Prednisolone Inhibits Proliferation of Cultured Pulmonary Artery Smooth Muscle Cells of Patients With Idiopathic Pulmonary Arterial Hypertension

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Background—Idiopathic pulmonary arterial hypertension (IPAH) is associated with proliferation of smooth muscle cells (SMCs) in small pulmonary arteries. There is no therapy that specifically inhibits SMC proliferation. Recent studies reported that prednisolone (PSL) inhibits the postangioplasty proliferation of SMCs in atherosclerotic arteries. In this study, we tested the hypothesis that PSL has antiproliferative effects on pulmonary artery SMCs of patients with IPAH.

Methods and Results—Pulmonary artery SMCs were harvested from the pulmonary arteries of 6 patients with IPAH who underwent lung transplantation. Control SMCs were obtained from 5 patients with bronchogenic carcinoma who underwent lung lobectomy. After incubation in the presence of platelet-derived growth factor (PDGF), PSL was added at different concentrations and cell proliferation was assessed by 3H-thymidine incorporation. PSL (2×10⁻¹² and 2×10⁻¹⁰ mol/L) significantly inhibited PDGF-stimulated proliferation (P<0.05) of SMCs from patients with IPAH but did not affect cell viability of SMCs, as confirmed by trypan blue staining. In cell cycle analysis using a microscope-based multiparameter laser scanning cytometer, PSL inhibited the progression of SMCs from G0/G1 to the S phase. This inhibition was associated with increased p27 expression level. PSL (2×10⁻¹² mol/L) also inhibited PDGF-induced SMC migration.

Conclusions—Our results indicate that PSL has an antiproliferative effect on cultured SMCs of pulmonary arteries from patients with IPAH and suggest that PSL may be potentially useful therapeutically in patients with IPAH. (Circulation. 2005;112:1806-1812.)

Key Words: prednisolone m myocytes m hypertension, pulmonary m muscle, smooth

Idiopathic pulmonary arterial hypertension (IPAH) is a disease of unknown cause associated clinically with dyspnea on effort, edema, and syncope. It is characterized by progressive elevation of pulmonary artery pressure and pulmonary vascular resistance. The pathology of IPAH includes increased thickness of the tunica media (increased number of smooth muscle cells [SMCs] in the arterial vasculature and muscularization of nonmuscularized arterioles). Calcium channel blockers and epoprostenol are used as potent dilators of pulmonary arteries. Although patients who respond to calcium channel blockers have favorable prognosis, they comprise only 10% to 15% of all patients with IPAH. There is no alternative therapeutic option to the majority of nonresponders to calcium channel blockers to reduce pulmonary artery pressure. Recent studies have indicated that continuous infusion therapy of epoprostenol improves hemodynamics, quality of life, and survival of patients with IPAH and has become a standard therapy. However, this therapy is expensive, and there are some serious complications associated with epoprostenol itself and the indwelling venous catheter used for the administration of epoprostenol. Thus, nonresponders to calcium channel blockers need effective and long-acting oral agents. There are some therapeutic options that are currently approved or under clinical investigation in the United States and in Europe, such as iloprost, treprostinil, endothelin receptor antagonists, and phosphodiesterase inhibitors. Because these agents are thought to work mainly as pulmonary artery dilators, there is a need for treatment that significantly inhibits SMC proliferation.

Coronary artery angioplasty is widely performed at present in patients with ischemic heart disease. However, restenosis after the procedure is the most difficult complication, which is predominantly caused by stimulation of SMC proliferation. Traumatic injury of the vessel wall during the proce-
dure may lead to SMC proliferation. Recent studies reported that prednisolone (PSL) exhibits an antiproliferative effect on SMCs of atherosclerotic arteries. This inspired us to consider the application of PSL to inhibit SMC proliferation in patients with IPAH. In the present study, we tested the hypothesis that PSL has an antiproliferative effect on pulmonary artery SMCs in patients with IPAH.

Methods

Isolation of Pulmonary Artery SMCs

Human lung and segments of pulmonary artery were obtained at lung transplantation from 6 patients with IPAH (1 man and 5 women; mean age, 24.5 ± 4.7 years, ± SEM). For control experiments, pulmonary artery samples were taken from 5 patients with bronchogenic carcinoma (4 men and 1 woman; mean age, 59.0 ± 4.1 years), who had no evidence of pulmonary hypertension at the time of lung lobectomy. Samples of the pulmonary arteries were obtained from the most distal area from the carcinoma in the resected lobe. Patients with bronchogenic carcinoma received no systemic chemotherapy or radiation therapy before lung lobectomy. All cell culture experiments were carried out after the approval of the Human Ethics Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, and written informed consent was obtained from all patients before the procedure.

