Interleukin-10 Expression Mediated by an Adeno-Associated Virus Vector Prevents Monocrotaline-Induced Pulmonary Arterial Hypertension in Rats

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Abstract—Pulmonary arterial hypertension (PAH) is a fatal disease associated with inflammation and pathological remodeling of the pulmonary artery (PA). Interleukin (IL)-10 is a pleiotropic antiinflammatory cytokine with vasculoprotective properties. We report the preventive effects of IL-10 on monocrotaline-induced PAH. Three-week-old Wistar rats were intramuscularly injected with an adeno-associated virus serotype 1 vector expressing IL-10, followed by monocrotaline injection at 7 weeks old. IL-10 transduction significantly improved survival rates of the PAH rats 8 weeks after monocrotaline administration compared with control gene transduction (75% versus 0%, P<0.01). IL-10 also significantly reduced mean PA pressure (22.8±1.5 versus 29.7±2.8 mm Hg, P<0.05), a weight ratio of right ventricle to left ventricle plus septum (0.35±0.04 versus 0.42±0.05, P<0.05), and percent medial thickness of the PA (12.9±0.3% versus 21.4±0.4%, P<0.01) compared with controls. IL-10 significantly reduced macrophage infiltration and vascular cell proliferation in the remodeled PA in vivo. It also significantly decreased the lung levels of transforming growth factor-β1 and IL-6, which are indicative of PA remodeling. In addition, IL-10 increased the lung level of heme oxygenase-1, which strongly prevents PA remodeling. In vitro analysis revealed that IL-10 significantly inhibited excessive proliferation of cultured human PA smooth muscle cells treated with transforming growth factor-β, or the heme oxygenase inhibitor tin protoporphyrin IX. Thus, IL-10 prevented the development of monocrotaline-induced PAH, and these results provide new insights into the molecular mechanisms of human PAH. (Circ Res. 2007;101:734-741.)

Key Words: pulmonary hypertension □ interleukins □ gene therapy □ inflammation □ vascular smooth muscle cell proliferation

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 for PAH has been established. The pathological process of PAH is characterized by abnormal remodeling of the pulmonary artery (PA) associated with excessive proliferation of pulmonary arterial smooth muscle cells (PASMCs). Accumulating evidence suggests important roles of vascular inflammation in its pathogenesis. For instance, serum levels of proinflammatory cytokines such as interleukin (IL)-1 and IL-6 reflect the disease activity in patients with idiopathic PAH. Furthermore, injection of IL-6 can produce PAH and PA remodeling in rats. The remodeled PA presents macrophage infiltration and increased expression of a variety of cytokines, including IL-6, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β,

Administration of steroids or immunosuppressive drugs decreases the level of PA pressure in patients with PAH. These observations suggest a therapeutic potential of targeting inflammation to prevent PAH progression. However, the precise mechanisms underlying the antiinflammatory effects on PA remodeling have not yet been fully investigated.

IL-10 is a multifunctional antiinflammatory cytokine with a vasculoprotective property. During the course of inflammation, IL-10 is produced by type-2 helper T (Th2) lymphocytes, and inhibits the production of various proinflammatory cytokines in macrophages and Th1 lymphocytes. Exogenous IL-10 prevents proliferative vasculopathy in vivo by inhibiting inflammatory cell infiltration, smooth muscle cell proliferation, and chemokine expression. However, clinical efficacy of systemic recombinant IL-10 administra-
tion are insufficient because of the lower local IL-10 levels resulting from its short bioactive half-life. In this study, we used an adeno-associated virus (AAV) vector for IL-10 expression because it is an efficient vehicle for systemic and sustained expression of therapeutic proteins. It also has an advantage over other viral vectors in the therapeutic or mechanistic analysis because it produces minimal inflammatory and immune responses in vivo.

