Interleukin-6–mediated trans-signaling inhibits transforming growth factor-β signaling in trabecular meshwork cells

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Glaucoma is one of the major causes of blindness, and transforming growth factor-β2 (TGF-β2) has been found to be elevated in the aqueous humor of eyes with primary open-angle glaucoma (POAG). TGF-β2 in aqueous humor causes the glaucoma-related fibrosis of human trabecular meshwork (HTM), suggesting an important role of TGF-β in POAG pathogenesis. Here, we sought to elucidate the effects of IL-6 trans-signaling on TGF-β signaling in HTM cells. Using a multiplex immunoassay, POAG patients decreased IL-6 levels and increased soluble IL-6 receptor (sIL-6R) levels compared with the controls. In in vitro experiments, we observed that the IL-6 level was increased in the conditioned medium of HTM cells after TGF-β2 stimulation. To elucidate the relationship between TGF-β2 and IL-6 in HTM cells, we conducted Western blotting and immunohistochemical analyses, and we noted that the combination of IL-6 and sIL-6R (IL6/sIL-6R) suppressed TGF-β–induced up-regulation of α-smooth muscle actin in HTM cells, whereas IL-6 alone did not. This suggests that trans-signaling, not classic signaling, of IL-6 suppresses TGF-β–induced fibrosis of HTM. IL6/sIL-6R also suppressed TGF-β–mediated activation of myosin light chain 2 (MLC2), Smad2, and p38. Of note, these inhibitory effects of IL6/sIL-6R on TGF-β were partly reduced by siRNA-mediated knockdown of STAT3. Moreover, IL-6/sIL-6R partly inhibited TGF-β–induced activation of the Smad-sensitive promoter detected with luciferase reporter gene assays and up-regulation of TGFR1 and TGFR2, evaluated by quantitative real-time RT-PCR. Strikingly, overexpression of TGFR1 and TGFR2 diminished these inhibitory effects of IL-6/sIL-6R. We conclude that IL-6–mediated trans-signaling potently represses TGF-β signaling in HTM cells.

Glaucoma is one of the major causes of blindness around the world (1). As elevated intraocular pressure (IOP) is a critical risk factor for the development and progression of glaucoma, reduction of IOP is regarded as the most important therapeutic modality (2). IOP is determined by the balance between inflow and outflow of aqueous humor. Elevated IOP is caused mainly by increased resistance to aqueous outflow. As transforming growth factor (TGF)-β was elevated in eyes with primary open angle glaucoma (POAG) and increased aqueous resistance by inducing up-regulation of the extracellular matrix (ECM) in the trabecular meshwork, TGF-β is thought to play an important role in the pathogenesis of POAG (3–9). It has also been reported that TGF-β increased α-smooth muscle actin (SMA) expression in trabecular meshwork cells (10, 11), suggesting the fibrotic changes in the trabecular meshwork may be related to the pathogenesis of POAG. Thus, controlling fibrosis in the trabecular meshwork may be a potent therapeutic target against glaucoma.

Interleukin (IL)-6 has various physiological and pathological functions. IL-6 signal transduction is mediated by binding the complex of the IL-6 receptor (IL-6R) and gp130 on target cells. Although gp130 is present on almost all cells, membrane IL-6R (mIL-6R) is present in only limited cell populations, including hepatocytes and some leukocytes (12). Signal transduction through mIL-6R is called classic signaling, whereas trans-signaling is mediated through soluble IL-6R (sIL-6R). sIL-6R is produced by shedding of mIL-6R or by translation of a differentially spliced mRNA (13, 14), and enables IL-6 to bind to gp130 on the cell membrane by forming a complex of IL-6 and sIL-6R. Thus, in the presence of sIL-6R, cells can respond to IL-6 even if the cells lack mIL-6R.

