Antifibrotic Effect of Pirfenidone on Orbital Fibroblasts of Patients with Thyroid-Associated Ophthalmopathy by Decreasing TIMP-1 and Collagen Levels

Hyunmi Kim,[1,2] *Youn-Hee Choi,*[*,2,3] *Soo Jung Park,*[1,2] *Sang Yeul Lee,*[4] *Sung Joo Kim,*[5] *Ilo Jou,*[1,2] *and Koung Hoon Kook*[*,2,6]

Purpose. The aim of this study was to determine the antifibrotic effects of pirfenidone in orbital fibroblasts of patients with thyroid-associated ophthalmopathy (TAO).

Methods. The effects of interleukin (IL)-1β and of fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and transforming growth factor (TGF)-β on the induction of tissue inhibitors of metalloproteinases (TIMP)-1 were assessed in orbital fibroblasts of TAO patients. TIMP-1 protein levels were measured by ELISA and Western blot analyses, and TIMP-1 activity was assessed by reverse zymography. The effect of pirfenidone on TIMP-1 induction in orbital fibroblasts was evaluated with the same methods using dexamethasone as a reference agent. A hydroxyproline assay was used to determine the effect of pirfenidone and dexamethasone on collagen production in orbital fibroblasts, and the tetrazolium-based MTT assay was used to assess pirfenidone cytotoxicity.

Results. IL-1β strongly and dose dependently increased the level of active TIMP-1 protein, whereas FGF, PDGF, and TGF-β did not significantly induce TIMP-1 protein. Pirfenidone was more effective than dexamethasone in inhibiting IL-1β–induced increases in TIMP-1, reducing TIMP-1 levels to less than those in untreated controls at a minimal concentration (5 mM). Moreover, pirfenidone effectively decreased hydroxyproline levels in orbital fibroblasts, whereas dexamethasone had no effect on hydroxyproline levels. Pirfenidone exhibited no toxicity in orbital fibroblasts at the concentrations used.

Conclusions. These results indicate that nontoxic concentrations of pirfenidone have significant antifibrotic effects on orbital fibroblasts from patients with TAO. (*Invest Ophthalmol Vis Sci.* 2010;51:3061–3066) DOI:10.1167/iovs.09-4257

Thyroid-associated ophthalmopathy (TAO) is an autoimmune component of Graves' disease, which is primarily caused by autoantibodies to thyroid-stimulating hormone (TSH) receptors.[1] Symptoms of TAO include cosmetic deficits, such as lid swelling, proptosis, and lid retraction, and functional deficits that manifest as limited ocular motion and visual loss. Inflammation dominates the early histopathology[2]; at this stage, corticosteroids remain the mainstay of medical treatment if specific therapy is indicated, despite the harmful side effects of these agents.[3] However, once fibrotic changes develop in orbital tissues, few effective medical interventions are available.[4]

Fibrosis is defined as overgrowth, hardening, or scarring of tissues, and it is attributed to excess deposition of extracellular matrix (ECM) components, including collagen.[5] Therefore, the amount of collagen produced can be an indicator of the extent of fibrosis. During the formation of collagen triple helices, prolyl 4-hydroxylase catalyzes the hydroxylation of proline in collagen to form 4-hydroxyproline residues, which stabilize the helices.[6] Thus, hydroxyproline, an amino acid unique to collagenous molecules, is an indicator of the amount of collagen and thus may serve as a characteristic biochemical marker of the extent of fibrosis.[7]

Two groups of enzymes, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), are involved in physiological homeostasis of the ECM; MMPs degrade ECM proteins to prevent excess deposition,[8] and TIMPs control the activities of MMPs to prevent excess degradation.[9,10] Pathologic increases in TIMP levels are associated with ECM deposition and result in fibrosis,[5,11] and regression of fibrosis is achieved by neutralization of TIMP-1.[12] It has recently been demonstrated that orbital fibroblasts, which are believed to be the major cells involved in the pathogenesis of TAO, express high levels of active TIMP-1 in response to interleukin (IL)-1β stimulation,[13] indicating that TIMP-1 may be an attractive therapeutic target to reduce fibrosis in TAO.

