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ORIGINAL ARTICLE

Antifibrotic Effect of Pirfenidone on Human Pterygium Fibroblasts

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ABSTRACT

Objective: The effects of pirfenidone were investigated on cultured human pterygium fibroblasts (HPFs).

Methods: HPFs were obtained from pterygium surgery and subjected to primary culture. After treatment with 0.5, 1.0 or 1.5 mg/mL pirfenidone, MTT and cell migration assays were performed, and procollagen secretion and TGF-β expression were measured by Western blotting and immunofluorescence analysis.

Results: Pirfenidone had a significant inhibitory effect on HPF proliferation, migration and collagen synthesis. There were no differences between the cells treated with 0.5, 1.0 and 1.5 mg/mL pirfenidone and the controls in the MTT assay. After 48 h of treatment with 1.0 or 1.5 mg/mL pirfenidone, TGF-β expression was significantly decreased.

Conclusions: These findings demonstrate that pirfenidone inhibits the proliferation, migration and procollagen secretion of HPFs at nontoxic concentrations by decreasing TGF-β expression. Thus, pirfenidone may be considered as a safe adjuvant for pterygium surgery to prevent recurrence.

Keywords: Antifibrotic effect, fibroblast, pirfenidone, pterygium, TGF-beta

INTRODUCTION

A pterygium is a triangular-shaped overgrowth of the fibrovascular conjunctiva onto the nasal or temporal cornea. It is a common condition among outdoor workers and people who live near the equator. It may cause injection, ocular irritation, cosmetic problems, astigmatism and visual impairment. Surgical removal is indicated for these symptomatic cases.[1,2] The exact pathogenesis of pterygium is unknown, but the destruction of limbal stem cells resulting from chronic exposure to ultraviolet radiation is thought to play a role.[1,3,4]

Although various techniques have been developed for pterygium surgery, the most important complication is disease recurrence. Simple excision, in which the underlying sclera is left uncovered (i.e. bare sclera technique), is thought to contribute to the high rate of recurrence (24–89%).[5,6] To reduce the recurrence rate, various surgical techniques, including covering the underlying sclera with amniotic membrane or a conjunctival graft, and adjunctive procedures such as radiation therapy and the application of chemical agents (e.g. mitomycin C, 5-fluorouracil and thiotepa) have been attempted.[6–11]

Conjunctival autografts are associated with relatively low recurrence rates (2–39%) and good cosmetic outcomes on long-term follow-up, and the application of mitomycin C with a conjunctival autograft has been shown to further reduce the recurrence rate of pterygia.[1,12–17] However, mitomycin C is associated with scleral thinning or ulceration as a vision-threatening complication.[18,19]

Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a new drug that has been reported to have antifibrotic effects in some experimental disease models, including pulmonary fibrosis[20–22] and liver cirrhosis.[23,24] Pirfenidone inhibits cellular proliferation and migration, and collagen contraction in human Tenon's fibroblasts.[25,26]

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The purpose of this study was to evaluate the antifibrotic effect of pirfenidone on primary cultured pterygeal fibroblasts and to examine the utility of pirfenidone as a potential adjunctive treatment to reduce the pterygium recurrence rate.

MATERIALS AND METHODS

Seven different human pterygium fibroblasts (HPFs) cell lines were cultivated from subconjunctival connective tissue gained from pterygium patients as a surgical specimen during the surgical removal of primary ones, with their informed written consent. Note, however, that the diagnosis of pterygium was purely clinical and no histological confirmation was performed. After surgical excision, subconjunctival connective tissue was propagated in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies), 100 U/mL penicillin, and 100 g/mL streptomycin (Gibco Life Technologies). The cells were maintained at 37 °C in 5% CO_2 in a humidified atmosphere, and the medium was changed every 3 days thereafter. Cells between the third and sixth passages were used for all experiments.

3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) Assay

Human pterygium fibroblasts were seeded in 96-well tissue culture plates at a concentration of 10^4 cells/well for 48 h in DMEM containing 10% FBS. These quiescent cells were then washed and immersed in DMEM containing 10% FBS supplemented with increasing concentrations of each pirfenidone (0, 0.5, 1.0 or 1.5 mg/mL) in triplicate for 48 h. After incubation with 180 µL of DMEM and 20 µL of 5 mg/mL MTT for 4 h at 37 °C, the MTT solution was discarded.

A total of 180 µL of DMSO (Amresco, Solon, OH) was used to dissolve the formazan precipitate by shaking the dishes for 10 min at 200 rpm on an orbital shaker. The absorbance was computed at a wavelength of 540 nm in each well using a microplate reader (Bio-Rad, Munich, Germany). Cells that stained positively with MTT were considered viable cells.

