Rosiglitazone Treatment Prevents Postoperative Fibrosis in a Rabbit Model of Glaucoma Filtration Surgery

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PURPOSE. To evaluate the potential antifibrotic effect of rosiglitazone (RSG), a peroxisome proliferator-activated receptor-γ (PPARγ)-selective agonist, on subconjunctival fibrosis in a rabbit model of glaucoma filtration surgery (GFS) in vivo, and to investigate the underlying mechanisms in human Tenon’s fibroblasts (HTFs) in vitro.

METHODS. GFS were performed on adult male New Zealand white rabbits with chronic ocular hypertension previously established by injections of 2% methylcellulose into the anterior chamber. Rabbits were treated by RSG, mitomycin C (MMC) or 5-fluorouracil (5-FU) intraoperatively. The morphology of filtering blebs was evaluated by Indiana Bleb Appearance Grading Scale (IBAGS) scoring. Expression of profibrotic genes was determined by quantitative PCR, immunoblot, and/or histochemical analysis. In vitro studies were performed in a transforming growth factor (TGF)-β1-based cell model of fibrosis. Autophagy was evaluated by the formation of autophagosomes and autolysosomes using fluorescent and transmission electron microscopy and by evaluation of key mediators in the autophagic pathway.

RESULTS. RSG treatment ameliorated a rebound intraocular pressure (IOP) elevation, prolonged the survival of filtering blebs, and attenuated subconjunctival fibrosis in rabbits following trabeculectomy. Pretreatment of HTFs with RSG inhibited TGF-β1-induced expression of profibrotic genes encoding specificity protein 1, connective tissue growth factor, and α smooth muscle actin. RSG augmented TGF-β1-induced autophagy in HTFs via a beclin1/VPS34-dependent mechanism. Augmentation of autophagy is associated with inhibition of TGF-β1-induced profibrotic gene expression by RSG.

CONCLUSIONS. RSG treatment prevents subconjunctival fibrosis after GFS by inhibition of profibrotic gene expression through a mechanism involved in promoting autophagy in local fibroblasts. RSG represents a novel antifibrotic drug with the potential to improve the success rate of GFS.

Keywords: rosiglitazone, fibrosis, glaucoma filtration surgery, autophagy, human Tenon’s fibroblast

Primary open angle glaucoma (POAG) is a complex neurodegenerative retinopathy manifested by irreversible visual field loss. It is associated with increased resistance of aqueous humor outflow at the trabecular meshwork region and damage to retinal ganglion cell (RGC) axons at the optic nerve head.1 Elevation of intraocular pressure (IOP) is currently the only modifiable risk factor associated with POAG. Lowering of IOP remains the only proven treatment of glaucoma. Glaucoma filtration surgery (GFS) is a surgical procedure that creates a passage for draining excess aqueous humor into the space between the sclera and conjunctiva to lower the IOP. However, subconjunctival tissue responses to GFS-induced injury promote aberrant fibroblast proliferation, myofibroblast differentiation, and excessive deposition of extracellular matrix (ECM) at the filtering blebs, resulting in postoperative scarring that limits the success rate of GFS.2 Antifibrotic agents, such as 5-fluorouracil (5-FU) and mitomycin C (MMC), have been widely used to inhibit fibrosis at the filtering site.3,4 The mechanisms of action by these drugs remain largely unknown. Additionally, the current antifibrotic treatments are also associated with a significant side-effect profile, including corneal toxicity, wound leak, blebitis, dysesthesia, and endophthalmitis.5 Better antifibrotic therapies are in need for the improvement of long-term outcomes of GFS.

Rosiglitazone (RSG) is a high-affinity agonist of nuclear peroxisome proliferator-activated receptor-γ (PPARγ), which has been used clinically as an antidiabetic drug to treat patients with insulin resistance.6 Recent studies reveal that RSG exerts multifaceted anticancer effects, including inhibition of cancer cell proliferation and metastasis, induction of apoptosis and autophagy, and reduction of angiogenesis and immune suppression, by both PPARγ-dependent and -independent mecha-
nisms.7 We have previously shown that RSG inhibits transforming growth factor (TGF)-β1-induced proliferation, migration, and myofibroblast differentiation of human Tenon’s fibroblasts (HTFs) through blocking the p38 signaling pathway.8 In a mouse model of glaucoma, treatment with RSG does not protect RGCs from apoptosis, suggesting that this drug may not function as a neuroprotective agent.9

The focus of this study is to examine the ability of RSG treatment to limit subconjunctival scarring after GFS. We investigated effects of RSG on stabilization of IOP reduction following trabeculectomy, the survival of filtering blebs, and expression of profibrotic genes in a rabbit model of GFS. We delineated the potential mechanisms by which RSG attenuates fibrogenesis in TGF-β1-treated HTFs.

