Pirfenidone attenuates the interleukin-1β-induced hyaluronic acid increase in orbital fibroblasts of thyroid-associated ophthalmopathy

Seung Ah Chung

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Directed by Professor Jong Bok Lee

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Seung Ah Chung

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This certifies that the Doctoral Dissertation of
Seung Ah Chung is approved.

Thesis Supervisor: Jong Bok Lee

Thesis Committee Member#1: Koung Hoon Kook

Thesis Committee Member#2: Sueng-Han Han

Thesis Committee Member#3: Sung Soo Kim

Thesis Committee Member#4: Hyung Keun Lee

The Graduate School
Yonsei University

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Seung Ah Chung

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ABSTRACT

Pirfenidone attenuates the interleukin-1β-induced hyaluronic acid increase in orbital fibroblasts of thyroid-associated ophthalmopathy

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Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Jong Bok Lee)

Thyroid-associated ophthalmopathy (TAO) is an autoimmune component of Graves' disease characterized by intense inflammation in the setting of volume expansion and tissue remodeling, leading to organ dysfunction. At the heart of this, tissue remodeling occurred by the disordered accumulation of glycosaminoglycan; hyaluronic acid (HA), which results in tissue edema due to its strong hydrophilic property. Because no reliable, specific and safe medical therapeutic agents are available for TAO, the development of specific therapies with minimal side effects is essential. Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a novel agent that has shown its anti-inflammatory and anti-fibrotic effects in animal models and clinical trials.

We investigated an effect of pirfenidone on interleukin (IL)-1β-induced HA expression in an *in vitro* model of TAO. The effect of pirfenidone on IL-1β-induced HA expression was assessed in primary cultured orbital fibroblasts of TAO patients. The level of HA in IL-1β-treated cells with or without pirfenidone was measured using an enzyme-linked immunosorbent assay (ELISA). The effect of pirfenidone on IL-1β-induced hyaluronic acid synthase (HAS) expression was evaluated by reverse transcription-polymerase

chain reaction (RT-PCR), and verified by Western blot. We then examined the role of mitogen-activated protein kinase (MAPK)s on IL-1β-induced HAS expression by RT-PCR, using specific inhibitors to p38 MAPK (p38), extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK). The level of phosphorylation of MAPKs in IL-1β-treated cells with or without pirfenidone was measured by immunoblot analysis. In addition, the effect of pirfenidone on HA production was compared with that of dexamethasone by ELISA.

Pirfenidone strongly attenuated the IL-1β-induced HA release with a dose -dependent manner. IL-1β-induced HAS mRNA and protein expression decreased significantly by co-treatment with pirfenidone. In the signaling pathway of IL-1β, *HAS* transcription was mediated by p38 and ERK dependent pathways, and the phosphorylations of them were nicely attenuated by co-treatments with pirfenidone. In our system, JNK pathways was not related with IL-1β-induced *HAS* transcription. Finally, pirfenidone was more effective than dexamethasone in inhibiting IL-1β-induced HA increases.

In summary, pirfenidone attenuates the IL-1β-induced HA production in orbital fibroblasts from patients with TAO, which is related to suppression of the MAPK-mediated HAS expression. These results support the potential use of pirfenidone in treatment of TAO.

Key words : hyaluronic acid, hyaluronic acid synthase, inflammation, interleukin-1β, mitogen-activated protein kinase, orbital fibroblasts, pirfenidone, thyroid-associated ophthalmopathy

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I. INTRODUCTION

Thyroid-associated ophthalmopathy (TAO) is the most important autoimmune component of the extra-thyroidal manifestations of Graves' disease which remains a vexing clinical problem.[1-3] Up to 50% of patients with Graves' disease develop pathologic manifestations in the eye: the cosmetic deficits, such as lid swelling, proptosis, and lid retraction, and the functional deficits that manifest as limited ocular motion and visual loss.[1,4,5] Approximately 3-5% of the patients with TAO suffer from severe morbidity, including intense pain with inflammation, diplopia related to limitation of ocular movement, or even sight-threatening compressive optic neuropathy.[1,4,5] Most of the clinical manifestations of TAO can be explained by the discrepancy between the increased volume of orbital tissues and the fixed volume of the bony orbit.[4,5]

The hallmark features of TAO include the volumetric expansion of orbital connective/fatty tissue and extraocular muscle: the tissue edema.[1-8] This remodeling of orbital tissues in TAO consists of intense inflammation and accumulation of hyaluronic acid (HA).[6-8] HA is a high-molecular-weight glycosaminoglycan composed of D-glucuronic acid and N-acetyl-D-glucosamine residues. Because of its profound hydrophilic nature and extreme molecular bulk when hydrated, the increase of HA, in conjunction with fat expansion, is currently believed to underlie much of the tissue dysfunction associated with TAO.[3,4,6,8]

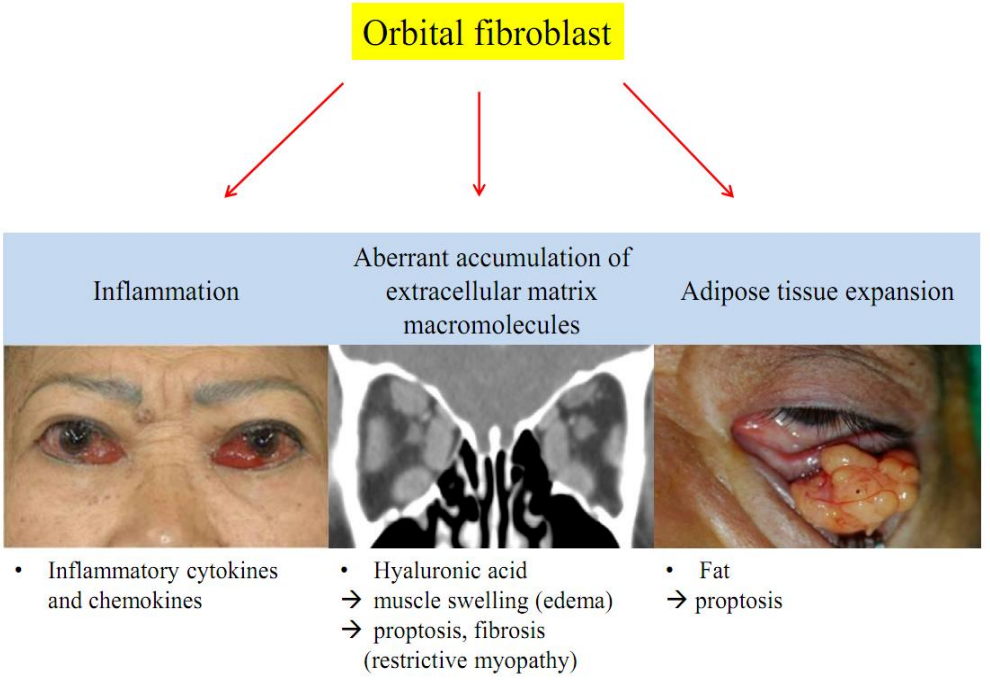

Figure 1. Three major pathological processes of thyroid-associated ophthalmopathy.