Cell Migration Assay

Cell migration was monitored from a confluent area to an area that was mechanically denuded of cells (i.e. scratch-wound assay). Initially, HPFs were grown to a confluent monolayer and then serum-deprived for 24 h. After the medium was discarded, a scratch was created in a straight line across the cells with a p1000 pipette tip. The plates were then rinsed with PBS to remove the suspended cells and incubated with DMEM supplemented with 0, 0.5, 1.0 or 1.5 mg/mL pirfenidone. Wound closure was monitored and photographed after 48 h under a light microscope and the number of migrating cells between the edges was counted.

Procollagen Type I C-peptide (PIP) Enzyme Immunoassay (EIA)

Cells were treated with 0, 0.5, 1.0 or 1.5 mg/mL pirfenidone for 48 h. The supernatants were processed for analysis using a PIP EIA kit (Takara, Tokyo, Japan) according to the protocol provided by the manufacturer. The color reaction was measured at 450 nm.

Western-Blot Assay

Human pterygium fibroblasts were treated with 0, 0.5, 1.0 or 1.5 mg/mL pirfenidone for 48 h. The cells were then lysed in mammalian protein extraction reagent (RIPA buffer; Sigma, St. Louis, MO). The final protein concentrations were determined with a BCA protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's specifications.

Prepared samples were heated to 100 °C for 5 min; for each sample the same amount of total protein was added to a well of a 12% acrylamide gel and resolved by SDS–PAGE. The separated proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Inc.). Nonspecific binding was blocked by incubation with 5% nonfat milk for 1 h prior to overnight incubation with 1:500 anti-TGF-β antibodies (Cell Signaling, Beverly, MA) at 4 °C. After washing, the membrane was incubated with a 1:1000 dilution of anti-rabbit IgG HRP-linked antibodies (Cell Signaling) in PBS-Tween. Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

Immunofluorescence

Human pterygium fibroblasts were treated with 0, 0.5, 1.0 or 1.5 mg/mL pirfenidone on coverslips for 48 h. They were then fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton in PBS for 20 min. The cells were then incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal anti-TGF-β (1:25 dilution; Abcam). The secondary antibodies (green) were

