Pirfenidone Attenuates the IL-1β–Induced Hyaluronic Acid Increase in Orbital Fibroblasts From Patients With Thyroid-Associated Ophthalmopathy

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PURPOSE. This study aimed to investigate the effect of pirfenidone on the IL-1β–induced hyaluronic acid (HA) increase in orbital fibroblasts from patients with thyroid-associated ophthalmopathy (TAO).

METHODS. Primary cultured orbital fibroblasts were obtained from patients with TAO, and the excreted levels of HA from IL-1β–treated cells with or without pirfenidone were measured. The effect of pirfenidone on IL-1β–induced hyaluronic acid synthase (HAS) expression was evaluated. The relevance of the mitogen-activated protein kinase (MAPK)–mediated signaling pathway in IL-1β–induced HAS expression was assessed using specific inhibitors to p38, extracellular signal-regulated kinase (ERK), or c-Jun N-terminal kinase (JNK). The phosphorylation level of each MAPK in IL-1β–treated cells with or without pirfenidone and the level of AP-1 DNA binding were measured. The inhibitory potency of pirfenidone on HA production was evaluated using dexamethasone as a reference agent.

RESULTS. Pirfenidone strongly attenuated the IL-1β–induced HA release in a dose-dependent manner. The IL-1β–induced HAS expression was decreased significantly following cotreatment with pirfenidone at the mRNA and protein levels. The production of mRNAs was halted by cotreatment with inhibitors of ERK and p38, but not by inhibitors of JNK. The IL-1β–induced ERK and p38 phosphorylation, and AP-1 DNA binding were attenuated in the presence of pirfenidone. Pirfenidone showed greater potency than dexamethasone in inhibiting increases in IL-1β–induced HA.

CONCLUSIONS. Pirfenidone attenuates the IL-1β–induced HA production in orbital fibroblasts from patients with TAO, at least in part, through suppression of the MAPK-mediated HAS expression. These results support the potential use of pirfenidone for treatment of patients with TAO.

Keywords: hyaluronic acid, hyaluronic acid synthase, IL-1β, orbital fibroblast, mitogen-activated protein kinase, pirfenidone, thyroid-associated ophthalmopathy

Thyroid-associated ophthalmopathy (TAO), one of the extrathyroidal manifestations of Graves’ disease, is an autoimmune component that remains a vexing clinical problem that includes proptosis, eyelid retraction, limitation of ocular motion, and even visual loss due to compressive optic neuropathy.1–2 The hallmarks of TAO include the volumetric expansion of orbital tissues, such as orbital fat and extraocular muscles, in the limited space of the bony orbit.2–4 Remodeling of these orbital tissues in TAO mainly consists of an accumulation of the glycosaminoglycan, hyaluronic acid (HA).1–4 Because of its important rheological property, which includes a profound hydrophilic nature and extreme molecular bulk when hydrated,5 the extracellular accumulations of HA result in edematous changes in corresponding tissues, which currently is believed to underlie much of the volumetric expansion noted in patients with TAO. Therefore, an effective suppression of HA increase could be a therapeutic target to halt the pathologic progression in TAO.5

Orbital fibroblasts, which are abundant in orbital connective tissues embedded in fatty compartments, are an important source of HA.6–8 Orbital fibroblasts have been shown to enhance HA synthesis when activated by pro-inflammatory cytokines, such as IL-1β and TGF-β.5,9,10 In addition, the magnitude of enhanced HA synthesis was greater in orbital than dermal fibroblasts.8,11 Dexamethasone attenuates IL-1β–induced increases of HA levels in orbital fibroblasts in vitro;5 clinically, glucocorticoids still are indicated as the first-line treatment for suppression of the disease activity regardless of their possible multiple side effects.12 Unfortunately, a pathogenic mechanism–based therapeutic agent for clinical use with few side effects remains elusive.

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a small molecule that exhibits novel antifibrotic effects not only in several experimental disease models, such as pulmonary fibrosis13 and liver cirrhosis,14 but also in clinical trials of idiopathic pulmonary fibrosis.15 Regarding TAO, we have
reported previously that pirfenidone attenuates IL-1β-induced tissue inhibitors of metalloproteinase (TIMP)-1/collagen levels in orbital fibroblasts without significant toxicity at the concentrations used. In addition to its antifibrotic effect, pirfenidone has been found to possess anti-inflammatory and antioxidant properties. Additionally, we reported recently that pirfenidone attenuates IL-1β-induced cyclooxygenase (COX)-2/prostaglandin (PG)E2 levels, suggesting the efficacy of this particular drug in attenuating the pathologic conditions of TAO.