Materials and Methods

Reagents

RSG was purchased from BioVision (Milpitas, CA, USA). T0070907 was purchased from Selleck Chemicals (Houston, TX, USA). Rapamycin, 3-Methyladenine (3-MA), and Chloroquine (CQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant Human TGF-β1 was purchased from PeproTech (Rocky Hill, NJ, USA). Methylcellulose was purchased from Solarbio (Beijing, China). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, puromycin, and trypsin, which were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Information for all antibodies used in this study is listed in Table 1.

Cell Culture and Treatment

Primary HTFs were isolated and characterized as described in our previous studies.8 Cells were used between passages 3 and 7. HTFs were incubated at 37°C in a humidified (95%) incubator under 5% CO2. Medium was changed every 2 to 3 days. Cells were divided into eight groups and treated as follows: (1) no treatment group; (2) TGF-β1 group in which cells were treated with 10 ng/mL TGF-β1 for 24 hours; (3) TGF-β1+dimethyl sulfoxide (DMSO) (a solvent of RSG) group in which cells were treated with 10 ng/mL TGF-β1 for 24 hours followed by 10 ng/mL TGF-β1 treatment for another 24 hours; (4) RSG + TGF-β1 group in which cells were pretreated by 10 µM RSG for 2 hours followed by 10 ng/mL TGF-β1 for another 24 hours; (5) T0070907 + TGF-β1 group in which cells were pretreated by 50 µM T0070907, a potent and selective PPARγ antagonist, for 2 hours followed by 10 ng/mL TGF-β1 for another 24 hours; (6) rapamycin + TGF-β1 group in which cells were pretreated by 100 nM rapamycin, a potent and specific mTOR inhibitor and activator of autophagy, for 1 hour followed by 10 ng/mL TGF-β1 for another 24 hours; (7) CQ + TGF-β1 group in which cells were treated by 10 ng/mL TGF-β1 for 16 hours followed by treatment with 50 µM CQ, an inhibitor of autolysosomes and lysosomal protein degradation, for another 8 hours; and (8) 3-MA + TGF-β1 group in which cells were pretreated with 5 mM 3-MA, an inhibitor of autophagosome formation, for 2 hours followed by 10 ng/mL TGF-β1 for 24 hours. All experiments were repeated at least three times.

Cytotoxicity Assay

RSG cytotoxicity assay was performed with Cell Counting Kit-8 assay cytotoxicity assay kit (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer’s recommendations. Briefly, HTFs were seeded in 96-well plates at 5 × 10^3 cells per well and were treated with 5, 10, 20, and 50 µM RSG for 24 hours. The colorimetric absorbance was measured at 570 nm using Varioskan Flash (Thermo Fisher Scientific).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was prepared with Trizol reagents (Sigma-Aldrich) according to the manufacturer’s recommendations. The quality and quantity of isolated RNAs were determined by NanoVue Plus (GE, Pittsburgh, PA, USA). Total RNA was subjected to reverse transcription to synthesize cDNA using a SuperScript II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). Quantitative RTPCR was performed using SYBR green expression master mix (Applied Biosystems, Foster City, CA, USA). The forward and reverse primer sequences were as follows: (a) smooth muscle actin (αSMA) (5’ to 3’) CCAAG CCACC ATCC TATCC TCTG CCTG ACTTC TCCTT GCATT; (b) connective tissue growth factor (CTGF) (5’ to 3’) CCTGG TCCAG ACCAC AGAGT; (c) TGGAG ATTTT GGGAG TACGG; (d) COL1 (5’ to 3’) CAGGG TGGTT TGGCA TGACC; (e) TCTTG GTGGG TGTTGG G; (f) specificity protein 1 (SP1) (5’ to 3’) GCACACT TATTC CCTCA TCCTT TC, TGTGG GTTGT TACTG.

Table 1. Summary of Antibodies Used in This Study

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TTCTG G; GAPDH (5’ to 3’) GAAGG TCGGA GTCAA CGGAT TT; CCTTG AAGAT GGTGA TGGGA TT. A ΔΔCT method (2\(^{-\Delta\Delta C_{T}}\)) was applied to calculate the relative differences between the control and treated groups. All experiments were repeated at least three times.

**Analysis of Autophagic Flux by Laser Scanning Confocal Microscopy**

HTFs were infected by lentiviruses harboring tandem fluorescent SensGFp-StubRFP-LC3 for 24 hours. Positive cells were selected by puromycin treatment for 3 days. Selected cells were then seeded in 96-well plates at 4 \times 10\(^3\) cells per well and were subjected to various treatments as indicated elsewhere. Red and green fluorescence were observed under a confocal quantitative image cytometer (YOKOGAWA CQ1, Kanazawa, Japan) and were quantified using GraphPad Prism (GraphPad, San Diego, CA, USA).