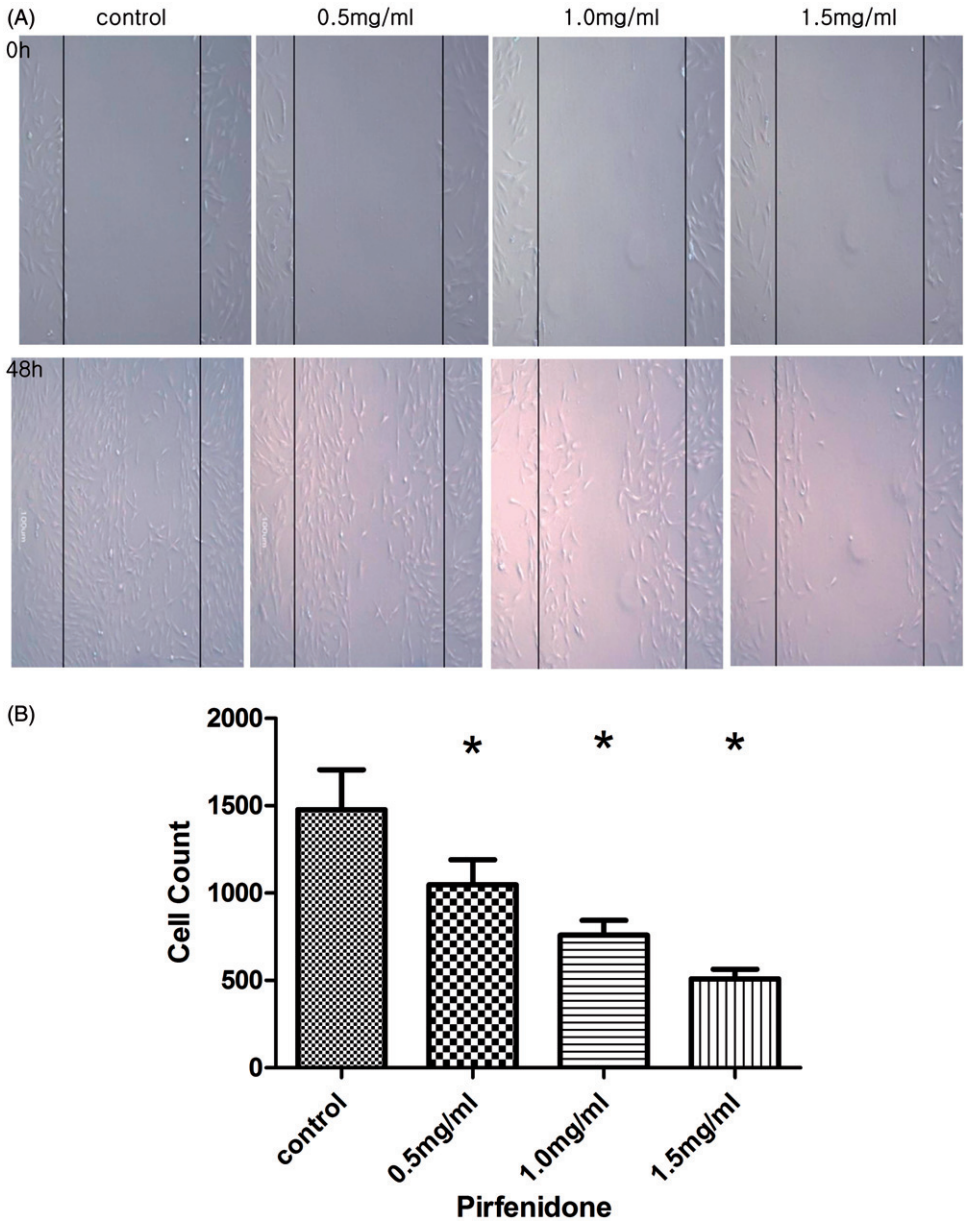

FIGURE 1. Effect of pirfenidone on HPF migration. (A) Light microscopic images showed a decreased number of migrated cells at 48 h, after a scratch wound was inflicted across the cells and treated with 0, 0.5, 1.0 and 1.5 mg/mL pirfenidone. (B) Each treated group had a statistically significant difference compared with the others (*$p < 0.05$). The most obvious suppression was shown at a concentration of 1.5 mg/mL pirfenidone. The data in each bar are the mean number of cells that migrated through the membrane in three separate experiments. Magnification, ×40.

Alexa Fluor 488 goat anti-rabbit IgG (H + L), used at a 1:500 dilution for 2 h. Nuclei were stained with Hoechst 33342. The cells, then, were analyzed with a fluorescence microscope.

Statistical Analysis

A one-way analysis of variance and a *post hoc* test (Scheffé's test) were used to identify significant differences between the control and pirfenidone-treated groups. $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Pirfenidone on HPF Motility

The most significant reduction in HPF motility was noted at pirfenidone concentration of 1.5 mg/mL (Figure 1). All treated and untreated cells migrated during 48 h of observation. At concentrations of 0 (control), 0.5, 1.0 and 1.5 mg/mL, the average migrated cell counts were 1478, 1048, 759 and 510, respectively; each had a significant difference from the others ($p < 0.05$).

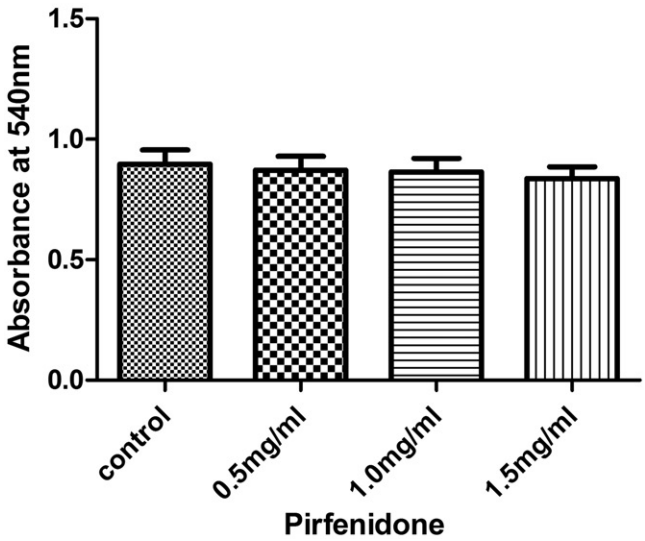

FIGURE 2. The toxicity of pirfenidone in HPFs. HPFs were treated with 0(control), 0.5, 1.0 and 1.5 mg/mL pirfenidone, for 48 h, and the toxicity was detected by an MTT assay. Absorbance was read at 540 nm. The error bars represent the SD of four experiments (*$p < 0.05$).