Previous studies indicated that various cytokines, including IL-6 and IL-8, and monocyte chemoattractant protein (MCP)-1, were present in the aqueous humor of patients with POAG and secondary glaucoma (15–18). Among the cytokines detected, IL-6 and MCP-1 increased aqueous outflow in ex vivo experiments (19, 20), suggesting that these cytokines are involved in the regulation of IOP. Although only the level of MCP-1 was higher in POAG than in controls, the levels of MCP-1 and IL-6 were positively correlated with each other. Interestingly, it has been reported that sIL-6R is also present in aqueous humor, and its concentration is elevated in patients with POAG and increased aqueous resistance.
with uveitis (21–23). Although trans-signaling of IL-6 is thought to be involved in the inflammatory response, its role in the pathology of glaucoma remains unknown.

In this study, we showed that the concentration of sIL-6R is elevated in the aqueous humor in eyes with POAG, and trans-signaling of IL-6 suppresses TGF-β signaling through activation of signal transducer and activator of transcription (STAT) 3 in trabecular meshwork cells.

**Results**

**Concentration of sIL-6R is higher in the aqueous humor of glaucoma patients**

A multiplex immunoassay was performed to examine the cytokine levels in the aqueous humor in eyes with cataracts (as controls) or POAG. Patient characteristics are shown in Table 1. The mean ± S.D. IL-6 levels were 34.6 ± 44.8 and 15.9 ± 54.7 pg/ml in eyes with cataracts (n = 17) and POAG (n = 22), respectively. The corresponding sIL-6R levels were 68.6 ± 30.9 and 118.7 ± 80.4 pg/ml, respectively (p = 0.025; Fig. 1A). The levels of IL-6 and sIL-6R were not correlated with each other in patients with POAG or cataracts (Fig. 1, B and C). These data suggest that aqueous sIL-6R was elevated in POAG patients independent of IL-6.

**TGF-β2 induces secretion of IL-6 from human trabecular meshwork (HTM) cells**

As TGF-β2 is involved in the pathology of glaucoma, we next investigated the effects of TGF-β2 on IL-6 secretion from cultured HTM cells. After treatment with TGF-β2 (1.0, 2.5, and 5.0 ng/ml) for 24 h, the IL-6 levels in the conditioned media of HTM cells were significantly elevated (28.4 ± 0.75, 29.3 ± 0.39, and 31.3 ± 0.69 pg/ml, respectively) compared with controls (6.8 ± 0.1 pg/ml; Fig. 1D). Thus, IL-6 secretion from HTM cells was shown to be up-regulated by TGF-β2.

**Trans-signaling is dominant in the transduction of IL-6 signaling in HTM cells**

Phosphorylation of STAT3 was investigated to confirm the effects of sIL-6R on the activation of IL-6 signaling in HTM cells. IL-6–induced phosphorylation of STAT3 was limited in the absence of sIL-6R (Fig. 2). In contrast, the combination of IL-6 and sIL-6R markedly enhanced STAT3 phosphorylation. Furthermore, the combination of IL-6 and sIL-6R suppressed the phosphorylation of Smad2, p38, and myosin light chain (MLC)-2, whereas IL-6 alone had no such effect. These results suggested that trans-signaling is dominant compared to trans-signaling outside the cell.
Inhibition of TGF-β by IL-6 trans-signaling

**Figure 3. Effects of TGF-β2, IL-6, and sIL-6R on STAT3 activation in HTM cells.** Cells were stimulated with TGF-β2 (2.5 ng/ml), IL-6 (100 ng/ml), and/or sIL-6R (200 ng/ml) for 24 h. A, data shown in the upper panels are results of representative Western blotting analyses of phospho-STAT3 (pSTAT3) and total STAT3. Relative changes in the ratio of STAT3 phosphorylation are shown in the lower graphs. Data are shown as means ± S.E., n = 6. *p < 0.05; **p < 0.01 calculated using the Tukey-Kramer HSD test. B–E, immunocytochemical analysis using anti-phospho-STAT3 antibody after treatment with vehicle (B), TGF-β2 (C), IL-6/sIL-6R (D), or TGF-β2/IL-6/sIL-6R (E). Scale bar, 100 μm.

with classic signaling in the transduction of IL-6 signals in HTM cells.

