SB-431542 Inhibition of Scar Formation after Filtration Surgery and Its Potential Mechanism

Yi-qin Xiao,1 Kun Liu,1 Jian-feng Shen,2 Guo-Tong Xu,3,2,3 and Wen Ye∗,1

PURPOSE. To explore the inhibitory effect of SB-431542 (an ALK5 inhibitor) on scar formation after glaucoma surgery and to identify the potential pharmacologic target(s).

METHODS. Twenty-four New Zealand rabbits underwent filtration surgery on the right eye and were divided into a control group and three experimental groups (n = 6). Human Tenon’s fibroblast monolayer was scraped to generate a single gap, and then the control medium with SB-431542 only or containing 10 μg/L TGF-β1 and SB-431542 (1–20 μM) was added. The cells were pretreated with SB-431542 or in control medium for 30 minutes before induction with 10 μg/L TGF-β1 or 1 μg/L TGF-β2. The expression of αSM-actin, CTGF, and Col I, as well as changes in the Smad, ERK, P38, and AKT signaling pathways were detected.

RESULTS. In comparison with the control rabbits, the IOPs in the experimental groups remained at lower levels until day 25 (P < 0.05) after the surgery. Histologic profiles showed that there was only a mild deposition of collagen in the subconjunctival space in the experimental groups. The cell growth and migration were inhibited effectively by SB-431542, regardless of whether TGF-β was present in the culture system. SB-431542 abrogated TGF-β-induced upregulation of αSM-actin, CTGF, and Col I. It effectively inhibited the phosphorylation of Smad2 stimulated by TGF-β but not that of the components of the MAPK pathways.

CONCLUSIONS. SB-431542 inhibits scar formation after glaucoma filtration surgery. The mechanism may be that SB-431542 interferes in the phosphorylation of Smad2, thus abrogating TGF-β-induced fibroblast transdifferentiation and then decreasing Col I synthesis. (Invest Ophthalmol Vis Sci. 2009;50: 1698–1706) DOI:10.1167/iovs.08-1675

Excess scarring of the conjunctiva after glaucoma filtration surgery is the major cause of the failure of such surgical treatments. Treatment with antimetabolites, such as mitomycin C (MMC) and 5-fluourouracil (5-FU), could decrease the scar formation and improve the IOP-lowering effect of the surgery. However, the treatments with MMC or 5-FU are often associated with serious adverse effects, like the loss of corneal endothelial cells and increases the risk of cataract.1,2 So, compounds with IOP-lowering effects but less toxic are desirable. Researchers have demonstrated that the transdifferentiation of human Tenon’s capsule fibroblasts (HTFs) to myofibroblasts (MFs) is one of the most crucial events in wound healing and scar formation after the surgery and is essential for subsequent tissue remodeling.3 Therefore, more specific compounds targeting HTF-MF transdifferentiation in the later stages of wound healing could be proper candidates for such postsurgical treatments.

Transforming growth factor (TGF)-β is essential for the transdifferentiation of HTF to MF.4 As reviewed by Roberts and Sporn,5 low levels of TGF-β signaling have been implicated in compromised wound healing, and inappropriately high levels of TGF-β signaling are associated with excessive scarring. Blockage of TGF-β with neutralizing antibody in rabbits that underwent eye surgery effectively suppressed the conjunctival scarring.6 There are three isoforms—β1, β2 and β3—in the TGF-β family, and they share similar abilities in regulating cell functions, such as proliferation, differentiation, apoptosis, and production of extracellular matrix. In the eye, the aqueous humor contains abundant TGF-β2, whereas TGF-β1 and -β2 are expressed in the cells in the filtering bleb.7,8 All the TGF-β isoforms take part in the wound healing and have similar effect in vitro9 and in vivo.10 Blocking TGF-β at the ligand level may impair other beneficial effects of TGF-β in wound healing after glaucoma surgeries. Selective enforcement of the blockade of TGF-β signaling at the downstream level in the signaling pathways may provide more therapeutic advantages.

