Sphingosine-1-Phosphate (S1P)-Related Response of Human Conjunctival Fibroblasts After Filtration Surgery for Glaucoma

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PURPOSE. To investigate levels of sphingosine-1-phosphate (S1P) in aqueous fluid samples taken before and after filtration surgery and S1P-induced human conjunctival fibroblast (HCF) responses.

METHODS. Levels of S1P and its related sphingophospholipids in aqueous fluid obtained immediately before and after filtration surgery were determined by liquid chromatography–tandem mass spectrometry. HCFs were used for all in vitro experiments. The expression of five S1P receptor subtypes in HCFs was examined by quantitative real-time PCR. The effect of S1P and receptor-specific antagonists on HCF viability and cell migration was assessed by WST-1 assay and scratch migration assay, respectively. Differentiation to myofibroblasts and extracellular matrix production was evaluated by examining changes in F-actin, α-smooth muscle actin (αSMA), and collagen expression with immunocytochemistry, Western blotting, and collagen accumulation assay, respectively.

RESULTS. No significant S1P levels in the aqueous fluid samples were detectable immediately before surgery, but postoperative levels of several lysophospholipids, including S1P, dehydro-S1P, and sphingosine, were significantly increased to bioactive concentrations in aqueous fluid in the blebs (P < 0.0001). mRNA expression of the three main S1P receptor subtypes was detected in HCFs. Although S1P levels did not influence HCF proliferation, S1P enhanced cell migration, which could be inhibited by the S1P2 antagonist JTE 013. Factin, αSMA, and collagen expression was significantly increased by S1P stimulation and was reduced by JTE 013.

CONCLUSIONS. Bioactive S1P concentrations were present in the aqueous fluid at the end of filtration surgery. S1P activated HCFs via S1P2 receptors. These results revealed the potential of S1P2 antagonists in preventing scarring after glaucoma filtration surgery.

Keywords: sphingosine-1-phosphate, fibrosis, filtration surgery, conjunctiva

High intraocular pressure (IOP) is associated with glaucomatous optic neuropathy. A large body of evidence indicates that sufficient reductions in IOP can suppress the progression of ganglion cell loss. Several approaches are now available to lower IOP including medications, laser treatments, and surgeries. Among the various glaucoma surgeries, filtration surgery is established as the most effective procedure to target and surgeries. Among the various glaucoma surgeries, filtration blebs,12,13 which can currently be treated by various approaches, including antimetabolite therapies involving mitomycin C (MMC) or 5-fluorouracil.12–14 Upon tissue damage, activated fibroblasts migrate to the damage site where they differentiate into α-smooth muscle actin (αSMA)-positive myofibroblasts, which synthesize extracellular matrix components such as collagen and contribute to the formation of contractile stress fibers that bind to the extracellular matrix. These fibrotic cascades precede the tissue fibrotic response15,16 that involves various liquid mediators, including transforming growth factor-beta (TGF-β), connective tissue growth factor (CTGF), fibroblast growth factor (FGF), and members of the matrix metalloproteinase (MMP) family.17–20 Among these mediators, TGF-β is related to fibrosis that occurs after glaucoma surgery.19,20 A clinical trial to investigate whether an antibody to TGF-β could suppress this fibrosis after trabeculectomy was not successful.21 Another factor involved in TGF-β crosstalk that has recently received increased attention for its therapeutic potential is the bioactive lipid mediator sphingosine-1-phosphate (S1P).22–25 S1P mediates diverse cellular responses such as proliferation, cytoskeletal organization and migration, immune cell regulation, adherence, and differentiation.24,25 S1P formation is mediated by sphingosine kinase (SphK), which phosphorylates sphingosine;24,26,27 it is reported that there exist two types of sphingosine kinases, SphK1 and SphK2, in ocular tissues.26 It is also reported that single knockout mice of SphK1 or SphK2 show normal
development and production, whereas double knockout mice lack completely S1P and are embryonic lethal. Thus far, fibrosis in various organs such as the lung, heart, liver, and dermis has been related to S1P. Lukowski et al. reported that in the rabbit eye, fibrosis was suppressed by treatment with an anti-S1P monoclonal antibody after trabeculectomy. However, the precise cellular mechanisms involved in S1P-induced fibrosis in the bleb and whether S1P is present in tissues and fluids affected by filtration surgery are unclear. In a majority of cells, S1P activity is mediated through a family of five G protein-coupled receptors, termed S1P1-5 receptors, which couple to a variety of G-proteins and initiate a multitude of downstream signaling cascades. We hypothesized that the S1P/SIP receptor system could play a crucial role in amplifying fibroblast activation, and that modulation of S1P acting as a bioactive mediator may have therapeutic applications in filtration surgery. In this report, we first characterized perioperative increases in the levels of several sphingolipids in filtration surgery, and then used an in vitro assay to investigate the role of S1P in wound fibrosis of human conjunctiva that can affect glaucoma filtering system could play a crucial role in amplifying fibroblast activation, and that modulation of S1P acting as a bioactive mediator may have therapeutic applications in filtration surgery. We also examined the antifibrotic effect of S1P receptor antagonists.