**Trans-signaling of IL-6 is active in HTM cells even in the presence of TGF-β2**

To clarify the effects of TGF-β2 on the activation of STAT3, we quantified the ratio of phospho-STAT3 in HTM cells. The combination of IL-6 and sIL-6R significantly increased the ratio of phospho-STAT3 in the presence or absence of TGF-β2 (p = 0.0029 and 0.0006, respectively; Fig. 3A). In addition, immunohistochemical studies demonstrated accumulation of phospho-STAT3 in the nuclei of HTM cells after treatment with IL-6 and sIL-6R regardless of the presence or absence of TGF-β2 (Fig. 3, B–E).

**Trans-signaling of IL-6 inhibits TGF-β2–induced formation of α-SMA and F-actin**

To elucidate the effects of IL-6 trans-signaling on fibrogenic changes, the expression of α-SMA, a representative marker of the epithelial-to-mesenchymal transition (EMT) and fibrosis under conditions of TGF-β2 stimulation, was investigated. α-SMA induction by TGF-β2 was significantly suppressed by treatment with both IL-6 and sIL-6R (p < 0.0001; Fig. 4A). Next, we assessed phosphorylation of MLC2 and F-actin formation, which are related to contractile properties. Phosphorylation of MLC2 was induced after treatment with TGF-β2, as expected, and its induction was significantly suppressed by activation of IL-6 trans-signaling (p = 0.0389; Fig. 4B). In addition, the TGF-β2–induced polymerization of actin was inhibited by the combination of IL-6 and sIL-6R in HTM cells (Fig. 5).

**Effects of IL-6 trans-signaling on the TGF-β–signaling pathway**

To elucidate the effects of IL-6 trans-signaling on canonical and noncanonical TGF-β–signaling pathways, the phosphorylation levels of Smad2 and p38 were investigated. TGF-β2 increased the relative level of phospho-Smad2 (pSmad2; p < 0.0001), and the induction was partly suppressed by the combination of IL-6 and sIL-6R (p = 0.0482; Fig. 6A), suggesting IL-6 trans-signaling may inhibit Smad2 activation. Similarly, TGF-β2–induced induction of p38 was partly inhibited by activation of IL-6 trans-signaling (p = 0.0404; Fig. 6B). Next, to clarify the effects of IL-6 trans-signaling on the transcriptional activity of regulator-Smad, the luminescence from a luciferase reporter gene fused with 12 repeats of the Smad-binding element (CAGA-12) was measured by luciferase assay. The relative levels of CAGA-12 promoter activity were 71.4 ± 5.6, 0.8 ± 0.1, and 40.4 ± 4.3-fold after treatment with TGF-β2 in the absence and presence of IL-6/sIL-6R, respectively (Fig. 6C; significantly different from each other, p < 0.0001). These results indicated that IL-6 trans-signaling suppressed canonical and noncanonical TGF-β2 signaling, at least partly, in HTM cells.

**STAT3 knockdown ameliorated the inhibitory effects of IL-6 trans-signaling on TGF-β2 signaling in HTM cells**

To confirm the inhibitory effects of IL-6 trans-signaling on TGF-β2–induced changes in HTM cells, small interfering RNA (siRNA)-mediated control of STAT3 production was induced.
We confirmed that control siRNA did not affect the activity of IL-6 or TGF-β2 signaling and that STAT3 siRNA efficiently knocked down the expression of STAT3 in HTM cells (Figs. 7 and 8). As described above, up-regulated levels of α-SMA, pSmad2, p-p38, and pMLC2 expression by TGF-β2 treatment were confirmed, and these effects were partly inhibited by knockdown of STAT3 (Figs. 7–9). In addition, TGF-β2-induced phosphorylation of ERK1/2 was suppressed by the combination of IL-6 and sIL-6R (Figs. 7 and 8). After knockdown of STAT3, the basal level of ERK1/2 phosphorylation was up-regulated, and the inhibitory effect of IL-6 trans-signaling was reduced in HTM cells. Taken together, the inhibitory effects of IL-6 trans-signaling on canonical and noncanonical TGF-β2 signaling were dependent on the activation of STAT3 in HTM cells.