In this study, we showed that pirfenidone effectively attenuates the IL-1β-induced HA increase in orbital fibroblasts in patients with TAO by suppressing hyaluronic acid synthase (HAS) expression. Moreover, this inhibitory effect is, at least in part, mediated by attenuating the activation of mitogen-activated protein kinases (MAPKs). These data suggested the possibility of future clinical use of pirfenidone in the treatment of TAO.

**MATERIALS AND METHODS**

**Reagents**

Pirfenidone and BSA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin/EDTA were purchased from Caisson (North Logan, UT, USA). Recombinant human IL-1β was purchased from PeproTech (Rocky Hill, NJ, USA), and TRIzol was obtained from Invitrogen (Carlsbad, CA, USA). The nuclear extraction kit was obtained from Thermo Scientific (Rockford, IL, USA). Antibodies against p38, phosphorylated (p-p)38, p44/42, p-p44/42, and actin were purchased from Cell Signaling Technology (Beverly, MA, USA). The HA-ELISA kit was obtained from Echelon Biosciences (Salt Lake City, UT, USA). The inhibitors of MAPKs; that is, SB 203580 (p38 MAPK [p38]), PD 98059 (MAPK kinase 1 [MEK1]), and SP 600125 (c-Jun N-terminal kinase [JNK]/stress activated protein kinase [SAPK]), were purchased from Sigma-Aldrich.

**Cell Culture**

Human orbital fibroblasts were cultivated from orbital fatty connective tissue obtained as surgical waste during decompression surgery in patients with TAO as described previously. The protocol for obtaining orbital tissue was approved by the Institutional Review Board of Ajou University Hospital and followed the tenets of the Declaration of Helsinki. Written informed consent was obtained from all donors. Five different orbital fibroblast culture strains were obtained from each patient with TAO. All patients with TAO had experienced at least 6 months of inactive disease status with a euthyroid condition before the decompression surgery. The patient characteristics are listed in the Table. Cell cultures were maintained in a humidified 5% CO2 incubator at 37°C in DMEM containing 10% FBS and antibiotics. Once a fibroblast monolayer was obtained, cultures were serially passaged after gentle treatment with trypsin/EDTA. The medium was changed every 3 days, and cells at passages 3 to 8 were used for experiments.

**ELISA Results**

The HA levels in cell culture supernatants of orbital fibroblasts were measured in cells seeded in six-well culture plates at a final concentration of 5 × 10^5/well. After stabilization for 48 hours, orbital fibroblasts were treated with 10 ng/mL IL-1β and 1, 5, 10 mM pirfenidone or 100 mM dexamethasone in fresh DMEM containing 1% charcoal-filtered FBS for 48 hours. Supernatants from the cell cultures were collected, and the concentrations of HA therein were determined using a competitive binding HA-ELISA kit according to the manufacturer’s instructions. Briefly, after adding 100 µL of standards and samples into the corresponding wells, 150 and 100 µL of diluent were added to the blank and zero HA control wells, respectively. Working detector (50 µL) was added to all wells except the blank, and the plate was covered and incubated for 1 hour at 37°C. Then, 100 µL of controls and samples were transferred to the corresponding wells of the HA-ELISA plate. After incubation for 30 minutes at 4°C, the solution was discarded, the wells were washed, and 100 µL of working enzyme suspension were added to each well. After incubation for 30 minutes at 37°C followed by washes, 100 µL of working substrate solution were added to each well, and the plate was incubated in the dark at room temperature for 30 minutes. Absorbance was measured spectrophotometrically at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The concentration of HA in each sample was determined by reference to a standard curve generated with known amounts of HA.

**RT-PCR Studies**

Orbital fibroblasts (5 × 10^5/well) were seeded in six-well cell culture plates and treated with 10 ng/mL IL-1β in the presence or absence of pirfenidone or inhibitors for MAPKs (SP 600125, SB 203580, PD 98059). Total RNA was extracted using TRIzol reagent at each designated time point, and cDNA was generated from RNA (1 µg) using the SuperScript First-Strand Synthesis System (Invitrogen). The PCR was performed using 1-µl cDNA, 0.25 mM dNTP, 1 U of Pfu DNA polymerase, and 10 pmole of the primer pair, with a thermal cycle. The specific primer sequences were as follows: HAST forward, 5'-TGT GTA TCC TGG ATC AGG GGT-3' and reverse, 5'-TCG GAG GTG TAC TTG GTA GCA TAA CC-3'; HAS2 forward, 5'-CTG GAG TTA ATC ATG TCG TTA CTT CC-3' and reverse, 5'-TGC ATA TGG TAC TCG TCT CTG TCT CCG C-3'; HAS3 forward, 5'-GCG ACC ATC AGA AGT TAC TGG TCC TCT CC-3' and reverse, 5'-GCG GAG AAT GAG CGA GAT CGC-3'; and b-actin forward, 5'-CCA AGG CCA ACC GGC AGA AGA TGA C-3' and reverse, 5'-AGG GTA CAT GGT GGT GCC GGC AGA C-3'. The PCR cycles were composed of one cycle of 95°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds.
minutes, 30 cycles of 45 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C. The reaction was terminated at 72°C for 5 minutes and quenched at 4°C. Band densities were quantified using the ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA). The measured intensities were corrected to the β-actin level in each lane and subjected to statistical analyses.

