EPITHELIAL AND MESENCHYMAL CELL BIOLOGY

VEGF Induces TGF- β 1 Expression and Myofibroblast Transformation after Glaucoma Surgery

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Subconjunctival fibrosis at the surgical site determines the outcome of glaucoma surgery. Myofibroblast transformation has a significant role in fibrosis, and vascular endothelial growth factor (VEGF) is reported to trigger myofibroblast transformation by inducing transforming growth factor (TGF)- β 1. In the present study, we used IHC, Western blot analysis, enzyme-linked immunosorbent assay, and electron microscopy to determine the contribution of VEGF to myofibroblast transformation in subconjunctival fibrosis after glaucoma surgery. A rabbit trabeculectomy model was generated, and VEGF stimulation or VEGF inhibition was performed during surgery. VEGF stimulation induced TGF- β 1 expression in a dose-dependent manner. Down-regulation of epithelial markers (E-cadherin and β -catenin) and up-regulation of mesenchymal marker (α -smooth muscle actin) were observed in the subconjunctival layers after trabeculectomy with VEGF stimulation. Up-regulations of Smad and Snail, which play a central role in myofibroblast transformation, were observed in the conjunctival and subconjunctival layers at the site of trabeculectomy. Electron microscopy revealed changes of the conjunctival epithelial cells, especially the presence of myofilaments and increased rough endoplasmic reticulum in the cytoplasm. Myofibroblast transformation was activated by VEGF stimulation and decreased by VEGF inhibition. These findings suggest that VEGF potentially affected the TGF- β 1/Smad/Snail pathway, thereby triggering myofibroblast transformation. Therapeutic approaches modulating VEGF may control myofibroblast transformation and reduce subconjunctival fibrosis after glaucoma surgery. (*Am J Pathol* 2013, 182: 2147–2154; <http://dx.doi.org/10.1016/j.ajpath.2013.02.009>)

Glaucoma is the worldwide leading cause of blindness. The mainstay of glaucoma treatment is control of the patient's intraocular pressure, which is achieved by medication or glaucoma filtration surgery. The success of glaucoma filtration surgery is crucial to prevent blindness. Trabeculectomy makes a filtering bleb under the conjunctival and subconjunctival space for the aqueous humor to be driven from the anterior chamber of the eye. Formation of the filtering bleb lowers the intraocular pressure after trabeculectomy; maintaining the filtering bleb after surgery is important for the success of the surgery. However, excessive subconjunctival scarring at the filtering bleb is the most common cause of failure after trabeculectomy.^{1,2} Tenon's fibroblasts are the main effector cells involved in the initiation and mediation of wound healing and fibrotic scar formation at the filtering bleb of trabeculectomy.³ Many studies investigated the role of transformation of Tenon's fibroblasts to myofibroblasts and the effect of transforming growth factor- β (TGF- β) to this process.^{4–6}

Vascular endothelial growth factor (VEGF) stimulates the growth of vascular endothelial cells and increases vascular permeability.⁷ VEGF may act as an initial activator of fibrosis and could play a role in the wound healing process, in addition to its effect on angiogenesis.^{8,9} Increased levels of VEGF induce scar formation in cutaneous wounds via the deposition of collagen.¹⁰ Several studies have shown that the inhibition of VEGF results in reduced scar formation at the trabeculectomy bleb and improves the success of glaucoma surgery.^{11–13} Although many reported the clinical significance of using anti-VEGF agents in trabeculectomy, the mechanism of its action is not well understood. There are possibilities that VEGF may trigger myofibroblast transformation by inducing TGF- β 1. This was investigated in several cases of organ fibrosis; however, it was not yet investigated in conjunctival and subconjunctival fibrosis after glaucoma surgery.^{14–16} Thus, we investigated whether myofibroblast transformation is affected by VEGF. Also, we

sought to determine the role of VEGF modulation and its influence on TGF- β 1, which may further modulate myofibroblast transformation after trabeculectomy.

