A PPAR-Gamma Agonist Rosiglitazone Suppresses Fibrotic Response in Human Pterygium Fibroblasts by Modulating the p38 MAPK Pathway

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Pterygium is an ocular disorder that clinically manifests as a benign growth of conjunctival epithelial tissues over the cornea. The characteristic origin and location of pterygium is the nasal limbus, from where it grows toward the cornea. Pterygium is characterized by extensive cell proliferation, connective tissue remodeling, angiogenesis and chronic inflammation resulting in redness and irritation in the area.1,2 The condition can significantly affect vision in advanced cases owing to a distortion of the shape in the cornea3 and can progress to ocular surface squamous neoplasia.4 Surgical excision is the standard treatment for pterygium. Wound healing after pterygium excision often leads to fibrosis characterized by recurrent pterygium. The bare sclera technique for instance is associated with high postsurgical recurrence rates reported to range from 38% to 88%.5-8

Because fibrovascular growth in recurrent pterygia is more extensive than that in primary disease,9 recurrence is of major concern as it worsens prognosis. To reduce postsurgical recurrences, mitomycin C application10 has been widely attempted, but numerous adverse effects such as scleral stromalysis11 and corneal melting12 have been reported. There is therefore the need for a safer and more targeted curative and preventive therapy.

Studies have shown the overexpression of transforming growth factor β1 (TGF-β1), a profibrotic agent, in pterygium tissues compared to normal conjunctiva cultures and in recurrent pterygium fibroblast cultures compared to primary pterygium fibroblast cultures.13,14 TGF-β1 signaling stimulates fibroblast migration, proliferation, and myofibroblast differentiation.15 The presence of myofibroblasts in pterygium is suggestive of the involvement of TGF-β1 in the pathogenesis and progression of the disorder.16 Other research findings also support the presence of myofibroblasts in the fibrovascular tissue of primary and recurrent pterygia.17,18

Previous research works have shown several synthetic ligands of peroxisome proliferator-activated receptor γ (PPAR-γ) that are effective in preventing fibrosis in various organs including the skin, kidney, and cornea.19-21 Notably, corneal fibrosis following...
laser refractive ablation was suppressed by the topical application of a PPAR-γ ligand. The topical application of these synthetic PPAR-γ ligands and the possibility of preventing corneal haze are advantageous to their potential use in ophthalmology. We thus aimed to determine the antiangiogenic properties of the PPAR-γ ligand rosiglitazone in cultured primary human pterygium fibroblasts and the possible signaling pathways mediating any observed effect.

**METHODS**

**Ethical Statement**

Pterygium tissues were obtained from patients who underwent pterygium excision surgery. This study protocol was approved by the Institutional Review Board of our institute (YWMR-15-0-053), and all subjects were treated in accordance with the Declaration of Helsinki. Written informed consent to participate in this research was obtained from each patient.

**Isolation and Culture of Primary Human Pterygium Fibroblasts**

The pterygium tissue sample was placed in a culture plate and allowed to attach. Dulbecco’s modified Eagle’s medium F12 (DMEM/F12; Welgene, Gyeongsan, Korea) with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Gibco Life Technologies, Foster City, CA, USA) was added and the tissue incubated at 37°C in a humidified atmosphere of 5% CO₂ during which cells migrated from the explant. Migrated cells were passaged with 0.25% trypsin (Gibco Life Technologies) and subcultured in DMEM (Welgene) with 5% FBS. Passage 3-5 cells were used for all experiments.