**Western Blot Analysis**

Orbital fibroblasts (5 × 10^4/well) were cultured and treated as described for ELISA. After treating fibroblasts for the designated incubation times, cells were scraped into a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Gendepot, Barker, TX, USA), and centrifuged at 14,171g for 20 minutes at 4°C. Proteins were separated by SDS-PAGE on 10% (wt/vol) gels and transferred to nitrocellulose membranes (Schleicher & Scuell Biotechnology, Keene, NH, USA). Each membrane was blocked by incubation at room temperature for 1 hour with Tris-buffered saline (TBS, pH 7.4) containing 5% (wt/vol) skim milk or BSA, and then was incubated at 4°C overnight with the primary antibody. After three washes with TBST (TBS containing 0.1% [vol/vol] Tween-20), membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies at room temperature for 1 hour. The immunoreactive proteins were detected by incubation with enhanced chemiluminescence blotting detection agent (Pierce Biotechnology, Rockford, IL) using the LAS Imaging System (Fujifilm, Tokyo, Japan). The band densities were quantified using the ImageJ software (NIH). The measured intensities were corrected to the actin level in each lane and subjected to statistical analyses.

**Nuclear Extracts (NEs) and Electrophoretic Mobility Shift Assay (EMSA)**

The EMSA was performed with 1 μg of NEs as described previously.20 The NEs from cells treated with IL-1β for 30 minutes were incubated with the AP-1 sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3'; SC-2505; Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was end-labeled with [γ-32P]ATP for 30 minutes. A 100 molar excess of cold oligonucleotide was added to the NEs for 30 minutes before adding labeled probe for the competition experiments. Bound and free DNA were then resolved by 5% PAGE and exposed to film for autoradiography.

**Statistical Analysis**

Data were analyzed using the SPSS software version 15.0 (SPSS, Inc., Chicago, IL, USA). The t-test was used to analyze the inhibitory effects of pirfenidone on the IL-1β–induced increase in HA and HAS levels. For comparison of inhibitory effects between pirfenidone and dexamethasone, the t-test was performed. P values of less than 0.05 were considered to indicate statistical significance.

**RESULTS**

**Pirfenidone Attenuates the IL-1β–Induced HA Increase in Orbital Fibroblasts**

Orbital fibroblasts from patients with TAO express relatively high levels of HA in response to IL-1β stimulation.5,8 Thus, we initially examined the effects of pirfenidone on IL-1β–induced HA expression in TAO orbital fibroblasts. Cells were cotreated with various concentrations of pirfenidone and 10 ng/mL of IL-1β for 48 hours, and the HA protein levels in culture media were measured by ELISA. Guided by our previous study showing an antifibrotic effect of pirfenidone in human orbital fibroblasts without significant toxicity,16 we used pirfenidone at concentrations up to 10 μM. The IL-1β induced a 6-fold increase in HA protein levels compared to that reached in untreated controls, and pirfenidone significantly reduced the IL-1β–induced HA release in a dose-dependent manner. As shown in Figure 1, pirfenidone attenuated the IL-1β–induced HA increase at 1, 5, and 10 μM, decreasing HA concentrations to 52%, 14%, and 5% of the values obtained by IL-1β stimulation, respectively. When treated with 10 μM pirfenidone, HA production was decreased dramatically below the untreated control level, even in the presence of IL-1β.

