $p < 0.05$ versus NYHA II; ‡ = $p < 0.05$ versus NYHA III.

2.3 Exercise Test

Cardiopulmonary exercise testing (CPX) allows reproducible assessment of functional capacity as well as ventilatory efficiency in patients with PPH.[73,74] Although CPX is a submaximal exercise test, most patients with PPH can safely undergo noninvasive cycle ergometer CPX to their maximal tolerance.[73] Reductions in peak O_2 uptake (peak $\dot{V}O_{2max}$), anaerobic threshold, peak O_2pulse, rate of increase in $\dot{V}O_{2max}$, and ventilatory efficiency were consistent and characteristic, and correlated well with NYHA class. Wensel et al.[74] have shown that CPX is a noninvasive prognostic substitute for hemodynamics. Patients with peak $\dot{V}O_{2max}$ ≤10.4 mL/kg/min and peak systolic blood pressure ≤120 mm Hg had poor survival rates at 12 months (23%), whereas patients with one or none of these risk factors had better survival rates (79% and 97%, respectively). These results suggest that peak $\dot{V}O_{2max}$ and peak systolic blood pressure are independent and strong predictors of survival in PPH patients.

The 6-minute walk test is a submaximal exercise test which can be performed even by a patient with heart failure not tolerating maximal exercise testing.[88,89] The test is very simple, requires inexpensive equipment, and is reproducible. It is considered safe because patients are self-limited during exercise. Six-minute walking distance correlated significantly with pulmonary hemodynamics and correlated strongly with peak oxygen consumption (exer-

cise capacity) determined by cardiopulmonary exercise testing.[72] Thus, the 6-minute walk test has been used as an indicator of the efficacy of vasodilator therapy in many studies.[7,34,43] In addition, we demonstrated that 6-minute walking distance was independently related to mortality in PPH by multivariate analysis. Patients walking <332m had a significantly lower survival rate than those walking farther, assessed by Kaplan-Meier survival curves.[72] Thus, the 6-minute walk test may serve not only as a potential marker for the efficacy of vasodilator therapy but also as a prognostic indicator in patients with PPH.

2.4 Echocardiography

Echocardiography is useful for the assessment of these patients. Recent studies have demonstrated prognostic value of echocardiography. Hinderliter et al.[75] have shown that pericardial effusion was noted in 43 of 79 patients (54%) with PPH. Larger effusion was associated with hemodynamic and echocardiographic evidence of right heart failure, impaired exercise tolerance, and a poor 1-year prognosis. Raymond et al.[76] have shown that pericardial effusion, right atrial enlargement, and septal displacement are echocardiographic abnormalities that reflect the severity of right heart failure and predict adverse outcomes in patients with severe PPH. These characteristics may help identify patients appropriate for more intensive medical therapy or earlier transplantation.

3. Future Perspectives

3.1 Adrenomedullin

Adrenomedullin is a potent vasodilator peptide that was originally isolated from human pheochromocytoma.[90] The vasodilating effect is mediated by cAMP- and NO-dependent mechanisms.[91,92] Immunoreactive adrenomedullin is detected in plasma and a variety of tissues, including blood vessels, heart, and lungs.[93,94] It has been reported that there are specific receptors for adrenomedullin in the lungs.[95] We have shown that plasma adrenomedullin level increases in proportion to the severity of pulmonary hypertension, and that circulating adrenomedullin is partially metabolized in the lungs.[96,97] These findings suggest that adrenomedullin plays an important role in the regulation of pulmonary vascular tone. In 2000, a randomized, placebo-controlled study by our group was the first to investigate the therapeutic use of adrenomedullin in the treatment of pulmonary arterial hypertension.[98,99] Short-term infusion of adrenomedullin produced a 44% increase in cardiac index and a 32% decrease in pulmonary vascular resistance with a 4% reduction in mean pulmonary arterial pressure in patients with PPH. Adrenomedullin also decreased plasma aldosterone levels without significant changes in plasma renin activity. These results suggest that intravenous infusion of adrenomedullin has beneficial hemodynamic and hormonal effects in patients with PPH.

3.2 Gene Therapy

Many researchers have already successfully transfected genes into bronchial epithelium, alveolar cells, and small pulmonary arteries by intratracheal delivery of genes using viral vectors. In 1999, Champion et al.[100] demonstrated that intratracheal transfer of the endothelial nitric oxide synthase (eNOS) gene selectively reduced pulmonary vascular resistance and pulmonary vasopressor responses to ET-1, angiotensin II, and hypoxia. Recently, they have shown that intratracheal gene transfer of calcitonin gene-related peptide to bronchial epithelial cells and alveolar cells attenuates chronic hypoxia-induced pulmonary hypertension in the mouse, suggesting lung cell transduction with a vasodilator peptide may be sufficient to alter vascular function.[101]

Earlier studies have shown that prostacyclin synthase (PGIS) deficiency in the lungs and impaired prostacyclin production are linked to the development of pulmonary hypertension in patients with PPH.[102,103] We demonstrated that intratracheal transfer of the PGIS gene augmented pulmonary prostacyclin synthesis, ameliorated monocrotaline-induced pulmonary hypertension, and thereby improved survival in rats.[104] These results suggest that gene therapy may hold great promise in the treatment of PPH. However, enormous hurdles still exist in the successful use of gene therapy in humans. Sustained expression is not yet possible, and concerns remain about an inflammatory reaction to the vectors. Therefore, the initial success of gene therapy should be confirmed by long-term experiments, and extensive toxicity studies in animals are needed before clinical trials.

4. Conclusions

Oral administration of calcium channel antagonists and intravenous infusion of epoproestenol are established as treatment of PPH. The dramatic success of long-term intravenous epoproestenol is now leading to the development of prostacyclin analogs using newer drug-delivery systems. Promising drugs including ET antagonists and type V phosphodiesterase inhibitors have been developed. Furthermore, gene therapy with eNOS gene or PGIS gene may hold great promise in the treatment of PPH. Accurate evaluation of disease severity and the efficacy of vasodilator therapy is important in the management of patients with PPH. In addition to invasive assessment by cardiac catheterization, we recommend repeated measurements of plasma BNP, serum UA, 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.

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