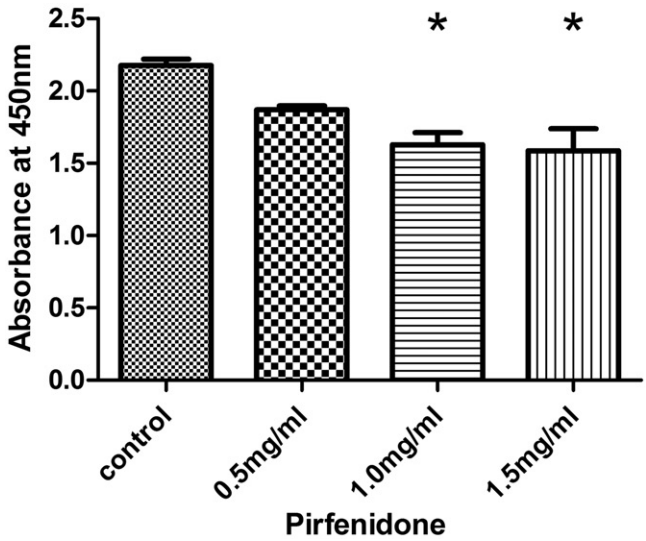

FIGURE 3. Effects of pirfenidone on type I procollagen secretion in HPFs. HPFs were treated with 0 (control), 0.5, 1.0 and 1.5 mg/mL pirfenidone, for 48 h and type I procollagen protein secretion was evaluated by an ELISA. The error bars represent the SD of three independent experiments (*$p < 0.05$).

Effect of Pirfenidone on HPF Viability

MTT assays were performed to analyze pirfenidone toxicity and to clarify whether the reported effects could have been influenced by harmful pirfenidone side effects. As shown in

Figure 2, HPF viability was not affected by 48 h of incubation with pirfenidone at 0, 0.5, 1.0 or 1.5 mg/mL.

Effect of Pirfenidone on Collagen Production in HPFs

To evaluate the effect of pirfenidone on collagen production by HPFs, we conducted an assay for PIP. Compared with the controls, there was a significant decrease in collagen synthesis at concentrations of 1.0 and 1.5 mg/mL ($p < 0.05$) (Figure 3).

Effect of Pirfenidone on TGF-β Expression

Human pterygium fibroblasts were treated with 0, 0.5, 1.0 or 1.5 mg/mL pirfenidone for 48 h. The cell lysates were subjected to immunoblot analysis for TGF-β expression. Compared with the controls, there was significant downregulation of TGF-β expression at concentrations of 1.0 and 1.5 mg/mL ($p < 0.05$) (Figure 4). We further examined the distribution of TGF-β in HPFs using immunocytochemistry. Figure 5 shows that TGF-β was expressed in the cells. Staining for TGF-β decreased significantly after exposure to pirfenidone.

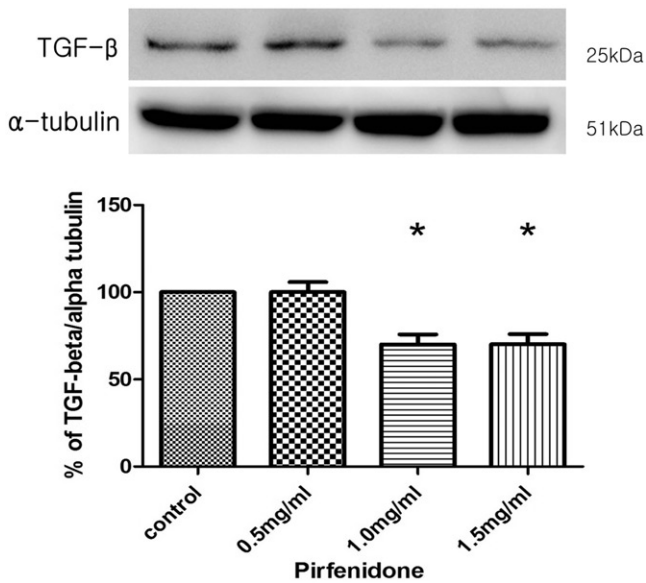

FIGURE 4. Expression of TGF-β in HPFs. Western blotting showed that pirfenidone reduced the protein level of TGF-β compared with control cells. . The error bars represent the SD of three independent experiments (*$p < 0.05$).