**Pirfenidone Attenuates the IL-1β–Induced HAS Expression in Orbital Fibroblasts**

The HA synthesis in mammals is regulated by three specific HAS enzymes that are encoded by individual genes: *HAS1*, *HAS2*, and *HAS3*.21,22 We investigated whether pirfenidone influences the expression of these HAS genes induced by IL-1β in TAO orbital fibroblasts. Orbital fibroblasts were cotreated with 10 ng/mL IL-1β and 10 μM pirfenidone for 4, 8, or 12 hours, and HAS mRNA levels were measured by RT-PCR. Responding to IL-1β treatment, mRNA expression of each *HAS* was enhanced significantly in a time-dependent manner, while pirfenidone diminished this inducing effect of IL-1β (Fig. 2A). In the case of *HAS1*, mRNA expression was increased up to 25-fold greater than control. The *HAS2* and *HAS3* mRNA levels also were enhanced by IL-1β, but to a lesser extent than *HAS1*. Pirfenidone significantly attenuated the IL-1β–induced *HAS1* mRNA levels at 8 and 12 hours (73% and 94% reduction of the IL-1β–induced levels, respectively) and those of *HAS2* and *HAS3* at 4, 8, and 12 hours (46%, 69%, and 71% reduction of the IL-1β–induced levels for *HAS2*, 69%, 75%, and 77% reduction of the IL-1β–induced levels for *HAS3*, respectively; Fig. 2B). This inhibitory effect of pirfenidone on the IL-1β–induced HAS increase was verified by Western blot (Fig. 2C).
IL-1β Induces HAS Expression Through the MAPK-Mediated Signaling Pathway in Orbital Fibroblasts

The IL-1β induces a number of genes in orbital fibroblasts. Induction of several of these is mediated through the activation of MAPK pathways. In addition, a previous study reported that HA synthesis in dermal fibroblasts is mediated by the MAPK signaling pathway. Therefore, we examined the contribution of MAPKs to IL-1β–induced HAS expression in orbital fibroblasts using specific inhibitors of three types of MAPKs: SB203580 for p38, PD98059 for extracellular signal-regulated kinase (ERK), and SP 600125 for JNK. The levels of HAS mRNA in cells cotreated with IL-1β, and each specific inhibitor for 4, 8, and 12 hours were measured by RT-PCR. As shown in Figure 3, inhibitors of ERK or p38 successfully abrogated the IL-1β–induced enhancement of all three HAS subtypes, while the JNK inhibitor did not yield a significant reduction in HAS expression levels compared to the other inhibitors.

Pirfenidone Attenuates the IL-1β–Induced Activation of the MAPK-Mediated Signaling Pathway in Orbital Fibroblasts

We next evaluated whether pirfenidone affects the IL-1β–induced activation of the MAPK-mediated signaling pathway,
particularly of p38 and ERK, in orbital fibroblasts. We performed Western blot analyses for phosphorylated and total levels of p38 and ERK protein. As shown in Figures 4A and 4B, IL-1β strikingly induced the phosphorylation of p38 at 5 minutes, and cotreatment with pirfenidone suppressed IL-1β-induced phosphorylation, while the decreased level at 10 minutes did not show significance. In the case of ERK, the IL-1β-induced increase in phosphorylation was observed at 10 minutes, and pirfenidone attenuated it. Although the increased phosphorylation of ERK was not observed at 5 minutes of treatment with IL-1β, pirfenidone decreased the level of ERK phosphorylation much below that of the untreated control. Pirfenidone or IL-1β itself showed no effect on the total levels of p38 or ERK. Since the p38 and ERK signaling pathways have been shown to affect AP-1 activity, which is a downstream target of MAPK, we performed EMSA to investigate the effect of pirfenidone on AP-1 binding to the consensus oligonucleotide in the presence of IL-1β. Responding to 10 ng/mL of IL-1β treatment, increased DNA binding of the AP-1 consensus oligonucleotide was observed in NEs from two independent TAO orbital fibroblasts (Fig. 4C, lanes 3 and 7), and the binding was reduced in cells cotreated with IL-1β and pirfenidone comparable to each control level (lanes 5 and 9). Competition with excess unlabeled AP-1 oligonucleotide abrogated the complex formation (lane 10), which confirmed a specific interaction between AP-1 and the AP-1 consensus oligonucleotide.

**Pirfenidone Has Greater Potency Than Dexamethasone in Attenuating the IL-1β-Induced HA Increase in Orbital Fibroblasts**

In light of a previous in vitro study showing that dexamethasone attenuates IL-1β-induced increases in HA levels in orbital fibroblasts, we compared the inhibitory effect of pirfenidone on HA expression to that of dexamethasone. After cotreating orbital fibroblasts with 10 ng/mL IL-1β and 10 mM pirfenidone or 100 nM dexamethasone for 48 hours, HA levels in the culture media were measured by ELISA. As shown in Figure 5, the inhibitory effect of pirfenidone on the IL-1β-induced HA increase was significantly greater than that of dexamethasone (96% and 72% reduction of the IL-1β-induced levels, respectively).