Orbital fibroblasts, which are abundant in orbital connective tissue and fatty compartment, are believed to play a major role in the pathologic processes of TAO (Figure 1) and to be an important source of HA.[8-10] Orbital fibroblasts have been shown to enhance HA synthesis when activated with pro-inflammatory cytokines such as interleukin (IL)-1β and transforming growth factor (TGF)-β.[8-15] In addition, the magnitude of enhanced HA synthesis was greater in orbital fibroblasts than that found in dermal fibroblasts.[10-16]

However, no reliable, specific, and safe therapeutic agents have yet been developed for TAO. Glucocorticoids have been used for decades and are still indicated as the first-line treatment due to their anti-inflammatory and immunosuppressive actions, even though their multiple side effects.[17-20] They are mostly effective in soft tissue inflammatory changes, recent-onset extraocular muscle involvement, and optic neuropathy, while proptosis and longstanding extraocular muscle involvement associated with fibrotic changes are poorly influenced.[17-20] A major drawback of systemic glucocorticoid therapy is indeed represented by its possible side effects and complications. Although transient, cushing-like features, diabetes, depression, reactivation of chronic diseases, infections, hypertension, osteoporosis, increased body weight, peptic ulcer, hirsutism, and cataract, have been reported during prolonged glucocorticoid therapy for TAO.[19,20] Therefore, the challenges to develop a new therapeutic agent which specifically targets the pathogenesis of TAO, such as inflammation,

adipose tissue expansion, aberrant accumulation of extracellular matrix macromolecules, or fibrosis, are mandatory.[20]

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a small molecule that has proved to exhibit novel anti-fibrotic effects not only in several experimental disease models, such as pulmonary fibrosis and liver cirrhosis, but also in clinical trials of idiopathic pulmonary fibrosis.[21, 22] Pirfenidone has also been found to possess anti-inflammatory and antioxidant properties.[22] Pirfenidone inhibits proliferation, migration, and collagen contraction in human Tenon's fibroblasts.[23,24] It also reduces fibronectin synthesis in cultured human retinal pigment epithelial cells.[25] Recently, it has been reported that pirfenidone attenuates IL-1β-induced tissue inhibitors of metalloproteinase (TIMP)-1/collagen levels and cyclooxygenase (COX)-2/ prostaglandin (PG)E2 levels without significant toxicity at the concentrations used, indicating the anti-fibrotic and anti-inflammatory effects of this agent in TAO.[26,27]

In this study, we showed that pirfenidone effectively decreases the level of HA induced by IL-1β in orbital fibroblasts from TAO patients through suppressing hyaluronic acid synthase (HAS) expression, and that this inhibitory effect is, at least in part, mediated by the suppression of mitogen-activated protein kinase (MAPK)s activities.

II. MATERIALS AND METHODS

1. Reagents and antibodies

Pirfenidone, dexamethasone (1,4 pregnadien-9-fluoro-16α-methyl-11β, 17α, 21- triol-3,20-dione), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A). Structure of pirfenidone used in this study is shown in Figure 2. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin/ethylenediaminetetraacetic acid (EDTA), penicillin, and streptomycin were purchased from Caisson (North Logan, UT, U.S.A). Recombinant human IL-1β was purchased from PeproTech (Rocky Hill, NJ, U.S.A). TRIzol® was obtained from Invitrogen (Carlsbad, CA, U.S.A). The nuclear extraction kit was obtained from Thermo scientific (Rockford, IL, U.S.A). Anti-human HAS1, HAS2, and HAS3 rabbit antibodies were purchased from Abcam (Cambridge, UK). Antibodies against p38, phosphorylated (p)-p38, p44/42, p-p44/42, and actin were purchased from Cell Signaling Technology (Beverly, MA, U.S.A). A HA-enzyme-linked immunosorbent assay (ELISA) kit was obtained from Echelon Biosciences (Salt Lake City, UT, U.S.A). The inhibitors of MARKs, SB 203580 (for p38 MAPK [p38]), PD 98059 (for MAPK kinase 1 [MEK1]), and SP 600125 (for c-Jun N-terminal kinase [JNK]/ stress activated protein kinase [SAPK]) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A).

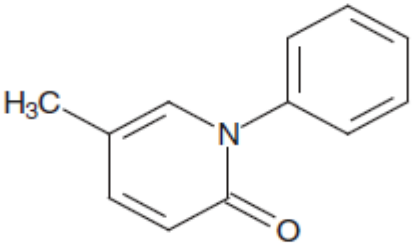

Figure 2. Chemical structure of pirfenidone.

2. Cell culture

Human orbital fibroblasts were cultivated from orbital fatty connective tissue obtained as surgical waste during decompression surgery for patients with TAO as described previously.[26,27] The protocol for obtaining orbital tissue was approved by the Institutional Review Board of Ajou University Hospital and following 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 of five patients with TAO. Two patients had been administered prednisone therapy (1 mg/kg for 3 days followed by tapering of dose over 4 to 8 weeks) for their previous active disease. All patients with TAO had experienced at least 6 months of inactive disease status with euthyroid condition before the decompression surgery. Patient characteristics are presented in Table 1. Cell cultures were maintained in a humidified 5% CO_2 incubator at 37℃ covered with 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.

Table 1. Characteristics of patients with thyroid-associated ophthalmopathy (TAO) from whom the fibroblast strains were obtained

Characteristics	Patients with TAO (n=5)
Mean age, yrs (range)	51.8 (41~64)
Gender (male/female)	1/4
Smoking (yes)	0
Treatment for Graves` disease	
Radioiodine therapy	0
Anti-thyroid drug	5
Surgery	0
Treatment for TAO	
Surgery	5
Prednisone	2
Radiation	0
Mean clinical activity score (range)	1 (0~2)
TSH-receptor antibodies (+)	5
Euthyroid	5

TSH: thyroid-stimulating hormone.

3. ELISA

HA levels in cell culture supernatants of orbital fibroblasts were measured in cells seeded in 6-well culture plates at a final concentration of 5 x 10^5 cells/well. After stabilization for 48 hours, orbital fibroblasts were treated with 10 ng/ml IL-1β and 1, 5, 10 mM pirfenidone or 100 nM dexamethasone in fresh DMEM containing 1% charcoal-filtered FBS for 48 hours. Supernatants from the cell cultures were collected, and the concentrations of HA in supernatants were determined using a competitive binding HA-ELISA kit according to the manufacturer's instructions. Briefly, after adding of 100 μl of standards and samples into the corresponding wells, 150 and 100 μl of diluent was added to the blank and zero HA control wells, respectively. A 50 μl of working detector was added to each well except the blank, and the plate was covered and incubated for 1 hour at 37℃. 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℃, the solution was discarded, and the wells were washed and 100 μl of working enzyme suspension was added to each well. After incubation for 30 minutes at 37℃ and washing, 100 μl of working substrate solution was 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, U.S.A). The concentration of HA in each sample was determined by reference

to a standard curve generated with known amounts of HA.