**Western Blot**

Total proteins were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing 1% protease inhibitor cocktail (Sigma-Aldrich) and 1% phosphatase inhibitor (Sigma-Aldrich). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime). An equal amount of cell lysates (20–40 μg) was subjected to gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (GE) followed by blocking in 5% nonfat dried milk in phosphate buffered saline + 0.1% tween 20 (PBST) at room temperature for 1 hour and incubated with primary antibodies overnight at 4°C. After washing with PBST five times for 30 minutes, the membranes were incubated with corresponding secondary antibodies at room temperature for 1 hour. Specific protein bands were visualized with an enhanced chemiluminescence (ECL) advanced Western blot analysis detection kit (Merck, Darmstadt, Germany). Band intensity was measured by ImageJ software (NIH, Bethesda, MD, USA).

**Transmission Electron Microscopy**

Cells were fixed with 3% glutaraldehyde (PH 7.4) in 0.1 M sodium cacodylate for 1 hour at room temperature followed by 1% osmium tetroxide in 0.1 M sodium cacodylate for 2 hours. After dehydration with graded alcohols, cells were embedded in EMbed 812 (Electron Microscopy Sciences, Hatfield, PA, USA) and polymerized at 60°C for 48 hours. Thin sections were made on a Leica Ultracut UCT microtome (Leica, Munich, Germany). Sections were stained with uranyl acetate and lead. Images were collected and analyzed with a HT7700 transmission electron microscope (TEM) (Hitachi, Ibaraki, Japan).

**Animal Procedures**

Adult male New Zealand white rabbits with a bodyweight of 2.5 to 3.5 kg were used in this study. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A protocol of animal usage in this study was approved by the Second Xiangya Hospital of Center South University.

To better simulate the pathological conditions where GFS is applied in human patients, chronic ocular hypertension was established in rabbits prior to GFS. Rabbits were anesthetized by intravenous injection of 30 mg/kg pentobarbital sodium (Solarbio); 0.1 mL 2% methylcellulose (Solarbio) was injected into the anterior chamber of right rabbit eye once a week for 4 weeks.

Trabeculectomy was performed in the nasal-superior quadrant of rabbit eyes. Briefly, an eyelid speculum was used to expose the surgical field. Eyes were fixed with a 7-0 Vicryl corneal traction suture (Ethicon, Bridgewater, NJ, USA). A half-thickness, limbal-based 4 × 4 mm scleral flap was made anterior to the limbus. The scleral flap was sutured with two suture line (10-0 nylon) (Alcon, Fort Worth, TX, USA). The conjunctiva was closed with 8-0 Vicryl suture (Ethicon) in a continuous fashion. To reduce inflammation, 0.5 mL dexamethasone was injected in the subconjunctiva of surgical eyes. Tobramycin Dexamethasone Eye Ointment (Alcon) (one time daily) and Tobraflex eye drops (four times daily) were applied on the surface of surgical eyes in the first week. All surgeries were performed by one investigator (E.Z.).

A pilot study was conducted to optimize the dose of RSG used in rabbit studies. Fifty milligrams per milliliter of RSG exerted antiobiotic efficacy with minimal complications. One hundred milligrams per milliliter of RSG caused tissue necrosis in both conjunctiva and sclera, severe corneal toxicity, and endophthalmitis. Ten and twenty milligrams per milliliter of RSG had no significant effects on the bleb survival times and stabilization of IOP reduction as compared with the surgery only group. Based on these findings, 50 mg/mL RSG was determined as an optimal concentration for treatment of rabbits.

Rabbits were randomly assigned into five groups (n = 6 per group) as follows: (1) no surgery control group; (2) surgery only group in which rabbits underwent GFS with no treatments; (3) RSG group in which rabbits underwent GFS with 50 mg/mL RSG cotton patting intraoperatively for 3 minutes; (4) MMC group in which rabbits underwent GFS with 0.4 mg/mL MMC cotton patting intraoperatively for 3 minutes; (5) 5-FU group in which rabbits underwent GFS with 5 mg/mL 5-FU cotton patting intraoperatively for 3 minutes. Rabbits were followed up for evaluations of IOP, bleb appearance and survival, and complications at POD1 to POD28. At the end of experiments, rabbits were killed by intravenous injection of overdose pentobarbital sodium. Bleb tissues were collected for histochemical and immunoblot analyses.

**IOP Measurement and Bleb Scores**

The IOP was measured with a Tonovet (Vantaa, Finland) at 10:00 AM daily. The final IOP readings were averaged from three measurements. All measurements were performed by one investigator (E.Z.). Bleb’s scoring was conducted based on an Indiana Bleb Appearance Grading Scale (IBAGS),10 in which Bleb height, extent, vascularity, and leakage with the Seidel test were evaluated on a scale from 0 to 3. Ten and twenty milligrams per milliliter of RSG exerted antifibrotic efficacy with minimal complications. One hundred milligrams per milliliter of RSG caused tissue necrosis in both conjunctiva and sclera, severe corneal toxicity, and endophthalmitis. Ten and twenty milligrams per milliliter of RSG had no significant effects on the bleb survival times and stabilization of IOP reduction as compared with the surgery only group. Based on these findings, 50 mg/mL RSG was determined as an optimal concentration for treatment of rabbits.