TGF-β superfamily members function via the same signal transduction network. The TGF-β receptor complex comprises type I and II receptors. Binding to the receptors leads to the phosphorylation of signal transducer proteins Smad2 and -3. The phosphorylated Smad2 and -3 form complexes with the signaling molecule Smad4, and then translocates into the nucleus to regulate gene transcription.11 Smad 7 gene introduction could block Smad2/3 nuclear translocation with suppression of α-smooth muscle actin (α-SM-actin) and thus modulate injury-induced conjunctival wound healing.12 Activin receptor-like kinase 5 (ALK5, one of the seven known type 1 TGF-β receptors) is the key point in the TGF-β signaling pathway. Blocking TGF-β binding or the phosphorylation of its substrate could inhibit cell signal transduction.

SB-431542 was developed as a potent inhibitor of ALK5. Inman et al.13 further demonstrated that SB-431542 selectively inhibited ALK4, -5, and -7, but not other ALKs or other TGF-β-related signal pathways.13 Consistent with this observation, they showed that SB-431542 inhibits all TGF-β- and activin-induced phosphorylation of Smad2, which is mediated by ALK5 and -4. The present study demonstrated the extent of the IOP-lowering effect of SB-431542 after filtration surgery in an animal model by suppressing the scar formation. An in vitro system that mimics the in vivo pathophysiological changes was also investigated for the potential mechanism of this inhibitor. This information could be helpful in developing new drugs for...
improving glaucoma filtration surgery and for understanding the mechanism of surgery-induced scar formation.

**Materials and Methods**

For the medical intervention of the postsurgical process in an antiglaucoma surgery model in the rabbit, all the animal-related procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The process of obtaining human Tenon’s capsule tissue for cell culture experiments was granted hospital Ethics Review Board approval and adhered to the Declaration of Helsinki.

**In Vivo Studies**

**Animals and Groups.** New Zealand rabbits, 3 to 5 months old and weighing 1.8 to 2.5 kg, were purchased from the experimental Animal Center of Shanghai First People’s Hospital and acclimatized for 1 week before the experiments.

Twenty-four rabbits were randomly divided into a surgical control (SC) group and three experimental groups. Standard filtration surgeries were performed on the right eyes of all the rabbits in the four groups. As summarized in Table 1, the rabbits in the SC group received only vehicle (0.1 mL physiologic saline solution, or PBS) after the surgeries, whereas the rabbits in the three experimental groups were treated either with 0.2 mg/mL MMC during the surgery as the treatment control (MMC group) or with low and high concentrations of SB-431542 (the SB-low and -high groups, respectively). For the SB-low and -high groups, SB-431542 (cat. no. 1614; Tocris Cookson, Inc., Ellisville, MO) solutions of 0.5 and 2 mM were given to the right eyes of the rabbits. The standard procedure for the treatments with PBS and SB-431542 were subconjunctival injections (in 0.1 mL), immediate after the surgery and on days 1, 2, 3, and 7, after surgery. The treatment with MMC, a current clinical procedure, serves as a benchmark to show the potential clinical benefit of SB-431542, if there is any.

**Surgical Procedure.** Surgery was performed on the right eye only under general anesthesia with intramuscular injections of ketamine (Ketaset; Parke Davis, Berlin, Germany) and xylazine (Rompun; Bayer, Leverkusen, Germany), plus local anesthesia with oxybuprocaine drops (Novesine 0.4%; Novartis, Nürnberg, Germany). After a lid speculum was placed, a partial-thickness 8-0 silk corneal traction suture (Ethicon, Edinburgh, UK) was applied superiorly to pull down and fix the eyeballs. A peritomy at 5 mm above limbus was performed to identify any cross-reaction of the two agents.

**Histologic Examinations.** All the rabbits were killed on day 28 after surgery. The eyeballs were enucleated, together with the conjunctiva to preserve the bleb, for histologic examination. The eyes were immediately fixed with stationary liquid for at least 24 hours. Tissue samples were then dehydrated and embedded in paraffin. Serial sections (5 µm thick) were cut, dehydrated, and stained with hematoxylin-eosin for light microscopic examination, to get a general impression of the histologic changes, with the Masson technique used to determine the collagenous extracellular matrix (ECM) deposition, and with immunohistochemistry of α-SM-actin used to mark the MF. A histologic examination was performed at the center of the sclerotomy site, as indicated by the location of the iridectomy.