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a small molecule that has proved to exhibit novel antifibrotic effects in several experimental disease models, such as pulmonary fibrosis[14–16] and liver cirrhosis.[17,18] Pirfenidone reduces fibronectin synthesis in cultured human retinal pigment epithelial cells[19] and inhibits proliferation, migration, and collagen contraction in human Tenon's fibroblasts.[20]

In this study, we showed that pirfenidone effectively decreases the levels of TIMP-1 and hydroxyproline in orbital fibroblasts of TAO patients. To the best of our knowledge, this study is the first to evaluate the antifibrotic effects of pirfenidone in orbital fibroblasts in TAO.

From the Departments of [1]Pharmacology and [6]Ophthalmology and the [2]Chronic Inflammatory Disease Research Center, Ajou University School of Medicine, Suwon, Korea; the [3]Department of Physiology, Ewha Womans University School of Medicine, Seoul, Korea; the [4]Department of Ophthalmology, Yonsei University College of Medicine, Seoul, Korea; and the [5]Department of Ophthalmology, Kim's Eye Hospital, Seoul, Korea.

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*Each of the following is a corresponding author: Koung Hoon Kook, Department of Ophthalmology, Ajou University School of Medicine, Suwon, Korea; drkook@ajou.ac.kr.

Youn-Hee Choi, Department of Physiology, Ewha Womans University School of Medicine, Seoul, Korea; yc@ewha.ac.kr.

Table 1. Characteristics of TAO Patients and Control Patients without Inflammatory Disease from Whom Fibroblast Strains Were Obtained

	Patients with TAO (n = 6)	Control Patients without TAO (n = 6)
Mean age, y (range)	41 (29–61)	45 (21–67)
Sex, m/f	4/2	2/4
Smoking, yes/no	1/6	2/6
Graves' disease	6/6	0/6
Radioactive iodine therapy	0/6	—
Surgery	1/6	—
Methimazole	5/6	—
Treatment TAO	6/6	0/6
Surgery	6/6	—
Prednisone	4/6	—
Radiation	0/6	—
Euthyroid	5/6	6/6
TSH-receptor antibodies	6/6	0/6
Clinical activity score (range)	3 (1–5)	—

Materials and Methods

Cell Culture

Human orbital fibroblasts were cultivated from orbital fatty connective tissue obtained as surgical waste during decompression surgery for severe TAO or during orbital surgery to resolve noninflammatory conditions. These activities were undertaken after informed consent was obtained from the donors according to procedures approved by the Institutional Review Board of Ajou University Hospital and the tenets of the Declaration of Helsinki. Six different orbital fibroblast culture strains were obtained from each of six individual patients with TAO and six control patients without TAO. At the point of decompression surgery, five patients were euthyroid; one was in a hypothyroid state because of previous total thyroidectomy. Four 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, and all six patients had experienced at least 6 months of inactive disease and no corticosteroid therapy before orbital decompression. Patient characteristics are presented in Table 1. Once obtained, cells were maintained in Dulbecco's modified essential medium (DMEM; HyClone, Logan, UT) containing 10% (vol/vol) fetal bovine serum (FBS; HyClone) and antibiotics in a humidified 5% CO_2 incubator at 37°C. Cells at passages 3 to 8 were used in the present study.

Enzyme-Linked Immunosorbent Assay

TIMP-1 protein 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 cells/well. After stabilization for 48 hours, cells were treated with IL-1β (Biosource, Camarillo, CA) or growth factors (fibroblast growth factor [bFGF]; Biosource), platelet-derived growth factor (PDGF)-BB (Biosource), and transforming growth factor (TGF)-β1 (R&D Systems, Minneapolis, MN). In some experiments, cells were cotreated with IL-1β and pirfenidone (Sigma-Aldrich, St. Louis, MO) or dexamethasone (Sigma-Aldrich), as described. After treatment for 48 hours, the concentration of TIMP-1 in supernatants was determined using a sandwich ELISA method. Briefly, 96-well plates were precoated overnight at room temperature with a capture antibody (R&D Systems). After three washes, plates were blocked with phosphate-buffered saline (PBS; pH 7.4) containing 5% (wt/vol) sucrose and 1% (wt/vol) bovine serum albumin, followed by incubation with detection antibody (R&D Systems). After washing, plates were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 20 minutes and then incubated with color reagents A (substrate, hydrogen peroxide) and B (tetramethylbenzidine; R&D Systems) for 30 minutes. Absorbance was measured spectrophotometrically at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). The concentration of TIMP-1 in each sample was determined by reference to a standard curve generated using known amounts of TIMP-1 (R&D Systems).