Recently, heme oxygenase (HO)-1, an inducible form of HO that promotes production of a vasodilator carbon monoxide (CO), was shown to mediate antiinflammatory and antiproliferative effects of IL-10 in a model of chronic vasculopathy. Increased HO-1 and CO levels attenuated PAH and PA remodeling by inhibiting PASMC proliferation. However, no study has explored a direct link between IL-10 and HO-1 in the pathogenesis of PAH. Thus, we examined the effects of IL-10, delivered via an AAV vector, on PA remodeling in a widely-used rat model of PAH induced by the pyrrolizidine alkaloid monocrotaline (MCT).

We also investigated the mechanisms underlying the effects of IL-10 on the following factors involved in the inflammatory and proliferative vascular changes in PAH: PASMC, macrophage, TGF-β, IL-6, and HO-1.

Materials and Methods

AAV Vector Production

DNA encoding rat IL-10 was polymerase chain reaction-amplified from rat splenocyte complementary DNA, using the primers 5′-GCAGGAACACACACACCA-3′ and 5′-GATTGATACGATTTTATCAAAAAAGAT-3′. For efficient transgene expression in the skeletal muscle, we constructed a recombinant AAV vector which carried the IL-10 gene (AAV-IL-10) or enhanced green fluorescent protein (eGFP) gene (AAV-eGFP), controlled by the modified chicken β-actin promoter with the cytomegalovirus-immediate early enhancer and the woodchuck hepatitis virus post-transcriptional regulatory element (a kind gift from Dr Thomas Hope, Infectious Disease Laboratory, Salk Institute). AAV vectors were prepared according to the previously described 3-plasmid method.

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Protein Expression

PASMCs were prepared as previously described. The cells were extracted in lysis buffer [10 mM Tris (pH 7.4), 0.15 mol/L NaCl] before injection.

Animal Models

All animal experiments were approved by the Jichi Medical University ethics committee and were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. To evaluate the efficiency of in vivo gene expression, 3-week-old male Wistar rats (Clea Japan Inc, Tokyo, Japan) weighing 45 to 55 g were injected with AAV-IL-10 (200 µL, 3×10^{10} genome copies [g.c.] per body) into the bilateral anterior tibial muscles (n=3 animals per group). For hemodynamic and histological analysis, we randomly formed 4 groups comprising 5 rats each: sham rats that were administered the HN buffer (1, NC group); MCT-treated rats administered the HN buffer (2, MCT group); MCT rats administered AAV-eGFP (3, MCT-eGFP group); and MCT rats administered AAV-IL-10 (4, MCT+IL-10 group). After anesthesia with a spontaneous inhalation of 1% isoflurane, the rats in the groups 3 and 4 received intramuscular injection of AAV-eGFP or AAV-IL-10 (200 µL, 6×10^{10} g.c. per body), respectively. Rats in groups 1 and 2 were injected with the HN buffer (200 µL). MCT (Wako Pure Chemicals) was dissolved in 0.1N HCl, and the pH adjusted to 7.4 with 1.0N NaOH. For hemodynamic and histological studies, all rats except those in the NC group were subcutaneously injected with MCT (30 mg/kg) under the spontaneous inhalation of 1% isoflurane at 4 weeks after vector treatment. For the survival study, rats (n=8 animals/group) were injected with a lethal dose of MCT (45 mg/kg) under the spontaneous inhalation of 1% isoflurane at 4 weeks after vector injection. Survival was estimated from the date of MCT injection until death or 8 weeks after injection.

Hemodynamic Analysis

Four weeks after MCT injection, the rats were anesthetized with spontaneous inhalation of 1% isoflurane, and a tracheotomy was performed. Then, they were mechanically ventilated using a respirator (SAR-830/AP, CWE; tidal volume: 10 mL/kg; respiratory rate: 30 breaths per min) and anesthetized with 0.5% isoflurane through a tracheotomy. After the thoracic cavity was opened using a midsternal approach, 2.0F high-fidelity manometer-tipped catheters (SPC-320, Millar Instruments Inc) were inserted directly into the right or left ventricle. The mean pulmonary arterial pressure (mPAP) or mean aortic arterial pressure (mAoP) was measured using the catheters that were advanced from the right or left ventricle, respectively. The heart rate (HR) was measured by unipolar lead electrocardiography.