**Statistical Evaluation.** Statistical analysis was performed to determine the differences of IOP, among the SC group and three experimental groups (SPSS 10.0; SPSS Sciences, Chicago, IL). An ANOVA was performed, and significance was assumed if $P < 0.05$.

**In Vitro Studies**

**Cell Culture.** HTFs were obtained from patients with cataract during surgery. Primary human Tenon’s fibroblasts were obtained as an expansion culture of the human Tenon’s explants and were propagated in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, Utah) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen-Gibco Life Technologies, Karlsruhe, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin (Biochrum, Berlin, Germany). The cells were maintained in the logarithmic growth phase. Cells from passages 5 to 15 were used in all the experiments. Fibroblasts from the same initial population were cultured in monolayers at 37°C, and the medium was renewed every 3 days.

**SB-431542 and TGF-β Treatments.** Stock solutions of SB-431542 were prepared in Me2SO (DMSO) at (5 mM, prepared in advance and stored at $-20^\circ$C until use). The solution was diluted in DMEM, added to the cell culture system 30 minutes before other treatments, and was present in the culture system at different concentrations (0, 1, 5, and 20 µM). When human recombinant TGF-β1 (CytoLab Ltd., Rehovot, Israel) and TGF-β2 (CytoLab Ltd.) were tested in the system, various concentrations of TGF-β were prepared as described previously. If in most of the typical experiments, 10 µg/L TGF-β1 (reconstituted in PBS containing 2 mg/mL albumin and stored at $-20^\circ$C) in DMEM and 1% bovine serum albumin (BSA) was used. The cells were preincubated with different concentrations of SB-431542 (0, 1, 5, and 20 µM) for 30 minutes before TGF-β1 stimulation. An SB-431542-alone group was included as a control, to identify any cross-reaction of the two agents.

**Scratch Wound Assay.** A scratch wound assay was performed to mimic the scarring process and to evaluate the effects of SB-431542 in regulating scar formation. The HTFs were grown on poly-ε-caprolactone (100 µg/mL coated 12-well plates. When the cells reached 80% confluence, the culture medium were replaced by serum-free DMEM, and the cells were starved for 12 hours to synchronize cell growth. After pretreatment with 0, 1, 5, and 20 µM SB-431542, the cell monolayer was scraped with a sterilized 1000-µL pipette tip, to generate a single gap. After the cells were washed with PBS, both the control medium with SB-431542 only and medium containing 10 ng/mL TGF-β in addition to SB-431542 were added to the indicated wells. The wound gap in each well was observed with phase contrast microscope every 8 hours. The distance between the edges of each gap was measured and evaluated (Image Pro plus 5.1 software; Media Cybernetics, Silver Spring, MD) and was normalized to the width of the gap in the 0-hour wells.

**Evaluation of TGF-β1 and/or SB-431542 on Transdifferentiation of HTFs to MFs.** Considering that the transdifferentiation of fibroblasts to MFs, characterized by the synthesis of α-SM-actin, a marker of MFs, and CTGF, the potent mediator of transdifferentiation, is a crucial step in wound healing, and Col I was an important component of local tissue remodeling in response to baseline and on the designated days after surgery. An average of three measurements was taken as the IOP readings. The general appearances of the eyes were examined with slit lamp and ophthalmoscope.

**Table 1. Animal Groups and Treatments**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>SC</td>
<td>PBS, standard procedure</td>
</tr>
<tr>
<td>MMC</td>
<td>0.2 mg/mL MMC, 3 minutes during the surgery, with washing</td>
</tr>
<tr>
<td>SB-low</td>
<td>0.5 mM SB-431542, standard procedure</td>
</tr>
<tr>
<td>SB-high</td>
<td>2 mM SB-431542, standard procedure</td>
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$n = 6$ in all groups.
In Vivo Studies

RESULTS

In Vivo Studies

General Eye Examination, Focused on Scar Formation. The rabbits’ eyes were evaluated daily in the first week after the surgery and twice a week thereafter. Inflammatory responses in the surgical eyes such as conjunctival hyperemia were mild in all groups and lasted for approximately 1 week. No endophthalmitis was observed. No corneal edema or other type of corneal damage was detected in any group. Rabbits in both the control and experimental groups showed no sign of cataract during the experimental period. In summary, there was no remarkable difference between the SC group and experimental groups in general eye examination, except the scar formation.