We isolated and cultured SMCs by using the methods described previously, with minor modifications. Pulmonary artery SMCs were isolated from the peripheral segments of the artery smaller than 1 mm in outer diameter. For the enzymatic disaggregation of SMCs, a mixture of 10 mL of phosphate-buffered saline (PBS) and 100 μg/mL collagenase was used in a shaking water bath for 15 minutes at 37°C. The adventitia and endothelial cell layers were removed. The vessels were cut into 2-mm-long sections and plated in 6-well culture plates containing Dulbecco modified Eagle’s medium (DMEM; Nissui) supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL kanamycin. They were incubated in a humidified 5% CO₂ atmosphere at 37°C. The medium was changed every 3 days. After reaching confluence, the cells were subcultured by a trypsin (0.05%/ethylenediaminetetraacetic acid (EDTA) (0.02%)) treatment.

Chemicals

Human recombinant platelet-derived growth factor (PDGF-BB) was purchased from Sigma. It was diluted in 4 mmol/L HCl containing 0.1% bovine serum albumin (Sigma), and the optimal final concentration of PDGF-BB was 10 ng/mL. PSL (Sigma) was dissolved in a mixture of 10 mL of phosphate-buffered saline (PBS) and 100 μg/mL collagenase was used in a shaking water bath for 15 minutes at 37°C. The adventitia and endothelial cell layers were removed. The vessels were cut into 2-mm-long sections and plated in 6-well culture plates containing Dulbecco modified Eagle’s medium (DMEM; Nissui) supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL kanamycin. They were incubated in a humidified 5% CO₂ atmosphere at 37°C. The medium was changed every 3 days. After reaching confluence, the cells were subcultured by a trypsin (0.05%/ethylenediaminetetraacetic acid (EDTA) (0.02%)) treatment.

Immunohistochemistry

For cell identification and examination of cytoskeletal components, cells of first subcultures were seeded on glass coverslips coated with pig collagen (type I collagen) (Nitta Gelatin Inc.). After the cells were incubated in culture media (DMEM, 10% FBS, and 0.1 mg/mL kanamycin) and completely spread out, they were fixed with 4% paraformaldehyde. After permeabilization in methanol, the cells were incubated with anti-α-smooth muscle actin (α-SMA) mouse monoclonal antibody (Sigma) at a dilution of 1:400 with antibody diluent as the secondary antibody. Staining with anti-myosin antibody (Sigma: 1:500) and anti-smoothelin antibody (Chemicon International, 1:200) was performed along with the same procedure.

Effects of PSL and Immunosuppressants on DNA Synthesis

To assess the SMC antiproliferative effect of PSL, measured by ³H-thymidine incorporation, the cultured cells were first made quiescent in low-serum media. The addition of PDGF-BB to quiescent SMC cultures results in a significant increase in ³H-thymidine uptake. On day 0 of the proliferation assay, SMCs were grown until approximately 80% confluence. Then, the cells were detached with trypsin (0.05%/EDTA (0.02%)) and seeded in 24-well plates at a density of 1 to 10×10⁶ cells/mL. After 16 hours of incubation (day 1), the culture media were replaced with low-serum culture media (DMEM, 0.1% FBS, and 0.1 mg/mL kanamycin), and the cells were cultured for another 48 hours. On day 3, PSL and PDGF-BB (10 ng/mL) were added to the cultures. In preliminary experiments, the antiproliferative effects of PSL on PDGF-BB-stimulated SMCs were examined at final concentrations of 2×10⁻⁸ mol/L to 2×10⁻⁹ mol/L. The basis of the results of these experiments, we set the final concentrations to 2×10⁻⁸ mol/L to 2×10⁻⁹ mol/L in the remaining experiments shown in Figure 3. On day 4, 21 hours after the addition of PSL, the cells were labeled with ³H-thymidine at 1 μCi/mL for 3 hours. After labeling was completed, the cells were washed in situ twice each with 500 μL of ice-cold PBS, 5% trichloroacetic acid, and 95% ethanol, and then lysed with 200 μL of 0.33 M NaOH. Aliquots of the cell lysates were neutralized with 1N HCl, and the radioactivity was measured in a liquid scintillation counter.

We also assessed the antiproliferative effect of immunosuppressants including cyclosporine A (Sigma), tacrolimus (Fujisawa Pharmaceutical Co, Ltd), azathioprine (Sigma), and rapamycin (Sigma), ranging from 10⁻⁶ to 10⁻⁴ mol/L. The same method applied to assess antiproliferative effect of PSL was used.