Materials and Methods

Rabbit Model of Trabeculectomy

All animal experiments complied with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. The animals were treated according to the regulations of the Catholic Ethics Committee of the Catholic University of Korea, Seoul, and the NIH Guide for the Care and Use of Laboratory Animals (publication 80-23, revised 1996). All efforts were made to minimize suffering and the number of animals used in this study.

Thirty-two male New Zealand white rabbits (aged 12 to 14 weeks, weighing 2 to 3 kg) were used in this study. Before surgery, rabbits were anesthetized by an i.m. injection of 50 mg/kg zolazepam (Zoletil; Virbac Laboratories, Carros, France) and 15 mg/kg xylazine hydrochloride (Rompun; Bayer Health Care, Pittsburgh, PA). Proparacaine hydrochloride ophthalmic solution (Alcaine; Alcon Laboratories, Fort Worth, TX), a topical anesthesia, was applied to the corneal and conjunctival surface. The ocular area was disinfected with povidone iodine, and the eyelids were retracted with a wire eyelid speculum. Surgery was performed using a previously described technique that results in a trabeculectomy filtering bleb.¹⁷ A partial-thickness 8-0 polyglactin 910 (Vicryl; Ethicon, Livingston, Scotland) corneal traction suture was placed superior to the eye, and the eye was pulled inferiorly. A conjunctival incision was then made, superotemporally approximately 8 mm from the limbus. A limbal-based subconjunctival flap was raised by a blunt dissection of the subconjunctival space to the limbus using Westcott scissors. A 1-mm blade was used to fashion a partial-thickness sclera flap approximately 3 mm from the limbus. A rectangular 1 $\times$ 1-mm sclerotomy was made, and two corners were sutured with 10-0 nylon. The conjunctival incision was closed with a continuous 8-0 polyglactin 910 suture. The anterior chamber was reformed with balanced salt solution. Immediately after surgery, VEGF stimulation was performed by injecting 0.2 mL of VEGF (1, 10, 20, and 50 ng/mL) into the anterior chamber with a 30-gauge needle. VEGF inhibition was performed by injecting 0.1 mL of 25 mg/mL bevacizumab (Avastin; Genentech, Basel, Switzerland) subconjunctivally into the filtration bleb with a 30-gauge needle. Ofloxacin eye drops and tetracycline ointment were applied to the eyes postoperatively.

Tissue Preparation

On days 0, 7, 14, and 30 after surgery, rabbits were sacrificed using a lethal i.v. injection of xylazine hydrochloride. Except for an enzyme-linked immunosorbent assay (ELISA) test, all

animals were sacrificed 7 days after surgery. Conjunctival and subconjunctival tissues at the site of trabeculectomy were sampled and stored immediately at -70°C and later used for ELISA ($n = 10$ for VEGF, and $n = 14$ for TGF- β 1) and Western blot analysis ($n = 6$ control eyes, $n = 6$ trabeculectomy eyes, $n = 6$ trabeculectomy eyes with VEGF stimulation, and $n = 6$ trabeculectomy eyes with VEGF inhibition). Eyes were enucleated for immunohistochemical (IHC) analysis and electron microscopic examinations. For IHC analysis, conjunctival and subconjunctival tissues at the site of trabeculectomy were fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin ($n = 3$ control eyes, $n = 3$ trabeculectomy eyes, $n = 3$ trabeculectomy eyes with VEGF stimulation, and $n = 3$ trabeculectomy eyes with VEGF inhibition). For electron microscopic examinations, samples were immediately fixed with 2.5% glutaraldehyde ($n = 2$ control eyes, and $n = 2$ trabeculectomy eyes).