Intraocular Pressure. As a key parameter in evaluating the results of surgery or glaucoma treatment, IOP was measured on the designated days to evaluate the effect of SB-431542 or with MMC (SB-low group, SB-high group, and MMC group, as shown in Figs. 1B–D).

Histologic Examination. Histologic profiles revealed massive subconjunctival scarring in the SC animals (Figs. 3A, 3B). The subepithelial connective tissue showed fibroblast hyperplasia. The sclerotomy site was infiltrated by hypercellular fibrotic tissue and a dense collagenous connective tissue. In the

TABLE 2. Primers and Conditions in PCR Analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer</th>
<th>Temp. (°C)</th>
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<tbody>
<tr>
<td>CTGF</td>
<td>Forward 5'-GACGGCTGCGCTGCTGCTGC-3'</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CACACCCACTGCTGGCAGCA-3'</td>
<td></td>
</tr>
<tr>
<td>α-SM-actin</td>
<td>Forward 5'-GCTCAGGGAGGAGACCCTGGA-3'</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTGATAGGACATTGTAGGAT-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5'-ACCAGAATCCATGGCCATCAG-3'</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TCACACCCTGTTGCGTGA-3'</td>
<td></td>
</tr>
</tbody>
</table>

In Vivo Studies

RESULTS

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General Eye Examination, Focused on Scar Formation. The rabbits’ eyes were evaluated daily in the first week after the surgery and twice a week thereafter. Inflammatory responses in the surgical eyes such as conjunctival hyperemia were mild in all groups and lasted for approximately 1 week. No endophthalmitis was observed. No corneal edema or other type of corneal damage was detected in any group. Rabbits in both the control and experimental groups showed no sign of cataract during the experimental period. In summary, there was no remarkable difference between the SC group and experimental groups in general eye examination, except the scar formation.

Wound healing was observed as the loss of conjunctival transparency and thickening due to the deposition of fibrotic tissue. In the SC group, scarring of the conjunctiva was prominent (Fig. 1A). The subconjunctival fibrotic tissue covered and impaired the view of the scleral flap. In contrast, less severe scar formation, as shown by the translucent conjunctiva, was observed in the surgical eyes of the rabbits treated with either SB-431542 or with MMC (SB-low group, SB-high group, and MMC group, as shown in Figs. 1B–D).

Histologic Examination. Histologic profiles revealed massive subconjunctival scarring in the SC animals (Figs. 3A, 3B). The subepithelial connective tissue showed fibroblast hyperplasia. The sclerotomy site was infiltrated by hypercellular fibrotic tissue and a dense collagenous connective tissue. In the
MMC group and SB groups (SB-low and -high), there were only mild fibrotic responses and depositions of collagen in the subconjunctival spaces.

There was a slight difference between MMC- and SB-431542-treated rabbits (both SB-low and -high groups). In the MMC group, the subepithelial connective tissue consisted of evident hypercellular fibrotic tissue (Fig. 4A), and degeneration of the corneal stroma was observed in one case (Fig. 4B). In the SB-low (Fig. 4C) and -high (Fig. 4D) groups, the conjunctival epithelium looked healthy, and the subepithelial connective tissue was loosely arranged and contained histologically clear spaces. Furthermore, the eyes did not reveal an intra- or extraocular inflammatory reaction. No toxic effect in the corneal epithelium, endothelium, lens, and ciliary body was detected by light microscope.

Because of the importance of MF in wound healing and scarring, we also detected MF in the surgery area. In the SC group, activated fibroblasts or MFs infiltrated the subepithelial field and the sclerotomy site (Fig. 5A). In the MMC group, MFs were located in the subepithelial connective tissue (Fig. 5B). However, there were rare MFs in the surgical area in both the SB-low and -high groups (Fig. 5C, 5D).