4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Orbital fibroblasts (5 x 10^5 cells/well) were seeded in 6-well cell culture plates and treated with 10 ng/ml IL-1β in the presence or absence of pirfenidone and 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 1 μg of RNA using the SuperScript® First-Strand Synthesis System (Invitrogen, Carlsbad, CA, U.S.A). PCR was performed using 1 μl cDNA, 0.25 mM dNTP, 1 U of Pfu DNA polymerase, and 10 ρmole of the primer pair with a thermal cycler. The following specific primers were used (Table 2).

Table 2. Primer sequences used for RT-PCR

Genes	Sense (5`-3`)	Antisense (3`-5`)
HAS1	TGTGTATCCTGCATCAGCGGT	CTGGAGGTGTACTTGGTAGCATAACC
HAS2	GTGTTATACATGTCGAGTTTACTTCC	GTCATATTGTTGTCCCTTCTTCCGC
HAS3	GGTACCATCAGAAGTTCCTAGGCAGC	GAGGAGAATGTTCCAGATGCG
β-actin	CCAAGGCCAACCGCGAGAAGATGAC	AGGGTACATGGTGGTGCCGCCAGAC

HAS: hyaluronic acid synthase.

PCR cycles consisted of 1 cycle of 95℃ for 5 min to denature all proteins, 30 cycles of 45 sec at 94℃, 45 sec at 55℃, and 45 sec at 72℃. The reaction was terminated at 72℃ for 5 min and quenched at 4℃. Band densities were quantified using the Image J software (National Institutes of Health, Bethesda, MD, U.S.A). The measured intensities were corrected to the β-actin level in each lane and subjected to statistical analyses.

5. Western blot analysis

Orbital fibroblasts (5 x 10^5 cells/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, 2mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Gendepot, Barker, TX, U.S.A), and centrifuged at 13,000 rpm for 20 min at 4℃. Proteins were separated by SDS-polyacryalmide gel electrophoresis (PAGE) on 10% (wt/vol) gels and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Keene, NH, U.S.A). 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℃ 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, U.S.A) using the LAS Imaging System (Fujifilm, Tokyo, Japan). The band densities were quantified using the Image J software (NIH, Bethesda, MD, U.S.A). The measured intensities were corrected to the actin level in each lane and subjected to statistical analyses.

6. Statistical analysis

Data were analysed using the SPSS software version 15.0 (SPSS Inc., Chicago, IL, U.S.A). The *t*-test was used in analyzing 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 same test was performed. P values of less than 0.05 were considered to be statistically significant.

III. RESULTS

1. IL-1β-induced HA release in orbital fibroblasts is reduced by pirfenidone

Orbital fibroblasts from patients with TAO express relatively high levels of HA in response to IL-1β stimulation.[11-15,28,29] Thus, we initially examined the effects of pirfenidone on IL-1β-induced HA expression in TAO orbital fibroblasts. Cells were co-treated with different concentrations of pirfenidone and 10 ng/ml of IL-1β for 48 hours, and HA levels in culture media were measured by ELISA. Guided by the previous study showing an anti-fibrotic effect of pirfenidone in human orbital fibroblasts without significant toxicity,[26] we used pirfenidone at concentrations up to 10 mM. IL-1β induced a marked increase in HA levels that reached 6-fold that of untreated controls, and pirfenidone significantly reduced the IL-1β-induced HA release in a dose-dependent manner. As shown in Figure 3, pirfenidone attenuated the IL-1β-induced HA increase at concentrations of 1, 5, and 10 mM, decreasing HA concentrations to 52%, 14%, and 5% of the values obtained after IL-1β stimulation. Pirfenidone of 5 and 10 mM dramatically suppressed HA production below the control level although IL-1β was co-treated.

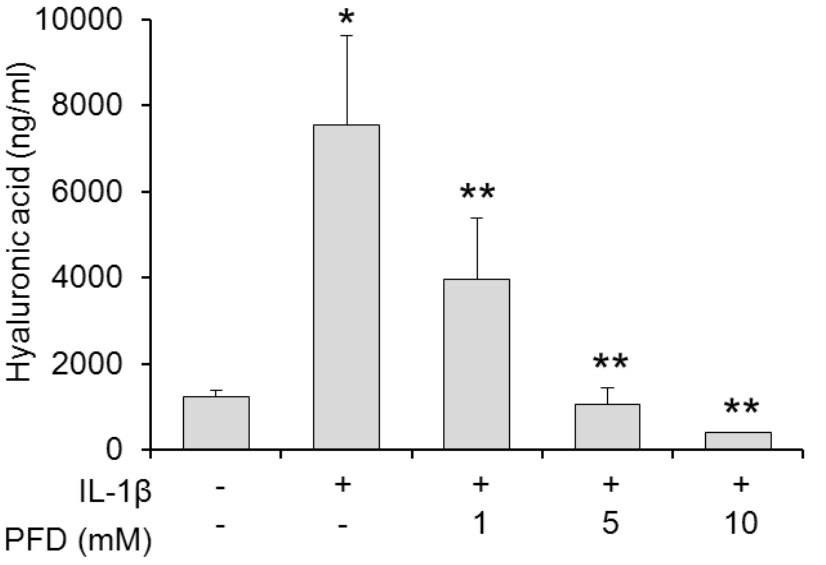

Figure 3. Effect of pirfenidone (PFD) on IL-1β-induced hyaluronic acid (HA) increase in orbital fibroblasts. Primary cultures of orbital fibroblasts from TAO patients were co-treated with 10 ng/ml of IL-1β and different concentrations of pirfenidone (1, 5, or 10 mM) for 48 hours. HA levels in culture supernatants were measured by ELISA. Data are expressed as the mean ± SD of quadruplicate determinations (*P < 0.05 *vs.* untreated control; **P < 0.05 *vs.* IL-1β-treated cells).

