Adenoassociated Virus–Mediated Prostacyclin Synthase Expression Prevents Pulmonary Arterial Hypertension in Rats

Takayuki Ito, Takashi Okada, Jun Mimuro, Hiroshi Miyashita, Ryosuke Uchibori, Masashi Urabe, Hiroaki Mizukami, Akihiro Kume, Masafumi Takahashi, Uichi Ikeda, Yoichi Sakata, Kazuyuki Shimada, Keiya Ozawa

Abstract—Prostacyclin synthase (PGIS) is the final committed enzyme in the metabolic pathway of prostacyclin production. The therapeutic option of intravenous prostacyclin infusion in patients with pulmonary arterial hypertension is limited by the short half-life of the drug and life-threatening catheter-related complications. To develop a better delivery system for prostacyclin, we examined the feasibility of intramuscular injection of an adenoassociated virus (AAV) vector expressing PGIS for preventing monocrotaline-induced pulmonary arterial hypertension in rats. We developed an AAV serotype 1–based vector carrying a human PGIS gene (AAV-PGIS). AAV-PGIS or the control AAV vector expressing enhanced green fluorescent protein was injected into the anterior tibial muscles of 3-week–old male Wistar rats; this was followed by the monocrotaline administration at 7 weeks. Eight weeks after injecting the vector, the plasma levels of 6-keto-prostaglandin F1α increased in a vector dose-dependent manner. At this time point, the PGIS transduction (1×10^10 genome copies per body) significantly decreased mean pulmonary arterial pressure (33.9±2.4 versus 46.1±3.0 mm Hg; P<0.05), pulmonary vascular resistance (0.26±0.03 versus 0.41±0.03 mm Hg·mL⁻¹·min⁻¹·kg⁻¹; P<0.05), and medial thickness of the peripheral pulmonary artery (14.6±1.5% versus 23.5±0.5%; P<0.01) as compared with the controls. Furthermore, the PGIS-transduced rats demonstrated significantly improved survival rates as compared with the controls (100% versus 50%; P<0.05) at 8 weeks postmonocrotaline administration. An intramuscular injection of AAV-PGIS prevents monocrotaline-pulmonary arterial hypertension in rats and provides a new therapeutic alternative for preventing pulmonary arterial hypertension in humans. (Hypertension. 2007;50:531-536.)

Key Words: hypertension ■ pulmonary ■ gene therapy ■ remodeling ■ prostacyclin synthase

Pulmonary arterial hypertension (PAH) is an intractable disease that leads to increased pulmonary arterial pressure, progressive right heart failure, and premature death; however, no satisfactory treatment has been established for PAH. Although intravenous prostacyclin (PGI2) therapy prolongs survival in patients with PAH, the use of this treatment option is limited by the short half-life of the drug, requirement for a continuous infusion system, and catheter-related complications. PGIS synthase (PGIS) is the final committed enzyme in the metabolic pathway of PGI2 production. PGIS gene transfer is a promising approach for the stable production of endogenous PGI2. However, previous strategies have several limitations both in the selection of delivery routes and in the efficiency of gene expression. For instance, intratracheal gene transfer may deteriorate respiratory function in critically ill subjects, and the intrahepatic approach may cause peritonitis as a result of direct liver puncture. Although an intramuscular approach seems to be safer than the previous approaches, the conventional plasmid-based strategies achieved only transient gene expression and required repeated gene transfer to inhibit pathological remodeling of the pulmonary artery (PA).

In this study, we used an adenoassociated virus (AAV) vector together with an intramuscular approach to obtain more efficient PGI2 expression. AAV vectors permit efficient and sustained gene expression in nondividing skeletal muscle cells with minimal inflammatory and immune responses. We reported previously that a stable serum concentration of a secretory protein was achieved over a 1-year period by using a single intramuscular injection of several AAV vector (AAV2 and AAV5) serotypes in mice. Currently, AAV1 is one of the most efficient serotypes for muscle transduction.
Single subcutaneous injection of a pyrrolizidine alkaloid, namely, monocrotaline (MCT), produces severe PAH and PA remodeling in rats. We examined the effects of sustained PGIS expression in preventing PAH development and progression by means of this widely used model and an AAV1 vector.