**METHODS**

**Measurement of Sphingolipid Levels in Human Aqueous Humor and Blebs**

Fluid samples from patients undergoing trabeculectomy were collected from the anterior chamber and blebs before and after surgery, respectively. This research adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of the University of Tokyo Hospital. Written informed consent was obtained from the subjects, who received an explanation of the possible consequences of the study before the operation. Patients who had undergone trabeculectomy or trabeculectomy with cataract surgery for primary open-angle glaucoma (POAG) and secondary open-angle glaucoma (SOAG) were recruited. None of the patients were using topical or systemic anti-inflammatory drugs, including corticosteroids. Preoperative aqueous humor was obtained by limbal paracentesis using a syringe with a 27-gauge needle (Nipro, Osaka, Japan) at the start of the surgery before any incisional procedures were undertaken. Postoperative aqueous fluid in the bleb was collected through a syringe with an attached 27-gauge micro cannula (Nipro). Approximately 70 to 100 and 10 to 30 μL fluid was collected preoperatively and postoperatively, respectively, in CryoTubes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The preoperative volume was chosen to avoid excessive hypotony. The samples were registered and stored at −80°C until processing. Sphingolipid levels in preoperative and postoperative aqueous fluids were determined by liquid chromatography–tandem mass spectrometry (LC/MS/MS) as previously described. Briefly, sphingolipids were separated by Nanospace LC (Shiseido, Tokyo, Japan) with a 1.5 × 250-mm C18 CAPCELL PAK AKR column using a gradient of solvent A (5 mM ammonium formate in water, pH 4.0) and solvent B (5 mM ammonium formate in 95% acetonitrile, pH 4.0). The MS system was a triple quadrupole mass spectrometer (TSQ Quantiva; Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated-electrospray ionization-II (HESH) source.

**Cell Culture and Passage**

Several fragments of Tenon’s capsule that were collected from donated human eyeballs (Eye Bank Association of America, Washington, DC, USA) were cultured on collagen gels (Collagen Culture Kit; Nitta Gelatin, Inc., Osaka, Japan) for several weeks. Human conjunctival fibroblasts (HCF) that migrated from the tissue were harvested for use in continuous cultures. Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% fetal bovine serum and Antibiotic Antimycotic Solution (100×) (Sigma-Aldrich Corp., St. Louis, MO, USA) was used as the culture medium. To ensure sufficient lots, human conjunctival fibroblasts (Sciencell Research Laboratories, San Diego, CA, USA) were purchased and cultured in Fibroblast Medium (Sciencell Research Laboratories) with Fibroblast Growth Supplement to ascertain that the cells retained the phenotypes during passages. Cells from passages 3 to 6 of both cell varieties were used for the experiments. Cell morphology and vimentin as a fibroblast marker (1:200; Sigma-Aldrich Corp.) were assessed to confirm that cells did not transform during the passages (data not shown).