IL-6 trans-signaling suppresses expression of the TGF-β receptors, TGFBR1 and TGFBR2

To explore the molecular mechanisms underlying the inhibitory effects of IL-6 trans-signaling on TGF-β signaling, mRNA levels of TGFBR1 and TGFBR2, encoding TGF-β receptors 1 and 2, respectively, were investigated. Our quantitative real-time reverse-transcription PCR (RT-PCR) analyses showed that TGFBR1 mRNA was induced by 10.0 ± 1.6-fold after treatment with TGF-β2 (2.5 ng/ml) alone, and this value was significantly reduced to 4.5 ± 0.5-fold after treatment with TGF-β2 and IL6/sIL-6R (100 and 200 ng/ml, respectively; \( p = 0.0180 \); Fig. 10A). The level of TGFBR2 mRNA was also increased by 1.5 ± 0.1-fold after treatment with TGF-β2, and its induction was reduced to the basal
level (1.1 ± 0.1-fold; p = 0.0057; Fig. 10B). These results suggested that the activation of IL-6 trans-signaling inhibited TGF-β receptor 1 expression, and thereby it suppressed TGF-β2–induced fibrogenic changes in HTM cells. 

**Overexpression of the TGF-β receptors, TGFB1 and TGFB2, partly eliminates the inhibitory effects of IL-6 trans-signaling**

To confirm the effects of down-regulation of TGF-β receptors on the inhibitory effects of IL-6 trans-signaling, we overex-

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**Figure 7. Effects of STAT3 knockdown on the TGF-β2–signaling pathway in HTM cells.** Cells were transfected with siRNA (control or targeting STAT3) and then cultured for 6, 12, or 24 h in culture medium. After serum starvation, cells were stimulated with TGF-β2 (2.5 ng/ml), IL-6 (100 ng/ml), and/or sIL-6R (200 ng/ml) for 24 h, and expression levels of signaling molecules were determined by Western blot analysis. Data are reproducible, n = 3.

**Figure 8. Quantification of effects of STAT3 knockdown on the TGF-β2–signaling pathway in HTM cells.** Cells were transfected with siRNA (control or targeting STAT3) and then cultured for 6 h (A–E), 12 h (F–J), or 24 h (K–O) in culture medium. After serum starvation, cells were stimulated with TGF-β2 (2.5 ng/ml), IL-6 (100 ng/ml), and/or sIL-6R (200 ng/ml) for 24 h, and expression levels of signaling molecules were determined by Western blot analysis. Data are reproducible, n = 3.
Inhibition of TGF-β by IL-6 trans-signaling

Figure 9. Effects of STAT3 knockdown on the phospho-relation level of MLC2 in HTM cells. Cells were transfected with siRNA (control or targeting STAT3) and cultured for 24 h in culture medium. After serum starvation, cells were stimulated with TGF-β2 (2.5 ng/ml), IL-6 (100 ng/ml), and/or sIL-6R (200 ng/ml) for 24 h, and expression levels of signaling molecules were determined by Western blot analysis. Data are reproducible, n = 3.

Discussion

In this study, we showed that the concentration of sIL-6R is elevated in the aqueous humor of eyes with POAG, and trans-signaling of IL-6 suppressed TGF-β–induced fibrogenic changes through activation of STAT3 in HTM cells. To the best of our knowledge, this is the first study to clarify the roles of IL-6 trans-signaling in glaucoma pathology. Moreover, the inhibitory effects of IL-6 trans-signaling on the expression of TGFBR1 and TGFBR2 have not been reported previously. Indeed, overexpression of both TGFBR1 and TGFBR2 partly suppresses the inhibitory effects of IL-6 trans-signaling, probably explaining the potential mechanism of the effect. Thus, the findings of this study suggest potential novel targets for glaucoma therapy.

