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WILEY
Potential Treatment for Vocal Fold Scar With Pirfenidone

Haruka Kodama, MD, PhD; Yoshihiko Kumai, MD, PhD; Kohei Nishimoto, MD, PhD; Yutaka Toya, MD, PhD; Satoru Miyamaru, MD, PhD; Shinobu Furushima; Eiji Yumoto, MD, PhD

Objectives/Hypothesis: Pirfenidone (PFD) is a strong antifibrotic agent that has been clinically approved in Japan for idiopathic pulmonary fibrosis. We examined the antifibrotic effects of PFD on fibroblasts isolated from scarred vocal folds (VFs) of ferrets in vitro.

Study Design: Prospective animal experiments with controls.

Methods: Scar fibroblasts (SFs) were isolated from scarred VFs that had been electrocauterized 2 weeks before harvesting (N = 4). Normal fibroblasts (NFs) were isolated from intact VFs (N = 4). SFs and NFs were incubated in the presence of 10 ng/mL transforming growth factor β1 (TGF-β1), with or without PFD. After the 48-hour incubation, mRNA expression levels of α smooth muscle actin (αSMA), TGF-β1, collagen type I, and hyaluronan synthase 2 (HAS2) were examined by real-time polymerase chain reaction. Immunohistochemistry with anti-αSMA anti-collagen type I and phosphorylated Smad (p-Smad)2/3 antibodies in SFs with or without PFD was performed. SFs and NFs were cultured in collagen gel with or without PFD for 48 hours, and the extent of gel contraction was examined quantitatively.

Results: PFD treatment significantly (P < .05) decreased mRNA expression of collagen type I, significantly increased mRNA expression of TGF-β1 and HAS2, and significantly suppressed collagen gel contraction. However, it did not have a significant effect on the expression of αSMA. The expression of p-Smad2/3 in the nucleus was faded with PFD, possibly demonstrating the suppression of translocation of p-Smad2/3 from cytoplasm to nucleus with PFD.

Conclusions: This is the first report to demonstrate the in vitro antifibrotic effects of PFD on fibroblasts isolated from scarred VFs of ferrets.

Key Words: Vocal fold scar; pirfenidone; in vitro; ferret; real-time polymerase chain reaction; immunohistochemistry.

Level of Evidence: NA.

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INTRODUCTION

Basic investigations of treatments that promote wound healing and fibrosis in vocal folds (VFs) is an active area of research in otolaryngology. Trauma and inflammation are the two main causes of VF scars, and the pathology remains a clinical challenge.1 Several recent reviews have provided pathological characterizations of VF scars and the array of possible solutions under investigation. Potential solutions include the use of autologous tissues, biomaterial implants, growth factors, antifibrotic agents2–4 and stem cells.5 However, current problems include the difficulty obtaining a consistent effect, commercial availability, and applicability to humans.

The superficial lamina propria (SLP) is a specialized layer of highly pliable connective tissue that is particularly important for supporting vibration during phonation. The extracellular matrix (ECM) of the SLP, which has a soft and resilient quality, similar to a gel,6 contains fine elastin and collagen fibers embedded in a mixture of different proteoglycans and hyaluronic acid (HA).7 The quality of the voice depends largely on SLP pliability, which in turn depends on a balance of amorphous ECM ground substance and fine fibrous proteins that are normally produced by VF fibroblasts. With VF injury and inflammation, myofibroblasts, which are largely responsible for wound contraction, predominate and produce an abundance of coarse collagen fibers that stiffen the VF ECM. Therefore, the goal in treating a VF scar is to restore the composition and mechanical properties of the ECM of the SLP to a more normal state by reducing the collagen deposition and ideally increasing HA production in the SLP.