Western Blot Analysis

TIMP-1 protein levels in supernatants of orbital fibroblasts (5×10^5 cells/well), cultured and treated as described for ELISA, were measured by immunoblotting. After treating fibroblasts for 48 hours, supernatants (normalized for cell numbers) were solubilized in 5× Laemmli buffer. Proteins were separated by SDS-PAGE on 12% (wt/vol) gels and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience, Keene, NH). 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 and then was incubated at 4°C overnight with anti–TIMP-1 antibody. After three washes with TBST (TBS containing 0.1% [vol/vol] Tween-20), membranes were incubated with HRP-conjugated anti–mouse secondary antibodies, and immunoreactive proteins were detected by incubation with Western blot substrate (AbFrontier, Seoul, Korea) followed by exposure to X-ray film.

Reverse Zymography

Orbital fibroblasts (5×10^5 cells/well) were seeded in six-well cell culture plates and treated according to the same protocol used for ELISA. After incubation for 24 hours, supernatants were collected and assayed by reverse zymography as described previously,[21] with minor modifications. Briefly, supernatants were diluted (with normalization for cell number), solubilized in 5× Laemmli buffer, and separated by electrophoresis on gels prepared to contain MMP-9 (20 ng/mL). Gels were shaken for 1 hour in 2.5% (vol/vol) Triton X-100 after electrophoresis, washed in distilled water, and incubated overnight in developing solution at 37°C. Gels were then washed in distilled water, stained with Coomassie Brilliant Blue for 2 hours, and incubated with shaking in destaining solution.

Cytotoxicity Assay

Cell growth and death were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) assays. Orbital fibroblasts (1×10^5) were seeded into 24-well culture plates and treated according to the same protocol used for ELISA. After washing cells with PBS (pH 7.4), 50 μL MTT solution (1 mg/mL in PBS) was added to each well, and plates were incubated for 4 hours at 37°C. Plates were then centrifuged at 3000g for 10 minutes, the supernatant was carefully removed, and the remaining adherent cells were dissolved in 200 μL dimethyl sulfoxide (DMSO). Optical density was measured at 570 nm using a microplate reader.

Hydroxyproline Assay

Total collagen production was assessed by measuring hydroxyproline levels in culture media using a hydroxyproline assay kit (Nanjing Jiancheng Biochemical Institute, Nanjing City, P.R. China) according to the manufacturer's instructions. Briefly, cells were seeded in six-well culture plates at a final density of 1×10^6 cells/well and were allowed to stabilize for 48 hours. After treatment with IL-1β or pirfenidone (or both) or dexamethasone for 48 hours, the levels of hydroxyproline in culture media were determined.

Statistical Analysis

Tukey's HSD tests were used in analyzing the stimulatory effects of IL-1β, FGF, PDGF, or TGF- β on TIMP-1 production and the inhibitory effects of pirfenidone or dexamethasone on TIMP-1 or collagen production. The effect of pirfenidone on the viability of cells was analyzed with one-way analysis of variance (ANOVA) test. $P < 0.05$ was considered statistically significant.

RESULTS

Increase of TIMP-1 Protein by IL-1β in Orbital Fibroblasts

We initially examined the influence of IL-1β and three other growth factors, FGF, PDGF, and TGF-β, on TIMP-1 levels in human orbital fibroblasts of patients with TAO. Cells were treated with 10 ng/mL of each cytokine or growth factor for 48 hours, and TIMP-1 protein levels in culture media were analyzed by ELISA, as described in Materials and Methods. IL-1β induced a marked increase in TIMP-1 protein levels that reached nearly 2.5-fold that of controls; in contrast, the addition of FGF, PDGF, or TGF-β did not substantially increase TIMP-1 levels (Fig. 1A). TIMP-1 protein levels were also increased in orbital fibroblasts of normal tissue, but to a much lesser extent (1.2-fold compared with control) than in those of TAO patients (Fig. 1B). Therefore, subsequent experiments were performed using orbital fibroblasts of TAO patients. Incubation of such cells for 48 hours with a range of IL-1β concentrations (1–20 ng/mL) caused a dose-dependent increase in TIMP-1 (Fig. 2A). The increase in TIMP-1 levels after treatment with 10 ng/mL IL-1β was verified by immunoblotting (Fig. 2B). This increase in TIMP-1 protein was associated with an increase in enzymatic activity, as revealed by reverse zymography analysis (Fig. 2C).