Assay for Cell Viability

Cell viability was examined by the trypan blue assay. SMCs were exposed to DMEM, PDGF-BB, and 2×10⁻⁴ mol/L of PSL for 24 hours and then stained with trypan blue. Viable cells with an intact membrane excluded trypan blue and were not stained.

Cell Cycle Analysis

Cultured SMCs were grown in 2-well chamber slides (Laboratory-Tek II; Nalgen Nunc International) for 24 hours. In one well, SMCs were treated with 2×10⁻⁴ mol/L of PSL, whereas no PSL was added to the other well. To analyze the cell cycle profile, nuclear DNA was stained with propidium iodide (PI, 50 μg/mL) and completely spread out, they were fixed with 4% paraformaldehyde. After permeabilization in methanol, the cells were incubated with a trypsin (0.05%/ethylenediaminetetraacetic acid (EDTA) (0.02%)) treatment.

Migration Assay

Assessment of SMC migration was conducted as described previously. Briefly, migration assays were performed with the use of the Falcon cell culture insert system (Becton Dickinson) cell culture chambers with polycarbonate (pore size, 8 μm) membrane. PDGF-BB (10% solution) was dissolved in DMEM and placed in the lower compartment in the presence or absence of 2×10⁻⁴ mol/L of PSL. Cultured SMCs (1×10⁵ cells/mL) were suspended in DMEM supplemented with 3% FBS and 0.1 mg/mL kanamycin and then loaded in the upper compartment and incubated for 16 hours in a humidified 5% CO₂ atmosphere at 37°C. Cells were fixed in methanol and stained with 4% Giemsa.

Western Blot Analysis

SMCs of IPAH were prepared in the same manner as described for analysis of DNA synthesis. They were treated in the presence or absence of 2×10⁻⁴ mol/L of PSL. Western blot analysis was performed as described previously. Briefly, total cell lysates of cultured SMCs were extracted in commonly used radioimmunoprecipitation buffer with 10 μg/mL phenylmethylsulfonyl fluoride (Sigma) and then concentrated by centrifugation at 12 000 rpm for 20 minutes. Protein samples (7 μg) were loaded on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and blotted onto nitrocellulose membranes. Blots were incubated with rabbit anti-p21 (1:200, Santa Cruz Biotechnology) or p27 (Santa Cruz Biotechnology; 1:200) and then reacted with donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences). Blots were developed by using the ECL Western blotting detection system.
system (Amersham Biosciences). Anti-GAPDH antibody (Chemicon; 1:200) was used to normalize for loading and transfer artifacts.

**Statistical Analysis**

Results are expressed as mean±SEM. Differences between groups were examined for statistical significance using one-way analysis of variance. Differences between control cells and drug-treated cells were examined for statistical significance by using Fisher’s protected least significant difference test. A probability value less than 0.05 denoted the presence of a statistically significant difference.

**Results**

**Characteristics of Patients With IPAH**

The diagnosis of IPAH was established according to standard diagnostic criteria.13 The clinical and demographic data of the 6 patients with IPAH are shown in the Table. Data were obtained just before the lung specimen was obtained. The range of systolic/diastolic pulmonary artery pressure was 70 to 113/30 to 59 mm Hg. Histopathologic examination of the lung tissue of patients with IPAH at transplantation showed characteristic pulmonary vascular remodeling. Marked medial hypertrophy and intimal thickening were evident in pulmonary arteries. Furthermore, marked intimal thickening and narrowing of the lumen were seen in smaller arteries surrounded by collateral arterioles. In some patients, the majority of small pulmonary arteries were damaged, and inflammatory infiltrates were noted.

**Identification and Characterization of Isolated Cells**

Derived cells of primary cultures or first subcultures from the pulmonary arteries of patients with IPAH were identified as SMCs by their positive staining with antibodies against α-SMA, myosin, and smoothelin, which are markers for SMCs (Figure 1).

### Table: Clinical Data of Patients With Idiopathic Pulmonary Arterial Hypertension

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>PAP (s/d/m)</th>
<th>mRAP</th>
<th>CI</th>
<th>BNP</th>
<th>Epoprostenol</th>
<th>Duration</th>
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<td>F</td>
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<td></td>
<td>334.1</td>
<td>8</td>
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<tr>
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<td>F</td>
<td>31</td>
<td>73/30/48</td>
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<td>325.0</td>
<td>3</td>
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<tr>
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<td>F</td>
<td>13</td>
<td>111/49/67</td>
<td>10</td>
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<tr>
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<td>M</td>
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</table>

PAP indicates pulmonary artery pressure (mm Hg); s/d/m: systolic/diastolic/mean; mRAP, mean right atrial pressure (mm Hg); CI, cardiac index (L/min per m²); BNP, plasma concentration of brain natriuretic peptide (pg/dL); epoprostenol, maximum dose of epoprostenol given (ng/kg per minute); and duration, period between diagnosis of IPAH and termination of epoprostenol therapy (years).