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**Histology and Immunohistochemistry**

The tissues of operation sites (bleb, conjunctiva, Tenon’s capsule and sclera) were fixed in 4% paraformaldehyde and embedded in paraffin. Hematoxylin and eosin (H&E) staining and Masson’s trichrome staining were performed by standard protocols.11 Results were observed and imaged by light microscopy (Nikon Eclipse Ci, Tokyo, Japan).

**Statistical Analysis**

Results were expressed as means ± standard deviation (SD) of at least three independent experiments. Statistical analyses
We sought to determine the potential effect of RSG treatment on subconjunctival scar formation and on stabilization of reduced IOP in a rabbit model of GFS. To better simulate the pathological conditions where GFS is applied to treat patients with glaucoma, we established chronic ocular hypertension in rabbit eyes prior to trabeculectomy by injections of 2% methylcellulose into the anterior chamber. Rabbits with ocular hypertension were randomly assigned into four groups in which each group of animals were treated by RSG, MMC, 5-FU, and saline (designated as surgery only), respectively (Fig. 1). Initial injection of 2% methylcellulose caused an acute elevation of IOP at day 1 in the group of no surgery, surgery only, RSG, MMC, and 5-FU (42.3 ± 2.5, 39.0 ± 1.0, 47.0 ± 1.0, and 47.0 ± 1.0 mm Hg, respectively) followed by an IOP reduction to the baseline level at day 3 to 5. Repeated injections of 2% methylcellulose again elevated IOP at day 7 in these five groups (45.0 ± 1.0, 43.5 ± 1.5, 47.7 ± 1.5, 43.0 ± 1.0, and 46.0 ± 1.0 mm Hg, respectively). Although IOP gradually reduced thereafter, it reached to a platform ranging from 27.7 ± 0.6 to 32.7 ± 1.5 mm Hg at day 21 to 35. The level of platform IOP was significantly higher than the baseline level, indicating an establishment of ocular hypertension in rabbit eyes. After GFS, a drastic decrease of IOP was observed at postoperative day 1 (POD1) with animals in all four surgical groups (surgery only 12.0 ± 1.0 mm Hg, RSG 12.7 ± 0.6 mm Hg, MMC 9.7 ± 0.6 mm Hg, 5-FU 12.0 ± 1.0 mm Hg). IOP in the no surgery group had no significant changes at POD21 to 28. Animals in the surgery only group (with no treatments) showed a complete rebound IOP elevation. However, the rebound occurred at a slower rate than that of no surgery group. Of which, RSG-treated rabbits showed the slowest rate of rebound IOP elevation in a period of 28 days after GFS. Collectively, these data suggest that RSG treatment benefits stabilization of IOP reduction after GFS.

RSG Treatment Prolongs Filtering Bleb Survival After GFS

Maintenance of a functional filtering bleb is critical to the control of IOP after GFS. Next, we evaluated the effect of RSG treatment on the survival of filtering blebs following GFS. Bleb survival analysis demonstrated a significant difference in the survival distribution among surgery only, RSG, MMC, and 5-FU-treated groups after GFS. Following GFS in rabbits, ocular hypertension was induced by injections of 2% methylcellulose into the anterior chamber. Rabbits with ocular hypertension were randomly assigned into four groups in which each group of animals were treated by RSG, MMC, 5-FU, and saline (designated as surgery only), respectively (Fig. 1). Initial injection of 2% methylcellulose caused an acute elevation of IOP at day 1 in the group of no surgery, surgery only, RSG, MMC, and 5-FU (42.3 ± 2.5, 39.0 ± 1.0, 47.0 ± 1.0, and 47.0 ± 1.0 mm Hg, respectively) followed by an IOP reduction to the baseline level at day 3 to 5. Repeated injections of 2% methylcellulose again elevated IOP at day 7 in these five groups (45.0 ± 1.0, 43.5 ± 1.5, 47.7 ± 1.5, 43.0 ± 1.0, and 46.0 ± 1.0 mm Hg, respectively). Although IOP gradually reduced thereafter, it reached to a platform ranging from 27.7 ± 0.6 to 32.7 ± 1.5 mm Hg at day 21 to 35. The level of platform IOP was significantly higher than the baseline level, indicating an establishment of ocular hypertension in rabbit eyes. After GFS, a drastic decrease of IOP was observed at postoperative day 1 (POD1) with animals in all four surgical groups (surgery only 12.0 ± 1.0 mm Hg, RSG 12.7 ± 0.6 mm Hg, MMC 9.7 ± 0.6 mm Hg, 5-FU 12.0 ± 1.0 mm Hg). IOP in the no surgery group had no significant changes at POD21 to 28. Animals in the surgery only group (with no treatments) showed a complete rebound IOP elevation. However, the rebound occurred at a slower rate than that of no surgery group. Of which, RSG-treated rabbits showed the slowest rate of rebound IOP elevation in a period of 28 days after GFS. Collectively, these data suggest that RSG treatment benefits stabilization of IOP reduction after GFS.

