Gene Transfer of Human Prostacyclin Synthase Ameliorates Monocrotaline-Induced Pulmonary Hypertension in Rats

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Background—Prostacyclin is a potent vasodilator that also inhibits platelet adhesion and cell growth. We investigated whether in vivo gene transfer of human prostacyclin synthase (PGIS) ameliorates monocrotaline (MCT)-induced pulmonary hypertension in rats.

Methods and Results—The cDNA encoding PGIS was intratracheally transfected into the lungs of rats by the hemagglutinating virus of Japan–liposome method. Rats transfected with control vector lacking the PGIS gene served as controls. Three weeks after MCT injection, mean pulmonary arterial pressure and total pulmonary resistance had increased significantly; the increases were significantly attenuated in PGIS–transfected rats compared with controls [mean pulmonary arterial pressure, 31 ± 1 versus 35 ± 1 mm Hg (−12%); total pulmonary resistance, 0.087 ± 0.01 versus 0.113 ± 0.01 mm Hg · mL · min⁻¹ · kg⁻¹ (−23%), both P<0.05]. Systemic arterial pressure and heart rate were unaffected. Histologically, PGIS gene transfer inhibited the increase in medial wall thickness of peripheral pulmonary arteries that resulted from MCT injection. PGIS immunoreactivity was intense predominantly in the bronchial epithelium and alveolar cells. Lung tissue levels of 6-keto-PGF₁α, a stable metabolite of prostacyclin, were significantly increased for ≥1 week after transfer of PGIS gene. The Kaplan-Meier survival curves demonstrated that repeated transfer of PGIS gene every 2 weeks increased survival rate in MCT rats (log-rank test, P<0.01).


Key Words: prostaglandins • gene therapy • hypertension, pulmonary • viruses

Prostacyclin, a metabolite of arachidonic acid, has vasorelaxant, antiplatelet aggregation, and inhibition of smooth muscle cell proliferation.¹,² Prostacyclin synthase (PGIS) catalyzes the conversion of prostaglandin H₂ to prostacyclin. In 1994, we determined the amino acid sequence of human endothelial PGIS by cDNA cloning.³ Recently, we showed that introduction of the PGIS cDNA into rat carotid artery after balloon-induced injury results in increased prostacyclin synthesis and inhibition of smooth muscle cell proliferation.⁴ These results raise the possibility that in vivo gene transfer of PGIS could have beneficial effects in patients with vascular disease.

Primary pulmonary hypertension (PPH) is a rare but life-threatening disease characterized by progressive pulmonary hypertension, ultimately producing right ventricular (RV) failure and death.⁵ Interestingly, PGIS deficiency in the lungs and impaired prostacyclin production have been linked to the development of pulmonary hypertension in this disease.⁶,⁷ As a result, continuous intravenous infusion of prostacyclin has become recognized as a therapeutic breakthrough that can improve hemodynamics and survival in patients with PPH.⁸–¹¹ Recently, transgenic mice with lung PGIS overproduction have been shown to be protected from the development of pulmonary hypertension after exposure to hypoxia.¹² However, whether in vivo gene transfer of human PGIS can ameliorate pulmonary hypertension remains unknown.

The hemagglutinating virus of Japan (HVJ)—liposome method is a liposome-based gene delivery system that enables introduction of the contents of liposomes directly into living cells by means of the virus-cell fusion machinery.¹³–¹⁶ Because HVJ-liposomes are prepared after ultraviolet irradiation of the virus, the gene transfer system is less toxic and less immunogenic than adenovirus and herpesvirus vectors.

Thus, the purpose of this study was to investigate whether intratracheal transfer of the human PGIS gene by use of HVJ-liposomes augments pulmonary prostacyclin synthesis,
ameliorates monocrotaline (MCT)-induced pulmonary hypertension, and improves survival in MCT rats.

Methods

Animals

Male Wistar rats weighing 100 to 120 g were used in this study. Rats were randomly assigned to receive intratracheal injection of either the HVJ-liposome complex with PGIS expression plasmid or control vector, followed by a subcutaneous injection of either 60 mg/kg MCT or 0.9% saline. This protocol resulted in the creation of 4 groups: MCT rats transfected with PGIS expression plasmid (MCT-PGIS, n=8); MCT rats transfected with control vector (MCT-CON, n=8); normal rats transfected with PGIS expression plasmid (NL-PGIS, n=8); and normal rats transfected with control vector (NL-CON, n=8). Another 40 rats injected with MCT were studied to assess the time course of prostacyclin synthesis after transfer of either PGIS expression plasmid or control vector. An additional 24 rats were studied to evaluate the effects of PGIS gene therapy on survival in MCT rats.