**Quantitative Real-Time PCR (qRT-PCR)**

Cells were lysed with ISOGEN (NIPPON GENE, Tokyo, Japan). mRNA was isolated using chloroform and isopropyl alcohol. cDNA was synthesized from the isolated mRNA with a PrimeScript RT reagent Kit (TaKaRa BIO, Inc., Shiga, Japan). mRNA levels were quantitated by qRT-PCR of cDNA with SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa BIO, Inc.) and the Thermal Cycler Dice Real Time System II (TaKaRa BIO, Inc.) with the ΔΔCt method. For real-time PCR, primers for glycerolaldehyde-3-phosphate dehydrogenase (GAPDH), S1P3, and S1P5 were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/; in the public domain) and were purchased from Hokkaido System Science Co., Ltd. (Sapporo, Japan). Primer sequences for S1P2 and S1P3 were taken from previously published sequences and theprimers were purchased from Hokkaido System Science Co., Ltd. S1P4 primers were purchased from TaKaRa BIO, Inc. The sequences of PCR primers used were GAPDH: forward 5′-GAGTCACCGGATTGGTCGTG-3′ and reverse 5′-TGTATTGGAGGGATCTCGC-3′; S1P1: forward 5′-AAATTCACCGCACCACGTGA-3′ and reverse 5′-AGTTATTGCTCCGGGTGTGTG-3′; S1P2: forward 5′-ACTGTGTCCTGCTCTCATGCC-3′ and reverse 5′-GTCTTGAGCAGGCTAGCGTC-3′; S1P3: forward 5′-ACCATCGTATGCTCTTACCGAC-3′ and reverse 5′-CTGTAGTGGTCTCG-3′; S1P4: forward 5′-TGTCAAGAGCAAGGTGCTAGT-3′ and reverse 5′-CCCCACACTCTTCTCTGTTT-3′. Data were normalized relative to GAPDH.

**Migration Assay**

The cells were starved by incubation for 24 hours in serum-free medium. Four scratches in each well were made with a pipette tip and were recorded photographically under a microscope (BZ-9000; Keyence Corp., Osaka, Japan). The starvation medium was then changed to medium containing S1P or S1P with antagonists at the indicated concentration. After the cells were incubated at 37°C for 9 hours, the scratches were recorded. Scratch widths were measured with ImageJ 1.49 (National Institutes of Health, Bethesda, MD, USA). Changes in scratch widths were recorded and used to calculate migration distances.

**Cell Viability Assay**

Cells (5 × 10⁴ cells/well) were put in 96-well plates and incubated at 37°C for 24 hours. The cells were then starved by...
incubation for 24 hours in serum-free medium, after which the starvation medium was changed to media containing S1P or antagonists at the indicated concentration. After the cells were incubated at 37°C for 24 hours, cell viability was assayed with the Premix WST-1 Cell Proliferation Assay System (TaKaRa BIO, Inc.) according to the manufacturer’s instructions.

Western Blotting

Cells were first starved by incubation for 24 hours in serum-free medium. The starvation medium was changed to media containing S1P or S1P with antagonists at the indicated concentration. After a 24-hour incubation, cells were collected in RIPA Buffer (Thermo Fisher Scientific K.K., Kanagawa, Japan) containing protease inhibitors (Roche Diagnostics, Basel, Switzerland), sonicated, and centrifuged. Protein concentrations in the supernatant were determined by a BCA assay using a BCA Protein Assay Kit (Thermo Fisher Scientific K.K.). Proteins were boiled in sample buffer (Thermo Fisher Scientific K.K.). The same amount of proteins was loaded onto 4% to 20% precast polyacrylamide gels (BIO-RAD Laboratories, Hercules, CA, USA) and separated by SDS-PAGE. Protein bands were transferred to polyvinylidene difluoride membranes (BIO-RAD Laboratories) and the membranes were immersed in Tris-buffered saline with Tween 20 (TBST) containing the first antibody. After washing, the membranes were immersed in TBST containing the second antibody and reacted with ECL substrate (Thermo Fisher Scientific, Waltham, MA, USA). The bands were quantified with ImageJ 1.49 (National Institutes of Health).