**Methods**

**Western Blot Analysis for PGIS Expression In Vitro**

Human embryonic kidney 293 (HEK293) cells were incubated in 10-cm dishes containing DMEM and nutrient mixture F12 (Invitrogen) with 2% FCS in an atmosphere of 5% CO2 in air at 37°C. The cells at 70% confluence were transfected with an AAV proviral plasmid encoding human PGIS (phPGIS, a kind gift from Dr Mimuro) or plasmid encoding enhanced green fluorescent protein (eGFP) by using a calcium phosphate method. The cells were harvested 72 hours after transfection, and cell lysates were prepared with a lysis buffer (10 mmol/L of Tris-HCl, 150 mmol/L of NaCl, and 1% NP40 [pH 7.6]) containing Complete Mini protease inhibitor (Roche Diagnostics). For Western blot analysis, 10 μg of the lysate was subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked and incubated with a 1:5000 dilution of rabbit anti-human PGIS polyclonal antibody (a gift from Dr Mimuro) and a 1:5000 dilution of peroxidase-linked anti-rabbit IgG antibody (Amersham Pharmacia Biotech), and immunoreactive bands were visualized using an enhanced chemiluminescence Western blotting kit (Amersham).

**AAV-PGIS Production and PG12 Expression**

We developed a recombinant AAV1-based vector containing the human PGIS or eGFP gene controlled by a modified chicken β-actin promoter with a cytomegalovirus immediate-early enhancer (AAV-PGIS or AAV-eGFP) to obtain efficient transgene expression in skeletal muscle cells. The AAV vectors were prepared according to the previously described 3-plasmid transfection adenovirus-free protocol with minor modifications for enabling the use of an active gassing system.10-11 In brief, 60% confluent HEK293 cells that were incubated in a large culture vessel with active air circulation were cotransfected with phPGIS, AAV-1 chimeric helper plasmid (p1RepCap), and adenoviral helper plasmid pAdeno (Avigen Inc). The crude viral lysate was purified with 2 rounds of cesium chloride-2% centrifugation.12 The titer of the viral stock was determined against plasmid standards by real-time PCR with primers 5'-CCCCGGAGGTTGTGTTGGAC-3' and 5'-ATGGCCGATGCG-TGAC-3'; subsequently, the stock was dissolved in a buffer (50 mmol/L of HEPES [pH 7.4] and 0.15 mol/L of NaCl [HN buffer]) before infection. The HEK293 cells cultured in 6-well plates containing DMEM and nutrient mixture F12 with 5% FCS were infected with AAV-PGIS at 1×10⁹ genome copies per cell to evaluate PG12 expression in vitro, and the supernatant was harvested after 72 hours. Concentrations of 5-keto-prostaglandin F₂α (5-keto-PGF₂α) in plasma or culture media were determined by enzyme immunoassay (R&D Systems) according to the manufacturer’s instructions. The minimum detectable dose of the assay was <1.4 pg/mL. Interassay and intra-assay precision of the kit was <10%.

**Animal Models**

All of the animal experiments were approved by the Jichi Medical University ethics committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. To evaluate the efficiency of gene expression in vivo, AAV-eGFP (200 μL; 1×10¹⁰ gene copies per body) or AAV-PGIS (200 μL; 1×10¹⁰ gene copies per body) was injected into the bilateral anterior tibial muscles (n=3 each) of 3-week–old male Wistar rats (Clea Japan Inc) weighing 45 to 55 g. For hemodynamics and histological analyses, the rats were divided into 4 groups: sham rats that were administered the HN buffer (group 1, negative control [NC] group; n=4); MCT-PAH rats administered the HN buffer (group 2, MCT group; n=6); MCT rats administered AAV-eGFP (group 3, MCT+eGFP group; n=6); and MCT rats administered AAV-PGIS (group 4, MCT+PGIS group; n=10). After the anesthesia with spontaneous inhalation of 1% isoflurane, the rats in groups 3 and 4 were intramuscularly injected with AAV-eGFP or AAV-PGIS (1×10¹⁰ gene copies per body), whereas those in groups 1 and 2 were injected with the HN buffer (200 μL). MCT (Wako Pure Chemicals) was dissolved in 0.1 N HCl, and the pH was adjusted to 7.4 with 1.0 N NaOH. After the anesthesia with spontaneous inhalation of 1% isoflurane, all of the rats except for those in the NC group were injected subcutaneously with MCT (40 mg/kg) 4 weeks after the injecting the vector. Blood samples were collected from the tail vein on ethylenediamine tetracetic acid tubes, and the concentrations of the leucocytes, platelets, hematocrit, alanine aminotransferase, and creatinine were determined by standard procedures.