Construction of Plasmid DNA

The expression vector for human PGIS was constructed as described previously. In brief, the blunted HindIII/BamHI fragment of the full-length human PGIS cDNA was ligated into the blunted XhoI site of the pUC-CAGGS expression plasmid. To verify that the pUC-PGIS construct encoded a biologically active PGIS protein, pUC-PGIS was transfected into 293 cells, and PGIS activity in the transfected cells was measured as conversion of [14C]PGH2 to 6-keto-[14C]PGF1α. The pUC-CAGGS vector lacking the insert encoding human PGIS served as the control vector.

Preparation of HVJ-Liposomes

Preparation of HVJ-anionic liposomes with a lipid composition similar to that of the viral envelope (AVE) has been described elsewhere. In brief, lipid mixtures were prepared by mixing lipid solutions (phosphatidycholine/dioleoylphosphatidyl ethanolamine/sphingomyelin/phosphatidylserine/cholesterol, 4:4:4:4:3:15 in molar ratio). Dried lipid mixture was propagated with plasmid DNA (200 μg) in 200 μL balanced salt solution (mmol/L: NaCl 137, KCl 5.4, Tris-HCl 8 [pH 7.6]). HVJ-liposomes were prepared by mixing the lipid with HVJ virus inactivated by ultraviolet irradiation. After incubation and sucrose gradient centrifugation, the HVJ-liposome complex was collected with a Pasteur pipette.

Experimental Protocol

After the rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg), tracheostomy was performed to introduce a polyethylene catheter (PE-50) into the left and right main bronchi. Through the catheter, 400 μL HVJ-liposome complex including 100 μg plasmid DNA was administered into the bronchi. Next, rats were intubated with a polyethylene tube (PE-240) and artificially ventilated with a volume-regulated respirator for 30 minutes. Then, the polyethylene tube was removed and the tracheotomy was closed. Finally, rats were given a single subcutaneous injection of either 60 mg/kg MCT or 0.9% saline 24 hours after gene transfer. Animals were maintained on standard rat chow. Hemodynamic studies were performed 22 days after gene transfer. Rats were anesthetized with intraperitoneal pentobarbital (30 mg/kg) and placed on a heating pad to maintain body temperature at 37°C to 38°C throughout the study. A polyethylene catheter (PE-10) was inserted into the right femoral artery to measure heart rate and mean arterial pressure. A 3.5F umbilical vessel catheter was inserted through the right jugular vein into the pulmonary artery for measurement of RV pressure and pulmonary arterial pressure. These hemodynamic variables were measured with a pressure transducer (model P 23 ID, Gould) connected to a polygraph and recorded with a thermal recorder (7758 B System, Hewlett-Packard). A thermomicroprobe was advanced into the ascending aorta via the right carotid artery and connected to a cardiac output computer (Cardiotherm-500, Columbus Instruments). Cardiac output was measured in triplicate by the thermodilution method. Total pulmonary resistance was calculated by dividing mean pulmonary arterial pressure by cardiac output. After completion of the above measurements, cardiac arrest was induced by injection of 2 mmol KCl through the catheter. The ventricles and lungs were excised, dissected free, and weighed. The measurement of the RV weight and body weight (RV/BW) and the ratio of left ventricular weight to body weight (LV/BW) were calculated as indexes of ventricular hypertrophy.

Morphometric Analysis of Pulmonary Arteries

Paraffin sections 4 μm thick were obtained from the middle region of the right lung and stained with hematoxylin and eosin for examination by light microscopy. Statistical analysis of the medial wall thickness of the pulmonary arteries was performed as described previously. In brief, the external diameter and the medial wall thickness were measured in 30 muscular arteries (ranging in size from 25 to 50 and from 51 to 100 μm in external diameter) per lung section. For each artery, the medial wall thickness was expressed as follows: % wall thickness=[(medial thickness/external diameter)×100]. A lung section was obtained from individual rats for comparison among 4 groups (MCT-PGIS, MCT-CON, NL-PGIS, and NL-CON groups, n=5 each).