Our results indicated up-regulation of IL-6 secretion from HTM cells after treatment with TGF-β2, a dominant isofrom in aqueous humor (24). Liton et al. (19) reported that IL-6 expression was elevated under conditions of cyclic mechanical stress partly by TGF-β1 in HTM cells. They also found that the TGF-β1–induced up-regulation of IL-6 was suppressed by inhibitors of p38, ERK, or JNK (25). As TGF-β1 and TGF-β2 share receptors, the findings of this study are consistent with previous reports. Thus, it was indicated that IL-6 production from HTM cells was induced by TGF-β, and this induction may represent part of a negative feedback system. As the aqueous TGF-β2 level was elevated in eyes with POAG, it may be confusing that the level of IL-6 in the aqueous humor in eyes with POAG was not different from that in controls. As the trabecular meshwork is downstream of aqueous flow in the anterior chamber, the IL-6 production from HTM cells may not be directly reflected in the concentration of IL-6 in the aqueous humor. It is also possible that the HTM cells from glaucoma patients do not produce IL-6, which could be a potential mechanism for glaucoma pathogenesis.

The level of STAT3 phosphorylation was elevated after treatment with IL-6 alone in this study. However, its induction was less prominent compared with combined treatment with IL-6 and sIL-6R. These results indicate that trans-signaling is dominant over classic signaling for IL-6 signal transduction in HTM cells. Although the limited reaction to treatment with IL-6 alone could be a result of signal transduction via classical signaling, mIL-6R is expressed in only limited cell types, including hepatocytes and some leukocytes (12). Thus, another explanation could be that external IL-6 binds to sIL-6R, which is produced by HTM cells, and thereby activates STAT3 in the absence of external sIL-6R. Further experiments are required to clarify the occurrence of IL-6 classical signaling in HTM cells.

To the best of our knowledge, this is the first report on the inhibitory effects of IL-6 trans-signaling on TGF-β signaling and its downstream targets, α-SMA and MLC2, in HTM cells. These results suggest that IL-6 trans-signaling could be involved in the regulation of IOP. First, TGF-β signaling has been shown to decrease aqueous outflow by ECM accumulation in ex vivo experiments (5). Second, it has been reported that α-SMA expression is a key marker of fibrogenic changes, such as cell contractility, attachment, motility, and EMT-like phenomena, in HTM cells (10, 11, 27). Third, recent investigations showed that activation of Rho-Rho kinase signaling induces cell contraction accompanied by phosphorylation of MLC2, F-actin formation, and α-SMA expression in HTM cells, and Rho-Rho kinase inhibitor lowered IOP by increasing aqueous outflow (11, 28–31). Notably, it was reported that a T104M mutation in IL-20 receptor 2 significantly impacts the function of this receptor, as shown by decreased pSTAT3 levels and generic matrix metalloproteinase activity (32). Considering the presence of IL-6 and sIL-6R in aqueous humor, the IL-6–STAT3 axis may contribute to the pathophysiology of glaucoma.

Our results showed that activation of IL-6 trans-signaling suppressed TGF-β2 signaling, Smad2 activation (canonical signaling pathway), ERK, and p38 activation (noncanonical signaling pathway). As knockdown of STAT3 decreased the inhibitory effects, this cross-talk between IL-6 and TGF-β2 depends on activation of STAT3. These results are consistent with previous studies indicating the inhibitory effects of STAT3 signaling on TGF-β signaling and tissue fibrosis (33–37). However, the cross-talk between IL-6 and TGF-β signaling is complicated, because STAT3 signaling was reported to be enhanced or required in TGF-β signaling and/or fibrogenic changes in some conditions (38–41). For example, Seong et al. (39) reported that IL-6 participated in TGF-β–induced trans-differentiation of
human Tenon’s fibroblasts to myofibroblasts. In addition, in lung cancer cells, JAK/STAT signaling is required for TGF-β–induced EMT (39). Currently, the reasons for this inconsistency are unclear, but it may be due to differences in cell types or the effects of other regulatory factors.