**Western Blotting**

The pterygium fibroblasts were seeded into culture plates in DMEM with 5% FBS. After attachment, serum-free media was added 8 hours prior to treatment to promote cellular quiescence. A 10-mM stock solution of rosiglitazone (Cayman Chemical, Ann Arbor, MI, USA) in dimethyl sulfoxide (DMSO) was added to cells at two concentrations (25 and 75 μM) for a 30-minute pretreatment. TGF-β1 (PeproTech, Seoul, Korea) was then added to a 2.5-ng/mL concentration. The cells were incubated for 48 hours after which total protein was extracted with the Pro-prep protein extraction kit (Intron Biotechnology, Korea). Proteins were resolved with 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Milan, Italy). The membrane was blocked in 5% skimmed milk solution and incubated overnight at 4°C with monoclonal antibodies against α-smooth muscle actin (α-SMA; Sigma-Aldrich Corp., St. Louis, MO, USA), fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), vimentin, and tubulin (Cell Signaling Technology, Beverly, MA, USA). The membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 1 hour at room temperature. Proteins were detected with enhanced chemiluminescence (ECL). Images were captured by UVP Bio-Spectrum 600 imaging system (Ultra-Violet Products Ltd. Cambridge, UK). The expression of target proteins relative to tubulin was evaluated by densitometry using ImageJ software (http://image.nih.gov/ij/index.html; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). To determine if the observed effects of rosiglitazone were PPAR-γ dependent, the cells were pretreated with 1 μM of a synthetic PPAR-γ antagonist GW9662 (Cayman Chemical) to block PPAR-γ receptor function. The effect of rosiglitazone on intrinsic TGF-β1 production was also investigated by western blotting with anti-TGF-β1 antibodies (Cell Signaling Technology). Major signaling pathways downstream of TGF-β1 were investigated to see their relation to the observed effects of rosiglitazone. Primary antibodies to p38 MAPK, p44/42MAPK (Erk1/2), and Smad2/3 (Cell Signaling Technology) were used. The p38 kinase inhibitor SB203580 (InvivoGen, San Diego, CA, USA) was used to further investigate the effect of blocking p38 MAPK signaling on myofibroblast differentiation and extracellular matrix (ECM) synthesis. The effect of rosiglitazone on the expression of matrix metalloproteinases (MMPs)-1, 3, and 9 were also investigated.

**Quantitative Real-Time PCR**

The pterygium fibroblasts were seeded and treated with GW9662, rosiglitazone, and TGF-β1 as described above. After 48 hours of incubation, the cells were harvested and total RNA extracted using an RNA extraction kit (Qiagen, Germany) according to the manufacturer’s protocol. The quality and quantity of the RNA extract were assessed by spectrophotometry. The qualified RNA was reverse-transcribed into cDNA with LaboPass cDNA synthesis kit (Cosmo Genetech, Seoul, Korea) according to the manufacturer’s protocol and used for real-time PCR. Primer pairs of target genes used are given in Supplementary Table S1. The reaction was performed in a 10-μL reaction volume containing SYBR green PCR mix (Applied Biosystems Life Technologies, Foster City, CA, USA), cDNA, primers, and nuclease free water.

**Immunofluorescence Staining and Indirect Flow Cytometry**

The pterygium fibroblasts were cultured on glass chamber slides and pretreated with rosiglitazone (75 μM) in serum-free DMEM, followed by TGF-β1 (2.5 ng/mL). After 48 hours of incubation, the cells were fixed with 4% paraformaldehyde (PFA) followed by peroxidase and protein blocking. The slides were then incubated with primary antibodies against α-SMA and fibronectin overnight at 4°C and then with Alexa Fluor labelled secondary antibodies (Thermo Fisher Scientific, Rockford, IL, USA) and incubated at room temperature in the dark for 2 hours. The nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI), and immunofluorescence was visualized and digitally captured using a confocal microscope (Leica Microsystems, GmbH, Wetzlar, Germany). Internal control was performed using PBS and secondary antibodies without the primary antibodies. The proportion of α-SMA positive cells was quantified using indirect flow cytometry. Briefly, cell suspensions (approximately 1 × 10^6 cells/100 μL) prepared in stain buffer (ice cold PBS, 2% FBS, 5% sodium azide) were fixed for 10 minutes with 1% PFA. After washing, the cells were suspended in 500 μL permeability buffer (stain buffer + 0.2% saponin), incubated in the dark for 5 minutes, and washed again. The cell pellets obtained after centrifugation at 1500 rpm for 5 minutes were resuspended in permeability buffer and incubated with primary antibodies against α-SMA for 30 minutes on ice in the dark. After washing, the cells were then incubated with an Alexa Fluor 488-conjugated secondary antibody for another 30 minutes, washed, and analyzed by flow cytometry.