Immunohistochemical Analysis

Seven days after transfer of PGIS expression plasmid or control vector, lungs of MCT rats were stained immunohistochemically with a rabbit polyclonal antibody raised against a partial sequence of the human PGIS sequence. Sequences 4 μm thick were incubated in 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity and to permeabilize the cells. Nonspecific binding of rabbit serum was prevented by preincubating the sections with 0.2% normal goat serum. The sections were sequentially incubated at 4°C overnight with rabbit antibody against human PGIS and a combination of 1:1000 and then with biotinylated goat anti-rabbit IgG (Dako Japan Co) for 30 minutes, followed by peroxidase labeling with streptavidin (LSAB kit, Dako Japan Co) for an additional 20 minutes at room temperature. Each incubation was followed by washing in Tris-buffered saline. Staining was visualized with a chromogen, 0.06% 3,3’-diaminobenzidine/0.03% hydrogen peroxide in 8 mmol/L Tris-HCl (pH 6.85). Hematoxylin was used as a counterstain. Control sections were incubated with nonimmune rabbit IgG at a concentration of 1:1000.

Measurement of 6-keto-PGF1α

Lung tissue levels of 6-keto-PGF1α, a stable metabolite of prostacyclin, were analyzed to assess prostacyclin production. To determine the time course of prostacyclin synthesis in the lung, a total of 80 lungs in MCT rats were evaluated 2, 7, 14, and 21 days after transfer of PGIS expression plasmid (n=10 each) or control vector (n=10 each). 6-keto-[3H]PGF1α (10 000 dpm, 6.55 TBq/mmol, Amersham Pharmacia Biotech) was added as a tracer for calculation of the recovery factor. After extraction in ice-cold ethanol and purification with a C-18 reverse-phase cartridge (Sep-Pak Plus, Waters), 6-keto-PGF1α was quantified with an enzyme immunoassay kit (Cayman Chemical Co). The protein content of the precipitate after ethanol extraction of the lung was determined by the Lowry method. The results were expressed as picograms of 6-keto-PGF1α per milligram of protein.

Survival Analysis

To evaluate the effects of PGIS gene therapy on survival in MCT-injected rats, 24 rats received repeated administration of HVJ-liposome complex with PGIS expression plasmid (n=12) or control vector (n=12) every 2 weeks. After the rats were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg), the repeated administration of 200 μL HVJ-liposome complex including 50 μg plasmid DNA was done via tracheal tube (PE-240). Rats were then ventilated with a volume-regulated respirator for 30 minutes.
Survival was estimated from the date of MCT injection to the death of the rat or 10 weeks after the injection.

Statistical Analysis
All data were expressed as mean±SEM unless otherwise indicated. Comparisons of parameters between 2 groups were made by unpaired Student’s t test. Comparisons of parameters among 4 groups were made by 1-way ANOVA, followed by Newman-Keuls test. Survival curves according to the presence or absence of PGIS gene transfer were derived by the Kaplan-Meier method and compared by log-rank tests. A value of P<0.05 was considered statistically significant.

Results
Physiological Profiles
The physiological profiles of the 4 experimental groups are summarized in the Table. Body weight and mean arterial pressure were significantly lower in MCT rats (MCT-CON and MCT-PGIS groups) than in normal rats (NL-CON and NL-PGIS groups). There was no significant difference in heart rate among the 4 groups. Both RV/BW and RV systolic pressure were significantly increased in rats injected with MCT.

Effect of PGIS Gene Transfer on MCT-Induced Pulmonary Hypertension
Three weeks after MCT injection, pulmonary hypertension developed in both MCT groups, but the rise in mean pulmonary arterial pressure was significantly smaller (by 12%) in the MCT-PGIS group than in the MCT-CON group (31±1 versus 35±1 mm Hg, P<0.05, Figure 1). Cardiac output was significantly higher (by 13%) in the MCT-PGIS than in the MCT-CON group (360±11 versus 320±16 mL min⁻¹ kg⁻¹, P<0.05). Cardiac output tended to be decreased in the MCT-CON compared with the NL-CON group, but no reduction in cardiac output was observed in the MCT-PGIS group. Therefore, total pulmonary resistance was significantly lower (by 23%) in the MCT-PGIS than in the MCT-CON group (0.087±0.01 versus 0.113±0.01 mm Hg mL⁻¹ min⁻¹ kg⁻¹, P<0.05). Similarly, increases in RV systolic pressure and RV/BW were significantly attenuated in the MCT-PGIS compared with the MCT-CON group (Table). In contrast, neither systemic arterial pressure nor heart rate differed between the 2 groups. There were no significant differences in hemodynamic parameters between the NL-CON and NL-PGIS groups.