Pirfenidone (PFD) (5-methyl-1-phenyl-2-[1H]-pyridone) has combined anti-inflammatory and antifibrotic effects, acting through the regulation of tumor necrosis factor (TNF)-α and TNF-β pathways, as well through modulation of cellular oxidation.8 Since the late 1990s, some studies have shown that PFD inhibits fibroblast proliferation and collagen synthesis and deposition, both
in vitro and in animal models.\textsuperscript{9,10} Idiopathic pulmonary fibrosis (IPF) is a rare, progressive disease of unknown etiology, characterized by dyspnea and deterioration of lung function, with poor quality of life and a median survival time of $\sim$3 years.\textsuperscript{11} Initial open-label trials showed the therapeutic potential of PFD,\textsuperscript{12} and the first prospective clinical studies demonstrated slowed deterioration of lung function, measured by the change in lowest saturation of peripheral oxygen (SpO$_2$) during a 6-minute exercise test and vital capacity.\textsuperscript{13} Based on these findings, in 2008, the Japanese Ministry of Health, Labor, and Welfare approved the use of PFD to treat mild-to-moderate IPF, which demonstrated potential for widening its clinical application to progressive fibrotic disorders of other organs, such as VF scars. Transforming growth factor $\beta$1 (TGF-$\beta$1) is one of the most studied profibrotic cytokines. We hypothesized that PFD would suppress TGF-$\beta$1 activity as described elsewhere.\textsuperscript{14,15} This would reduce both collagen production and collagen gel contraction in vitro. We chose the ferret as an animal model because Kumai et al.\textsuperscript{9} established a model with a thicker SLP layer than that of rats, and demonstrated that the fibroblasts isolated from scarred VFs are myofibroblasts, which maintain a different phenotype from normal fibroblasts (NFs) isolated from normal VFs.

We examined the in vitro antifibrotic effects of PFD on fibroblasts isolated from scarred VFs.

**MATERIALS AND METHODS**

**Ferret VF Fibroblast Isolation**

Eight young-adult male ferrets (1.25–1.4 kg) were used. All experiments were performed in accordance with the guidelines of our Animal Care and Use Committee, and complied with the dictates of the Animal Research Center of Kumamoto University.

Scar fibroblasts (SFs) were isolated from the scarred right VF of four ferrets, and NFs were isolated from the intact VF of the remaining four ferrets. The ferrets were anesthetized with intramuscular injection of ketamine (30 mg/kg body weight) and xylazine (1.5 mg/kg body weight). For NF isolation, animals with normal VFs were sacrificed via the administration of intracardiac ketamine and xylazine; the larynges were harvested. SFs were isolated from the VF scar tissue induced by electrocauterization in four male ferrets. The experimental time point (2 weeks) was set because previous studies\textsuperscript{11} examined ferret VFs 2 weeks after electrocauterization with endoscopic, histological, and ultrastructural studies and validated the cauterized ferret VF acute scar model as a source of myofibroblasts at 2 weeks after electrocauterization. Each VF was minced and seeded into a 10-cm petri dish in modified Dulbecco modified Eagle’s medium (DMEM) (Wako, Osaka, Japan), supplemented with 10% fetal bovine serum (FBS) (Cosmo Bio, Tokyo, Japan) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO$_2$. Two weeks later, fibroblasts that migrated out of the cultured VF explants were subcultured.

Cells were used at passage three or four. TGF-$\beta$1 (10 ng/mL, human recombinant TGF-$\beta$1; T7039; Sigma-Aldrich, St. Louis, MO) was added to mimic the inflammatory environment of fibrosis at the same time as PFD (provided by Shionogi & Co., Osaka, Japan) treatment at 25% to 50% confluence and incubated for 48 hours.

**Real-Time Quantitative Polymerase Chain Reaction**

NFs and SFs were treated with 10 ng/mL TGF-$\beta$1 and PFD at concentrations of 0 or 1.0 mg/mL for 48 hours, and total RNA was extracted using a spin column method employing a NucleoSpin RNA kit (MACHEREY-NAGEL, Duren, Germany). These concentrations were determined following studies that have used PFD in vitro.\textsuperscript{9,10} Total RNA was reverse-transcribed into complementary DNA (cDNA) using a Super Script VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, CA) employing a Dice TaKaRa polymerase chain reaction (PCR) Thermal Cycler (Takara-Bio, Otsu, Japan). Quantitative polymerase chain reaction was performed using the reagents and protocol of the TaqMan Fast Advanced Master Mix (Applied Biosystems, Foster City, CA). The primer pairs used are listed in Table I. These primer pairs were designed with Primer Express 2.0 software (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. All reactions were performed in 20-µL volumes containing 4 µL of cDNA solutions. The amplification program featured an initial step at 50.0°C for 2 minutes, then a second step at 95.0°C for 20 seconds, followed by 40 cycles of denaturation at 95°C for 3 seconds, and annealing and elongation at 62°C for 30 seconds. Data were collected and analyzed on an ABI 7300 HT platform (Applied Biosystems) based on the ferret mRNA sequences database obtained from National Center for Biotechnology Information. The relative expression levels of target genes were determined using the quantitative 2$^{-\Delta\Delta Ct}$ method.