Attenuation of TIMP-1 by Pirfenidone in Orbital Fibroblasts

To examine the effects of pirfenidone on the IL-1β–induced increase in TIMP-1 level, we cotreated orbital fibroblasts with different concentrations of pirfenidone and 10 ng/mL IL-1β and measured the levels of TIMP-1 in culture media by ELISA. Guided by a previous in vitro study showing an antifibrotic effect of pirfenidone in human Tenon's fibroblasts at concentrations up to 1 mg/mL (5.4 mM),[20] we used pirfenidone at 1, 5, and 10 mM. As shown in Figure 3A, pirfenidone significantly attenuated the IL-1β–induced TIMP-1 increase at concentrations of 5 and 10 mM, decreasing TIMP-1 protein concentrations to 18% and 14% of the values obtained after IL-1β stimulation. Strikingly, the levels of TIMP-1 protein in cells treated with these two concentrations of pirfenidone were lower than those in non–IL-1β–treated control cells (44% and 34% of control values at 5 and 10 mM, respectively). This inhibitory effect of pirfenidone on the IL-1β–induced TIMP-1 increase was verified by immunoblotting (Fig. 3B).

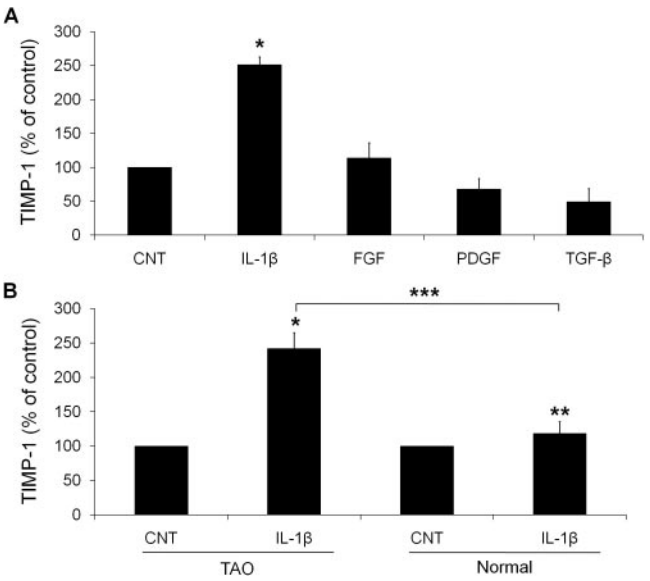

FIGURE 1. Effect of IL-1β and growth factors (bFGF, PDGF BB, and TGF-β) on TIMP-1 levels in orbital fibroblasts. (**A**) Primary cultures of orbital fibroblasts (5×10^5) of TAO patients were incubated with or without 10 ng/mL IL-1β, bFGF, PDGF BB, or TGF-β for 48 hours. After treatment, supernatants were collected and TIMP-1 protein levels were measured in each culture supernatant by ELISA. Results are expressed as percentage of untreated control values presented as mean ± SD; normalized values are shown. (*$P < 0.05$ vs. untreated control [CNT]; $n = 6$.) (**B**) Orbital fibroblasts obtained from control patients without TAO and patients with TAO were treated with or without 10 ng/mL IL-1β for 48 hours, and TIMP-1 levels were measured by ELISA. Data are expressed as percentage of control values presented as mean ± SD. (*$P < 0.05$ and **$P < 0.05$ vs. each untreated control [CNT]; ***$P < 0.05$ between orbital fibroblasts of patients and controls.)