Morphometric Analysis of Pulmonary Arteries
Representative photomicrographs showed that the hypertrophy of the pulmonary vessel wall was attenuated in MCT-PGIS rats compared with MCT-CON rats (Figure 2). Quantitative analysis of peripheral pulmonary arteries demonstrated significant increases in percent wall thickness in both MCT groups, but these changes were significantly attenuated in the MCT-PGIS compared with the MCT-CON group (25±2% versus 31±2% in the vasculature with external diameters of 25 to 50 μm; 26±2% versus 33±3% in the 51- to 100-μm vasculature, both P<0.05, Figure 3).
was no significant difference in either parameter between the NL-CON and NL-PGIS groups.

Immunohistochemical Analysis
PGIS immunoreactivity was intense in distal bronchial epithelium in MCT rats transfected with PGIS gene (Figure 4). Diffuse immunostaining for PGIS was detected in alveolar cells after PGIS gene transfer. In addition, intense immunoreactivity for PGIS was observed in the bronchial epithelium near the pulmonary vasculature. No major adverse effects of gene expression were observed in the transfected lung segments.

Lung Tissue Level of 6-keto-PGF$_{1\alpha}$
Lung tissue content of 6-keto-PGF$_{1\alpha}$ in MCT rats was significantly increased 2 days (999±173 versus 534±103 pg/mg protein, $P<0.05$) and 7 days (1045±133 versus 716±68 pg/mg protein, $P<0.05$) after a single transfer of PGIS expression plasmid (Figure 5). Lung 6-keto-PGF$_{1\alpha}$ levels remained elevated 14 days after PGIS gene transfer, although this was statistically not significant.

Survival Analysis
Kaplan-Meier survival curves demonstrated that MCT rats transfected with PGIS gene had a significantly higher survival rate than those transfected with control vector alone (33% versus 0% in 10-week survival, log-rank test, $P<0.01$, Figure 6). No definite adverse effects were detected after repeated transfer of PGIS gene.

Discussion
In the present study, we demonstrated that (1) intratracheal transfer of the human PGIS gene by use of HVJ-liposomes induced overexpression of PGIS protein in bronchial epithelium and alveolar cells in MCT rats, resulting in augmenting prostacyclin synthesis in the lungs; and (2) transfer of the PGIS gene ameliorated MCT-induced pulmonary hypertension and pulmonary vascular remodeling. Finally, we demonstrated that (3) repeated transfer of the PGIS gene improved survival in MCT rats without definite adverse effects.

Patients with PPH have significantly decreased production of prostacyclin relative to that of thromboxane. Patients with PPH have significantly decreased production of prostacyclin relative to that of thromboxane. Recently, PGIS expression has been shown to be particularly decreased in remodeled pulmonary arteries containing plexiform lesions in patients with PPH. These findings raise the possibility that impaired prostacyclin synthesis resulting from decreased PGIS may be implicated in the pathogenesis of PPH. In fact, continuous intravenous infusion of prostacyclin markedly lowers pulmonary vascular resistance in patients with PPH and improves survival beyond that attained with conventional therapy alone. However, prostacyclin is metabolized rapidly, requiring continuous intravenous administration. Quality of life still remains an important issue to be resolved. Therefore, it would be more desirable to enhance endogenous prostacyclin production.

In the present study, transfer of human PGIS gene to the lung could be achieved by intratracheal injection of HVJ-liposome complex. Intense immunoreactivity for PGIS was

![MCT-PGIS and MCT-CON](http://circ.ahajournals.org/)

**Figure 2.** Representative photomicrographs of peripheral pulmonary arteries in MCT-PGIS rats and MCT-CON rats. PGIS gene transfer attenuated hypertrophy of vessel wall in MCT rats. Magnification ×400. Abbreviations as in text.

**Figure 3.** Effects of PGIS gene transfer on percent wall thickness of peripheral pulmonary arteries with external diameter of 25 to 50 μm (A) or 51 to 100 μm (B). Percent wall thickness was calculated as [(medial thickness×2)/external diameter]×100. Abbreviations as in text. Data are mean±SEM.

* $P<0.05$ vs NL-CON; † $P<0.05$ vs MCT-CON.
observed predominantly in bronchial epithelium and alveolar cells. Earlier studies have shown that HVJ-liposomes have strong affinity for bronchial epithelium and alveolar cells.\textsuperscript{20,21} Recently, Saeki et al\textsuperscript{15} developed a novel anionic liposome with a lipid composition similar to the AVE, which showed up to a 10-fold improvement in transduction efficiency in vivo over conventional HVJ-liposomes. Thus, in the present study, this HVJ-AVE liposome was used for successful intratracheal gene transfer. The human PGIS gene discovered in our institute was used in the present study because the cDNA for human PGIS has high identity with its rat counterpart.\textsuperscript{3,22}