Ventricular Weight Measurement and Morphometric Analysis of the PA

After hemodynamic analysis, the rats were euthanized using an overdose isoflurane (5%). The lungs and PAs were perfused with 5 mL of saline followed by 10 mL of cold 4% paraformaldehyde. Each ventricle and the lungs were excised, dissected free, and weighed. The weight ratio of right ventricle to the left ventricle plus septum [RV/(LV+S)] was calculated as an index of right ventricular hypertrophy (RVH). The tissues were fixed in 4% paraformaldehyde for 4 hours, transferred to 30% sucrose in 0.1 mol/L phosphate buffer (pH 7.4) for cryoprotection, and stored at 4°C overnight. Lung tissue was frozen in Tissue-Tek OCT compound (Sakura Finetechnical Co) at −20°C. Then, 7-μm sections were cut using a cryostat. Hema-toxylin and eosin (HE) staining was performed on sections from the middle lobe of the right lung, and these were examined using light microscopy. Morphometric analysis was performed in PAs with an external diameter of 25 to 50 and 51 to 100 µm. The medial wall thickness was calculated with the following formula: medial thickness (%)=[medial wall thickness/external diameter]×100. For quantitative analysis, 30 vessels from each rat were counted and the average was calculated.

Immunohistochemistry

Immunohistochemical staining was performed with monoclonal antibodies against ED1 (1:100; Serotec) and proliferating cell nuclear antigen (PCNA, 1:200; Zymed), using the streptavidin-biotin-peroxidase method, as described previously. ED1 recognizes the lysosomal membrane antigen expressed by a majority of tissue macrophages. Irrelevant mouse immunoglobulin G (Vector Laboratories) was used as a negative control. Reactions were visualized using Vector SG (Vector Laboratories) or 3,3′-diaminobenzidine (Zymed) and counterstained with nuclear fast red or hematoxylin. The number of ED1-positive cells was counted in 250×250-μm fields under 400× magnification and expressed as cells per mm². The number of PCNA-positive cells was quantitatively evaluated as a percentage of total vascular cells in the fields under 1000× magnification. For each rat, the average number or percentage of each cell in 15 randomly selected fields was used for statistical analysis.

Protein Assay

Protein samples were prepared by homogenization of the frozen lung tissue in lysis buffer [10 µmol/L Tris/Cl (pH 8.0), 0.2% NP-40,
1 μmol/L EDTA (pH 7.6) supplemented with protease inhibitor cocktail Complete Mini (Roche Diagnostics). After centrifugation of the homogenates (3000g for 10 minutes), the supernatants or serum samples were used for measurement. To activate latent TGF-β, to an immunoactive form, the samples were treated with acid according to the manufacturer’s instructions (R&D Systems Inc.). IL-10 or IL-6 concentrations in the sera and TGF-β, IL-6, HO-1, or TNF-α in the lung extracts were measured using enzyme-linked immunosorbent assay (ELISA) kits (Amersham Pharmacia Biotech; R&D Systems). The minimum detectable dose was 3, 3, 16, and 5 pg/mL or 0.78 ng/mL for IL-10, TGF-β, IL-6, and TNF-α, or HO-1, respectively. Inter- and intraassay precision of these kits was <10%. The total protein concentrations in the lung extracts were estimated using a BCA Protein Assay kit (PIERCE). The levels of TGF-β, IL-6, HO-1, or TNF-α in the lung were expressed as pg per mg protein.

Cell Culture and Proliferation Assay

Human PASMCs were obtained from Clonetics Corp and grown in SmGm-2 medium (Clonetics Corp). PASMCs with a passage between 4 and 6 were used in the experiments. Cells (1×10^4 per well) were incubated in 96-well plates with serum-free Dulbecco’s modified Eagle’s medium and nutrient mixture F12 (DMEM-F12, In-vitrogen) in an atmosphere of 5% CO2 in the air at 37°C. A tetrazolium-based colorimetric proliferation assay (XTT assay; Cell Proliferation Kit II, Roche Diagnostics) was performed 2 days after adding tin protoporphyrin IX (SnPP; Frontier Scientific), human recombinant TGF-β, IL-6, or IL-10 (PeproTech Inc). The optical density between 450 and 650 nm were measured to estimate the number of viable cells.