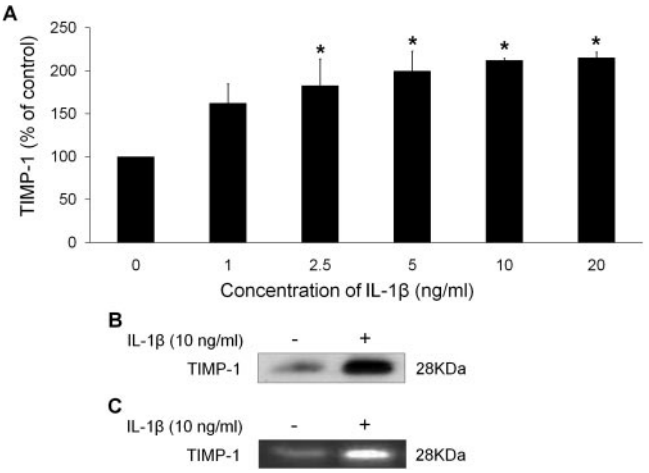

FIGURE 2. IL-1β dose dependence of TIMP-1 induction in orbital fibroblasts and activity of induced TIMP-1. (**A**) After treatment of orbital fibroblasts (5×10^5) of TAO patients with the indicated concentrations of IL-1β (0–20 ng/mL) for 48 hours, TIMP-1 levels in culture supernatants were measured by ELISA. Data are expressed as percentage of control values presented as mean ± SD. (*$P < 0.05$ vs. untreated control; $n = 6$). (**B**) Western blot analysis was performed to verify TIMP-1 protein levels in cell culture supernatants of orbital fibroblasts. Orbital fibroblasts (5×10^5) of TAO patients were treated with 10 ng/mL IL-1β for 48 hours using the same protocol described for ELISA. After treatment for 48 hours, supernatants of orbital fibroblasts of TAO patients were collected and analyzed by immunoblotting using anti–TIMP-1 antibody. Data are representative of at least three experiments. (**C**) The enzymatic activity of TIMP-1 expressed in orbital fibroblasts was assessed by reverse zymography. After treatment with 10 ng/mL IL-1β for 24 hours, supernatants of orbital fibroblasts (5×10^5) were collected and analyzed in gels containing MMP-9 (20 ng/mL). Representative data from three independent experiments are shown.

Effect of Pirfenidone on the Viability of Orbital Fibroblasts

MTT assays were performed to analyze pirfenidone toxicity and to clarify whether the effects reported could have been influenced by harmful pirfenidone side effects. As shown in Figure 4, the viability of primary orbital fibroblasts was not affected by 48-hour incubation with pirfenidone at concentrations of up to 10 mM.

Comparative Efficacy of Pirfenidone and Dexamethasone in Decreasing of TIMP-1 in Orbital Fibroblasts

In light of a previous report that dexamethasone attenuates IL-1β–induced increases in TIMP-1 levels,[22] we compared the inhibitory efficacy of pirfenidone on TIMP-1 expression with

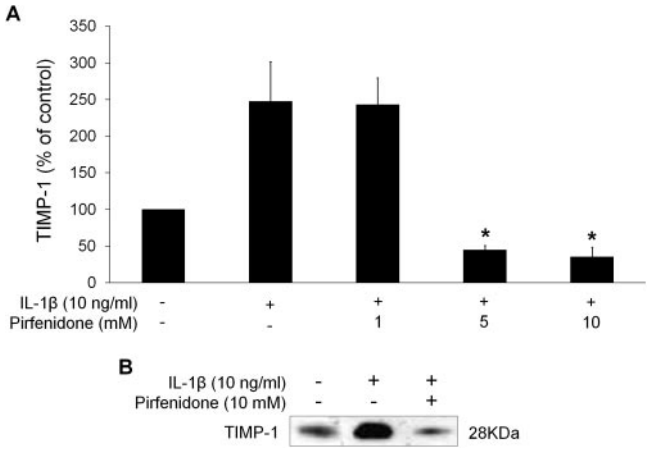

FIGURE 3. Effect of pirfenidone on IL-1β–induced TIMP-1 levels in orbital fibroblasts. Primary cultures of orbital fibroblasts of patients with TAO (5×10^5) were cotreated with 10 ng/mL IL-1β and different concentrations of pirfenidone (1, 5, or 10 mM) for 48 hours. (**A**) TIMP-1 levels in culture supernatants were measured by ELISA. Data are expressed as percentage of control values presented as mean ± SD. (*$P < 0.05$ vs. IL-1β–treated cells; $n = 6$.) (**B**) Western blot analysis for TIMP-1 protein in culture supernatants of orbital fibroblasts treated with IL-1β with or without 10 mM pirfenidone. Data are representative of at least three experiments.