**Cell Viability Test**

The pterygium fibroblasts were seeded into a 96-well plate at a density of 5000 per well and incubated for 24 hours. The cells were then treated with rosiglitazone (25, 50, 75, and 100 μM).
Control cells were vehicle treated. After 48 hours of incubation, the cell counting kit 8 (CCK-8) assay was performed according to kit manufacturer’s protocol (Dojindo Laboratories, Kumamoto, Japan). After a 2-hour incubation, the optical absorbance was read at 450 nm on a microplate reader. The experiment was done in triplicate for each concentration of rosiglitazone.

Apoptosis Test

The pterygium fibroblasts were pretreated with rosiglitazone (25 and 75 μM) in serum-free DMEM after which TGF-β1 (2.5 ng/mL) was added. Cells with only TGF-β1 treatment were used as control. After 48 hours of incubation, the cells were washed with cold PBS and resuspended in 1X binding buffer at a concentration of 1 x 10^6 cells/mL. One hundred microliters of the solution with approximately 1 x 10^5 cells was transferred to a 5-mL culture tube and 5 μL each of FITC Annexin V and propidium iodide (PI) (BD Pharmingen, San Jose, CA, USA) added. After 15 minutes of incubation at room temperature in the dark, 400 μL 1X binding buffer was added to each tube and analyzed by flow cytometry within 1 hour.

Cell Migration Test

The pterygium fibroblasts were seeded into culture plates and incubated for 24 hours to allow the cells to attach to the plate surface. Further, scratch wounds were induced with a pipette tip (1000 μL), and the suspended cells were washed off. The cells were then pretreated with rosiglitazone (75 μM), followed by treatment with TGF-β1 (2.5 ng/mL) in serum-free media. The wounds were monitored using phase-contrast microscopy and photographed at 12, 24, and 48 hours. The ImageJ software was used to determine the wound area. Vehicle-treated cells were used as controls.

Statistical Analysis

The data were analyzed using Prism 5.0 software. Statistical comparisons were done using Student’s t-test or 1-way analysis of variance (ANOVA), as appropriate. All results are expressed as mean ± SD. Statistical significance was defined as P < 0.05 in all cases.

RESULTS

Rosiglitazone Decreased TGF-β1-Induced Synthesis of ECM Proteins and Myofibroblast Differentiation in Pterygium Fibroblasts Independent of PPAR-γ

Basal levels of α-SMA, a key marker for myofibroblast differentiation, and fibronectin, an ECM protein, in the pterygium fibroblasts were detectable (Fig. 1). There was a marked increase of approximately threefold in the expression of α-SMA (P < 0.0001, F[5, 12] = 31.75), (C) fibronectin (P < 0.0001, F[5, 12] = 145.1), and (D) vimentin (P = 0.1989, F[5, 12] = 1.745). #Represents P < 0.05 compared to vehicle-treated control cells; *represents P < 0.05 compared to only TGF-β1-treated cells.
the expression of vimentin (Fig. 2). Immunostaining results showed decreased expression of α-SMA and fibronectin in rosiglitazone-treated cells (Fig. 3A). Indirect flow cytometry results revealed that rosiglitazone treatment decreased the proportion of α-SMA-positive cells, which further confirmed its antifibrotic effect in pterygium fibroblasts (Fig. 3B).

Rosiglitazone Decreased Intrinsic TGF-β1 Production in Pterygium Fibroblasts Independent of PPAR-γ

Our results showed a dose-dependent decrease in the intrinsic expression of TGF-β1 when the cells were treated with rosiglitazone, indicating a modulatory effect of rosiglitazone on TGF-β1 protein synthesis. In addition, this effect was independent of PPAR-γ receptor, as cotreatment with rosiglitazone and GW9662 did not reverse the decreased synthesis of TGF-β1. The basal levels of αSMA and fibronectin proteins also tended to decrease in a dose-dependent manner subsequent to the decreased TGF-β1 expression (Fig. 4).