2. Pirfenidone attenuates IL-1β-induced HA synthases (HAS) expression in orbital fibroblasts

HA synthesis in mammals is regulated by three specific HAS, encoded by individual genes; *HAS1*, *-2*, and *-3*.[30,31] We determined whether pirfenidone influences the expression of these *HAS* genes, induced by IL-1β in TAO orbital fibroblasts. Orbital fibroblasts were co-treated with 10 ng/ml IL-1β and 10 mM pirfenidone for 4, 8, or 12 hours, and *HAS* mRNA levels were measured by RT-PCR. Responding to IL-1β treatment, mRNA expression of all *HAS* isoforms was significantly enhanced in a time-dependent manner while pirfenidone diminished this increasing effect of IL-1β about the control level (Figure 4A). In the case of *HAS1*, mRNA expression was increased up to 25 times greater than control. *HAS2* and *HAS3* mRNA expressions were also enhanced but not as much as that of *HAS1*. Pirfenidone significantly attenuated the IL-1β-induced *HAS1* mRNA expression at 8 and 12 hours (73% and 94% reduction of the IL-1β-induced levels), and those of *HAS2*, *-3* at 4, 8, and 12 hours (46%, 69% and 71% reduction of the IL-1β-induced levels for *HAS2*, 69%, 73%, and 77% reduction of the IL-1β-induced levels for *HAS3*, respectively; Figure 4B). This inhibitory effect of pirfenidone on the IL-1β-induced HAS protein increase was verified by immunoblotting (Figure 4C).

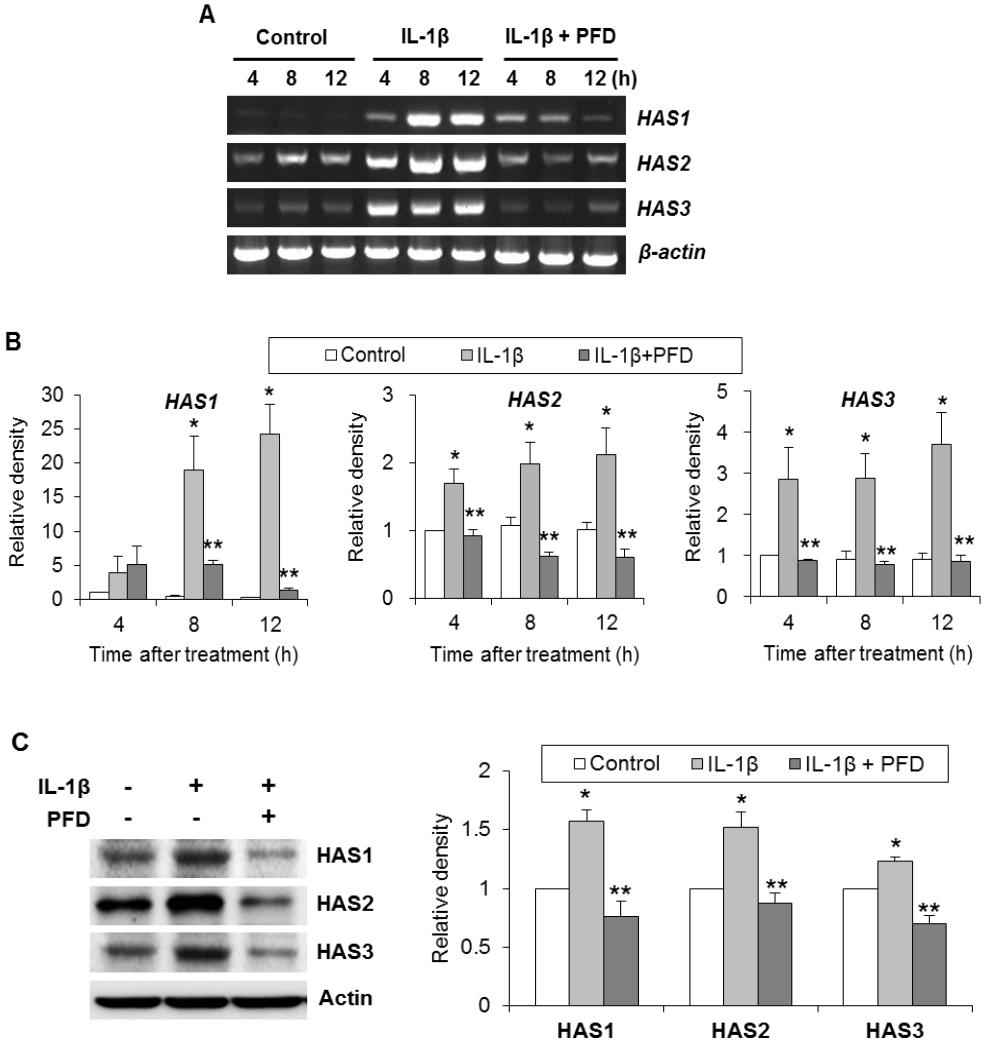

Figure 4. Effect of pirfenidone (PFD) on IL-1β-induced hyaluronic acid synthase (HAS) expression in orbital fibroblasts. (A) Orbital fibroblasts of TAO patients were co-treated with 10 ng/ml of IL-1β and 10 mM of PFD for indicated times (4, 8, or 12 hours) and RT-PCR analysis for three types of *HAS (HAS1, HAS2, and HAS3)* mRNA levels was performed. Representative bands from independent

experiments of five strains of TAO cells are shown. (B) Relative signal intensities of *HAS* mRNA from the results of (A) are shown. The measured intensities of *HAS* mRNA were corrected for their respective *β-actin* signals and analyzed (bar graphs, mean ± SD; *P < 0.05 vs. untreated controls; **P < 0.05 vs. IL-1β-treated cells). (C) Western blot analysis was performed to verify HAS protein levels in cell culture supernatants of orbital fibroblasts. TAO cells were co-treated with 10 ng/㎖ of IL-1β and 10 mM of PFD for 24 hours. Representative blots (left panel) and relative signal intensities of HAS protein (right panel) from the independent experiments of five strains of TAO cells are shown. The measured intensities of HAS protein were corrected for their respective actin signals and analyzed (bar graph, mean ± SD; *P < 0.05 *vs.* untreated controls; **P < 0.05 *vs.* IL-1β-treated cells).

3. IL-1β induces HAS expression through the p38 and ERK-mediated signaling pathways in orbital fibroblasts

IL-1β induces a number of genes in orbital fibroblasts.[10-15,27-29,32-34] Several of these inductions are mediated through the activation of MAPK pathways.[32-37] In addition, previous study reported that MAPK signaling pathway was related to HA synthesis in other fibroblasts.[38] Therefore, we examined the contribution of MAPKs to IL-1β-induced HAS secretion in orbital fibroblasts using specific inhibitors of the three types of MAPK; SB 203580 for p38, PD 98059 for ERK,

and SP 600125 for JNK. The levels of *HAS* mRNA in cells treated with IL-1β and specific inhibitors for 4, 8, and 12 hours were measured by RT-PCR. Inhibitors of ERK (PD 98059) and p38 (SB 203580) successfully abrogated IL-1β-induced enhancing effect of all *HAS* isoforms, especially in *HAS1*. However, JNK inhibitor (SP 600125) did not significantly reduce *HAS* expression level compared to other inhibitors. The suppression of the IL-1β-induced *HAS1* mRNA by inhibitors of ERK and p38 was greatest among three isoforms of *HAS*, but the effects occurred at 8 and 12 hours (Figure 5).