VEGF and TGF- β 1 ELISA

Concentrations of VEGF and TGF- β 1 were determined by an ELISA (Quantikine ELISA kit; R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Proteins were extracted from the conjunctival and subconjunctival tissue samples before the ELISA. Tissues were homogenized in radioimmunoprecipitation assay buffer [1% Triton X-100, 5% sodium dodecyl sulfate, 5% deoxycholic acid, 0.5 mol/L Tris-HCl (pH 7.5), 10% glycerol, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 5 $\mu\text{g/mL}$ aprotinin, 1 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ pepstatin, 200 mmol/L sodium orthovanadate, and 200 mmol/L sodium fluoride]. The tissue extracts were incubated for 10 minutes on ice and clarified by centrifugation at $10,000 \times g$ for 25 minutes at 4°C . The amount of total protein from tissue samples was determined using a standard bicinchoninic acid assay (Pierce, Rockford, IL). Tissue extracts (30 μg total protein) were used for the ELISA. To determine TGF- β 1 levels, samples were activated with 1 N HCl for 60 minutes at 4°C , followed by neutralization with 1 N NaOH. The optical density at 540 to 570 nm was measured with a spectrophotometer (DU-530; Beckman Instruments Inc., Fullerton, CA).

Western Blot Analysis

Samples for Western blot analysis were homogenized in radioimmunoprecipitation assay buffer. Tissue extracts were incubated for 10 minutes on ice and clarified by centrifugation at $10,000 \times g$ for 25 minutes at 4°C . Total protein from conjunctival and subconjunctival extracts was measured using a standard bicinchoninic acid assay. Sample extracts (40 μg total protein) were resuspended in $5\times$ sample buffer [60 mmol/L Tris-HCl (pH 7.4), 25% glycerol, 2% SDS, 14.4 mmol/L 2-mercaptoethanol, and 0.1% bromophenol blue] at a 4:1 ratio, boiled for 5 minutes, and resolved by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane, and

blots were stained with Ponceau S (Sigma, St. Louis, MO) to visualize the protein bands and ensure equal protein loading and uniform transfer. Blots were washed and blocked for 45 minutes with 5% non-dried skim milk in TBST buffer [20 mmol/L Tris-HCl (pH 7.6), 137 mmol/L NaCl, and 0.1% Tween 20]. Blots were then probed for 24 hours using antibodies against TGF- β 1 (Ab Frontier, Rantoul, IL), E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), β -catenin (Chemicon, Temecula, CA), Smad 2/3 (Abbiotec, San Diego, CA), phosphorylated Smad 2/3 (Santa Cruz Biotechnology), Snail (Abgent, San Diego), and actin (Sigma). Blots were then probed with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence system (ECL, Amersham, MA) and X-ray film. Relative intensity was measured using an ImageMaster VDS (Pharmacia Biotech, San Francisco, CA). Fold changes in protein levels are indicated. Results are representative of six independent experiments. Data are expressed as means $\pm$ SD.

Histological Examinations and IHC

Paraffin blocks were cut into sections (6 μ m thick). Sections were deparaffinized, rehydrated, and stained with H&E. These sections were observed by light microscopy (Nikon, Tokyo, Japan). For fluorescent staining, sections were pre-treated with 3% hydrogen peroxide in methanol to decrease endogenous peroxidase activity. After washing with PBS, sections were incubated with 10% normal horse serum in PBS for 1 hour at room temperature to block non-specific binding activity, followed by incubation with rabbit anti-TGF- β 1 (1:1000; Ab Frontier), mouse anti- α -smooth muscle actin (SMA; 1:4500; Sigma), rabbit anti-E-cadherin (1:100; Santa Cruz Biotechnology), rabbit anti- β -catenin (1:100; Chemicon), rabbit anti-Smad 2/3 (1:100; Abbiotec), and rabbit anti-Snail (1:100; Abgent) at 4°C overnight in a humid chamber. The following day, sections were incubated with either a goat anti-rabbit Alexa 546 or anti-mouse Alexa 488 antibody (Molecular Probes, Carlsbad, CA) for 1 hour at room temperature after several washes with PBS. After additional washes in 0.1 mol/L PBS for 30 minutes, sections were mounted using VECTARSHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Sections were then washed, coverslipped, and examined by confocal laser scanning microscopy (Zeiss, Oberkochen, Germany).

Transmission Electron Microscopy

Electron microscopy was conducted on samples from two eyes of a control rabbit and two eyes of a rabbit that underwent a trabeculectomy. Samples were immediately fixed with 2.5% glutaraldehyde. Ultrathin sections (0.1 μ m thick) were cut and mounted on grids coated with Formvar and were examined with a transmission electron microscope (model 1200EX; JEOL, Tokyo, Japan).