The molecular mechanisms underlying the inhibitory effects of IL-6 trans-signaling on TGF-β signaling in HTM cells are still under investigation. Although not yet conclusive, we found a potential novel mechanism in HTM cells: activation of IL-6 trans-signaling caused down-regulation of TGF-β receptors. Moreover, the combined overexpression of TGFβRI and TGFβRII rescued the inhibitory effect of IL-6 trans-signaling on CAGA promoter activity, which is sensitive to active Smad2/3. Wang et al. (33) reported that STAT3 interacts directly with Smad3 in vivo and in vitro, resulting in attenuation of the Smad3–Smad4 complex formation and suppression of the DNA-binding ability of Smad3 in HaCaT cells. Taken together, the cross-talk between IL-6 and TGF-β signaling pathways may be mediated by multiple steps.

In conclusion, this study indicated that the aqueous humor of patients with POAG contains high levels of sIL-6R. The combination of IL-6 and sIL-6R activates STAT3 and down-regulates TGF-β receptors, and thereby inhibits TGF-β–induced changes via down-regulation of canonical and noncanonical TGF-β signaling in HTM cells. Our results suggest that IL-6 trans-signaling is an important regulatory system of aqueous outflow.

**Experimental procedures**

**Materials**

Recombinant human TGF-β2 was purchased from R&D Systems (Minneapolis, MN). Recombinant human IL-6 and sIL-6R were purchased from Wako Pure Chemical Industries (Osaka, Japan). The anti-MLC2 (1:800 dilution), anti-phospho-MLC2 (1:700 dilution; Thr-18/Ser-19), anti-Smad2 (1:2000 dilution), anti-phospho-Smad2 (1:800 dilution; Ser-465/467), anti-STAT3 (1:2000 dilution; 4904), anti-phospho-STAT3 (1:1000 dilution; Tyr-705), anti-p38 (1:2000 dilution), and anti-phospho-p38 (1:1000 dilution) antibodies and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibodies (1:2000 dilution) were purchased from Cell Signaling Technology.
Inhibition of TGF-β by IL-6 trans-signaling

(Danvers, MA). The anti-β-actin (1:5000 dilution) and anti-α-SMA mouse monoclonal antibodies (1:2000 dilution) were obtained from Sigma. HRP-conjugated anti-mouse IgG antibodies (1:5000 dilution) were obtained from GE Healthcare (Little Chalfont, UK). siRNA targeting STAT3 (Invitrogen™ Stealth RNAi™ siRNA) was purchased from ThermoFisher Scientific (Rockford, IL). pCMV TβRI-His was a gift from Dr. Joan Massague (Addgene plasmid 19161). pCMV5B-TβRII WT was a gift from Dr. Joan Massague and Jeff Wrana (Addgene plasmid 11766). pCMV-HA was a gift from Dr. Tetsuya Taga.

Patients

The study to analyze cytokine levels in the aqueous humor was approved by the Institutional Review Board of Kumamoto University. All of the procedures conformed to the Declaration of Helsinki. Written informed consent was obtained from each patient. Patients that had undergone trabeculectomy for POAG aged ≥20 years old were recruited. Cataract patients without systemic diseases (other than hypertension and hyperlipidemia), ocular diseases other than cataracts, a history of ocular surgeries, or IOP >21 mm Hg were included as controls. IOP was determined using a noncontact tonometer in cataract cases and a Goldmann tonometer in glaucoma cases. When both eyes of a patient met the inclusion criteria, only the eye treated first was included in the analysis. In all of the participants, the anterior eye segment was examined by glaucoma specialists using a slit-lamp biomicroscope, and all of the changes were recorded. The optic disc was evaluated through dilated pupils with a slit-lamp biomicroscope, and all of the changes were recorded. In all of the participants, the anterior eye segment was examined by glaucoma specialists using a slit-lamp biomicroscope, and all of the changes were recorded. The optic disc was evaluated through dilated pupils with a slit-lamp biomicroscope, and all of the changes were recorded.