**Hemodynamics Analysis**

Four weeks after the MCT injection, the rats were anesthetized with spontaneous inhalation of 1% isoflurane, and a tracheotomy was performed. Then, they were mechanically ventilated with 1% isoflurane (tidal volume, 10 mL/kg; respiratory rate, 30 breaths per minute) through a tracheostomy. After the thoracic cavity was opened using a midsternal approach, 2F high-fidelity manometer-tipped catheters (SPC-320; Millar Instruments Inc) were inserted directly into the right or left ventricle, and an ultrasonic flow probe (FlowLab Probe 2.5S176; Transonic Systems Inc) was placed on the ascending root of the aorta. The heart rate, mean pulmonary arterial pressure (mPAP), aortic systolic arterial pressure, left ventricular end-diastolic pressure (LVEDP), and mean aortic flow indicating the cardiac output (CO) were measured. Cardiac indices (CI) and pulmonary vascular resistance (PVR) were calculated using the following formula: CI (mL·min⁻¹·kg⁻¹) = CO/body weight, PVR (mm Hg · mL⁻¹·min⁻¹·kg⁻¹) = (mPAP–LVEDP)/CI.

**Ventricular Weight Measurement and Morphometric Analysis of the PA**

After the hemodynamic analysis, the rats were killed with an overdose (5%) of isoflurane through a tracheostomy. Their lungs were perfused with 5 mL of saline followed by 10 mL of cold 4% paraformaldehyde. Each ventricle and the lungs were then excised, dissected free, and weighed. The weight ratio of the right ventricle to the left ventricle plus septum [RV/(LV+SV)] was calculated as an index of right ventricular hypertrophy (RVH). The lung tissues were fixed overnight at 4°C in 4% paraformaldehyde and frozen in Tissue-Tek OCT compound (Sakura Finetechnical Co) at −20°C. Hematoxylin and eosin staining was performed on 7-μm-thick sections that were subsequently examined using light microscopy. A morphometric analysis was performed on a PA having an external diameter of 25 to 50 μm or 51 to 100 μm. The medial wall thickness was calculated using the following formula: medial thickness (%) = medial wall thickness/external diameter×100.13 For the quantitative analysis, 30 vessels of each rat were measured and averaged randomly by the 2 external observers.

**Survival Analysis**

The 3-week–old Wistar rats were divided into 3 groups (MCT, MCT+eGFP, and MCT+PGIS; n=8 each). After the anesthesia with spontaneous inhalation of 1% isoflurane, the rats in the MCT+eGFP or MCT+PGIS group were intramuscularly injected with AAV-eGFP or AAV-PGIS at 1×10¹⁰ genome copies per body, respectively. Under the same anesthetic condition, all of the rats were injected subcutaneously with MCT (40 mg/kg) at 4 weeks after injecting the vector. The survival rate was estimated from the date of the MCT administration until death or after 8 weeks of the injection. Survival curves were analyzed using the Kaplan-Meier method and compared by log-rank tests.
Effects of PGI2 Expression on Hemodynamics and RVH

Four weeks after the MCT administration, the mPAP levels were significantly elevated in the treated rats as compared with the untreated controls (Figure 3A). Treatment with AAV-PGIS but not AAV-eGFP significantly inhibited this increase (Figure 3A). In addition, the expression of PGI2 significantly mitigated an increase in PVR and a decrease in CI that were induced by MCT (Figure 3B and 3C, respectively); however, it produced no significant changes in the heart rate and aortic systolic arterial pressure (Table). PGI2 expression also had a beneficial effect on RVH. Treatment with AAV-PGIS but not AAV-eGFP significantly inhibited the MCT-induced increase in RV/(LV+S) (Figure 3D).