**Immunohistochemistry**

The SFs and NFs were plated (7.5 × 10$^6$ cells per well) in 8-well chamber slides (Matsunami, Osaka, Japan) and treated with 10 ng/mL TGF-$\beta$1 and 0 or 1.0 mg/mL PFD for 48 hours, fixed with 4% paraformaldehyde for 20 minutes, and washed with 0.1 mol/L phosphate-buffered saline (PBS). Cells were incubated in 0.1 mol/L PBS with 10% (v/v) normal donkey serum and 1% (v/v) Triton X-100 for 20 minutes, followed by incubation with primary antibodies against $\alpha$ smooth muscle actin (zSMA) (rabbit polyclonal, 1:100; Abcam, Cambridge, United Kingdom), collagen type I (rabbit polyclonal, 1:200; Abcam), and phosphorylated Smad (p-Smad)2/3 (Ser 423/425, goat polyclonal, 1:100; Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. After washing with PBS, sections were
incubated with Alexa Fluor 555-labeled donkey anti-rabbit immuno-

noglobulin (IgG; 1:200; Invitrogen, Carlsbad, CA) or Alexa Fluor

555-labeled donkey anti-goat IgG (1:200; Invitrogen) for 2 hours

at 37°C. Nuclei were counterstained with Hoechst stain (Dojindo

Laboratories, Kumamoto, Japan). After washing with PBS, sec-

tions were mounted using Fluoromount (Diagnostic BioSystems,
Pleasanton CA) X-100. All cells were viewed under a microscope

(BZ9000 Biorevo; Keyence, Osaka, Japan).

**Collagen Contraction Assay**

Collagen contractility, a characteristic of fibroblasts, can be evaluated using the collagen gel contraction assay. Collagen gel contraction induced by NFs and SFs was assessed using a Cell Contraction Assay Kit (Cell Biolabs, San Diego, CA). Briefly, cells were suspended in culture medium at 4 × 10^6 cells/mL, and the collagen lattice was prepared by mixing two parts of cell suspension and eight parts of cold collagen gel.

**Table I. Primers Used in This Study.**

<table>
<thead>
<tr>
<th>Sense Primer</th>
<th>Antisense Primer</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>CTGAGCTGAATAGGGAGCACACT</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>GATATCTCTCAGACCTGCTCAACTC</td>
</tr>
<tr>
<td>ACTA2 (αSMA)</td>
<td>ATGCTCCAGGGGTGTTTT</td>
</tr>
<tr>
<td>Col1A1 (collagen 1)</td>
<td>GCTTGCCCATCCTAGATGT</td>
</tr>
<tr>
<td>HAS2</td>
<td>TCCTGGGTCTTCGGAAGTCTT</td>
</tr>
</tbody>
</table>

ACTA2(αSMA) = α smooth muscle actin; Col1A1 (collagen 1) = collagen type I, alpha1; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HAS2 = hyaluronan synthase 2; TGF-β1 = transforming growth factor β1.

**Fig. 1.** Effects of pirfenidone (PFD) on the mRNA expression of transforming growth factor β1 (TGF-β1), α smooth muscle actin (αSMA), collagen type I (collagen 1), and hyaluronan and synthase 2 (HAS2) in normal fibroblasts (NFs) and scar fibroblasts (SFs) stimulated with TGF-β1. In NFs stimulated with TGF-β1, the mRNA expression of αSMA was not significantly affected by PFD; however, the mRNA expression of TGF-β1, collagen 1, and HAS2 was significantly increased with PFD than in those without PFD. In SFs stimulated by TGF-β1, mRNA expression of collagen 1 was decreased significantly (P < .05) with PFD paradoxically, and mRNA expression of TGF-β1 and HAS2 was increased significantly (P < .01) with PFD. The mRNA expression of αSMA with PFD was not significantly different to that without PFD. Data are expressed as the mean ± standard deviation of the four cell lines of each group. N.S. = not significant.

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solution. Subsequently, 500 μL of the cell–collagen mixture was loaded into a 24-well plate and incubated at 37°C for 1 hour. After collagen polymerization, gels were incubated in culture medium with 10 ng/mL TGF-β1 and 0 or 1.0 mg/mL PFD for 48 hours, during which collagen contractility developed. At the same time, collagen gel containing NFs was incubated without TGF-β1 or PFD, for a normal control. To initiate gel contraction, gels were gently separated from the walls of the wells. To determine the degree of collagen gel contraction, pictures of the gels were taken, and the area of each gel was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). Data were calculated as a percentage of each well size.

**Statistical Analysis**

Data are presented as mean ± standard deviation. Paired t tests were used to compare the changes with or without PFD in both the NFs and SFs. Unpaired t tests were used to compare between NFs and SFs for statistical analyses. A difference was considered statistically significant at P < .05.