Statistical Analysis

Comparisons between the two groups were performed using a *U*-test. SPSS, version 11.0 (SPSS, Chicago, IL), was used for all analyses, with $P < 0.05$ indicating significance.

Results

VEGF Is Elevated after Trabeculectomy and Induces TGF- β 1 Expression

The level of VEGF in the conjunctival and subconjunctival layers, as determined by ELISA, was elevated early after trabeculectomy (Figure 1A). VEGF was significantly elevated at 3 days ($P = 0.032$) and peaked at 7 days ($P = 0.008$) after the surgery. At 1 and 2 months after the surgery, the level of VEGF in the conjunctival and subconjunctival

Figure 1 TGF- β 1 is induced by VEGF. **A:** The level of VEGF after trabeculectomy increased and peaked 1 week after trabeculectomy, followed by a decrease to preoperative levels 1 and 2 months after trabeculectomy. **B:** By VEGF stimulation with various doses (1, 10, 20, and 50 ng/mL), TGF- β 1 showed a dose-dependent response. Induction of TGF- β 1 peaked with 20 ng/mL of VEGF. VEGF inhibition by 25 mg/mL of bevacizumab, with or without VEGF stimulation, significantly decreased TGF- β 1. For measurement of VEGF level, $n = 2$ for each time period (total $n = 10$); for measurement of TGF- β 1 level, $n = 2$ for each group (total $n = 14$). * $P < 0.05$, *U*-test.

layers decreased to the preoperative level. When VEGF stimulation was performed by injecting various doses of VEGF (1, 10, 20, and 50 ng/mL) into the anterior chamber during surgery, a dose-dependent response of TGF- β 1 was observed (Figure 1B). TGF- β 1 in the conjunctival and subconjunctival layers was significantly increased after VEGF stimulation at doses of 10 ng/mL ($P = 0.016$), 20 ng/mL ($P = 0.004$), and 50 ng/mL ($P = 0.008$). The induction of TGF- β 1 peaked at 20 ng/mL VEGF; therefore, this dose was used for VEGF stimulation in subsequent experiments. In trabeculectomy eyes with VEGF inhibition by 0.1 mL of 25 mg/mL bevacizumab, TGF- β 1 was significantly decreased compared with the trabeculectomy eyes ($P = 0.032$). When VEGF stimulation (20 ng/mL) was combined with VEGF inhibition (0.1 mL of 25 mg/mL bevacizumab) during surgery, the level of TGF- β 1 significantly decreased ($P = 0.008$).

VEGF Leads Excessive Subconjunctival Fibrosis after Trabeculectomy

Eyes without surgery show normal conjunctival epithelium with loose connective tissues beneath the epithelial layer (Figure 2A). In trabeculectomy eyes with VEGF stimulation, subconjunctival fibrosis, which is stained densely by hematoxylin, was observed (Figure 2B). After VEGF inhibition by bevacizumab, subconjunctival fibrosis was decreased (Figure 2C), and increased cystic spaces in the subconjunctival stroma, which indicates a functioning trabeculectomy bleb, was observed (Figure 2C).

To examine whether a mesenchymal marker is expressed after trabeculectomy, immunostaining for α -SMA was performed. Staining was not observed in the eyes without surgery in the conjunctival and subconjunctival layers (Figure 2D). Immunostaining for α -SMA was increased throughout the subconjunctival layers in trabeculectomy eyes with VEGF stimulation (Figure 2E). After VEGF inhibition, immunostaining for α -SMA was decreased throughout the subconjunctival layers (Figure 2F).

Eyes without surgery showed no immunostaining for TGF- β 1 in the conjunctival and subconjunctival layers (Figure 2G). In trabeculectomy eyes with VEGF stimulation, immunostaining for TGF- β 1 was increased throughout the subconjunctival layers, especially in the deeper layers of the subconjunctiva (Figure 2H). The stained layers by TGF- β 1 were similar with immunostaining for α -SMA. After VEGF inhibition, TGF- β 1 expression decreased in the superficial subconjunctival layers and no expression was found in the deeper layers of the subconjunctiva (Figure 2I). These results support that VEGF induces TGF- β 1 production, and this increases the mesenchymal phenotype of the cells in the subconjunctiva after trabeculectomy.