Effects on Medial Hypertrophy of the PA

Medial hypertrophy is a hallmark of pathological vascular remodeling in PAH. Four weeks after the MCT injection, the

Figure 1. Expression of PGIS and PGI2 in vitro. A, Western blot analysis of PGIS expression in HEK293 cells after plasmid transfection. The cells were harvested 72 hours after transfection with phPGIS or eGFP. B, AAV vector-mediated PGI2 expression in HEK293 cells. The PGI2 levels were estimated by measuring the amount of 6-keto-PGF1α, a stable metabolite of PGI2, in the culture supernatant by enzyme immunoassay 72 hours after infecting the cells (n=4 each) with AAV-PGIS (1×10⁴ genome copies per cell). Data are presented as mean±SEM. **P<0.01. NC indicates untreated negative control.

Figure 2. AAV vector-mediated systemic expression of PGI2 in vivo. The concentration of plasma 6-keto-PGF1α was determined by enzyme immunoassay after a single injection of AAV-PGIS into the anterior tibial muscle of 3-week-old male Wistar rats. A, Time course of plasma 6-keto-PGF1α levels after injection of AAV-PGIS at 1×10⁵ genome copies per body. B, Vector dose dependency of plasma 6-keto-PGF1α levels 8 weeks after the injection. The rats injected with AAV-eGFP (1×10⁴ genome copies per body) were used as controls. Data are presented as mean±SEM (n=3 animals per group). *P<0.05 vs NC; **P<0.01.

Figure 3. Effects of PGI2 on hemodynamics and RVH. A quantitative analysis was performed using MCT-induced PAH rats 8 weeks after injecting the vector. A, mPAP (mm Hg); B, PVR (mm Hg·mL⁻¹·min⁻¹·kg⁻¹); C, CI (mL·min⁻¹·kg⁻¹). D, Weight ratio of the right ventricle to the left ventricle plus septum [RV/(LV+S)] presented as an index of RVH. Data are presented as means±SEM (n=4 to 10 animals per group). *P<0.05; **P<0.01. NS indicates not statistically significant; NC, untreated negative control.
medial thickness of the PA was greater in the MCT-administered rats than in the untreated controls (Figure 4A). Treatment with AAV-PGIS but not AAV-eGFP prevented the MCT-induced increase in the percentage of medial thickness significantly (Figure 4B, 25 to 50 μm; Figure 4C, 51 to 100 μm in external diameter).

Effects on the Survival of the MCT-PAH Rats and Their Organ Dysfunctions
The PGIS-transduced rats exhibited significantly improved survival rates as compared with the eGFP-transduced rats (Figure 5). The MCT administration produced a slight but not significant decrease in the body weight of the rats, and PGIS gene transfer prevented this decrease. Although the MCT group showed only a slight but not significant increase in the leukocyte count and serum alanine aminotransferase levels as compared with the NC group, the AAV-PGIS treatment caused no additional change in these parameters (Table).

Discussion
The present study demonstrates that sustained PGI₂ expression by a single intramuscular injection of AAV-PGIS prevents the development of MCT-PAH in rats. PGI₂ expression not only increased the cardiac output significantly but also prevented the progression of PVR, RVH, and medial hypertrophy of the PA that was induced by the MCT administration. The PGIS-transduced rats also exhibited significantly improved survival rates as compared with the controls. Furthermore, the PGIS expression observed in this study caused no additional adverse effects on hematologic data and serum indicators of hepatorenal function (alanine aminotransferase and creatinine levels) in the MCT-PAH rats.