**RESULTS**

**Effects of PFD on mRNA Levels of TGF-β1, αSMA, and Collagen Type I in NFs and SFs Stimulated With TGF-β1**

Compared to untreated samples, TGF-β1 mRNA expression was significantly higher in both the NFs (P < .05) and SFs (P < .01) after treatment with PFD. In addition, expression was significantly higher in the SFs than in NFs with (P < .05) or without (P < .01) PFD. In both the NFs and SFs, αSMA mRNA expression did not differ in either NFs or SFs with or without PFD. Expression was significantly higher (P < .01) in the SFs than in the NFs with or without PFD. In the NFs, collagen type I mRNA expression was significantly higher with PFD (P < .05) compared to without PFD; in the SFs, however, it was significantly lower with PFD (P < .05) than without PFD (Fig. 1). Hyaluronan synthase 2 (HAS2) mRNA expression was significantly higher in both NFs and SFs with PFD (P < .05) than in those without PFD. The expression was not significantly different in NFs and SFs.

Effects of PFD on immunohistochemical expression of αSMA, collagen type I, and p-Smad2/3 in SFs stimulated with TGF-β1. Immunohistochemical expression of αSMA in SFs without PFD presented formation of stress fibers composed of αSMA being suppressed with PFD dose dependently, which did not accord with the data analyzed by real-time polymerase chain reaction (PCR) (A). The expression of collagen 1 decreased with PFD compared to that without PFD, which did accord with the real-time PCR data (B).

**Effects of PFD on Collagen Gel Contraction**

Representative images of the collagen gel contraction assay of SFs with or without PFD (Fig. 4a) and NFs incubated without TGF-β1 and PFD as control are...
shown. PFD inhibited the gel contraction that was induced by TGF-β1. The area of gels divided by each well area with each concentration of PFD in NFs and SFs were measured (data are expressed as the mean ± SD of five separate experiments per group [Fig. 4b]). Collagen gel contraction induced by TGF-β1 with either NFs or SFs was suppressed by PFD, and the extent of gel contraction with NFs or SFs was significantly inhibited by PFD ($P < .01$ [Fig. 4b]).

**DISCUSSION**

This is the first report demonstrating the antifibrotic effects of PFD, especially the reduction of collagen type I expression and gel contraction in fibroblasts...
isolated from ferret VF scar. Many animal models support the conclusion that excessive collagen deposition is a major pathological issue in VF scars.\textsuperscript{16,17} More specifically, the abundant production of type I collagen is a significant component of fibrosis during and after the healing process that impairs VF function. Type I collagen produced during VF healing differs in molecular and macroscopic structure from the native collagen, which is largely type III. Type III–containing fibers are of a finer caliber than type I–containing fibers and are well organized in the normal VF.\textsuperscript{18} Myofibroblasts are largely responsible for the synthesis of collagen type I for wound contracture and the development of fibrosis.\textsuperscript{19} Kumai et al.\textsuperscript{5} examined fibroblasts in both normal and scarred ferret VFs by light and electron microscopy. A VF scar was induced by electrocautery using a needle electrode. At 2 weeks after treatment, the cauterized VF showed whitish scar tissue with prominent vascularization, in contrast to the normal translucent VF. Histological and ultrastructural studies of the SLP showed an increased density of coarse collagen and large numbers of large fibroblasts in the VF scar, as seen in the present study. In contrast, normal VF tissue is composed of a loose array of collagen and smaller fibroblasts. These observations indicate that the fibroblasts in ferret VF scars are probably myofibroblasts and that myofibroblasts are involved in VF fibrosis. The challenge, therefore, in treating VF scars is to reduce the excessive collagen to a normal state and ideally to increase hyaluronic acid (HA) for the restoration of SLP pliability. Mesenchymal stem cells, HA-based biomaterials, decellularized scaffold materials, hepatocyte growth factor, and platelet-rich plasma have been used for restoration of SLP pliability. Despite several advantages, these materials are still far from being used routinely in clinical settings.

PFD is an orally available pyridone analog (5-methyl-1-phenyl-1H-pyridin-2-one).\textsuperscript{14} Oku et al.\textsuperscript{9} investigated the antifibrotic effect of PFD in bleomycin-induced lung fibrosis in mice using prednisolone as a reference agent. Both PFD and prednisolone suppressed lung inflammation in this model; however, only PFD significantly suppressed the subsequent lung fibrosis, as evaluated by hydroxyproline content representing the amount of collagen type I and histological fibrotic score. The antifibrotic action of PFD has also been reported in several animal models of progressive fibrotic disorders of other organs, including the kidney, liver, and heart.\textsuperscript{14,15} Thus, one advantage for applying PFD as an new antifibrotic medicine for VF scars is that PFD has already been approved for clinical use in progressive fibrotic disorders of other organs.