Quantification of Total Collagen Amounts by Sirius Red Staining

To evaluate the effect of S1P and its receptor antagonists on soluble collagen levels in the cells, cells were treated with increasing concentrations of S1P and S1P receptor antagonists. The amount of total collagen in cell lysates was measured using a Sirius Red Total Collagen Detection Kit (Chondrex Inc., Redmond, CA, USA) according to the manufacturer’s instructions. The ratio of the amount of total collagen to total protein was calculated for each sample.

Immunocytochemistry

Cells were grown on chamber slides. Cells were first starved by incubation for 24 hours in serum-free medium. Then the starvation medium was changed to media containing 1 μM S1P. The S1P2 antagonist JTE 013 (Tocris Bioscience, Bristol, UK) (1 μM) was added 30 minutes before adding S1P. After a 24-hour incubation, the cells were fixed with ice-cold 4% paraformaldehyde for 15 minutes, permeabilized with 0.3% Triton X-100 for 5 minutes, and blocked with 3% bovine serum albumin (BSA) for 30 minutes. The primary antibodies were anti-α-SMA (1:1000; Sigma-Aldrich Corp.) and anti-β-tubulin (1:1000; Wako Pure Chemical Industries, Ltd.). Horseradish peroxidase–conjugated secondary antibody (1:10,000) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The bands were detected all S1P receptor subtypes and found that the relative expression of S1P receptor in primary cultured HCFs by qRT-PCR (Fig. 2). We next quantified the relative mRNA level of each S1P receptor subtype and found that the relative expression of S1P receptor in primary cultured HCFs by qRT-PCR (Fig. 2). We next quantified the relative mRNA level of each S1P receptor subtype and found that the relative expression of S1P receptor in primary cultured HCFs by qRT-PCR (Fig. 2). We next quantified the relative mRNA level of each S1P receptor subtype and found that the relative expression of S1P receptor in primary cultured HCFs by qRT-PCR (Fig. 2). We next quantified the relative mRNA level of each S1P receptor subtype and found that the relative expression of S1P receptor in primary cultured HCFs by qRT-PCR (Fig. 2). We next quantified the relative mRNA level of each S1P receptor subtype and found that the relative expression of S1P receptor in primary cultured HCFs by qRT-PCR (Fig. 2).

S1P and Antagonists

S1P (Enzo Life Sciences, Inc., Exeter, UK) was dissolved in methanol (0.5 mg/mL). Prior to the experiments, the methanol was evaporated with a stream of nitrogen and the S1P deposit was dissolved in water with 4 mg/mL BSA. The S1P1/3 antagonist VPC 23019 (Tocris Bioscience) was dissolved in acidified dimethyl sulfoxide (DMSO) (5% 1N HCl in DMSO) to make a 5 mM stock solution. The S1P2 antagonist JTE 013 was dissolved in ethanol to make a 10 mM stock solution. The S1P3 antagonist suramin (Sigma-Aldrich Corp.) was dissolved in water to make a 10 mM stock solution. Each S1P antagonist was added 30 minutes before adding S1P.