that of dexamethasone. After cotreating orbital fibroblasts with 10 ng/mL IL-1β and 10 mM pirfenidone or 100 nM dexamethasone for 48 hours, TIMP-1 levels in the culture medium were measured by ELISA. As shown in Figure 5A, the inhibitory effect of pirfenidone on IL-1β–induced TIMP-1 upregulation (52% reduction of IL-1β–induced levels) was significantly greater than that of dexamethasone (42% reduction of IL-1β–induced levels). Pirfenidone also inhibited TIMP-1 expression in orbital fibroblasts not treated with IL-1β; again, the inhibitory effect of pirfenidone (76% reduction compared with untreated control) was significantly greater than that of dexamethasone (45% reduction compared with untreated control). To confirm the inhibitory effects of pirfenidone and dexamethasone on TIMP-1 expression, we immunoblotted the culture media for TIMP-1 protein after treating cells as described for the experiment in Figure 5A. Consistent with the ELISA results, Western blot analysis showed that the inhibitory effect of pirfenidone on TIMP-1 protein levels was significantly greater than that of dexamethasone (Fig. 5B). The 28-kDa band of

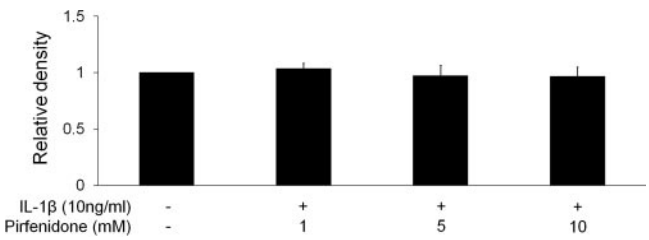

FIGURE 4. Effect of pirfenidone on the viability of orbital fibroblasts. Orbital fibroblasts of TAO patients (1×10^5) were seeded into 24-well culture plates and cotreated with 10 ng/mL IL-1β and different concentrations of pirfenidone (1, 5, or 10 mM) for 48 hours. After treatment, cells were washed, incubated with 50 μL MTT solution (1 mg/mL) for 4 hours at 37°C, dissolved in DMSO, and analyzed spectrophotometrically, to assess cell proliferation/cytotoxicity. Results are expressed as mean ± SD in relative OD units (compared with controls); normalized values are shown. Assays were performed at least three times in quadruplicate; data from a representative experiment are shown.

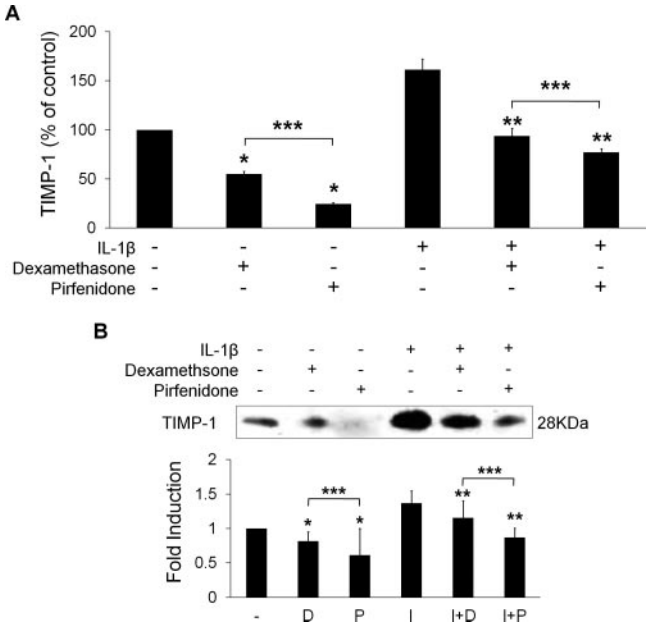

FIGURE 5. Comparison of the efficacy of pirfenidone and dexamethasone in decreasing TIMP-1 protein levels. Orbital fibroblasts of TAO patients were treated with 10 mM pirfenidone or 100 nM dexamethasone, cotreated with 10 ng/mL IL-1β and 10 mM pirfenidone or 10 ng/mL IL-1β and 100 nM dexamethasone, or left untreated, as indicated, for 48 hours. (**A**) TIMP-1 protein levels in culture medium were measured by ELISA. Results are expressed as percentage of untreated control values presented as mean ± SD; normalized values are shown. (*$P < 0.05$ vs. untreated control; **$P < 0.05$ vs. IL-1β–treated cells; ***$P < 0.05$ between pirfenidone and dexamethasone.) (**B**) Western blot analysis was performed to assess TIMP-1 protein levels under the same conditions. *Upper*: representative blots from four independent experiments. *Lower*: densitometric quantification of protein bands on blots. Data in bar graphs are expressed as percentage of untreated control values presented as mean ± SD. (*$P < 0.05$ vs. untreated control [-]; **$P < 0.05$ vs. IL-1β–treated cells [I]; ***$P < 0.05$ between pirfenidone [P] and dexamethasone [D]; $n = 6$.)