RSG Prevents Fibrosis After GFS

Subconjunctival fibrosis/scarring following GFS disrupts aqueous humor drainage, resulting in a rebound IOP elevation that is responsible for failure of the filtering surgery in most cases. We sought to determine the potential effect of RSG treatment on subconjunctival scar formation and on stabilization of reduced IOP in a rabbit model of GFS. To better simulate the pathological conditions where GFS is applied to treat patients with glaucoma, we established chronic ocular hypertension in rabbit eyes prior to trabeculectomy by injections of 2% methylcellulose into the anterior chamber. Rabbits with ocular hypertension were randomly assigned into four groups in which each group of animals were treated by RSG, MMC, 5-FU, and saline (designated as surgery only), respectively (Fig. 1). Initial injection of 2% methylcellulose caused an acute elevation of IOP at day 1 in the group of no surgery, surgery only, RSG, MMC, and 5-FU (42.3 ± 2.5, 39.0 ± 1.0, 47.0 ± 1.0, and 47.0 ± 1.0 mm Hg, respectively) followed by an IOP reduction to the baseline level at day 3 to 5. Repeated injections of 2% methylcellulose again elevated IOP at day 7 in these five groups (45.0 ± 1.0, 43.5 ± 1.5, 47.7 ± 1.5, 43.0 ± 1.0, and 46.0 ± 1.0 mm Hg, respectively). Although IOP gradually reduced thereafter, it reached to a platform ranging from 27.7 ± 0.6 to 32.7 ± 1.5 mm Hg at day 21 to 35. The level of platform IOP was significantly higher than the baseline level, indicating an establishment of ocular hypertension in rabbit eyes. After GFS, a drastic decrease of IOP was observed at postoperative day 1 (POD1) with animals in all four surgical groups (surgery only 12.0 ± 1.0 mm Hg, RSG 12.7 ± 0.6 mm Hg, MMC 9.7 ± 0.6 mm Hg, 5-FU 12.0 ± 1.0 mm Hg). IOP in the no surgery group had no significant changes at POD21 to 28. Animals in the surgery only group (with no treatments) showed a complete rebound IOP elevation. However, the rebound occurred at a slower rate than that of no surgery group. Of which, RSG-treated rabbits showed the slowest rate of rebound IOP elevation in a period of 28 days after GFS. Collectively, these data suggest that RSG treatment benefits stabilization of IOP reduction after GFS.

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groups (P < 0.0001) (Fig. 2). Rabbits in the surgery only group displayed a rapid loss of filtering blebs after trabeculectomy and few filtering blebs were observed in this group at POD16 (Fig. 2). Compared with the surgery only group, filtering blebs in RSG, MMC, and 5-FU groups survived a significantly longer period of time. Loss of filtering blebs in 5-FU group started at POD16, and the filtering blebs completely lost at POD22. In contrast, the time periods of filtering bleb survival in RSG and MMC groups were significantly longer than that of 5-FU group. Loss of filtering blebs in MMC group started at POD24, and 88.3% blebs remained at POD28. Loss of blebs in RSG group occurred at POD22, and 66.7% blebs survived at POD28. RSG treatment significantly improved the survival of blebs as compared with surgery only (P < 0.001) and MMC (P < 0.05) and 5-FU (P < 0.001) treatment. Evaluation of the morphology of filtering blebs by IBAGS was summarized in Table 2. Taken together, these data suggest that RSG treatment benefits the survival of filtering blebs following trabeculectomy and the beneficial effects appear to be greater than that of 5-FU.

**RSG Treatment Attenuates Subconjunctival Fibrosis After GFS**

To evaluate the subconjunctival fibrotic response to GFS-induced injury, we analyzed expression of αSMA (a marker of myofibroblasts), CTGF (a potent fibrogenic cytokine), and SP1 (a profibrotic transcription factor) in bleb tissues isolated from rabbit eyes. Immunoblot analysis showed that protein levels of αSMA, CTGF, and SP1 were significantly elevated after FIGURE 3. RSG treatment attenuates subconjunctival fibrosis after GFS in rabbits. Rabbit bleb tissues in five groups were harvested at POD28. (A) Protein levels of SP1, αSMA, and CTGF were determined by immunoblot. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Immunoblots were scanned, and the band intensity of each specific protein was normalized to that of corresponding GAPDH. Results are mean ± SEM (n = 3 per group). *P < 0.05 and **P < 0.01. (B) Histological sections were stained with H&E and Masson’s trichrome. Scale bar: 100 μm.