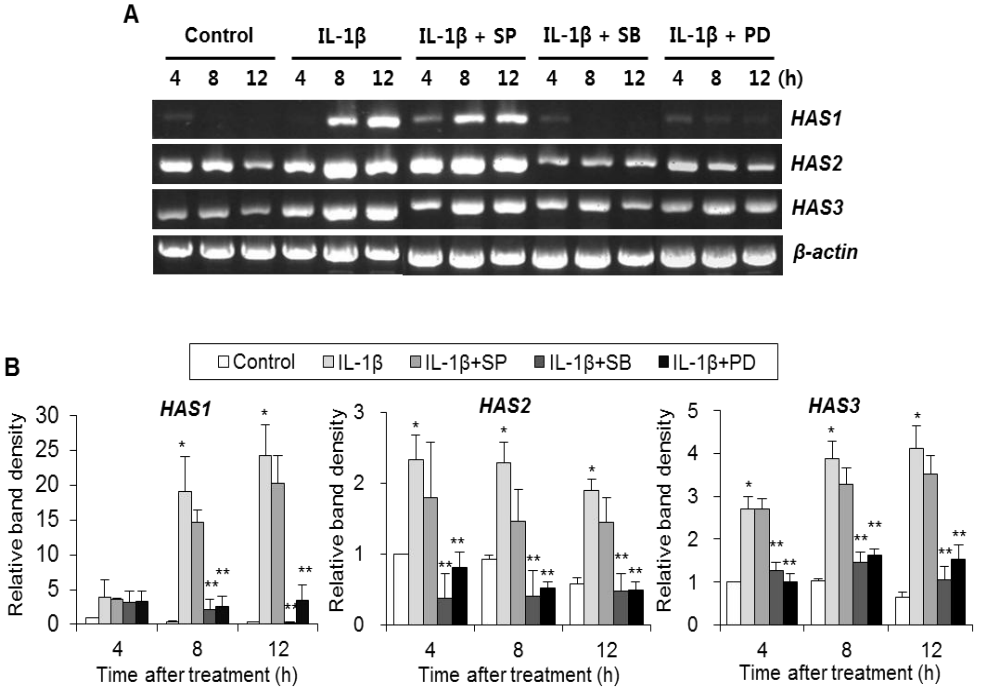

Figure 5. Involvement of MAPK-mediated signaling pathway in IL-1β-induced *HAS* expression in orbital fibroblasts. (A) Orbital fibroblasts of TAO patients were

co-treated with 10 ng/ml IL-1β and 20 μM of each specific inhibitor for MAPK-mediated signaling pathway (SP 600125 (SP), a JNK inhibitor; SB 203580 (SB), a p38 inhibitor; PD 98059 (PD), a ERK inhibitor) for indicated times (4, 8, or 12 h) and RT-PCR analysis for three types of *HAS* mRNA was performed. Representative bands from independent experiments of five strains of TAO cells are shown. (B) Signal intensities of *HAS* from the results of (A) are shown. The measured intensities of *HAS* mRNA were corrected for their respective *β-actin* signals and analyzed (bar graphs, mean ± SD; *P < 0.05 *vs.* untreated control; **P < 0.05 *vs.* IL-1β-treated cells).

4. Pirfenidone reduces IL-1β-induced activation of MAPK-mediated pathway in orbital fibroblasts

We next evaluated whether pirfenidone affects the MAPK signaling activation particularly in p38 and ERK in IL-1β-stimulated orbital fibroblasts. We performed immunoblot analyses for phosphorylated and total levels of p38 and ERK (p44/42) protein. IL-1β strikingly induced p38 phosphorylation at 5 min, and co-treatment with pirfenidone suppressed IL-1β-induced p38 phosphorylation. IL-1β induced ERK phosphorylation at 10 min and pirfenidone also attenuated the IL-1β-induced ERK phosphorylation (Figure 6).

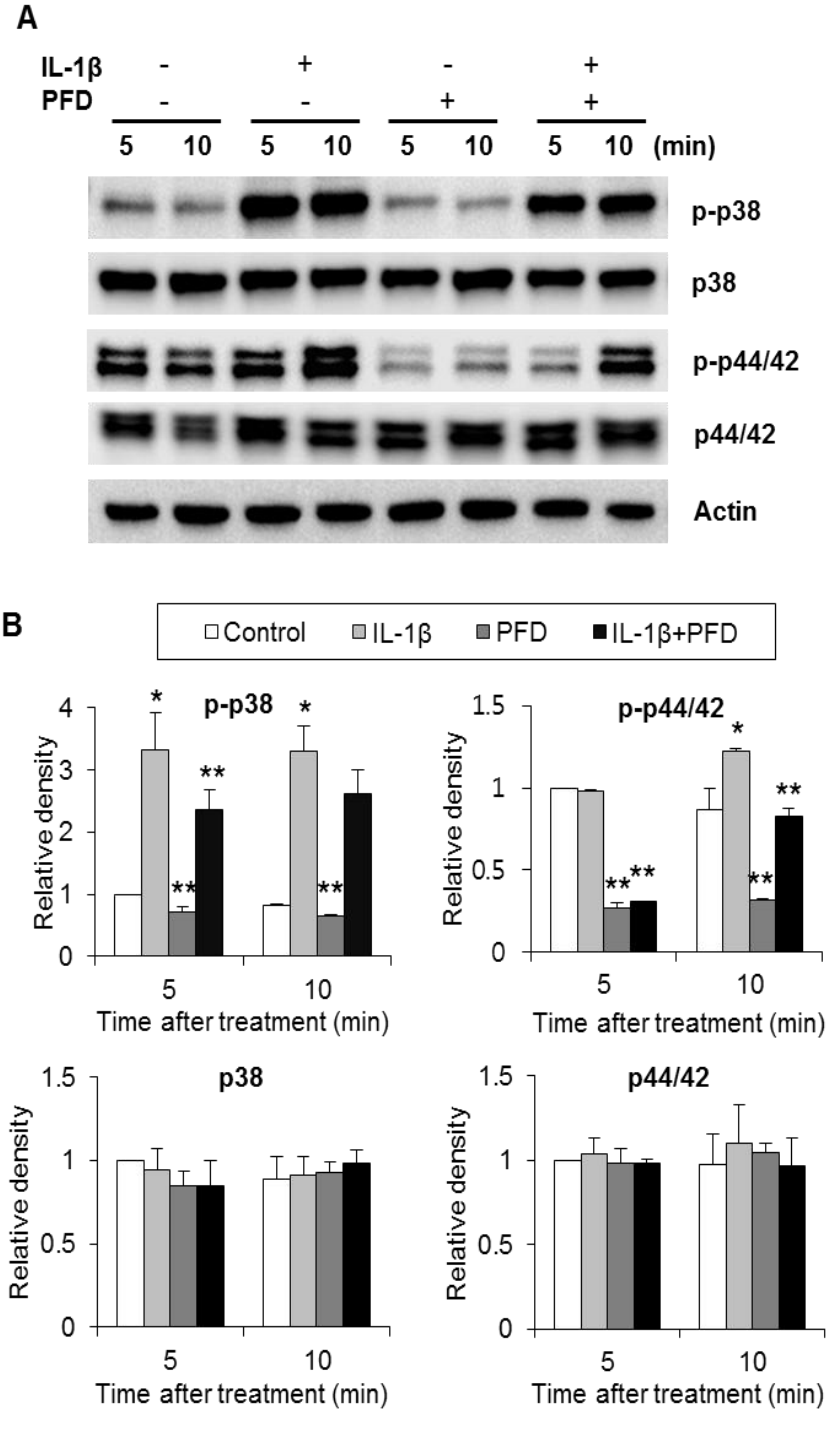
A
IL-1β - + - +
PFD - - + +
5 10 5 10 5 10 5 10 (min)
p-p38
p38
p-p44/42
p44/42
Actin
B
Control
IL-1β
PFD
IL-1β+PFD
p-p38
p-p44/42
p38
p44/42
Relative density
Time after treatment (min)
5
10