To examine the expression of epithelial markers, we performed immunostaining for E-cadherin, which is a key epithelial cell adhesion molecule. Eyes without surgery showed characteristic staining of E-cadherin at the intercellular junctions of conjunctival epithelial cells (Figure 3A). In trabeculectomy eyes with VEGF stimulation, intercellular staining of E-cadherin was diminished in the conjunctival epithelium compared with normal conjunctival epithelium (Figure 3B). In

Figure 2 Subconjunctival fibrosis after trabeculectomy (Trabe) is aggravated by VEGF stimulation. **A–C:** H&E staining of the trabeculectomy. Thick subconjunctival fibrosis (arrow, **B**) is observed in trabeculectomy eyes with VEGF stimulation (**B**) compared with the conjunctiva and subconjunctiva in eyes without surgery (**A**). VEGF inhibition results in decrease of subconjunctival fibrosis (arrow, **C**) with increased cystic spaces (asterisk, **C**), which indicates better function of the trabeculectomy bleb. **D–F:** Immunostaining for α -SMA of the trabeculectomy bleb. No immunostaining for α -SMA was found in the eyes without surgery (**D**). However, α -SMA expression was increased throughout the subconjunctiva in trabeculectomy eyes with VEGF stimulation (**E**). After VEGF inhibition, α -SMA expression was decreased (**F**). **G–I:** Immunostaining for TGF- β 1 of the trabeculectomy bleb. No staining was found in the eyes without surgery (**G**). In trabeculectomy eyes with VEGF stimulation, TGF- β 1 expression was increased in the subconjunctival layers (**H**). TGF- β 1 staining in the subconjunctival layers decreased after VEGF inhibition (**I**). Each $n = 3$ for eyes without surgery, trabeculectomy eyes with VEGF stimulation, and trabeculectomy eyes with VEGF inhibition. The epithelial layer is outlined with a white line (**D–I**). Original magnification: $\times 400$ (**A–C**); $\times 200$ (**D–I**).

Figure 3 Loss of epithelial markers in the conjunctival epithelium after VEGF stimulation. **A–C:** A characteristic E-cadherin staining pattern was observed at the intercellular junctions of the epithelial cells in the eyes without surgery (**A**). Intercellular staining of E-cadherin diminished in the conjunctival epithelium after trabeculectomy (Trabe) with VEGF stimulation (**B**). Immunostaining of E-cadherin showed expression in the conjunctival epithelium after VEGF inhibition (**C**). **D–F:** A normal β -catenin staining pattern was observed in the eyes without surgery (**D**). Intercellular staining of β -catenin diminished in the conjunctival epithelium after trabeculectomy with VEGF stimulation (**E**). Recovery of β -catenin staining was observed after VEGF inhibition (**F**). Each $n = 3$ for eyes without surgery, trabeculectomy eyes with VEGF stimulation, and trabeculectomy eyes with VEGF inhibition. The epithelial layer is outlined with a white line (**A–F**). Original magnification, $\times 400$ (**A–F**).

contrast, when VEGF was inhibited by bevacizumab, expression of E-cadherin was maintained in some layers of the conjunctival epithelium (Figure 3C). In eyes without surgery, β -catenin stained similar with E-cadherin (Figure 3D). After trabeculectomy with VEGF stimulation, staining of β -catenin was decreased in the conjunctival epithelium (Figure 3E). When VEGF was inhibited by bevacizumab, expression of β -catenin was maintained in some layers of the conjunctival epithelium compared with trabeculectomy eyes with VEGF stimulation (Figure 3F). Results from epithelial and mesenchymal markers show that loss of epithelial phenotype and gain of mesenchymal phenotype occur after trabeculectomy with VEGF stimulation.