Sample collection

Preoperative aqueous humor was obtained at the start of the phacoemulsification surgery and/or trabeculectomy before any incisional procedures, as described previously (16). Briefly, aqueous humor was obtained gently at the start of surgery from the anterior chamber, through limbal paracentesis using a syringe with a 30-gauge needle attached. Approximately 70–100 µl was collected in CryoTubes, registered, and stored at −80 °C until processing.

Multiplex immunoassay analysis

Concentrations of IL-6 and sIL-6R in the aqueous humor samples and supernatant of HTM cells were determined using multiplex bead-based immunoassays, xMAP, and human cytokine/chemokine panel (Luminex, Austin, TX), as described previously (16). Briefly, a 25-µl aliquot of aqueous humor sample or supernatant of HTM cells was transferred to a plate, and some of each aliquot was placed into one of the capture microsphere multiplexes. After incubation at 4 °C for 18 h, multiplexed cocktails of biotinylated reporter antibodies were mixed and then incubated at room temperature for 1 h. Multiplexes were developed using an excess of streptavidin/phycoerythrin solution. The solution was mixed into each multiplex and then incubated at room temperature for 30 min. Vacuum filtration was performed to reduce the volumes of the multiplexed reagents, and then the volume of each reaction was increased by dilution in matrix buffer. A Luminex 200 instrument (Luminex) was used for the analysis, and data were interpreted using proprietary data analysis software (DNASIS Plex, version 2.5; Hitachi Software Engineering, Tokyo, Japan).

Cell culture

Primary HTM cells were obtained from ScienCell (Carlsbad, CA), and maintained in Trabecular Meshwork Cell Medium (TMCM; ScienCell) containing 5% fetal bovine serum (FBS) and supplements (undisclosed growth factors and antibiotics; ScienCell), according to the manufacturer’s protocol as described previously (42). Briefly, cells cultured without serum were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako) supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin sulfate (Invitrogen) and GlutaMAX-I supplement (Life Technologies, Inc.). HTM cells were used between passages 6 and 8 in this study. After 24 h of serum starvation, cells were treated with TGF-β2, IL-6, and/or sIL-6R for 24 h.

Immunocytochemistry

Immunocytochemical analysis was conducted as described previously (42). Briefly, glass coverslips in 12-well plates were coated with gelatin for 30 min at room temperature and then washed with phosphate-buffered saline (PBS). After drying the plates, HTM cells were grown on gelatin-coated glass coverslips, starved of serum for 24 h, and then stimulated with TGF-β2, IL-6/sIL-6R, or TGF-β2 with IL-6/sIL-6R. After a 24-h period of stimulation, HTM cells were washed twice in PBS and then fixed in methanol at −20 °C for 15 min. After fixation, the cells were washed three times in PBS, permeabilized, and blocked with 3% FBS in PBS. Subsequently, the cells were incubated with primary antibody (anti-phospho-Smad3; 1:200; Cell Signaling) overnight at 4 °C, and then with Alexa-Fluor-conjugated secondary antibodies (1:1000 dilution; Life Technologies, Inc.) for 1 h at room temperature. Phalloidin-FITC (1:200 dilution; Life Technologies, Inc.) was used for F-actin staining. After cells were washed with PBS, they were mounted with VECTASHIELD mounting medium with 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and the slides were observed under a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Western blot analysis