Recently, Champion et al\textsuperscript{23} showed 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 that lung cell transduction with a vasodilator peptide may be sufficient to alter vascular function. Geraci et al\textsuperscript{12} showed that pulmonary prostacyclin synthase overexpression in transgenic mice protects against development of hypoxic pulmonary hypertension. Because they chose to use the epithelial cell–specific promoter instead of a vascular system–specific promoter, PGIS gene was expressed in bronchial epithelial cells and alveolar cells but not in pulmonary vessels. Nevertheless, PGIS-expressing mice did inhibit a pulmonary hypertensive response to acute hypoxia. In clinical settings, inhaled aerosolized prostacyclin acts transepithelially with pulmonary selectivity and improves pulmonary hypertension and oxygenation.\textsuperscript{24,25} These findings suggest that transepithelial delivery of prostacyclin to the vasculature may have beneficial effects on pulmonary hemodynamics. In the present study, lung tissue content of 6-keto-PGF\textsubscript{1\alpha} was significantly increased for at least 1 week after transfer of PGIS gene, suggesting increased prostacyclin synthesis in the lung. The consequence of this synthesis in rats injected with MCT

**Figure 4.** Immunohistochemical demonstration of PGIS in lungs of MCT rats after PGIS gene transfer. Intense immunostaining for PGIS was observed in distal bronchial epithelium (A, arrows), in alveolar cells (B, arrows), and bronchial epithelium near pulmonary vasculature (C, arrows) after transfer of PGIS expression plasmid, which was hardly observed in those with control vector (D, E). Magnification \( \times 400 \). Abbreviations as in text.

**Figure 5.** Lung tissue levels of 6-keto-PGF\textsubscript{1\alpha} in MCT rats after single injection of PGIS expression plasmid (MCT-PGIS) or control vector (MCT-CON). Data are mean±SEM. *\( P<0.05 \) vs MCT-CON.

**Figure 6.** Kaplan-Meier survival curves showing that MCT rats transfected with PGIS expression plasmid (MCT-PGIS) have a significantly higher survival rate than those treated with control vector (MCT-CON) (log-rank test, \( P<0.01 \)).
was a significant decrease in mean pulmonary arterial pressure and total pulmonary resistance. Importantly, the improvement in pulmonary hemodynamics was not accompanied by systemic hypotension, paralleling earlier results with gene transfer of endothelial nitric oxide synthase to the lung.26,27 Considering increased PGIS in the bronchial epithelium near the pulmonary vasculature, it is possible that endogenous prostacyclin produced by epithelial PGIS acts transepithelially with pulmonary vascular selectivity and ameliorates MCT-induced pulmonary hypertension.

Histological examination of MCT rats revealed that PGIS gene transfer inhibited an increase in the medial wall thickness of peripheral pulmonary arteries. In MCT-injected rats, endothelial cell injury caused by MCT activates platelets and vasoconstrictive factors, resulting in pulmonary hypertension and pulmonary vascular remodeling.28 Given the known potent vasoprotective effects of prostacyclin, such as vasodilation, antiplatelet aggregation, and inhibition of smooth muscle cell proliferation,1,2 it is interesting to speculate that endogenous prostacyclin produced by epithelial PGIS overexpression may act as a paracrine factor in the regulation of progressive pulmonary vascular remodeling in MCT rats.

Single gene transfer with HVJ-liposomes induced overexpression of PGIS and increased production of 6-keto-PGF1α for at least 1 week. The HVJ-liposome method has been shown to be less toxic and less immunogenic than viral vectors such as adenovirus and herpesvirus because HVJ-liposome complexes with nuclear protein in adult rat liver.29 They have been used for intracellular gene transfer into cells using HVJ (Sendai virus) liposomes with gangliosides.30 Repetitive transfer of PGIS gene significantly improved survival in MCT rats without definite inflammation in the lungs. Thus, MCT rats received repeated administration of the HVJ-liposome complex with PGIS gene every 2 weeks. Interestingly, repetitive transfer of PGIS gene significantly improved survival in MCT rats without definite inflammation in the lungs. Thus, PGIS gene therapy may be a promising treatment for severe pulmonary hypertension. However, the initial success of PGIS gene therapy reported here should be confirmed by long-term experiments, and extensive toxicity studies in animals are needed before clinical trials.

Conclusions

Intratracheal transfer of the human PGIS gene with HVJ-liposomes augmented pulmonary prostacyclin synthesis, inhibited MCT-induced pulmonary hypertension, and thereby improved survival in MCT rats. PGIS gene therapy may be a new therapeutic strategy for the treatment of pulmonary hypertension.

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