**DISCUSSION**

Our data demonstrated that pirfenidone attenuated IL-1β-induced HA production in primary cultured orbital fibroblasts from patients with TAO by interfering with the MAPK-mediated HAS expression. The pathology of TAO is characterized by the volumetric expansions of extraocular muscles and orbital fat within the limited bony orbital space, which result in congestion and finally organ dysfunction. At the heart of this tissue remodeling is the disordered accumulation of the glycosaminoglycan, HA. Because HA-accumulated tissues can expand by hydration due to its remarkable avidity for water, the abnormal HA accumulation in orbital tissues is thought to be the major cause of edematous changes in orbital tissues noted in patients with TAO. In addition, since HA lacks a core protein, protein synthesis is not required, and the production of HA proceeds rapidly. Of note, orbital fibroblasts express extraordinary levels of HA in an anatomic site-selective manner with exaggerated responses to pro-inflammatory cytokines, such as IL-1β. Therefore, effective attenuation of this exaggerated HA production in orbital...
FIGURE 4. Effect of PFD on the IL-1β–induced MAPK-mediated signaling pathway. (A) Orbital fibroblasts from patients with TAO were treated with 10 ng/mL of IL-1β in the presence or absence of 10 mM of PFD for the indicated times (5 or 10 minutes) and Western blottings were performed for phosphorylated p38 (p-p38) or phosphorylated p44/42 (p-p44/42). Representative bands from independent experiments using five strains of cells are shown. (B) Relative signal intensities from the results of (A) are shown. The measured intensities of p-p38, p-p44/42, p38, and p44/42 were corrected for their respective actin signals and analyzed (bar graphs; mean ± SD; *P < 0.05 versus untreated control; †P < 0.05 versus IL-1β–treated cells). (C) Orbital fibroblasts from a patient with TAO were treated with 10 ng/mL IL-1β in the presence or absence of 10 mM PFD for 30 minutes. Then, nuclear extracts were obtained and the levels of AP-1 DNA binding were evaluated by EMSA. Representative blots from three independent experiments are shown.
is mediated by ERK and p38, but not by JNK. These discrepancies may be due to the different characteristics of the particular stimulants and cell types used.

Pirfenidone is a novel agent that has shown anti-inflammatory and antifibrotic potential in animal models and clinical trials. It exerts its downregulating effects on a series of cytokines, including TGF-β, connective tissue growth factor, platelet-derived growth factors, TNF-α, IFN-γ, intercellular adhesion molecule-1, IL-6, and IL-1. Regarding TAO, we reported previously that pirfenidone exerts antifibrotic and anti-inflammatory effects in orbital fibroblasts by inhibiting TIMP-1 and COX-2 expression without causing significant cytotoxicity. In addition, pirfenidone showed superior potency compared to that of dexamethasone. Herein, we revealed an additional effect of pirfenidone at a concentration that is corresponding to the ones used in our previous reports, the attenuation of IL-1β–induced HA production through the inhibition of MAPK-mediated HAS expression. However, this inhibitory effect of pirfenidone on MAPK activity, particularly on p38, was relatively incomplete when compared to its effect on HA production. These results suggested the existence of other pharmacologic mechanisms of pirfenidone in attenuating the IL-1β–induced HA production in orbital fibroblasts, in addition to the inhibition of the MAPK signaling pathway shown here. In human umbilical vein endothelial cells, IL-1β induces HAS2 transcription via the nuclear factor (NF)-κB–dependent pathway. Taken together with our previous report of the inhibitory effect of pirfenidone on IL-1β–induced activation of NF-κB signaling pathway in orbital fibroblasts, this pharmacologic action mechanism might be applicable in our present results.

In this study, we demonstrated that pirfenidone showed a superior potency in inhibiting IL-1β–induced HA production in orbital fibroblasts compared to dexamethasone. In contrast with the well-known side effects of corticosteroids, oral pirfenidone was generally well tolerated with few side effects in several large-scale phase III clinical trials, indicating that pirfenidone could be safer than high-dose glucocorticoids. The most frequent side effects include photosensitivity rash, nausea, dyspepsia, and dizziness, which appear to be dose-related and typically resolve completely once the drug is withdrawn. Further research and clinical studies are necessary to ensure the safety of pirfenidone treatment and to ascertain the optimum doses for treatment of TAO.

In summary, our results provided initial evidence that pirfenidone has inhibitory effects on IL-1β–induced HA production by attenuating HAS expression in orbital fibroblasts from patients with TAO. These results suggested that pirfenidone may be a promising candidate for future use as a therapeutic agent in TAO.

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**References**