**In Vitro Studies**

As a key component in wound healing and scar formation, HTFs were studied in a cell culture system. This system at least partially mimics the process in wound healing and scarring. The growth and migration capacity of the HTFs was examined for their behavior in wound healing and for the effects of

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**FIGURE 2.** The effect of SB-431542 and MMC on post-surgical maintenance of IOP. In comparison with the IOP from the same rabbits before surgery (pre), IOP was lowered until day 14 ($P < 0.05$) in the SC group. The three treatments maintained IOP at low levels until day 25 ($P < 0.05$). When comparisons were made against the SC group, the IOP in all the three experimental groups were significantly lower between days 14 and 25 after surgery ($P < 0.05$). There is no significant difference between the MMC and SB-431542 groups.

**FIGURE 4.** Histologic analysis of the specimens of rabbits in the MMC and SB groups. (A) MMC-treated rabbit. Hypercellular fibrotic tissue was identified in the subepithelial connective tissue. (B) MMC-treated rabbit. Degeneration of corneal stroma occurred in one of the rabbits in this group. (C, D) Rabbits treated with low- and high-dose SB-431542. The conjunctival epithelium looked healthy, and the subepithelial connective tissue was loosely arranged and contained histologically clear spaces. HE stain; magnification, ×100.

**FIGURE 3.** Histologic examination of the eye tissues of the rabbits. (A, B) In SC group, massive subconjunctival scarring could be seen. (C–H) In the experiment groups, only mild fibrotic responses were found. (A, C, E, G) HE stain; (B, D, F, H) Masson stain; magnification, ×40.

**FIGURE 5.** Immunohistochemistry of α-SM-actin of the specimens of rabbits in the control and experimental groups. Sections were labeled with a primary antibody against α-SM-actin at a dilution of 1:400 in blocking buffer. A biotin-conjugated secondary antibody against mouse IgG was used at a 1:200 dilution in blocking buffer. The positive staining (yellow) demonstrates a reduction in the number of MFs. In the SC group and MMC group, the activated-fibroblasts or MFs infiltrated the surgery areas; however, there were rare MFs in SB-431542 treated groups. DAB stain; magnification, ×40.
different compounds on the behavior. Biochemical and molecular mechanisms of these compounds were also explored in a simple but effective system.

**Scratch Wound Assay.** In the scratch wound assay, the data showed that 10 μg/L of TGF-β1 significantly stimulated HTF growth and migration when compared with normal control cells (Figs. 6A, 6D). Cell growth and migration were inhibited effectively by SB-431542, regardless of the presence of TGF-β1 in the culture system. In the absence of TGF-β1, SB-431542 inhibited cell migration in a dose- and time-dependent manner (B, C, E, F). In the absence of TGF-β, the cell migration was also inhibited by SB-431542 in a dose- and time-dependent manner (B, representative images; E, statistics).

**Figure 6.** Scratch wound assay. The treatment of 10 μg/L TGF-β1 effectively stimulated the cell migration when compared with normal control (A, representative images; D, statistics). Such presence of SB-431542 could block the cell migration stimulated by TGF-β1 (C, representative images; F, statistics). In the absence of TGF-β, the cell migration was also inhibited by SB-431542 in a dose- and time-dependent manner (B, representative images; E, statistics).
manner (Figs. 6B, 6E). The presence of TGF-β1 in the culture system stimulated cell migration, which was also significantly blocked by the addition of SB-431542 (Figs. 6C, 6F).

**TGF-β1 Upregulation of α-SM-actin, CTGF, and Col I in Cultured HTFs.** On stimulation with TGF-β1 (10 μg/L), the expression of α-SM-actin mRNA by the cultured HTFs increased after 12 hours and peaked at 24 hours. The expression of CTGF mRNA increased faster, beginning 1 hour after TGF-β1 stimulation, and was pronounced at 12 hours (Fig. 7C). At the protein level, when the HTFs were stimulated with different concentrations of TGF-β1 or -β2 for different times, the expressions of α-SM-actin, CTGF, and Col I all increased (Fig. 7A), in a time-dependent manner. Under the stimulation of 10 μg/L TGF-β1, the expression of CTGF increased after 12 hours and was pronounced at 24 hours, although the expression of α-SM-actin peaked at 48 hours. The increase in Col I expression started late, and at a less evident level (Fig. 7B). The two isoforms of TGF-β showed similar action in this in vitro system.