The expression of PGI₂ and PGIS decreased in the remodeled PAs of the idiopathic PAH patients.14,15 Impaired PGI₂ synthesis resulting from a decrease in PGIS expression may be implicated in the pathogenesis of PAH. In fact, continuous intravenous infusion of exogenous PGI₂ markedly lowers PVR and improves survival in PAH patients. However, this system requires lifelong infusion with a central venous catheter because of the short biological half-life of PGI₂. Furthermore, because this system is associated with life-threatening complications (eg, shock and sepsis) that may result in poor survival and quality of life of patients, stable...

<table>
<thead>
<tr>
<th>Factor</th>
<th>NC</th>
<th>MCT</th>
<th>MCT + eGFP</th>
<th>MCT + PGIS</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Heart rate, per minute</td>
<td>294.0±10.6</td>
<td>281.2±14.7</td>
<td>268.0±9.0</td>
<td>274.8±8.7</td>
<td>NS</td>
</tr>
<tr>
<td>ASAP, mm Hg</td>
<td>99.5±1.6</td>
<td>97.3±2.0</td>
<td>96.3±2.4</td>
<td>94.7±4.4</td>
<td>NS</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>358.5±11.5</td>
<td>328.3±7.2</td>
<td>328.0±11.4</td>
<td>342.5±9.8</td>
<td>NS</td>
</tr>
<tr>
<td>Leukocyte, per mL</td>
<td>6725±372</td>
<td>7917±732</td>
<td>8800±849</td>
<td>8030±852</td>
<td>NS</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>48.2±0.7</td>
<td>48.9±1.9</td>
<td>51.0±3.0</td>
<td>47.8±1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Platelet, ×10⁴/mm³</td>
<td>88.3±8.7</td>
<td>79.2±8.8</td>
<td>80.4±3.6</td>
<td>84.6±6.3</td>
<td>NS</td>
</tr>
<tr>
<td>ALT, IU/L</td>
<td>37.8±2.5</td>
<td>49.5±8.4</td>
<td>52.5±6.8</td>
<td>44.1±4.3</td>
<td>NS</td>
</tr>
<tr>
<td>Cr, mg/dL</td>
<td>0.52±0.04</td>
<td>0.59±0.05</td>
<td>0.48±0.03</td>
<td>0.53±0.04</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as means±SEM (n=4 to 10 animals per group). ASAP indicates aortic systolic arterial pressure; ALT, serum alanine aminotransferase; Cr, serum creatinine; NS, not statistically significant.

![Figure 4](http://hyper.ahajournals.org/)

Figure 4. Effects of PGI₂ on medial hypertrophy of the peripheral PA. A, Representative cross-sections of the peripheral PA 4 weeks after the MCT administration (hematoxylin and eosin staining, original magnification, ×1000; scale bar=20 μm). B and C, Quantitative analysis of percentage of medial thickness (B, 25 to 50 μm; C, 51 to 100 μm in external diameter). Data are presented as means±SEM (n=4 to 10 animals per group). *P<0.05, **P<0.01. ns indicates not statistically significant; NC, untreated negative control.
production of endogenous PGI2 would be more desirable. Consistent with previous gene therapy studies, our strategy presented high levels of endogenous PGI2 expression. In addition, this strategy caused no systemic hypotension and hyperdynamic heart failure, which are the major adverse effects arising from uncontrolled blood levels during intravenous delivery of exogenous PGI2.3,4,6

In this study, we used an AAV serotype 1 vector because it is effective not only in efficient muscle transduction but also in long-term secretion of therapeutic proteins into the systemic circulation. The cDNA for human PGIS shares a high identity with its rat counterpart.16 In fact, the administration of a plasmid or hemagglutinating virus of the Japanese vector encoding human PGIS successfully ameliorated MCT-PAH. However, the use of these vectors requires repeated administration for achieving sustained gene expression.3-5 In contrast, the AAV vector used in this study achieved PGIS expression with a single intramuscular injection, and this expression was sustained for 1 year.7

Furthermore, gene transfer was believed to be safer when performed via an intramuscular approach as opposed to the intratracheal or intrahepatic approaches.6 Currently, researchers are using adenoviral gene transfer in most clinical trials because of its high efficiency for gene expression. However, the potential toxic effects of adenoviruses, such as strong immunogenicity, are well known. In contrast, the intramuscular administration of AAV vectors is a promising strategy for delivering therapeutic proteins safely and efficiently, and their use has been examined in clinical trials for hemophilia.17