TIMP-1 protein that was evident in culture medium was not detectable in whole-cell lysates analyzed by immunoblotting (data not shown).

Effect of Pirfenidone on Collagen Production in Orbital Fibroblasts

To evaluate the effect of pirfenidone on collagen production in orbital fibroblasts, we assayed cell cultures for hydroxyproline content, as described in Materials and Methods. After cotreatment of orbital fibroblasts with 10 ng/mL IL-1β and 10 mM pirfenidone or 100 nM dexamethasone for 48 hours, the level of hydroxyproline in cell culture medium was measured. Cells treated with 10 mM pirfenidone or 100 nM dexamethasone only, without IL-1β, were also included in the assay to determine the separate effects of these agents on the basal level of collagen produced by fibroblasts. As shown in Figure 6, in both IL-1β–treated and untreated orbital fibroblasts, pirfenidone significantly decreased the expression of hydroxyproline, reducing hydroxyproline levels to 41% and 44% of control levels, respectively. In contrast, dexamethasone had no significant inhibitory effect in IL-1β–treated or untreated fibroblasts. Notably, in contrast to the significant effect of IL-1β on the induction of TIMP-1 expression shown, the difference in hydroxyproline content between IL-1β–treated and untreated fibroblasts did not reach statistical significance.

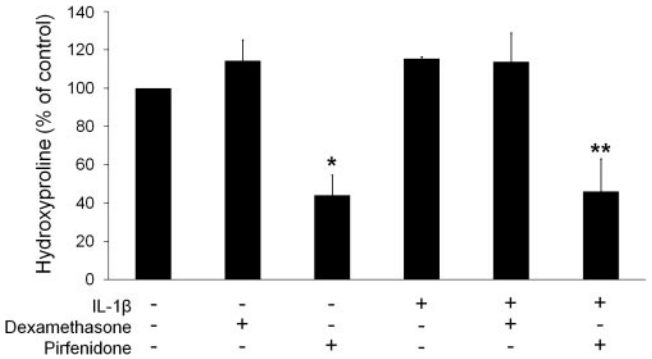

FIGURE 6. Effect of pirfenidone and dexamethasone on the amount of collagen produced in orbital fibroblasts. After cotreating orbital fibroblast of TAO patients with 10 ng/mL IL-1β and 10 mM pirfenidone or 100 nM dexamethasone for 48 hours, the level of collagen in culture supernatants was assessed using a hydroxyproline assay. Results are expressed as percentage of untreated control values presented as mean ± SD. (*$P < 0.05$ vs. untreated control; **$P < 0.05$ vs. IL-1β–treated cells; $n = 6$.) Data are representative of three independent experiments.

DISCUSSION

TIMPs inhibit the activity of MMPs, which degrade ECM materials such as collagen, and thus have an important role in maintaining the balance between ECM production and degradation. A pathologic increase in TIMP levels contributes to ECM deposition and leads to fibrosis.[5,11] In orbital fibroblasts, which are thought to be major players in the pathogenesis of TAO,[13] IL-1β induces TIMP-1 expression, largely by increasing the transcriptional activity of the corresponding *TIMP-1* gene.[22] In agreement with this previous report, our results demonstrated that IL-1β is a potent inducer of TIMP-1 in orbital fibroblasts. A number of in vitro studies have demonstrated that various stimulants increase TIMP-1 in other types of cells, such as bFGF in lung fibroblasts,[23] PDGF-BB in chondrocytes,[24] and TGF-β1 in mesothelial cells.[25] However, in our hands, none of these three growth factors produced a noticeable effect on TIMP-1 levels in orbital fibroblasts. Interestingly, unlike other site-specific fibroblasts that are derived from the mesoderm, orbital fibroblasts are known to originate from the neuroectoderm.[26] Orbital fibroblasts have unique site-specific characteristics compared with other types of fibroblasts.[1] Proinflammatory cytokines, such as leukoregulin and IL-1β, potentiate the induction of orbital fibroblast genes that play important roles in orbital inflammatory responses but do not stimulate the inflammatory response in dermal fibroblasts.[27,28] Such site-specific characteristics of orbital fibroblasts may be relevant to our results.