**SB-431542 Prevention of the Expression of α-SM-actin, CTGF, and Col I.** When SB-431542 was added into the cultured HTF system, it effectively diminished α-SM-actin, CTGF, and Col I expressions induced either by TGF-β1 or -β2 (Fig. 8A). In the SB-431542 alone groups (without extragenous TGF-β stimulation), when a low concentration was used (1 μM), the expression of Col I was downregulated to a relatively lower level than that of CTGF. The inhibitor alone did not affect the expression of α-SM-actin (Fig. 8B).

**Effects of SB-431542 on Related Signaling Pathways in HTFs.** When the HTFs were stimulated with TGF-β1, SB-431542 prevented the phosphorylation of Smad2, but showed no inhibitory effect on the components of the ERK, p38 MAPK, or AKT pathways (Figs. 9A, 9B). However, in the absence of extragenous TGF-β in the culture system, the inhibitor alone diminished the expressions of ERK, p38, and AKT at high concentrations (20 μM; Figs. 9A, 9C).

**DISCUSSION**

Postoperative scarring is a major cause of the failure of glaucoma surgery observed in long-term follow-up. Experimental rabbits exhibit a more aggressive healing response when compared with that of human tissue. The demonstration of efficacy of a drug in such models is therefore likely to be reproduced in the clinical setting. In our rabbit experiment, surgical failure was observed within 14 days, as indicated by the IOP increase in the surgery-alone group. Both the antifibrotic drug MMC and the ALK5 inhibitor SB-431542 prolonged the normal IOP period between the surgery and the failures. In another words, local application of SB-431542 after the surgery maintained the surgical effect for longer time and was as effective as MMC.

One key process in wound-healing and scar formation is the transdifferentiation of fibroblasts to MFs. MFs have high contractility and also deposit extracellular matrix proteins, serving as the main agent of scarring. The relationship between scar formation and the failure of surgery to lower IOP was also observed in the rabbit model in the present study. In the SC group, MFs infiltrated the subepithelial field and the sclerotomy site, and mean IOP increased 14 days after the surgery. On the contrary, in both the SB-low and -high groups, there were rare MFs in the surgical area, and the mean IOPs were maintained at lower levels up to 28 and 25 days, respectively, indicating that SB-431542 lowered IOP by preventing the transdifferentiation of MFs and thus decreasing scar formation. In an in vitro experiment with HTFs which could mimic the behavior of wound healing and scarring, regardless of the presence of TGF-β, SB-431542 was also shown to inhibit cell migration in a dose-dependent manner.
and time-dependent manner and attenuated the formation of α-SM-actin. MFs share ultrastructural features of both fibroblasts and smooth muscle cells. Both exert increased contractile activity, which is associated with the expression of α-SM-actin. The increased amounts of α-SM-actin are incorporated into actin stress fibers as part of the contractile apparatus, to ensure sufficient wound closure.3 Moreover, MFs represent an "activated" fibroblast phenotype with increased synthesis of ECM proteins.3 The presence of MFs suggests long-standing scar modulation.17 So, any medicine, such as SB-431542, that can slow down the formation of MFs from HTFs, may improve the long-term effect of filtration surgery.

In the study of the relationship between TGF-β level and the development of a filtering bleb in primary open-angle glaucoma (POAG) eyes showed that favorable bleb development had low TGF-β2 levels, whereas POAG eyes with unfavorable bleb development had higher mean TGF-β2 levels.18 The functional significance of TGF-β is complex. In the early phases of wound healing, TGF-β is secreted by inflammatory cells and acts as a chemoattractant to promote wound healing. But the persistence of TGF-β stimulates the transdifferentiation of fibroblasts to highly contractile MFs. TGF-β is essential for the transdifferentiation of HTF to ME.4 TGF-β has three isoforms, β1, β2, and β3. All the three had a similar effect in wound healing and scar formation in the present study (Fig. 7A), as well as in other in vitro5 and in vivo6 studies. It is worth noting that, in addition to the aqueous humor, TGF-β was also generated locally at the wound site. HTFs have been reported to generate TGF-β1 protein by autosecretion.19 The profile of TGF-β activity at the wound site may be altered by filtration surgery, complicated by the fact that the passage of aqueous through the filtration, resulting in a constantly changing environment. The role of TGF-β during HTF-mediated conjunctival wound healing depends on its concentration at the wound site. Under physiological conditions, the level of TGF-β1 remains low (~1 μg/L). However, in pathophysiological conditions such as in filtering blebs, this concentration was reported to be effective in one study.14 With either high or low concentrations of TGF-β1, SB-431542 showed inhibitory effect against the stimulation of both TGF-β1 and -β2.