Figure 6. Effect of pirfenidone (PFD) on IL-1β-induced phosphorylation of p38 and ERK (p44/42). (A) Orbital fibroblasts from TAO patients were treated with 10 ng/ml IL-1β in the presence or absence of 10 mM of PFD for the indicated time point (5 or 10 min) and immunoblottings were performed for phosphorylated p38 (p-p38) or phosphorylated p44/42 (p-p44/42). Representative bands from independent experiments of 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 *vs.* untreated control; **P < 0.05 *vs.* IL-1β-treated cells).

5. Pirfenidone has superior potency in decreasing of IL-1β-induced HA increase in orbital fibroblasts compared to dexamethasone

In light of a previous report that dexamethasone attenuates IL-1β induced increases in HA levels,[11] we compared the inhibitory effect of pirfenidone on HA expression to that of dexamethasone. After co-treating 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. The inhibitory effect of pirfenidone on IL-1β-induced HA increase (96% reduction of the IL-1β-induced levels) was significantly greater than that of dexamethasone (72% reduction of the IL-1β-induced levels; Figure 7).

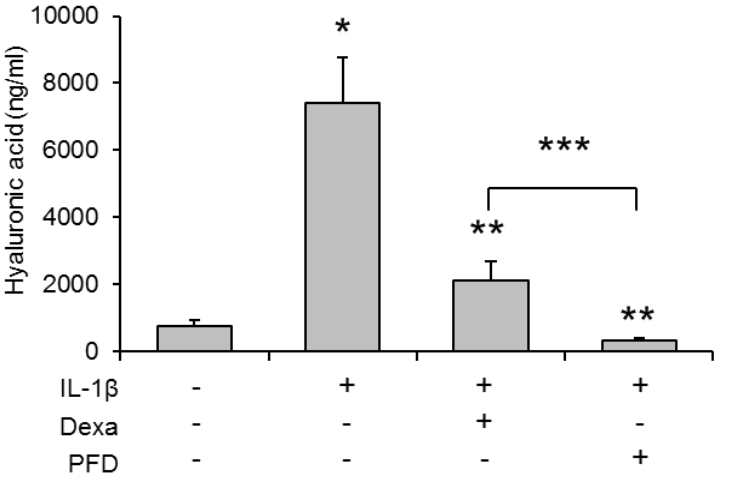

Figure 7. Comparison of the efficacy of pirfenidone (PFD) and dexamethasone (Dexa) in decreasing hyaluronic acid (HA) levels. Orbital fibroblasts of TAO patients were treated with 10 mM PFD or 100 nM Dexa; co-treated with 10 ng/ml IL-1β and 10 mM PFD, or 10 ng/ml IL-1β and 100 nM Dexa, or left untreated, as indicated, for 48 hours. Then, HA levels in the culture media were measured by ELISA. Data are expressed as the mean ± SD of four independent replicates (*P < 0.05 *vs.* untreated control; **P < 0.05 *vs.* IL-1β-treated cells; ***P < 0.05 between PFD and Dexa).

IV. DISCUSSION

Our results demonstrate that pirfenidone inhibits IL-1β-induced HA production in primary cultured orbital fibroblasts from TAO patients by interfering with the MAPK-mediated HAS expression. Considering the possible roles of HA and HAS in the pathogenesis of TAO, prevention of abnormal expression of these molecules could be an effective treatment for TAO.

TAO is an autoimmune component of Graves' disease characterized by intense inflammation in the setting of volume expansion.[1-8] Orbital connective tissues and extraocular muscles of TAO undergo an often dramatic remodeling, resulting in organ dysfunction.[6-8] At the heart of this, tissue remodeling is related with the disordered accumulation of the pivotal glycosaminoglycan; HA, in orbital connective tissues.[6,39] HA is originally a natural and abundant component of the extracellular matrix (ECM) of most tissues, where it serves a function in maintaining structure, flexibility, and the hydrated environment of tissue.[40] However, under conditions of stress, HA is produced excessively and contribute to inflammation.[40-42] HA in the native ECM exists in large polymeric form that does not promote inflammation, whereas, in fragmented/depolymerized form, HA induces gene expression in a variety of cell types and signals many inflammatory responses by leukocytes, endothelium and mesenchymal cells.[40-42] Because chronic inflammation is widely considered a major predisposing factor to fibrosis in all

organs, HA appears to affect the process of both inflammation and fibrosis.[40,42] HA exhibits a remarkable avidity for water. When hydrated, each molecule can occupy a volume 10,000 times greater than that of an equivalent mass of albumin. Thus, tissues accumulating HA often expand greatly.[40] In addition, unlike many other complex carbohydrates, it lacks a core protein.[43] Since protein synthesis is not required, HA production proceeds rapidly.[43] Furthermore, orbital fibroblasts exhibit a novel phenotype including exaggerated responses to proinflammatory cytokines such as IL-1β.[9-12] The magnitude of enhanced HA synthesis by IL-1β was considerably greater in orbital fibroblasts than that found in their dermal counterparts.[9-12,16] This exaggerated capacity of orbital fibroblasts to express high levels of HA in an anatomic site-selective manner could represent an important basis for immune responses localized to the orbit in Graves' disease.[9-12,16] Therefore, we believe that the development of successful remedies targeting this HA synthesis is essential in treatment for TAO.