In this preliminary study, we demonstrated that PFD significantly decreased the mRNA expression of

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**Fig. 4. Effects of pirfenidone (PFD) on collagen gel contraction containing normal fibroblasts (NFs) or scar fibroblasts (SFs) stimulated with transforming growth factor β1 (TGF-β1).** (a) Representative images of the collagen gel contraction assay of NFs without TGF-β1 or PFD (control), and SFs with or without PFD with 10 ng/mL TGF-β1. PFD inhibited the gel contraction that was induced by TGF-β1. (b) The areas of gels for each well area with each concentration of PFD were measured. Data are expressed as the mean ± standard deviation of five separate experiments per group. Collagen gel contraction induced by TGF-β1 with NFs or SFs was suppressed by each concentration of PFD. Contraction of collagen gels containing NFs or SFs was inhibited by PFD dose dependently. The extent of gel contraction with NFs or SFs was significantly inhibited by adding 1 mg/mL PFD (NFs: \( P < .05 \), SFs: \( P < .01 \)).
collagen type I and inhibited collagen contractility in fibroblasts isolated from ferret VF scars. Moreover, immunohistochemical expression levels of collagen type I, and zSMA, were reduced with 1.0 mg/mL of PFD. These data suggest that PFD could be a significant new antifibrotic medicine for the treatment of VF scars. In previous literature, the antifibrotic activity of PFD is mainly considered to be due to the suppression of TGF-β1. TGF-β1 is one of the most studied profibrotic cytokines.14,15 In the lung, TGF-β1 is produced by a wide variety of cell types, including alveolar macrophages, neutrophils, activated alveolar epithelial cells, endothelial cells, fibroblasts, and myofibroblasts. TGF-β1 induces the proliferation of macrophages and fibroblasts. In these cells, TGF-β1 also stimulates the expression of several other proinflammatory and fibrogenic cytokines, such as TNF-α, interleukin (IL)-1β, and IL-13, further enhancing and perpetuating the fibrotic response.16 We hypothesized that, as described elsewhere,17 PFD could work negatively on the TGF-β1 pathway, by decreasing the expression of TGF-β1 and consequently reducing the expression of zSMA dose-dependently; however, our results were contradictory to this hypothesis. In keloid fibroblasts stimulated with TGF-β1, PFD significantly inhibited the mRNA expression of zSMA; however, paradoxically, PFD significantly enhanced the mRNA expression of TGF-β1 and had no effect on the expression of collagen type I. We assume that the characterization of the myofibroblasts isolated from VF scars might differ from those isolated from fibrous tissues of other organs, especially with respect to the sensitivity to the PFD or severity of fibrous tissue. Previous articles demonstrated that PFD inhibited TGF-β1-activated Smad signaling by preventing nuclear accumulation of phosphorylated Smad2/3, which can suppress Smad signaling without affecting other pathways regulated by TGF-β1.20 Data in our study may follow this pathway demonstrating the suppression of translocation of p-Smad2/3 from the cytoplasm to the nucleus with PFD. Interestingly, HAS2 mRNA expression, which induces HA production, was significantly higher with PFD. As described previously,7 HA is the key component for the VF pliability, and our preliminary results demonstrated the positive favorable effect of PFD in terms of restoration of VF pliability.

The fundamental limitation of this study is that ferret VFs are not representative of human VFs, which lack the three subepithelial layers found in ferrets. On the other hand, the length of the ferret VF membrane is relatively longer than that of rat or mouse (data not shown), which is advantageous for injection experiments to VFs. Although the key target molecule for PFD to suppress collagen expression in VF scar fibroblasts is not yet clear, we need to establish the pathway of collagen type I suppression; therefore, further research is needed to determine its precise mechanism of action.

Because it was previously demonstrated that introral administration of PFD induced side effects such as nausea, skin problems, or gastroesophageal reflux,21 inhalation or intralesional routes might be ideal than intraoral administration. Therefore, in the future, we should test the effect of this medicine in vivo by inhalation, intralesionally, using the ferret model before future clinical application.

CONCLUSION

This is the first report demonstrating the in vitro antifibrotic effects of PFD (especially suppression of collagen expression and gel contraction) in fibroblasts isolated from ferret VF scar. Further research for its clinical application, especially in vivo by inhalation and intralesional routes, is needed to determine its precise mechanism of action, and to show its inhibitory effects on the pathway(s) that lead to the development of the pathology of VF scars.

BIBLIOGRAPHY