In addition, unlike some fibroblasts, orbital fibroblasts display cell surface expression of CD40, a receptor originally described in B lymphocytes.[29] The level of CD40 in TAO orbital fibroblasts is high compared with that in normal orbital fibroblasts.[29] Activation of CD40 by its ligand, CD154, on the surfaces of T lymphocytes promotes the upregulation of several inflammatory cytokines, including IL-6, IL-8, and macrophage chemoattractant protein-1, exclusively in TAO orbital fibroblasts.[29] Consistent with these TAO-dependent differences in orbital fibroblast behavior, our study showed that the IL-β–induced increase in TIMP-1 was significantly greater in orbital fibroblast of TAO patients than in those of controls without TAO.

Controlling a pathologic increase in TIMP levels could be a novel therapeutic strategy for halting the progression of fibrosis. In an animal model of chronic liver disease characterized by the development of hepatic fibrosis, TIMP-1 promoted liver fibrosis in association with decreased levels of the active form of MMP-2,[30] and fibrosis regression was achieved by neutralization of TIMP-1.[12] In the present study, we showed that pirfenidone effectively decreased TIMP-1 protein levels not only in IL-1β–treated orbital fibroblasts but also in untreated orbital fibroblasts of TAO patients. Moreover, pirfenidone significantly reduced collagen production of orbital fibroblasts. Our results are in agreement with those of previous studies using other fibrosis disease models. In studies using a cyclosporine-induced renal fibrosis model, increases in both TIMP-1 and collagen III mRNA expression were attenuated and reversed by treatment with pirfenidone.[31] Using a hepatic fibrosis model, Di Sario[17] reported that pirfenidone administration downregulated TIMP-1 in association with a 70% reduction of collagen deposition. In addition to its antifibrotic effects, which are mediated by reduction of TIMP-1, pirfenidone has been shown to attenuate the expression of heat shock protein 47 in a bleomycin-induced pulmonary fibrosis murine model[15] and to downregulate intercellular adhesion molecule-1 in cultured human synovial fibroblasts.[32]

Although the detailed mechanisms by which pirfenidone decrease the levels of TIMP-1 and hydroxyproline remain to be determined, it is unlikely that its inhibitory effects on orbital fibroblasts are the result of cytotoxicity. This conclusion is supported by the results of our MTT assay showing no influence of pirfenidone on cellular viability and the results of previous in vivo and in vitro studies. For example, in a clinical trial of pirfenidone in patients with multiple sclerosis, most participants were able to tolerate this agent at a high dose (2400 mg/d) for 1 year without any significant adverse effects.[33] Another trial conducted on 73 patients with idiopathic pulmonary fibrosis who were enrolled to receive pirfenidone for 9 months revealed no significant difference in safety profiles between patients receiving pirfenidone and those receiving placebo; both groups reported photosensitivity, stomach discomfort, anorexia, and nausea.[34] Similar results have been obtained by in vitro studies, which demonstrated that pirfenidone inhibits proliferation, migration, and collagen contraction in human Tenon's fibroblasts without causing significant cytotoxicity.[20] Despite these extremely promising indications, the possible adverse effects of pirfenidone should be systematically addressed in advance of clinical application.

In this study, we demonstrated that pirfenidone showed a superior inhibitory effect on both TIMP-1 expression and hydroxyproline accumulation in orbital fibroblasts compared with dexamethasone. 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.[4] 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.[14] In agreement with these previous results, we found that dexamethasone, unlike pirfenidone, did not significantly reduce collagen production in orbital fibroblasts, measured by the hydroxyproline assay. Recently, other agents that hold promise in treating TAO have been described, including the tyrosine kinase inhibitors imatinib mesylate and nilotinib (AMN107), which effectively block PDGF receptor phosphorylation in orbital fibroblasts[35] and have been reported to have antifibrotic effects in other types of diseased cells.[36,37]

In summary, our results provide initial evidence that pirfenidone reduces the levels of TIMP-1 protein and hydroxyproline in orbital fibroblasts of TAO patients. These results indicate that pirfenidone modulates orbital fibroblast reactions in TAO

and thus may be a promising candidate for future use as an antifibrotic agent.

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