Statistical Analysis

Data from multiple experiments are expressed as mean±SEM. Statistical analysis and correlations were performed using StatView (Abacus Concepts, Inc). Survival curves were analyzed using the Kaplan–Meier method and compared by log-rank test. Differences in other parameters were evaluated by analysis of variance combined with Fisher test. The correlation test was used to measure the association between 2 variables. A value of P<0.05 was considered statistically significant.

Results

AAV Vector-Mediated IL-10 Expression Improves Survival of MCT-PAH Rats

Eight weeks after AAV-IL-10 injection, serum IL-10 concentrations were elevated in a vector dose-dependent manner (Figure 1A). We determined that injection with AAV-IL-10 ((6×10^10 g.c. per rat) significantly increased serum IL-10 levels as compared with untreated controls (184.1±47.6 versus 18.8±1.3 pg/mL, P<0.05, n=3 each). In contrast, injection with MCT (Figure 1A) or AAV-eGFP alone (data not shown) caused no significant change in serum IL-10 levels. Therefore, we used this dosage for all vectors in subsequent experiments. For survival analysis, the rats were injected with a lethal dose of MCT, after 4 weeks of vector injection. The survival in IL-10–transduced rats was significantly improved as compared with the eGFP-transduced rats 8 weeks after MCT injection (75% versus 0%, P<0.01, n=8 each; Figure 1B).

Effects of IL-10 on PAH and RVH

Four weeks after MCT injection, the mPAP levels were significantly higher than those of the untreated controls (30.1±4.0 versus 20.0±2.1 mm Hg, P<0.01, n=5 each; Figure 2A). Treatment with AAV-IL-10 but not AAV-eGFP significantly inhibited the elevation of mPAP (22.8±1.5 versus 29.7±2.8 mm Hg, P<0.01, n=5 each; Figure 2A). Moreover, serum IL-10 concentrations correlated negatively with mPAP in MCT-treated rats (r=−0.75, P<0.01, n=15; Figure 2B). In contrast, this IL-10 expression caused no significant change in HR (data not shown) and mAoP (76.7±2.1 versus 74.6±6.8 mm Hg, MCT+IL-10 versus MCT+eGFP group, n=5 each). IL-10 expression also has a beneficial effect on RVH. Four-week MCT treatment significantly increased the RV/(LV+S) values as compared with the untreated controls (P<0.01, n=5 each; Figure 2C). Treatment with AAV-IL-10 but not AAV-eGFP inhibited MCT-induced increase of RV/(LV+S) significantly (P<0.05, n=5 each; Figure 2C). Furthermore, serum IL-10 concentrations correlated negatively with RV/(LV+S) in MCT-treated rats (r=−0.57, P<0.05, n=15; Figure 2D). These results indicate that sustained IL-10 expression prevented the development of MCT-induced PAH and RVH.

Effects of IL-10 on Histological Changes of the PA

Medial hypertrophy is a hallmark of pathological vascular remodeling in PAH. Four weeks after MCT injection, the medial thickness of PAs was markedly increased in the MCT-treated rats compared with untreated controls (P<0.01,
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Levels as compared with untreated controls (eGFP or MCT alone significantly increased the lung HO-1 expression caused no change in the lung TNF-α levels (Figure 4H).

Effects of IL-10 on PASMC Proliferation

To determine whether IL-10 directly inhibits PASMC proliferation, we performed an in vitro colorimetric XTT assay using cultured human PASMCs. Treatment of PASMCs with SnPP, which inactivates HO-1, and treatment with TGF-β1 or IL-6 dose dependently promoted cell proliferation (n=4 each, P<0.05; Figure 5A through 5C). Treatment with IL-10 alone had no significant effect on PASMC proliferation (Figure 5D). On the other hand, pretreatment with IL-10 significantly inhibited PASMC proliferation induced by SnPP or TGF-β1 (n=4 each, P<0.05; Figure 5E) but not that induced by IL-6.