### Effect of PSL on Proliferation of SMCs

In preliminary experiments of dose adjustment, the antiproliferative effects of PSL on PDGF-BB–stimulated SMCs were examined at final concentrations of $2 \times 10^{-5}$ to $2 \times 10^{-7}$ mol/L. Higher concentrations of PSL tended to induce a more marked antiproliferative effect. At peak concentration of PSL ($2 \times 10^{-3}$ mol/L), SMC proliferation was significantly inhibited compared with the control dishes ($P<0.05$). On the basis of the results of these experiments, we set the final concentrations to $2 \times 10^{-5}$ to $2 \times 10^{-3}$ mol/L in the remaining experiments.

PDGF stimulation caused a higher rate of growth of SMCs in patients with IPAH than control patients; the increase in $^{3}$H-thymidine incorporation in IPAH (489±291 cpm) was significantly larger than the control (72±39 cpm) (Figure 2). On the other hand, PSL induced a more profound and significant growth inhibition of SMCs of patients with IPAH ($434±214$ cpm) than the control patients (48±26 cpm, $P<0.05$), and such inhibition was dose-dependent in IPAH patients within the dose range used in the study ($2 \times 10^{-3}$ to $2 \times 10^{-5}$ mol/L). PSL had no antiproliferative effect on control cells (Figure 3A) but caused a significant inhibition at $2 \times 10^{-4}$ and $2 \times 10^{-3}$ mol/L in IPAH ($P<0.05$, Figure 3B). We assessed the antiproliferative effect of immunosuppressants including cyclosporine A, tacrolimus, azathioprine, and rapamycin ($10^{-5}$ to $10^{-3}$ mol/L). The results showed none of these agents exhibited SMCs antiproliferative effects (data not shown).

### Cell Viability

Trypan blue staining confirmed that PSL-treated SMCs excluded the trypan blue staining similar to control samples, indicating identical viability (Figure 4) and that the antiproliferative effects of PSL were independent of cell death.

### Cell Cycle Analysis

In the cell cycle analysis, PSL at $2 \times 10^{-4}$ mol/L decreased the percentage of SMCs in the S phase (Figure 5). PSL also

![Figure 1](https://example.com/figure1.png) Characterization of cultured SMCs from pulmonary arteries of patients with IPAH by immunohistochemical staining with anti-α-smooth muscle actin mouse monoclonal antibody (A), anti-myosin antibody (B), and anti-smoothelin antibody (C).
inhibited the progression of SMCs from G0/G1 to the S phase of the cell cycle, at which time cells are committed to cell replication. These results indicate that PSL induced G1 arrest.

Migration Assay
Representative data are shown in Figure 6. After 16-hour incubation, the migration assay showed migration of only a few SMCs. PDGF-BB increased the migration of SMCs, and PSL (2\times10^{-4} \text{ mol/L}) inhibited PDGF-induced SMC migration.

Western Blot Analysis
The results of semiquantitative analysis of p27 and p21 expression in SMCs of IPAH are shown in Figure 7. PSL treatment for 24 hours significantly increased p27 expression in SMCs (P<0.05). In contrast, the same treatment failed to produce a significant change in p21 expression.

Discussion
The pathological changes in the pulmonary arteries of patients with IPAH include SMC proliferation, but the mechanism of these changes remains poorly understood. The underlying cause of IPAH is also not clear, although several pathogenic mechanisms have been proposed, such as genetic predisposition, thrombosis, inflammation, infection, and angiogenesis. Thus, IPAH is likely to be a complex of different pathogenic mechanisms rather than a single disease entity.