The mechanism of signaling by TGF-β family members is now understood in some detail.20 The ligands bring together a type II TGF-β receptor (TβRII) with a type I receptor (TβRI), both serine/threonine kinases. The TβRII phosphorylates and activates the TβRI in the complex. In most cell types, TGF-β signals through the combination of TβRII and ALK5 (one of the TβRls).21 In an animal model,22 both TβRI and -II were found to be coexpressed and increased in the epidermis, epidermal appendages, and dermis after wounding. This finding is consistent with the phenomena that TβRII cannot be expressed without TβRII, and they function together to initiate the cascade of signal transduction.23,24 Meyer-Ter-Vehn et al.25 studied the cell-type-spe-
protein production. It has been reported that TGF-β functions, such as fibroblast activation and stimulation of ECM appeared 3 to 5 days later than the ligands during tissue repair. It is strongly induced by TGF-β1 and CTGF and Col I expression to different degrees, in the absence of extragenous TGF-β1. This delay in receptor expression suggests that upregulation of TGF-β receptor that results in an enhanced TGF-β response. SB-431542 as an inhibitor of ALK5, could break up this activation of such receptors and diminish the response in the cells.

During tissue repair, the expressions of connective tissue growth factor (CTGF) are coordinately regulated. Our data showed that 2-hour exposure to TGF-β is sufficient to induce CTGF gene transcription for up to 24 hours in fibroblasts. CTGF is a cysteine-rich, heparin-binding protein whose gene expression is strongly induced by TGF-β in fibroblasts. SB-431542, in the present study, suppressed the expression of CTGF and type-I collagen in the cultured HTFs. CTGF has been implicated as downstream of TGF-β. It is the increasing expression of the TGF-β receptor that results in an enhanced TGF-β response. SB-431542 as an inhibitor of ALK5, could break up this activation of such receptors and diminish the response in the cells.

Figure 9. Effects of SB-431542 on related signaling pathways. HTFs were treated with 0 (DMSO), 1, 5, or 20 µM of SB-431542 and with or without TGF-β1 (10 µg/L). Activation of Smad2, p38, Erk, and AKT was detected with phosphorylated antibodies. Antibodies against total proteins served as the control. SB-431542 prevented TGF-β-induced phosphorylation of Smad2 (A), but had no effect on components of the ERK, p38, or AKT pathways (B–D). Left, representative images; right, statistics.

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Other data show that P38 signal pathway is also important in the MF transdifferentiation process. Meyer-Ter-Vehn et al. showed that specific p38 signal pathway inhibitors (SB-203580, SB-202190, and SB-200252) could prevent TGF-β-induced transdifferentiation. This finding was not contradicted by the present observations because non-Smad signaling cascades were reported to be relevant to TGF-β signaling in addition to classic Smad signaling. Ohshima and Shimotohno showed that PI3K (protein inhibitor of activated signal transducers and activators of transcription)-mediated sumoylation of Smad4 is regulated by the p38 MAPK pathway. On the other hand, a study of A498 renal epithelial carcinoma cells showed that...
SB-203580 and SB-202190 could serve as ALK5 inhibitors, which could inhibit phosphorylation of Smad3 by ALK5.36 Thus, subconjunctival application of SB-431542, which has a favorable effect in terms of inhibition of fibroblast transdifferentiation and CTGF expression, is beneficial in suppressing excessive conjunctival scarring and maintaining the IOP-lowering effects of glaucoma filtration surgery.

Acknowledgments

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References

24. Gold LI, Sung JJ, Siebert JW, Longaker MT. Type I (RI) and type II (RII) receptors for transforming growth factor-beta isoforms are expressed subsequent to transforming growth factor-beta ligands during excisional wound repair. Am J Pathol. 1997;150:209–222.