Discussion

The present study demonstrates that IL-10, delivered by an intramuscular injection of an AAV1 vector, prevented the development of MCT-PAH in rats. Systemic IL-10 expression also improved survival in rats and prevented the development of RVH and medial hypertrophy of PA. IL-10 also reduced macrophage accumulation, vascular cell proliferation, and pulmonary tissue levels of TGF-β1 and IL-6, all of which play pivotal roles in progression of PA remodeling. Further, IL-10 enhanced HO-1 levels in the lung and TCPA rats compared with untreated controls, IL-10 expression caused no change in the lung TNF-α levels (Figure 4H).
ported. Evidence of right heart failure is involved in the mortality of MCT-PAH rats. In this study, all rats treated with a lethal dose of MCT exhibited symptoms of right heart failure such as pleural effusion and body weight decrease. In the setting of severe PAH and right heart failure, cytokine networks may orchestrate disease progression. Thus, blockades of multiple inflammatory signals might be responsible for the prosurvival effect of IL-10.

IL-10 has gained significant attention because of its suppressive influence on inflammatory and proliferative vasculopathy. The IL-10 receptor is expressed on vascular smooth muscle cells (VSMCs). IL-10 inhibits inflammation and VSMC proliferation in arterial remodeling after balloon injury or transplant rejection.\textsuperscript{12,13} Consistent with previous studies using MCT-PAH,\textsuperscript{6,7} we demonstrate that increased levels of TGF-\(\beta_1\) and IL-6 are related to PASMC proliferation and PA remodeling progression. Although treatment with IL-10 alone caused no significant effects on PASMC proliferation,\textsuperscript{27} IL-10 significantly inhibited the lung TGF-\(\beta_1\) expression and TGF-\(\beta_1\)-induced PASMC proliferation. TGF-\(\beta_1\) enhances PASMC proliferation of idiopathic PAH patients but not that of normal subjects or secondary PAH patients.\textsuperscript{28}

Figure 3. Antiinflammatory and antiproliferative effects of IL-10 on the remodeled pulmonary artery (PA). The 7-week-old Wistar rats were treated with MCT 4 weeks after vector injection. Representative cross-sectional views of the peripheral PAs stained with HE or immunohistochemistry (ED1 or PCNA) 4 weeks after MCT treatment (A; original magnification ×1000). Scale bar =20 \(\mu\)m. Blue arrows indicate ED-1–positive cells and red arrows, PCNA-positive cells. Quantification of percent medial thickness for vessels 25 to 50 \(\mu\)m (B) and 51 to 100 \(\mu\)m (C) in external diameter. Quantitative analysis of the number of perivascular macrophages (ED-1–positive cells, D) and proliferating vascular cells (PCNA-positive cells, E). Data represent mean±SEM (\(n=5\) animals per group, **\(P<0.01\)). ns indicates not statistically significant.
Additionally, TGF-β1 is accumulated in the hypertrophic PA of both human PAH and MCT-PAH and exacerbates PA remodeling. IL-6, a multifunctional proinflammatory cytokine, acts as a strong mitogen to promote VSMC proliferation. Macrophage infiltration is a hallmark of PAH progression, and activated macrophages produce substantial amounts of IL-6 in MCT-PAH rats. In this study, IL-10 treatment inhibited perivascular macrophage infiltration and the lung IL-6 expression in vivo but not IL-6-induced PASMC proliferation in vitro. These results suggest that IL-10 may attenuate IL-6 function indirectly through the decreased accumulation of perivascular macrophages and IL-6. Furthermore, the serum IL-6 levels significantly correlated with the lung IL-6 levels. Because serum IL-6 level reflects the disease activity of idiopathic PAH, it can be a useful biomarker of antiinflammation therapy of PAH. On the other hand, IL-10 did not affect the MCT-induced TNF-α expression in the lung. However, previous studies demonstrated that IL-10 prevents TNF-α-induced VSMC proliferation in vitro. These observations suggest that IL-10 might modulate the downstream signal of TNF-α but not its expression in the setting of MCT-PAH. Overall, IL-10 affects the dynamics of cytokine networks involved in PA remodeling, and its site of action may differ according to the cytokine signal.