Induction of Smad and Snail after Trabeculectomy

Normal conjunctival epithelium is a stratified squamous epithelium, which is well demarcated from the subconjunctival

layers, where loose connective tissues are located (Figure 4A). After trabeculectomy, the structure of conjunctival epithelium is disarranged and the border of conjunctival epithelium is not clearly seen. Cuboidal or spindle-shaped cells are increased in the subconjunctival layer after trabeculectomy, compared with normal control eyes (Figure 4, B and D). The IHC staining for Smad is not observed in normal conjunctival and subconjunctival layers (Figure 4A). In trabeculectomy eyes, Smad-positive cells were observed in the conjunctival epithelium (Figure 4B). After stimulation with VEGF, Smad-positive cells increased at the basal cells in the conjunctival epithelium and mostly in the subconjunctival layers (Figure 4C). The IHC staining for Snail shows positive cells in the conjunctival epithelium after trabeculectomy (Figure 4D). This was increased in the subconjunctival layers after VEGF stimulation (Figure 4E). The locations of the increased expression of Smad and Snail were observed in the subbasal layer of the

Figure 4 Change of Smad and Snail after trabeculectomy (Trabe). **A–C:** Normal conjunctival epithelium nearly expresses Smad (**A**). After trabeculectomy, Smad expression was increased in a cell at the conjunctival epithelium (arrowheads, **B**). In trabeculectomy eyes with VEGF stimulation, Smad-positive cells were found in both conjunctival epithelium and deeper layers of the subconjunctiva (arrowheads, **C**). **D and E:** In trabeculectomy eyes, Snail expression was observed in the conjunctival epithelium and subconjunctival layers (arrowheads, **D**). After VEGF stimulation, Snail-positive cells were increased in the deeper layers of the subconjunctiva (arrowheads, **E**). Each $n = 3$ for trabeculectomy eyes and trabeculectomy eyes with VEGF stimulation. Original magnification: $\times 400$ (**A–D**); $\times 200$ (**C and E**).

Figure 5 Electron microscopy of the conjunctival epithelium. Conjunctival epithelium in eyes without surgery shows a regular arrangement of spindle-shaped conjunctival epithelial cells with close contact by interdigitation between cells (**A**). Desmosomes, which maintain close contact between basal conjunctival epithelial cells, were observed in eyes without surgery (**arrowheads, A**). After trabeculectomy, disarrangement of the conjunctival epithelial cells with widened intercellular spaces was observed (**B**). Desmosomes are decreased after trabeculectomy (**arrowheads, B**). Each $n = 2$ for eyes without surgery and trabeculectomy eyes. Original magnification: $\times 5000$ (**A and B**); $\times 10,000$ (**boxed, A and B**) for magnification.

conjunctival epithelium and in the deeper layers of the subconjunctival tissue, respectively. These findings suggest that the activation transformation of myofibroblast was found after trabeculectomy, and this is increased by VEGF stimulation.

Change of the Conjunctival Epithelial Cells after Trabeculectomy

Unlike the regular arrangement and close contact of conjunctival epithelial cells in eyes without surgery (**Figure 5A**), disarrangement and loosening between the conjunctival epithelial cells was observed after trabeculectomy (**Figure 5B**). The number and contact length of desmosomes, which is important for maintaining the close contact between conjunctival epithelial cells, were decreased after trabeculectomy, compared with eyes without surgery.

At the basal layer of the conjunctival epithelium, epithelial cells undergoing mesenchymal transformation were found just above the basement membrane (**Figure 6A**). Disruption of the basement membrane with abnormal collagen bundles was present in the subepithelial stroma (**Figure 6B**). The presence of myofilaments (**Figure 6C**) and increased rough endoplasmic reticulum (**Figure 6D**), within the cytoplasm of conjunctival epithelial cells just above the basement membrane, indicated change of the basal conjunctival epithelial cells after trabeculectomy.