Rosiglitazone Inhibited TGF-β1-Induced Activation of p38 MAPK Signaling Pathway in Pterygium Fibroblasts

To determine the possible mechanisms underlying the antifibrotic effect of rosiglitazone, the major signal transduction pathways downstream of TGF-β1 were examined. As shown, TGF-β1 increased the activation of p38 MAPK, ERK1/2, and Smad2/3 from the basal level indicating increased signaling. However, the p38 MAPK signaling was decreased by rosiglitazone treatment as seen by the decreased phosphorylated p38 MAPK protein levels. This effect of rosiglitazone was not observed with ERK1/2 and Smad2/3 signaling as the phosphorylated protein levels remain unaltered (Fig. 5).

TGF-β1 Induces Synthesis of ECM Proteins and Differentiation of Myofibroblasts via p38 MAPK Signaling in Pterygium Fibroblasts

To test whether p38 was involved in the TGF-β1-dependent phenotypic transformation of myofibroblasts in pterygium fibroblasts, we first investigated the time course of p38 MAPK activation. As shown in Figure 6A, the TGF-β1-induced activation of p38 MAPK signaling peaked at 45 minutes and lasted for at least 24 hours. This indicated that rosiglitazone downregulated the p38 MAPK pathway. Maintenance of this downregulation by rosiglitazone on p38 activation for at least 24 hours (Fig. 6B) confirmed the role of p38 signaling in the antifibrotic effect of rosiglitazone. Further, the kinase inhibitor, SB203580, blocked the p38 signaling pathway and led to a dose-dependent decrease in the expression of αSMA and fibronectin proteins (Figs. 6C, 6D, 6E). This indicated that TGF-β1-induced myofibroblast transformation and ECM synthesis occurs at least partly through p38 MAPK signaling in pterygium fibroblasts.

FIGURE 2. Real-time PCR analysis. Quantitative analysis of mRNA expression levels of (A) α-SMA (P < 0.0001, F[5, 12] = 18.78), (B) collagen 1 (P = 0.0396, F[5, 12] = 3.471), (C) fibronectin (P = 0.0011, F[5, 12] = 9.447), (D) SRF (P = 0.0398, F[5, 12] = 9.447), and (E) vimentin (P = 0.2412, F[5, 12] = 1.593). #Represents P < 0.05 compared to vehicle-treated control cells; *represents P < 0.05 compared to only TGF-β1-treated cells.

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Rosiglitazone Reduced Pterygium Fibroblasts Proliferation and Cell Migration, But Did Not Induce Cell Death

Results of fluorescence-activated cell sorting (FACS) analysis of cells treated with rosiglitazone compared with vehicle-treated controls and cells treated with only TGF-β1 showed approximately the same percentage of distribution of live cells (80%), cells at early apoptosis (6%), cells at late apoptosis (11%), and necrotic cells (3%), indicating that rosiglitazone treatment did not induce apoptosis. However, CCK-8 assay results showed that rosiglitazone treatment significantly caused a reduction in cell number dose dependently (Fig. 7) indicative of an inhibitory effect of rosiglitazone on pterygium fibroblast proliferation. Rosiglitazone also suppressed the TGF-β1-induced cell migration in pterygium fibroblasts (Fig. 8).

Rosiglitazone Reduced the TGF-β1-induced Increase in MMP-9 in Pterygium Fibroblasts

We investigated the effect of rosiglitazone on the expression of MMP-1, -3, and -9, which are known to be highly expressed in pterygium fibroblasts. Results showed that TGF-β1 increased the expression of MMP-3 and -9, but not MMP-1. In addition, rosiglitazone significantly suppressed the TGF-β1-induced expression of MMP-9. However, it had no effect on the expression of MMP-1 and -3 (Supplementary Fig. S1).

DISCUSSION

Recent studies identifying PPAR-γ as an important cellular antifibrotic mechanism and its importance in the negative regulation of connective tissue biosynthesis during both physiologic and pathologic matrix remodeling have increased research focus in this area in the quest to finding potential therapeutic agents for the myriad chronic debilitating fibrotic disorders. In this regard, PPAR-γ ligands have also been reported to be effective corneal antifibrotics in vitro and in vivo in a PPAR-γ-independent manner. However, their effects on other ocular fibrotic disorders, such as pterygium, which is known to overexpress TGF-β1, is not known. Our results showed that the PPAR-γ ligand rosiglitazone is an effective antifibrotic agent in pterygium fibroblasts as well in vitro.