**FIGURE 4.** RSG inhibits TGF-β1-induced expression of profibrotic genes in HTFs. (A) Cytotoxicity assay was performed by incubation of an increasing concentration (0–50 μM) of RSG with HTFs. (B–D) HTFs were treated with 10 ng/mL TGF-β1 for 0, 6, 12, 24, and 48 hours. Effects of TGF-β1 on profibrotic SP1, CTGF, and αSMA expression were determined at both the mRNA (B) and protein levels (C, D). (E–G) HTFs were pretreated with RSG or DMSO (vehicle control) for 2 hours followed by treatment with 10 ng/mL TGF-β1 for 0, 6, 12, 24, and 48 hours. Effects of RSG on TGF-β1-induced expression of profibrotic SP1, CTGF, and αSMA were determined at both the mRNA (E) and protein levels (F, G). GAPDH was used as internal reference control (quantitative PCR) and loading control (immunoblot). Results are mean ± SEM of four independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001.
trabeculectomy (Fig. 3A), indicating that GFS activates expression of profibrotic genes. RSG, MMC, or 5-FU treatment significantly attenuated GFS-induced expression of these genes, suggesting that RSG, similar to MMC and 5-FU, functions to inhibit subconjunctival scar formation after trabeculectomy. Consistent with the immunoblot findings, Masson's trichrome staining demonstrated reduced collagen deposition in the subconjunctival region in animals of three treatment groups, in particular RSG and MMC groups, as compared with animals in surgery only group (Fig. 3B). Evaluation of additional compli-

![Graph showing quantitative analysis of autophagy](image)

**Figure 5.** RSG enhances TGF-β1-induced autophagy in HTFs. (A) HTFs were transfected by SensGFP-StubRFP-LC3 lentiviruses (green, red). Cells were treated by TGF-β1 in the presence of DMSO (vehicle control), RSG, T0070907, rapamycin (Rap), CQ, or 3MA. Cells were imaged by confocal fluorescent microscopy. (B) Quantification of autophagosomes (green dots and red dots) and autolysosomes (red dots) were performed by GraphPad Prism. *P < 0.05; **P < 0.01; ***P < 0.001 (n = 200 cells per treatment). Scale bar: 10 μm.

**Table 2.** Evaluation of Filtering Bleb Morphology by IBAGS

<table>
<thead>
<tr>
<th>Group</th>
<th>POD7</th>
<th>POD14</th>
<th>POD21</th>
<th>POD28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery only</td>
<td>H1,E2,V3,S0</td>
<td>H1,E2,V2,S0</td>
<td>H0,E0,V1,S0</td>
<td>H0,E0,V1,S0</td>
</tr>
<tr>
<td>RSG</td>
<td>H1,E1,V2,S0</td>
<td>H2,E3,V1,S0</td>
<td>H2,E2,V1,S0</td>
<td>H1,E2,V1,S0</td>
</tr>
<tr>
<td>MMC</td>
<td>H1,E2,V2,S0</td>
<td>H1,E2,V1,S0</td>
<td>H1,E1,V1,S0</td>
<td>H1,E1,V1,S0</td>
</tr>
<tr>
<td>5-FU</td>
<td>H1,E2,V2,S0</td>
<td>H1,E1,V1,S0</td>
<td>H0,E0,V1,S0</td>
<td>H0,E0,V1,S0</td>
</tr>
</tbody>
</table>

H, Height; H0 (flat) to H4 (high); E, Extent; E0 (less than 1 clock hour) to E3 (more than 4 clock hours); V, Vascularity; V0 (avascular) to V4 (extensive vascularity); S, Seidel, S0 (no leak) to S2 (streaming).
cations associated with GFS complications, including hyperemia, chemosis of the conjunctiva, corneal toxicity, wound leak, blebitis, bleb leakage, and endophthalmitis, was summarized in Table 3.

### RSG Inhibits TGF-β1-Induced Expression of Profibrotic Genes in HTFs

To determine the mechanisms by which RSG treatment attenuates scar formation after GFS, we performed cell-based studies in a well-established TGF-β1 model in primary HTFs. To optimize the RSG concentration for HTF studies, we conducted a cytotoxicity assay by culturing HTFs in the presence of an increasing concentration of RSG (Fig. 4A). HTFs remained viable and healthy at 5 and 10 μM RSG, but became ill-looking and started to die at higher concentrations (20 and 50 μM). Based on this observation, we considered 10 μM of RSG as an optimal concentration for treatment of HTFs in vitro.