DISCUSSION AND CONCLUSIONS

Studies have shown that there may be a benefit to using both a conjunctival or limbal autograft and mitomycin C. An overview of these studies suggested that increased exposure (dose or duration) to intraoperative and post-operative mitomycin C is associated with greater efficacy along with an increased risk of complications. Scleral ulceration and delayed

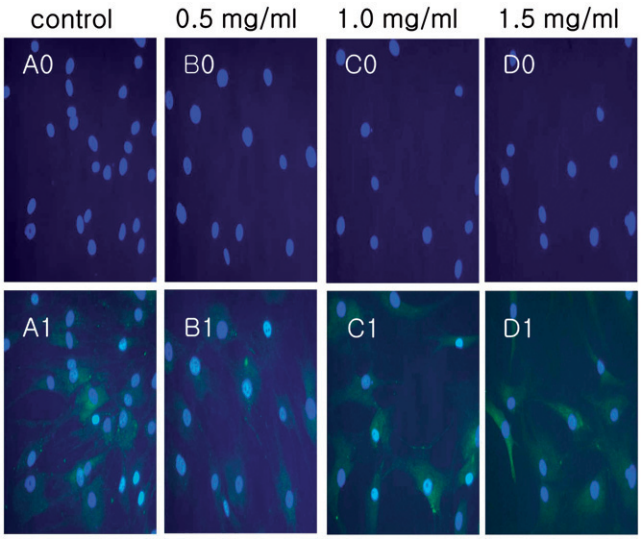

FIGURE 5. Detection of TGF-β using immunofluorescence. Immunofluorescence showed marked cytoplasmic and nuclear staining for TGF-β in control HPFs (A1) and relatively moderate staining after treatment with 0.5 (B1), 1.0 (C1) and 1.5 (D1) mg/mL pirfenidone, respectively. The images show a reduction in TGF-β after treatment with pirfenidone. A0, B0, C0 and D0 are the corresponding negative controls. Immunofluorescence was performed with Alexa Fluor 488-conjugated antibodies (green). The nuclei were counterstained with Hoechst 33342 (blue). Magnification, ×400.

conjunctival epithelialization were associated with both intraoperative and post-operative mitomycin C use, and there is evidence that increased complications are related directly to an increased concentration and extent of exposure.[27]

We would like to find efficient antifibrotic agents for use as adjuvants in pterygium surgery with reduced toxicity to prevent recurrence. Pirfenidone is an antifibrotic agent whose activity and safety has been established in tissues such as lung,[28,29] liver,[30] and kidney.[31] In ophthalmology, it also has antifibrotic effects on human orbital fibroblasts in thyroid-associated ophthalmopathy[25] and glaucoma filtration surgery.[26]

In this study, we demonstrated the antifibrotic effect of pirfenidone on the migration of, and collagen synthesis by HPFs obtained from patients undergoing pterygium surgery. Our analyses showed that pirfenidone exhibits its inhibitory effects on HPFs in a dose-dependent manner. In the case of mitomycin C, the antifibrotic effect is related to toxic side effects. A single exposure of human Tenon's capsule fibroblast to mitomycin C for 5 min at the clinical concentration (0.4 mg/mL) resulted in 62% of cells death after 48 h. On the contrary,[32] pirfenidone in the human eye is likely to be safe because our MTT assay showed no statistically significant change in cell viability between the control- and pirfenidone-treated groups. Moreover, in patients with pulmonary fibrosis, the drug has been applied orally at a daily dosage of 1800 mg with minimal adverse effects.[28] However, our study possesses some limitations in that an *in vitro* experiment is not enough to assess the toxicity of pirfenidone. The *in vivo* toxicity of pirfenidone compared with mitomycin C should be explored in an animal model.

Lin et al.[26] suggested that pirfenidone suppressed the mRNA and protein expression of TGF-β isoforms in a dose-dependent manner in human Tenon's fibroblasts. Similar to their findings, our ELISA, Western blot and immunofluorescencel data showed that the antifibrotic mechanism of pirfenidone in HPFs involves the suppression of TGF-β expression and decreased collagen synthesis. It is believed that we are the first to use pirfenidone in primary cultured HPFs, and we showed the possible use of pirfenidone as an adjuvant for reducing the recurrence of pterygium.

In summary, pirfenidone had inhibitory effects on the proliferation and migration of, and collagen synthesis by primary cultured HPFs. Its antifibrotic effects may be related to the suppression of TGF-β expression. Thus, pirfenidone may be a potential adjuvant for pterygium surgery. Further studies on animal models are, however, necessary to confirm pirfenidone's safe use as pterygium adjuvant therapy.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article. This study was supported by a faculty research grant of Ajou University School of Medicine for 2009.

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