Notably, our results also showed that rosiglitazone can reduce the basal levels of α-SMA and fibronectin, thereby making it a very suitable candidate for therapy. This assertion is further consolidated by the additive downregulation of the intrinsic expression of TGF-β1 in pterygium fibroblasts, which
**Figure 4.** Effect of rosiglitazone on intrinsic TGF-$eta$1 synthesis in pterygium fibroblast. (A) Representative western blot images of $\alpha$-SMA and fibronectin protein expression. Quantitative analysis of immunoblots with summary data from three independent experiments from (B) $\alpha$-SMA ($P = 0.0004$, $F[4, 10] = 14.05$) and (C) fibronectin ($P = 0.0007$, $F[4, 10] = 12.18$). (D) Representative western blot images of TGF-$\beta$1 expression. (E) Quantitative analysis of immunoblots with summary data from three independent experiments ($P = 0.0068$, $F[4, 10] = 6.714$). *Represents $P < 0.05$ compared to control cells.

**Figure 5.** Effects of rosiglitazone on signaling pathways following exposure to TGF-$\beta$1 for 2 hours. (A) Representative western blot images. Quantitative analysis of immunoblots for (B) P-P38/T-P38 ($P = 0.0008$, $F[5, 12] = 9.578$), (C) P-ERK/T-ERK ($P = 0.1599$, $F[5, 12] = 1.946$), and (D) P-Smad/T-Smad ($P = 0.0638$, $F[5, 12] = 2.848$), with summary data from three independent experiments. *Represents $P < 0.05$ compared to only TGF-$\beta$1-treated cells.
FIGURE 6. Role of p38 signaling in the TGF-β1-mediated fibrosis and the effect of rosiglitazone. (A) Representative western blot images of the time course of p38 protein activation, (B) effect of rosiglitazone on p38 signaling following exposure to TGF-β1 for 24 hours, and (C) protein expression of α-SMA and fibronectin after blocking p38 signaling with SB203580. Quantitative analysis of the immunoblots for (D) α-SMA ($P < 0.0001$, $F[5, 12] = 36.09$) and (E) fibronectin ($P < 0.0001$, $F[5, 12] = 97.39$) with representative data from three independent experiments. *Represents $P < 0.05$ compared to vehicle-treated control cells; #represents $P < 0.05$ compared to only TGF-β1-treated cells.

FIGURE 7. The effect of rosiglitazone on cell viability. FACS results of (A) vehicle-treated control cells, (B) cells treated with only TGF-β1, (C) cells treated with TGF-β1 and 25 μM rosiglitazone, (D) cells treated with TGF-β1 and 75 μM rosiglitazone, and (E) their quantitative representation. (F) Quantitative representation of CCK-8 assay results after treatment with different concentrations of rosiglitazone compared with vehicle-treated control cells ($P < 0.0001$, $F[4, 10] = 56.74$). *Represents $P < 0.05$ compared to vehicle-treated control.
suggests that rosiglitazone modulates TGF-β1 induced fibrogenesis. Given that pterygium fibroblasts exhibit aberrant ECM remodeling and are associated with MMPs, the effect of rosiglitazone on the production of MMPs provides another promising perspective. Investigation of the expression of MMP-1, -3, and -9 in pterygium fibroblasts showed that rosiglitazone significantly suppressed the TGF-β1-induced expression of MMP-9. However, it had no effect on MMP-1 and -3. Because MMP production is mainly associated with NF-κB pathway, further studies to understand the underlying mechanism are warranted. The noncytotoxic and antiproliferative effects of rosiglitazone treatment as shown by the cell viability assays are also an added advantage compared to the currently used therapeutic applications. Thus, rosiglitazone’s effect appears to be mediated by limiting the proliferation of pterygium fibroblasts and their transformation into myofibroblasts.