Primary HTFs were pretreated by RSG for 2 hours followed by TGF-β1 treatment for an additional 48 hours. We observed that TGF-β1 treatment promoted time-dependent increases in expression of SP1, CTGF, and αSMA at both the mRNA and protein levels (Figs. 4B–D). Significant increases in SP1, CTGF, and αSMA mRNAs were observed at 12, 24, and 48 hours. Significant increases in SP1 and αSMA proteins were observed at 12, 24, and 48 hours, while a significant increase in CTGF protein was observed at only 48 hours. Pretreatment with RSG blocked TGF-β1-induced mRNA and protein expression of SP1, CTGF, and αSMA (Figs. 4E–G). Additionally, RSG inhibited baseline expression of SP1 and CTGF mRNA at 24 hours, SP1 protein at 24 and 48 hours, and CTGF protein at 24 hours. In contrast, RSG did not alter mRNA and protein expression of αSMA at baseline.

### RSG Augments TGF-β1-Induced Autophagy in HTFs

A number of autophagy-inducing compounds derived from plants exert their antifibrotic properties by regulating the autophagic signal pathway. Since RSG functions to regulate autophagy, we investigated the effect of RSG pretreatment on HTF autophagy in response to TGF-β1. The formation of autophagosomes and autolysosomes was evaluated by lentivirus harboring tandem fluorescent SensGFP-StubRFP-LC3. TGF-β1 treatment per se increased the total number of autophagosomes and autolysosomes as indicated by red fluorescent dots in the cell body (Fig. 5A) and promoted autophagic flux or autophagic degradation activity as indicated by increased proportion of red fluorescent dots (autophagosomes plus autolysosomes) versus green fluorescent dots (autophagosomes only) (Fig. 5B). Pretreatment of HTFs with RSG further increased the formation of autophagosomes and autolysosomes and promoted autophagic flux, suggesting that RSG augments TGF-β1-induced autophagy in HTFs. As expected, rapamycin, an agonist of autophagy, drastically promoted TGF-β1-induced autophagy, whereas T0070907, CQ, and 3MA, antagonists of autophagy, blocked TGF-β1-induced autophagy. Furthermore, transmission electron microscopic analysis demonstrated increased formation of autophagosomes/autolysosomes in HTFs by TGF-β1 treatment; RSG and rapamycin enhanced, whereas T0070907, CQ, and 3MA inhibited, TGF-β1-induced formation of autophagosomes/autolysosomes (Fig. 6). Collectively, these data suggest that pretreatment with RSG augments TGF-β1-induced autophagy in HTFs.

### RSG Promotes TGF-β1-Induced Autophagy Via a Beclin1/Vps34-Dependent Pathway

To further elucidate the signal pathway that mediates RSG-regulated autophagy in HTFs, we examined effects of RSG on expression of key mediators in the autophagic pathway. RSG promoted expression of beclin1, Vps34, and Atg14L in the presence of TGF-β1 as compared with vehicle (DMSO) control

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**Table 3. Incidence of Common Complications After GFS by Different Treatments**

<table>
<thead>
<tr>
<th>Complications</th>
<th>Surgery Only</th>
<th>RSG</th>
<th>MMC</th>
<th>5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperemia</td>
<td>5/6</td>
<td>5/6</td>
<td>5/6</td>
<td>4/6</td>
</tr>
<tr>
<td>Chemosis of the conjunctiva</td>
<td>2/6</td>
<td>3/6</td>
<td>4/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Corneal toxicity</td>
<td>0/6</td>
<td>2/6</td>
<td>4/6</td>
<td>2/6</td>
</tr>
<tr>
<td>Bleb leakage</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Blebitis</td>
<td>0/6</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Endophthalmitis</td>
<td>0/6</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Number of animals with a complication/total # of animals.
group (Fig. 7). TGF-β1 alone did not alter expression of these mediators in HTFs. Similar to RSG, rapamycin promoted expression of beclin1, Vps34, and Atg14L in the presence of TGF-β1. T0070907 and CQ inhibited expression of Vps34 and Atg14L, but had no effects on beclin1 expression. 3MA inhibited Vps34 expression but not expression of beclin1 and Atg14L. TGF-β1 negatively regulated p62 (also known as SQSTM1) expression. Inhibition of p62 by TGF-β1 was preserved when cells were pretreated by RSG or rapamycin, but diminished when cells were pretreated by T0070907, CQ, or 3MA. Furthermore, pretreatment with CQ or 3MA not only reversed TGF-β1-dependent downregulation of p62, but even promoted p62 expression above the baseline. Conversion of nonlipidated LC3I to lipidated LC3II is known as a marker of autophagic activation. RSG increased the ratio of LC3II/LC3I similar to rapamycin, suggesting that pretreatment by RSG promotes autophagic activation in HTFs in the presence of TGF-β1. Taken together, these results suggest that RSG regulates TGF-β1-dependent autophagy in HTFs via a beclin1-VPS34-Atg14L-mediated pathway.