Although SMC proliferation is observed in all patients with IPAH, the pathogenic mechanism of this process is poorly understood. On the other hand, several studies have examined coronary artery SMC proliferation after percutaneous coronary angioplasty. Traumatic injury of the vessel wall caused by angioplasty leads to activation of SMCs, followed by SMC proliferation, migration to the subintima, and further proliferation. Some evidence suggests that the expression of interleukin (IL)-6 may be related to the formation of restenotic lesions after percutaneous coronary angioplasty as well as to the instability of atheromatous plaques. Recent studies have focused on the use of PSL and immunosuppressive agents in the prevention of restenosis. PSL was reported to have antiproliferative effects on SMCs of atherosclerotic arteries. On the basis of these observations, we hypothesized that PSL could suppress the development of IPAH. Specifically, we hypothesized that proliferation of SMCs of the pulmonary arteries in patients with IPAH is due to an inflammatory process similar to that of restenosis after coronary artery angioplasty and that PSL could inhibit SMC proliferation in IPAH.

The main findings of the present study were that cultured SMCs of pulmonary arteries from patients with IPAH showed greater proliferation than control cells and that PSL inhibited the excessive proliferation of SMCs in IPAH (Figure 2). The effective concentrations of PSL that caused significant suppression of SMCs proliferation in this study were 2\times10^{-4} and 2\times10^{-3} \text{ mol/L}. These concentrations are comparable to the reported plasma levels of PSL when given to human in clinical use.

Studies have unveiled mutations in the gene encoding the bone morphogenetic protein (BMP) type II receptor in famil-
Furthermore, this mutation can be detected in at least a quarter of apparently sporadic PAH cases. As to the excessive proliferation of SMCs, we have already reported in the same cell line as those included in the present study that signaling pathway under BMP receptor played a key role in increased proliferation of SMCs induced by PDGF in IPAH. The pathway works through activin receptor-like kinase-6, which leads to activation of mitogen-activated protein kinase. The favorable effect of PSL on IPAH disease suppression suggests the involvement of inflammation in disease progression. The association of IPAH with inflammatory cytokines has been well documented. Inflammatory cells such as T- and B-lymphocytes and macrophages have been identified in plexiform lesions, and elevated serum levels of inflammatory cytokines including IL-1\beta and IL-6 have been reported. High concentrations of circulating monocyte chemoattractant protein-1, which is recognized as a potent chemotactic and activating factor for monocytes and leukocytes, have also been reported in patients with IPAH. Chemokine RANTES (regulated on activation, normal T-cell expressed and se-
pressure and blood flow preferentially directed to the transplanted lung may result in regression of the vascular changes of the native lung. Alternatively, the regression is assumed to be due to the application of PSL and immunosuppressants for the contralaterally transplanted lung. Moreover, there are no reported cases of recurrent IPAH after lung transplantation. This might be attributed to the application of PSL and immunosuppressants besides the limited disease process, which had been removed with bilateral lung transplantation. In the present study, PSL exhibited antiproliferative effect on SMCs, but immunosuppressants including cyclosporine A, tacrolimus, azathioprine, and rapamycin did not. These findings suggest that PSL exclusively participate in preventing proliferation of SMCs.

The present in vitro study revealed that PSL caused cell cycle arrest of SMCs and subsequently led to suppression of proliferation. Cell proliferation requires the interaction of cyclins and cyclin-dependent kinase (CDK) to drive cells through the cell cycle. In general, CDK inhibitors p27 and p21 can bind cyclin-CDK complexes and cause G1 arrest. In the case of pulmonary artery SMC proliferation, an important role for p27 but not p21 in regulating cell cycle has been reported. In rat pulmonary artery SMCs, p27 expression decreased 24 hours after serum stimulation, which was associated with an increase in both 3H-thymidine incorporation and the percent of cells in S phase. In our study, PSL significantly increased p27 expression but not that of p21 in SMCs of IPAH (Figure 7). This implies that p27 rather than p21 plays a key role in regulating cell cycle of SMCs in IPAH, and it can be a critical target of the antiproliferative effect of PSL.

Limitations
The antiproliferative effect of PSL on cultured pulmonary artery SMCs from patients with IPAH was demonstrated in this study. The effective concentrations of PSL that caused significant suppression of SMC proliferation were 2\times10^{-4} and 2\times10^{-3} mol/L. Although these concentrations are comparable to the reported plasma levels of PSL as pulse therapy, they may be high as maintenance dose. Therefore, further experiments to assess the effectiveness of PSL at lower doses are needed before clinical application of PSL to treat IPAH.

Conclusions
Our study demonstrated the antiproliferative effects of PSL on cultured SMCs from the pulmonary arteries of patients with IPAH. The results suggest that PSL may be useful in patients with IPAH, although further analysis of the role of inflammatory mechanisms in IPAH is necessary to understand whether this component of the disease is relevant to its pathophysiology. Furthermore, translational research using an animal model of PAH is needed to gain information for clinical application, including dose and duration of PSL treatment.

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References


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