Figure 4. Effects of IL-10 on expression of transforming growth factor-β1 (TGF-β1), IL-6, heme oxygenase-1 (HO-1), and tumor necrosis factor-α (TNF-α) in the lung. The 7-week-old Wistar rats were treated with MCT 4 weeks after vector injection. Concentrations of active TGF-β1 (A), IL-6 (C), HO-1 (E), and TNF-α (H) in the lung extracts were detected using ELISA 4 weeks after MCT treatment. Data represent mean±SEM (n=5 animals per group; *P<0.05, **P<0.01). ns indicates not statistically significant. Correlation between the percent medial thickness and lung levels of TGF-β1 (B) or IL-6 (D) in rats (groups: NC, MCT, MCT+eGFP, or MCT+IL-10; n=5 animals per group; r=0.84, P<0.01 and r=0.87, P<0.01, respectively). Correlation between the HO-1 and IL-6 (F) levels in the rat lung (groups: MCT, MCT+eGFP, or MCT+IL-10; n=5 animals per group; r=-0.85, P<0.01). Correlation between the lung and serum IL-6 levels (G) in rats (groups: NC, MCT, MCT+eGFP, or MCT+IL-10; n=5 animals per group; r=0.69, P<0.01).
CO induced by HO-1 blocks PASMC proliferation not only directly by inhibiting the expression of a cell cycle–specific transcription factor but also indirectly by attenuating mitogen signaling.16 Interestingly, the transgenic mice that constitutively express HO-1 are protected from the development of hypoxia-induced PAH and excessive expression of a mitogen IL-6.33 In this study, AAV-IL-10 administration increased the HO-1 level that negatively correlated with the IL-6 level in the lung of MCT-PAH rats. These observations suggest a dynamic relationship between IL-6 and HO-1 in PA remodeling progression. Chen et al12 reported that AAV-IL-10 injection enhanced the activity and protein levels of HO-1, but SnPP treatment that inactivates HO-1 reversed the vasculoprotective effects of IL-10 in vivo. Here, we show that pretreatment with recombinant IL-10 suppressed the excessive PASMC proliferation induced by HO-1 inactivation with SnPP. Thus, IL-10 may sustain CO levels by maintaining HO-1 from inactivating, leading to the prevention of PA remodeling.

Finally, we will discuss the clinical implication 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. In this study, AAV-IL-10 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). Thus, our results are completely based on a prevention protocol, which may be rare in a clinical setting. Intramuscular AAV-IL-10 injection is an attractive candidate for antiinflammation therapy of PAH because inflammatory cytokine expression is associated with the clinical course of the disease. In addition, this strategy exhibited no life-threatening complications such as shock and sepsis which may occur in intravenous prostacyclin infusion therapy. However, therapeutic effects of IL-10 in established PAH has not been determined. Therefore, it should be further examined in studies using a treatment protocol. MCT-PAH is a widely-used and suitable model for exploring inflammatory mechanisms in PAH progression. However, how IL-10 affects other pathogenesis in PAH remains unknown. In the future, IL-10 function needs to be examined in other PAH models such as hypoxia-induced PAH.

In conclusion, AAV vector-mediated sustained IL-10 expression prevented the development of MCT-PAH in rats. The antiremodeling effects of IL-10 are related to the reduction of macrophage infiltration and pathological cytokine expression as well as increased HO-1 levels in the lung. Although the therapeutic role of IL-10 should be further investigated, our results provide new insights into molecular mechanisms underlying the development of human PAH.
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Disclosures

None.

References

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