**Autophagy Mediates TGF-β1-Induced Expression of Profibrotic Genes in HTFs**

To investigate whether autophagy is potentially involved in inhibition of TGF-β1-induced profibrotic gene expression by RSG in HTFs, we tested effects of autophagy agonist (rapamycin) and antagonists (T0070907, CQ, and 3MA) on expression of SP1, CTGF, and zSMA at the mRNA and protein levels. Pretreatment with either RSG or rapamycin inhibited
TGF-β1-induced expression of SP1, CTGF, and αSMA at both the mRNA and protein levels (Fig. 8), suggesting that augment of autophagy prevents TGF-β1-dependent profibrotic gene expression in HTFs. In contrast, TGF-β1-induced transcription of SP1, CTGF, and αSMA protein levels were preserved when cells were pretreated by autophagy antagonist T0070907, CQ, or 3MA. TGF-β1-induced expression of CTGF protein was also preserved when cells were pretreated by T0070907 or 3MA. Interestingly, TGF-β1-induced expression of SP1 protein was either completely or partially lost when cells were pretreated by T0070907, CQ, or 3MA. Furthermore, TGF-β1-induced expression of CTGF protein was completely lost when cells were pretreated by CQ. These findings suggest that augmentation of autophagy inhibits TGF-β1-induced profibrotic gene expression in HTFs, whereas inhibition of autophagy can result in complex outcomes. Taken together, our data suggest that RSG inhibits TGF-β1-induced expression of profibrotic SP1, CTGF, and αSMA genes by promoting autophagy.

**DISCUSSION**

Antimetabolites, particularly MMC and 5-FU, are commonly applied intraoperatively and/or postoperatively as an addition to inhibit subconjunctival fibrosis following GFS. However, undesirable effects can occur due to nonselctive nature of these antimitaboles drugs. Moreover, individual variations also make the results of using antimetabolites unpredictable. Recent studies into alternative methods of preventing subconjunctival fibrosis have focused on novel molecular targets, including VEGF, TGF-β receptor, microRNAs, interferon, and cyclosporine A. Although some of these new strategies have proven effective in reduction of scar formation in animal models, very few have translated into treatments of human patients undergoing GFS. This urges continuous research efforts for the development of specific, safe, and more effective therapies against ocular tissue fibrosis associated with GFS. HTF plays a critical role in the pathogenesis of bleb scarring by differentiation into an activated, profibrotic myofibroblast phenotype. In the previous study, we reported that RSG, a PPARγ agonist, inhibits TGF-β1-induced activation of HTFs via a p38-dependent signaling pathway. In the current study, we provided further in vivo evidence that RSG treatment inhibits profibrotic gene expression and subconjunctival fibrosis in a rabbit model of GFS. In vitro studies suggest that RSG inhibits TGF-β1-induced profibrotic gene expression by promoting autophagy. The findings provide novel mechanistic insights into the antifibrotic properties of RSG. Previous studies have shown that RSG attenuates inflammation in several tissues and organs, including adipose tissues and lungs. It remains to be determined whether it exerts similar anti-inflammation effects on the eye following GFS.

Autophagy is an evolutionarily conserved process for the destruction of harmful protein aggregates, intracellular pathogens, and organelles via a lysosome-dependent pathway. Although autophagy was initially discovered as a survival mechanism for starving cells, it is associated with a number of human diseases and models of cell death. Studies have shown that activation of autophagy can be either beneficial or detrimental under pathological conditions. In this study, TGF-β1 promotes HTF differentiation into myofibroblasts by induction of autophagy. This contributes to subconjunctival scarring after GFS. RSG treatment further enhances TGF-β1-
induced autophagy. Augmentation of autophagy, conversely, prevents TGF-β1-induced profibrotic gene expression and myofibroblast differentiation, thus inhibiting ocular tissue fibrosis after HTFs. Our findings support a context-dependent action of autophagy on subconjunctival scarring after GFS. Further studies are needed to delineate the differential roles of autophagy in the process of ocular tissue injury and repair after trabeculectomy.

In summary, the current study reveals the potential efficacy of RSG in treatment of subconjunctival fibrosis associated with GFS. It provides a novel antiscarring drug candidate to prevent the most common cause of failed trabeculectomy and increase the success rate of GFS. Developing RSG-based treatments including design and optimization of RSG-specific drug delivery system is the next focus of our ongoing research.

CONCLUSIONS
We conclude that RSG treatment prevents scar formation in a rabbit model of GFS by inhibition of profibrotic gene expression through a mechanism involved in promoting autophagy in local fibroblasts. RSG represents a novel antiscarring drug candidate to improve the success rate of GFS.

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References
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