Although PGI2 is known to be a short-acting vasodilator, recent studies have shown its antiremodeling effects when used in high doses. The administration of PGI2 analogues cicaprost and iloprost in high concentrations (>10^-7 mol/L) inhibits mitogen-induced proliferation of rat primary PA smooth muscle cells in a CAMP-dependent manner.18 Interestingly, another PGI2 analogue, treprostinil, also inhibits the proliferation of human and mouse primary lung fibroblasts through the activation of a peroxisome proliferator-activated receptor-β/δ when used in equivalent doses.19 These observations suggest that high levels of PGI2 may attenuate PA remodeling in vivo through antiproliferative effects. Consistent with previous studies, we demonstrated that high levels of endogenous PGI2 successfully attenuated medial hypertrophy of the PA.3,4,6 To discover new drug targets, the roles of peroxisome proliferator-activated receptors and high-level PGI2 in PAH therapy should be determined, because peroxisome proliferator-activated receptors are associated with many inflammatory and proliferative disorders, including PAH.2,20

Finally, we will discuss the clinical implications and limitations of this study. Consistent with previous studies, maximum gene expression was noted 6 to 8 weeks after the intramuscular injection of AAV vectors. AAV-PGIS was injected 4 weeks before MCT administration for the transgene expression to reach plateau levels when MCT-PAH was fully developed (3 to 4 weeks after the injection). Our results are completely based on a preventive protocol, which may be rare in a clinical setting. However, PGI2 is an established therapeutic molecule, and the advantage of early initiation of PGI2 therapy for improving survival in patients with idiopathic PAH has been demonstrated in a large clinical trial.21 These observations convinced us to propose the possible preemptive use of AAV-PGIS as a strategy to maintain basal levels of PGI2 in patients with mild symptoms of PAH or in those identified as high-risk subjects who have not experienced PAH. As an alternative, the combined use of AAV-PGIS and an initial infusion of intravenous PGI2 might be promising; the intravenous infusion should be tapered when sufficient levels of PGI2 are attained. To evaluate the efficacy of AAV-PGIS in a therapeutic protocol (ie, vector injection after the development of PAH), use of a chronic hypoxic PAH model or newly developed self-complementary AAV vectors that can express transgenes earlier than the conventional vectors should be considered.22

Perspectives
The present study has demonstrated that the single intramuscular injection of AAV-PGIS achieved a sustained expression of PGI2. This expression retarded the progression of MCT-PAH in rats without causing significant adverse effects. Thus, this strategy provides a new therapeutic alternative for PAH patients. However, the system in this study lacks the ability to regulate excessive transgene expression. Therefore, regulatory mechanisms to ensure adequate gene expression should be established to facilitate successful translation of this strategy in a clinical setting.

Acknowledgment
We thank Miyoko Mitsu for her encouragement and technical support.

Sources of Funding
This work was supported in part by grants from the Ministry of Health, Labor and Welfare of Japan; Grants-in-Aid for Scientific Research; grant for the 21st Century Centers of Excellence Program; “High-Tech Research Center” Project for Private Universities, matching fund subsidy, from the Ministry of Education, Culture,
Sports, Science and Technology of Japan; and the Research Award to Jichi Medical School Graduate Student.

Disclosures

None.

References


Adenoassociated Virus–Mediated Prostacyclin Synthase Expression Prevents Pulmonary Arterial Hypertension in Rats

Takayuki Ito, Takashi Okada, Jun Mimuro, Hiroshi Miyashita, Ryosuke Uchibori, Masashi Urabe, Hiroaki Mizukami, Akihiro Kume, Masafumi Takahashi, Uichi Ikeda, Yoichi Sakata, Kazuyuki Shimada and Keiya Ozawa

_Hypertension_. 2007;50:531-536; originally published online July 16, 2007;
doi: 10.1161/HYPERTENSIONAHA.107.091348
_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/50/3/531