Our evaluation of the possible molecular mechanisms underlying the observed antifibrotic effects of rosiglitazone indicated that rosiglitazone inhibits TGF-β1-induced fibrosis in pterygium fibroblasts at least in part by modulating the noncanonical p38 MAPK cellular signaling pathway. As noticed, TGF-β1-induced phosphorylated Smad protein level remained unaltered with rosiglitazone treatment, while phosphorylated p38 protein levels were downregulated with a consequent significant reduction in ECM synthesis and myofibroblast differentiation in a dose-dependent manner, albeit not to the baseline levels. Blocking of the p38 signaling in pterygium fibroblasts led a dose-dependent decrease in myofibroblast differentiation and ECM synthesis similar to the effect of rosiglitazone treatment. The MAPK signaling pathways, which include ERK1/2, Jun kinase (JNK/SAPK), and p38 pathways, are known to play a key role in the transduction of extracellular signals to elicit cellular responses including proliferation, differentiation, development, inflammation, and apoptosis. These pathways relay, amplify, and integrate signals from different stimuli, such as growth factors by phosphorylating proteins, which then translocate to the nucleus to activate transcription factors to regulate the expression of target genes. Indeed, TGF-β1 stimulated p38 MAPK activation has been reported to be involved in fibrosis and scar tissue formation, and research findings in human cornea and tenon fibroblasts indicate that rosiglitazone inhibits TGF-β1-induced activation of these fibroblasts via the p38 MAPK signaling pathway. The involvement of this pathway in the TGF-β1 stimulated fibrotic process in pterygium fibroblasts as our results also indicate is consistent with these reports. However, both rosiglitazone and p38 inhibition failed to completely block myofibroblast differentiation and ECM synthesis. This indicated that the TGF-β1-induced fibrosis is only partially dependent on p38 MAPK signaling. Previous studies have shown that the canonical Smad2/3 signaling and several other noncanonical pathways are involved in TGF-β1-induced fibrosis, thus indicating a complex regulatory linkage between TGF-β1 signaling and the development of fibrosis.

The observed antifibrotic effects of rosiglitazone were also seen to be independent of PPAR-γ receptors as treatment with the PPAR-γ receptor antagonist GW9662 did not reverse the effects. This is consistent with results obtained in cornea fibroblasts treated with rosiglitazone and other PPAR-γ ligands. PPAR-γ agonists modulate multiple cellular functions both in a PPAR-γ-dependent and PPAR-γ-independent manner as indicated by several research findings with different PPAR-γ ligands from different tissues. The dependence or otherwise of rosiglitazone on PPAR-γ signaling with regard to its antifibrotic effects seems to be tissue specific as similar results demonstrated in autosomal dominant polycystic kidney

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**FIGURE 8.** Effect of rosiglitazone on cell migration in pterygium fibroblasts. (A) Phase-contrast microscopic images of the scratch wound area of cells treated with vehicle, TGF-β1 (2.5 ng/mL) only, and TGF-β1 (2.5 ng/mL) along with rosiglitazone (75 μM), taken at 12, 24, and 48 hours after treatment. (B) Quantitative analysis of the scratch wound area with representative data from three different sites of the vehicle-treated control cells ($P = 0.0292, F(2, 6) = 6.739$), cells treated with only TGF-β1 ($P < 0.0001, F(2, 6) = 432.2$), and cells treated with TGF-β1 and rosiglitazone ($P = 0.0101, F(2, 6) = 10.89$). *Represents $P < 0.05$ compared to the scratch wound area at 12 hours after treatment.
nary fibroblasts, which when disrupted interfere with ECM synthesis. In corneal fibroblasts, activation of transient receptor potential cation channel subfamily V member 1 (TRPV1) with a subsequent increase in intracellular Ca\(^{2+}\) has also been reported, the receptors possibly targeted and underlying mechanisms are not fully understood. We speculate that Ca\(^{2+}\)/Ca\(^{2+}\) homeostasis and other techniques: meta-analysis.

In summary, our study demonstrated that rosiglitazone is effective in reducing ECM production and myofibroblast differentiation independent of PPAR-\(\gamma\). These findings may present the basis for its use as a promising candidate drug for adjuvant therapy after surgery to prevent recurrent pterygium.

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