TAO remains a poorly understood process, in large part because access to tissues early in the disease is limited and because no robust and complete animal models of Graves' disease have yet been devised.[14] The orbital fibroblasts used in this current experiments were all derived from orbital tissues of patients with inactive stage of TAO. All TAO patients were biochemically euthyroid with anti-hyperthyroid medications at the time of surgery, and their clinical activity scores were two or less. This could cause the similar responses between TAO and

normal cells.[32,36,44] Because previous reports showed that IL-1β mRNA is appreciably overexpressed in orbital tissues of patients in the active stage compared to those in the inactive stage,[45] and that IL-1β promoted all three pathological aspects of active TAO: inflammation, HA production, and adipogenesis, a stimulation of orbital fibroblasts with IL-1β in vitro can mimic orbital inflammation in TAO.[12-14,27,36] This was the reason we used IL-1β to stimulate HA production in orbital fibroblasts from TAO patients.

Synthesis of HA occurs at the cell surface and involves the activities of three synthetic enzymes, HAS1, -2, and -3.[30,31,40] Each isoform is encoded by a separate gene localizing to a different human chromosome.[30,31,40] Moreover, each isoform is associated with a characteristic pattern of tissue distribution and product chain length.[30,31,40] All three enzymes are expressed by orbital fibroblasts from patients with TAO in response to IL-1β.[11] However, little is known about differences in regulation and biological functions of the three enzymes of HAS in TAO. Testing the effects of drugs with inhibition for HA synthesis could help to clarify these issues. In this present study, orbital fibroblasts of TAO expressed much lower levels of *HAS1* mRNA under basal culture conditions than those of *HAS2* and *HAS3* in agreement with previous report by Kaback and Smith.[11] Of note, when activated by IL-1β, the relative expression of *HAS1* mRNA was higher than those of *HAS2* and *HAS3*. As a result, the attenuating effect of pirfenidone on IL-1β–induced *HAS1* mRNA was more prominent than those of *HAS2* and *HAS3*.

Similarly, in the study for unfettered HA production in fibroblast-like synoviocytes of rheumatoid arthritis,[46] *HAS2* and *HAS3* mRNA were constitutively expressed, while *HAS1* mRNA was very low or undetectable. Stimulation with IL-1β revealed that *HAS1* mRNA was readily inducible, resulting in a manifold increase in *HAS1* mRNA as well as in significantly elevated levels of HA as determined by ELISA. On the other hand, this induction of *HAS1* by IL-1β occurred at later time points although inductions of all isoforms of *HAS* by IL-1β were time-dependent. The differences in inducibility observed in this study suggest that each isoform may play a distinct role in the inflammatory response of orbital fibroblasts with regard to magnitude and timing.

In this study, IL-1β–induced *HAS* mRNA expression in orbital fibroblasts, at least in part, appears to be mediated through the two components of the MAPK-mediated signaling pathway, ERK and p38. These observations are in line with previous reports on signaling pathways utilized by IL-1β in orbital fibroblasts: up-regulation of prostaglandin E2 synthesis and induction of IL-6 expression.[32,33,35] In addition, pirfenidone abolished the increasing effect on p38 phosphorylation by IL-1β at the time point of 5 min but not 10 min, suggesting that p38 phosphorylation for HA production might be transient and earlier than ERK. These results are in agreement with the previous report, induction of IL-6 by IL-1β in orbital fibroblasts using the p38 and ERK-mediated pathway: the very rapid, transient phosphorylation of p38.[32]

However, the inhibitory effect of pirfenidone on the ERK and p38 MAPK activities was relatively incomplete when compared with its distinct effect on HA production. These results suggest the existence of other pharmacological mechanisms of pirfenidone in attenuating the IL-1β-induced HA production in orbital fibroblasts, in addition to inhibition of the MAPK signaling pathway shown here. Another major route by which IL-1β regulates cell activation is the Smad signaling cascade.[47,48] Especially, c-Abl–independent Smad signaling has been shown to be involved in HA production in different cell types.[49] Thus, it will be interesting to study this pathway in orbital fibroblasts of TAO.

Pirfenidone is an orally available pyridine derivative that has anti-fibrotic and anti-inflammatory effects in organs such as lung, liver, and kidney.[21,22] Since its discovery as an anti-fibrotic agent in a hamster model of bleomycin-induced pulmonary fibrosis, pirfenidone has been clinically evaluated for its safety and efficacy in numerous fibrotic disorders such as idiopathic pulmonary fibrosis, multiple sclerosis, primary sclerosing cholangitis, chronic hepatitis C, myelofibrosis, neurofibromatosis, and fibrotic renal disorders.[22] Although the precise pharmacological action mechanism of pirfenidone is incompletely understood, it exhibits both anti-inflammatory and anti-fibrotic effects.[22] An important aspect of the anti-fibrotic mechanism of pirfenidone is associated with its inhibition of both production and activity of TGF-β, whereas that of the anti-inflammatory effects in part involves its ability to inhibit the expression of

potent pro-inflammatory cytokines, TNF-α and IL-1.[22] In the murine endotoxin shock model, pirfenidone potently suppresses production of TNF, IL-6, and IFN-γ at the translation level.[50] Furthermore, it has recently reported that pirfenidone showed anti-fibrotic and anti-inflammatory effects in orbital fibroblasts from patients with TAO by inhibiting TIMP-1 and COX-2 expression with superior potency compared to that of dexamethasone.[26,27] In concordance with these previous reports, our results showing the inhibitory effects of pirfenidone on the IL-1β-induced increase in HA and HAS suggest the anti-inflammatory effect of this agent on orbital fibroblasts from patients with TAO. The inhibitory effect of pirfenidone on HA production was not associated with nonspecific drug cytotoxicity (data not shown), as shown by MTT analysis of cell viability in the previous study.[26] Similar results of drug safety have been obtained by another *in vitro* study, which demonstrated that pirfenidone inhibits proliferation, migration, and collagen contraction in human Tenon's fibroblasts without causing significant cytotoxicity.[23] In addition, Choi et al. showed that the co-treatment with pirfenidone did not influence the induction of IL-1 receptor 1 in orbital fibroblast.[27]

Corticosteroids are widely accepted as the first-line drug for treating TAO, particularly when the disease is in the active inflammatory stage. However, when fibrosis occurs, corticosteroids are of limited clinical benefit.[17-20] Although both prednisolone and pirfenidone were effective in reducing lung inflammatory edema

in a bleomycin-induced murine pulmonary fibrosis model, only pirfenidone significantly suppressed pulmonary fibrosis.[51] Kim et al. found that dexamethasone, unlike pirfenidone, did not significantly reduce collagen production in orbital fibroblasts, measured by the hydroxyproline assay.[26] In this study, we demonstrated that pirfenidone showed a superior inhibitory effect on IL-1β-induced HA production in orbital fibroblasts compared with even 10-fold dose of dexamethasone to that used in the previous study.[11] Therefore, pirfenidone may be a promising candidate for future use in TAO with superior potency compared with corticosteroid.