Western blot analysis was performed as described previously (42). Briefly, HTM cells were grown on 6-cm dishes, starved of serum for 24 h, and stimulated with TGF-β2 with or without IL-6 or IL-6/sIL-6R for 24 h. The cells were then washed three times with ice-cold PBS on ice and lysed with radioimmunoprecipitation buffer (ThermoFisher Scientific) containing protease inhibitors (ThermoFisher Scientific) and phosphatase inhibitors (Nacalai Tesque, Kyoto, Japan). Cell extracts were then centrifuged at 15,000 rpm for 10 min at 4 °C. Supernatants were collected, and the protein contents were determined using a BCA protein assay kit (ThermoFisher Scientific). Samples were resolved using SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membranes by electroblotting. Mem-
branes were blocked with 5% skim milk (Nacalai Tesque) in TBS containing 0.1% Tween 20 (TBS-T) for 30 min at room temperature. After washing with TBS-T, they were incubated with primary antibodies diluted with 5% BSA (Wako) in TBS-T overnight at 4 °C. After washing three times for 5 min each time with TBS-T, the membranes were incubated with HRP-conjugated anti-rabbit IgG (1:2000 dilution; Cell Signaling Technology) or HRP-conjugated anti-mouse IgG (1:5000 dilution; GE Healthcare) for 1 h at room temperature. After washing three times for 5 min each time with TBS-T, signals were enhanced using a chemiluminescence system (ImmunoStar LD; Wako); ECL Prime or ECL Western blotting detection reagents (GE Healthcare), and exposed using an LAS-4000 EPUV Mini (Fuji Film, Tokyo, Japan) imager. Densitometry of immunoreactive bands was performed using ImageJ software (National Institutes of Health).

**Luciferase assay**

Luciferase assay was conducted as described previously (42). Briefly, transcriptional activity was assessed by transient transfection of a luciferase reporter gene fused with CAGA-12. As an internal control, a plasmid containing Renilla luciferase (pRL-TK; Promega, Madison, WI) was cotransfected. Transfection of HTM cells was performed at 80% confluence using GeneJuice® transfection reagent (Merck Millipore, Billerica, MA) according to the manufacturer’s protocol. HTM cells were seeded the day before transfection in 6-well plates. Twenty four hours after transfection, the medium was changed to serum-free DMEM. Twenty four hours later, cells were stimulated with TGF-β2 with or without IL-6/sIL-6R for 24 h.

**RNA interference targeting STAT3**

HTM cells were plated at 70% confluence before transfection. siRNAs targeting STAT3 were transfected into cells using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer’s protocol. HTM cells transfected with siRNA were put into serum-free medium for 24 h and stimulated with TGF-β2 or IL-6/sIL-6R for 6, 12, or 24 h. The cell lysates were collected for Western blot analysis.

**RNA isolation and real-time RT-PCR**

Serum-starved HTM cells were stimulated with TGF-β2 with or without IL-6/sIL-6R for 24 h. Total RNA was extracted using NucleoSpin® RNA (Takara Biotechnology, Shiga, Japan). The RNA was reverse-transcribed using ReverTra Ace® qPCR RT master mix with gDNA remover (Toyobo, Osaka, Japan), according to the manufacturer’s instructions. Quantitative real-time RT-PCR (PCR) was performed in 20-μl reaction mixtures containing 10 μl of PCR master mix (THUNDERBIRD SYBR qPCR Mix; Toyobo), 1 μl of cDNA samples, and 0.3 μM primer pairs using an Applied Biosystems 7000 (Life Technologies, Inc.) according to the manufacturer’s instructions. The thermal cycling conditions were 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 31 s. PCR was performed in duplicate, and β-glucuronidase (Takara Biotechnology; sequence not disclosed) was used as a control. PCR was performed using the following primers: TGFBR1 (sense sequence, 5′-CTGCTCGGAGCAGATT-3′) and TGFBR2 (sense sequence, 5′-CAACACCAGGGGCTTCA-3′, and antisense sequence, 5′-TGTGGTGCACCAGACTCA-3′) (26).

**Statistical analysis**

Each experiment was repeated a minimum of three times. Data were analyzed using the JMP version 8 statistical software package (SAS Institute, Cary, NC). All data represent the means of at least three independent experiments. Quantitative data were analyzed using the Tukey-Kramer honest significance difference test. In all analyses, p < 0.05 was taken to indicate statistical significance.

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