Statistical Analyses

Differences in the data among the groups were analyzed by 1-way ANOVA and Tukey test as a post hoc test. Sphingolipid levels in aqueous humor before and after surgery were compared with the Wilcoxon signed-rank test. Differences between postoperative levels for trabeculectomy alone and phacotrabeculectomy were compared with the Mann-Whitney U test. All statistical analyses were done using EZR on R commander 1.31.40

RESULTS

Clinical Study: Increased Phospholipid Levels in Postoperative Aqueous Fluid Samples From Filtration Surgery

To examine the effect of filtration surgery on phospholipid levels in study subjects, the amount of phospholipids including S1P, dihydrosphingosine 1-phosphate (dhS1P), which is the other metabolic product of sphingosine kinase, and sphingosine in the aqueous humor before and after surgery was determined by LC-MS/MS (Table). No significant amount of sphingolipid was detectable in aqueous samples collected at the beginning of the surgery. However, by the end of the surgery, S1P, dhS1P, and sphingosine (Sph) levels in aqueous fluid samples from blebs significantly increased to bioactive concentrations (0.07 ± 0.07, 0.02 ± 0.01, and 0.02 ± 0.02 μM, respectively [P < 0.001]) (Fig. 1). Although there were no significant differences in sphingolipid levels between patients with POAG or SOAG (P > 0.1), postoperative sphingolipid levels were significantly higher after trabeculectomy alone relative to phacotrabeculectomy (P = 0.002). Since the postoperative samples had S1P levels ranging between 0.02 and 0.5 μM, we investigated the effect of ≥0.1 μM S1P on HCFs in vitro.

mRNA Expression of S1P Receptors in HCFs

We next quantified the relative mRNA level of each S1P receptor in primary cultured HCFs by qRT-PCR (Fig. 2). We detected all S1P receptor subtypes and found that the relative expression of S1P2 mRNA in HCFs was significantly higher than that of other subtypes (P < 0.02).

Effects of S1P and S1P Receptor Antagonists on Cell Viability

To examine the influence of S1P and S1P antagonists on HCF proliferation or viability, we performed cell viability assays. The number of viable HCFs was unchanged following a 24-hour stimulation with S1P at various concentrations (P = 0.05, 1-way ANOVA; P > 0.1, Tukey test) (Fig. 3A). However, the number of...
S1P-Related Fibrosis After Trabeculectomy

**TABLE. Clinical Characteristics of Aqueous Humor Samples**

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Operative Procedure</th>
<th>Type of Glaucoma</th>
<th>Med Score</th>
<th>Pseudophakia or Phakia</th>
<th>Surgical History</th>
<th>Ocular Past History</th>
<th>MD</th>
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<tr>
<td>76</td>
<td>F</td>
<td>TLE + phaco</td>
<td>POAG</td>
<td>3</td>
<td>Phakia</td>
<td>Cataract</td>
<td>–</td>
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<tr>
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<td>TLE + phaco</td>
<td>POAG</td>
<td>2</td>
<td>Phakia</td>
<td>Cataract</td>
<td>–</td>
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<td>2</td>
<td>Pseudophakia</td>
<td>Phaco</td>
<td>–</td>
<td>–9.7</td>
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<tr>
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<tr>
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<td>TLE + phaco</td>
<td>SOAG (XFG)</td>
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<td>Cataract</td>
<td>–</td>
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</table>

F, female; M, male; TLE, trabeculectomy; phaco, phacoemulsification; EX-PRESS, EXPRESS (Alcon Laboratories, Fort Worth, TX, USA) shunt implantation; med score, medication score; MD, mean deviation of the Humphrey visual field; XFG, exfoliation glaucoma.

Viable HCFs increased in the presence of the S1P1/3 antagonist VPC 23019 ($P < 0.05$) and the S1P2 antagonist JTE 013 ($P < 0.05$). In contrast, HCF numbers decreased in the presence of the S1P3 inhibitor suramin at 10 μM ($P < 0.05$), whereas 1 μM suramin had no effect ($P = 1.0$) (Fig. 3B).

**Effects of S1P and S1P Receptor Antagonists on Cell Migration**

We next performed a cell migration assay to examine the effect of S1P on HCF motility (Fig. 4). The migration of HCFs after 9 hours was dose-dependently suppressed with S1P ($P < 0.001$). When VPC 23019 was added to the HCFs with 1 μM S1P, cell migration was suppressed equally to the level seen for 1 μM S1P alone ($P = 0.99$). However, when JTE 013 at 1 or 10 μM was added to cells with 1 μM S1P, cell migration was increased ($P < 0.001$). Meanwhile, suramin with 1 μM S1P did not affect S1P-induced suppression of cell migration ($P = 0.4$).