Most clinical trials describe pirfenidone as being generally well tolerated in doses up to 2400 mg daily.[52,53] The most common adverse effects include gastrointestinal ones (nausea, dyspepsia, diarrhea, abdominal discomfort, vomiting, and anorexia), fatigue, sedation, and rash by photosensitivity. They appear to be dose-related and typically resolve completely once the drug is withdrawn. Pirfenidone has shown its excellent oral or intravenous absorption and its pharmacokinetic behavior has been studied in mice, beagle dog, horse, sheep, and human. Treatment with pirfenidone could be safer and have fewer side effects than high-dose glucocorticoids.[51-53] Further research and clinical studies are necessary to ensure the safety of pirfenidone treatment and to ascertain the optimum doses for prevention and treatment of TAO.

V. CONCLUSION

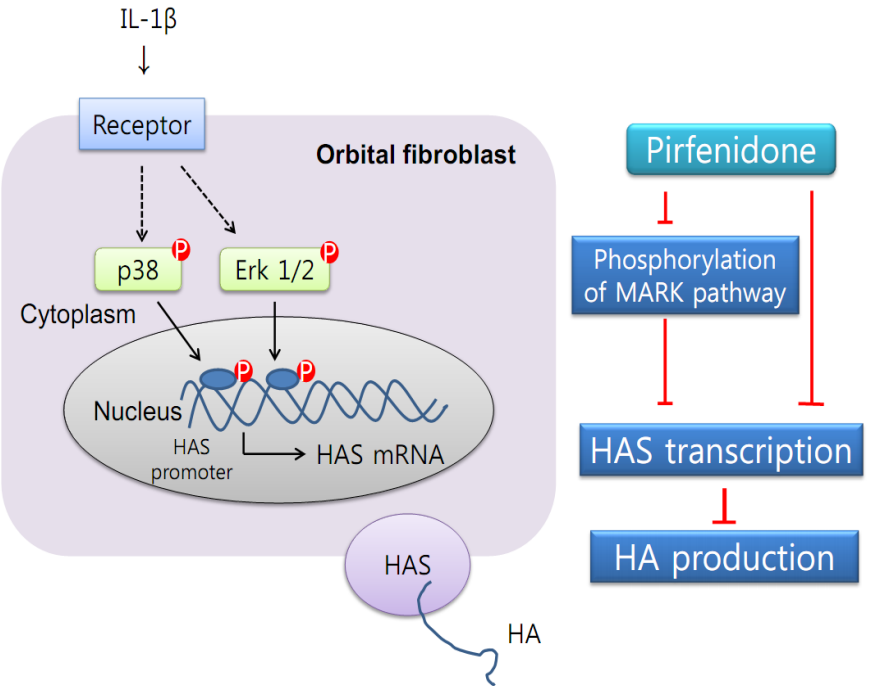

Figure 8. Proposed model for the inhibitory effects of pirfenidone on IL-1β-induced HA production.

In summary, pirfenidone has inhibitory effects on IL-1β-induced HA production in orbital fibroblasts from patients with TAO. Its pharmacological effect is, at least in part, related to the interruption of MAPK-mediated HAS expression (Figure 8). These results propose that pirfenidone may be a promising candidate for treatment of TAO that exerts its therapeutic effects by modulating orbital fibroblasts.

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ABSTRACT (IN KOREAN)

갑상샘눈병증 안와섬유모세포에서 interleukin-1β로 유도된 히알루론산 증가에 대한 pirfenidone의 억제 작용

<지도교수 이 종 복>

연세대학교 대학원 의학과

정 승 아

갑상샘눈병증은 그레이브스씨병과 연관되어 발생하는 자가면역 질환으로, 과도한 염증반응, 안와 내 지방결체조직과 외안근의 부피 팽창을 특징으로 하며 이로 인한 여러 시기능장애를 일으킨다. 특히, 친수성인 히알루론산이 안와조직 내 과도하게 침착되는 것이 부피 팽창의 주된 원인으로 생각되고 있다. 현재까지는 비선택적 광범위 면역억제제인 스테로이드가 활동성 갑상샘눈병증의 주요 치료제로 쓰이지만, 섬유화가 진행되면 치료효과가 급감하고 고용량 투여로 인한 부작용으로 사용에 한계가 있다. 최근 비교적 적은 부작용으로 항섬유화, 항염증 효과를 나타내는 것으로 알려지고 있는 pirfenidone이 갑상샘눈병증 안와섬유모세포에서 히알루론산 생성을 억제할 수 있는지 알아보았다.

갑상샘눈병증 환자에서 안와감압술 때 제거하게 되는 안와 지방결체조직으로부터 안와섬유모세포를 일차배양하였다. 세포독성이 없는 농도의 pirfenidone (10nM)을 안와섬유모세포에 처치하여, interleukin-1β (IL-1β) 자극에 의해 유도된 히알루론산 생성증가가 억제되는지 ELISA를 이용하여 확인하였다. IL-1β 자극에 의해 증가되는 히알루론산 합성효소 발현이 pirfenidone에 의해

억제되는지는 RT-PCR과 Western blot으로 확인하였다. 또한, IL-1β로 증가되는 히알루론산 합성효소 발현에 mitogen-activated protein kinase (MAPK)s가 관여하는지를 각각의 억제제를 이용하여 RT-PCR로 확인하였다. Pirfenidone이 IL-1β에 의해 활성화된 MAPKs의 인산화를 억제하는지는 Western blot으로 확인하였다. 마지막으로, pirfenidone의 히알루론산 합성에 대한 억제 효과를 ELISA를 이용하여 dexamethasone과 비교하였다.

Pirfenidone은 IL-1β 자극에 의해 증가되는 히알루론산 생성을 농도 비례하여 강력하게 억제하였다. IL-1β에 의해 증가되는 히알루론산 합성효소의 발현 역시 효과적으로 억제하였다. IL-1β로 증가되는 히알루론산 합성효소의 발현은 MAPKs 중 p38와 ERK경로를 통해 유도되었고, 이들의 인산화는 pirfenidone에 의해 감소되었다. Pirfenidone는 IL-1β에 의한 히알루론산 증가를 억제하는데 있어 dexamethasone보다 우월하였다.

Pirfenidone는 갑상샘안병증 안와섬유모세포에서 IL-1β 자극에 의해 증가된 히알루론산 생성을 효과적으로 억제할 수 있었고, 여기에는 MAPKs에 의해 매개되는 히알루론산 합성효소 발현을 억제하는 과정이 관여하는 것으로 나타났다. 본 연구를 통해 갑상샘눈병증에서 pirfenidone의 치료제로서의 가능성을 확인할 수 있었다.

핵심되는 말 : 갑상샘눈병증, 안와섬유모세포, 염증, 히알루론산, 히알루론산 합성효소, interleukin-1β, mitogen-activated protein kinase, pirfenidone