**Effects of S1P on αSMA Expression and Collagen Production**

We used Western blotting to examine how S1P affects HCF differentiation into myofibroblasts. After a 24-hour stimulation with 1 μM S1P, levels of the myofibroblast marker αSMA were significantly increased ($P < 0.001$) (Fig. 5). Adding VPC 23019 or suramin together with 1 μM S1P slightly decreased αSMA levels in HCF cultures ($P < 0.01$ and $P < 0.001$, respectively), but αSMA levels in the presence of JTE 013 were remarkably decreased ($P < 0.0002$). Since myofibroblasts play a role in fibrotic responses by producing extracellular matrix components such as collagen, we checked whether collagen production in HCF cultures was also increased. The amount of total collagen was indeed significantly increased by stimulation with 1 μM S1P ($P = 0.05$) (Fig. 6). The total collagen levels were significantly reduced ($P < 0.05$) following the addition of VPC 23019 (1 or 5 μM) or JTE 013 with S1P, but suramin had no effect ($P = 0.5$).

**S1P2 Receptor Antagonist Inhibition of F-Actin, αSMA, and Collagen Expression**

We also used immunocytochemistry to assess the fibrotic response of HCFs induced by S1P and the S1P2 receptor antagonist JTE 013, which was the most effective antagonist in the previous assays (Fig. 7). The amount of αSMA, collagen, and F-actin expression in HCFs was higher in the presence of S1P, but decreased with JTE 013 addition.

**DISCUSSION**

To prevent fibrotic responses after glaucoma filtering surgery, a better understanding of the detailed mechanisms of fibrosis is needed. Here we focused on the role of S1P/S1P receptors in mediating fibrotic responses.

Although S1P is thought to be involved in fibrotic responses in the eye, to date no studies have reported clinical measurement of sphingolipid levels in aqueous humor and how these levels are related to conjunctival fibroblasts. In this study, we first examined sphingolipid levels in aqueous fluid obtained before and after filtration surgery. We found that levels of Sph, as well as the dephosphorylated form of S1P and dihydrosphingosine 1-phosphate (dhsS1P), the other metabolic product of sphingosine kinase, and αSMA were significantly increased after filtration surgery (Fig. 1). We focused on the effect of S1P because S1P levels reached bioactive concentrations and were the highest of the three sphingolipid types detected ($P = 0.0001$). Previous reports indicated that S1P concentrations in the blood fluid ranged between 0.2 and 0.9 μM, which is high, but the levels were lower in tissues (0.5–75 pmol/mg), suggesting that S1P is secreted by cells in blood vessel walls or by blood cells. Thus, the high S1P concentrations that we observed in this study might have arisen because of bleeding during the operation. S1P may be present in the bleb in sufficient amounts to trigger the excessive fibrotic responses. However, whether these high S1P concentrations persist or S1P is metabolized after the operation is unclear. In addition, since Sph or dihS1P which may also be involved in postoperative fibrosis, additional investigation on sphingosine or dihS1P function in particular is needed.

Based on our hypothesis that S1P function in conjunctiva is mediated through S1P receptors, we used qRT-PCR to determine the mRNA expression of S1P receptors 1 to 5 in HCFs. All S1P receptor types were detected (Fig. 2), and, consistent with earlier reports, S1P2 levels were particularly high. Together these results suggest that S1P acting through...
S1P receptors could postoperatively trigger fibrotic responses in HCFs. We also examined what responses were caused by S1P and which receptors mediated these responses. Since treatment with 0.1 to 10 μM S1P for 24 hours had no effect on HCF cell viability (Fig. 3A), we used those concentrations for subsequent assays. Moreover, because 10 μM suramin reduced HCF viability (Fig. 3B), we used lower dose.

**Figure 1.** Clinical levels of S1P (A), dhS1P (B), and Sph (C) in human aqueous humor before and after trabeculectomy (n = 15).

**Figure 2.** qRT-PCR quantitation of HCF mRNA expression of all S1P receptor subtypes relative to GAPDH (n = 4).

**Figure 3.** (A) WST-1 assay of HCF proliferation in the presence of 0, 0.1, 1, or 10 μM S1P for 24 hours (n = 7). The baseline represents proliferation of cells without S1P stimulation. (B) WST-1 assay of HCF proliferation with 1 μM S1P for 24 hours (n = 7). The baseline represents proliferation of cells without S1P stimulation. *P < 0.0001, compared to the cells with no stimulation of antagonists.
In a migration assay, cell motility over 9 hours decreased with S1P treatment and was recovered by treatment with the S1P2 antagonist JTE 013 at 1 or 10 μM (Fig. 4). This result suggests that HCF migration was suppressed mainly by S1P2 and its downstream factors. Indeed, previous studies indicated that several other types of cells express much higher S1P2 levels. For example, vascular smooth muscle cells dominantly express S1P2, and the motility of these cells was suppressed by S1P. Arikawa et al. reported that B16 melanoma cells expressed more S1P2 than S1P1 or S1P3, and that the migration and invasion of these cells was reduced by S1P and restored in the presence of JTE 013. Mouse embryonic fibroblasts with deletion of the S1P2 receptor had a dramatic induction of migration, whereas S1P2 increased the motility of human hepatic stellate cells. Following those results, we considered two possibilities: S1P promotes fibrotic responses of HCF through S1P2 to strengthen cell–cell adhesion or cell–ECM adhesion that in turn reduces cell motility, or S1P2 and its downstream factors might work to prevent wound healing. The results from our assays support the first possibility. The expression of the myofibroblast marker αSMA increased with S1P treatment and decreased by adding JTE 013 (Figs. 5, 7). This result suggests that S1P promoted differentiation of fibroblasts to myofibroblasts through S1P2. As a result, increase of F-actin and the ECM component collagen (Figs. 6, 7) directed a fibrotic response. This finding is consistent with an earlier study showing that myofibroblasts express contractile stress fibers that bind to ECM proteins to decrease cell motility. Our results also corroborate previous reports on various other organs. In skin fibroblasts and normal rat kidney interstitial fibroblast cells, S1P stimulation enhanced differentiation to myofibroblasts and induction of ECM protein expression. Previous studies examined the effect of S1P on ocular tissue. One showed that S1P was important for fibrosis of
retinal pigmented epithelial cells, whereas another found that an anti-S1P monoclonal antibody had antifibrotic action. Furthermore, in a model of Graves' orbitopathy, S1P treatment promoted differentiation of orbital fibroblasts to adipocytes through the S1P receptor 1. However, the detailed mechanisms of S1P-related conjunctival fibrosis were unclear. Our finding that S1P concentrations were increased in the bleb formed after glaucoma filtering surgery suggests that S1P might affect wound fibrosis through S1P2 receptors.

This study has several limitations. First, we measured S1P concentrations only immediately after the filtration surgery. Thus, long-term changes in postoperative sphingolipid levels should be followed in future studies. Second, we focused on S1P in this study. Since other sphingolipids such as dhS1P or Sph, as well as related enzyme activities such as SphK or carriers, may simultaneously contribute to wound scarring, further biological analyses of these factors should be conducted. In addition, the effect of S1P and S1P antagonists on fibrosis must be examined using an animal model of filtration surgery, and results from these studies could be used to improve outcomes of this surgical procedure.

In conclusion, we revealed that aqueous fluid in the bleb at the end of filtration surgery had bioactive concentrations of S1P and that S1P activated conjunctival fibroblasts via S1P2 receptors. Thus, inhibition of S1P2 receptors has potential to prevent bleb failure and improve filtration surgery outcomes.

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References