Change of Related Proteins by VEGF Modulation

Western blot analysis demonstrated that the TGF- $\beta 1$ protein was significantly elevated after trabeculectomy ($P = 0.016$). The expression of epithelial markers, E-cadherin and β -catenin, significantly decreased after trabeculectomy,

compared with eyes without surgery ($P = 0.032$ and $P = 0.024$, respectively). With VEGF stimulation, TGF- $\beta 1$ was significantly up-regulated, whereas the expression of epithelial markers significantly decreased. Application of bevacizumab down-regulated TGF- $\beta 1$ expression, and epithelial markers were elevated to a similar level of the eyes without surgery. After application of bevacizumab, TGF- $\beta 1$ ($P = 0.520$), E-cadherin ($P = 0.478$), and β -catenin ($P = 0.494$) did not show any significant differences compared with the eyes without surgery. After trabeculectomy, Smad ($P = 0.012$), phosphorylated Smad ($P = 0.040$), and Snail ($P = 0.024$) proteins were significantly elevated. With VEGF stimulation, these proteins were further increased ($P = 0.008$, $P = 0.016$, and $P = 0.008$, respectively). Application of bevacizumab decreased Smad, phosphorylated Smad, and Snail protein expression (**Figure 7**).

Discussion

Herein, we observed that VEGF induces TGF- $\beta 1$ in the subconjunctival tissue, resulting in excessive subconjunctival fibrosis after trabeculectomy. Up-regulation of Smad and Snail was observed at the conjunctival and subconjunctival tissues, indicating myofibroblast transformation. This myofibroblast transformation was decreased with VEGF inhibition. Disarrangement of the conjunctival epithelium, disruption of the basement membrane, and conjunctival epithelial cells expressing mesenchymal phenotype by electron microscopy may show changes in cell-to-cell junction that represent migration properties of myofibroblasts.

Figure 6 Electron microscopy of the basal layer of conjunctival epithelium after trabeculectomy. Basal conjunctival epithelial cells adjacent to irregular and disrupted basement membrane (**arrowheads, A and B**) were surrounded by abnormal collagen bundles (**black arrow, B**). Myofilaments (**white arrows, C**), which are characteristics of myofibroblasts, were found in the cytoplasm of basal conjunctival epithelial cells. Other conjunctival epithelial cells showed increased rough endoplasmic reticulum in the cytoplasm (**black arrows, D**). Trabeculectomy eyes ($n = 2$). Original magnification: $\times 2000$ (**A**); $\times 5000$ (**B–D**).

Figure 7 Western blot analysis shows that the TGF-β1/Smad/Snail pathway is involved in myofibroblast transformation after trabeculectomy. **A:** TGF-β1 protein was increased significantly after trabeculectomy and further increased with VEGF stimulation. TGF-β1 expression returned to preoperative levels after VEGF inhibition. **B:** Epithelial markers, E-cadherin and β-catenin, decreased after trabeculectomy and further decreased with VEGF stimulation. Both epithelial markers were recovered by VEGF inhibition. **C:** Smad, phosphorylated Smad, and Snail were up-regulated after trabeculectomy. VEGF stimulation significantly increases the expression of Smad, phosphorylated Smad, and Snail, whereas VEGF inhibition down-regulated expression levels. Each $n = 6$ for eyes without surgery, trabeculectomy eyes, trabeculectomy eyes with VEGF stimulation, and trabeculectomy eyes with VEGF inhibition. * $P < 0.05$, U-test.

The cytokine, TGF-β1, is a key mediator of wound healing and is critically involved in postoperative scarring.^{18,19} On the cellular level, TGF-β1 plays a key role in the fibrotic process by driving the conversion of fibroblasts to myofibroblasts, which is the same for Tenon's fibroblasts.^{3,20} Immediately after ligand binding, activated TGF-β1 receptors form a transient complex with Smad 2/3, in which the C-terminal phosphorylation of Smad occurs.²¹ Subsequently, activated Smad translocates into the nucleus and activates Snail, a transcriptional factor that triggers myofibroblast transformation, converting target cells into mesenchymal cells with migratory properties.¹⁴ Recently, VEGF has been reported to have a critical role in TGF-β1 production and, in turn, may result in excessive fibrosis by activating myofibroblast transformation in various diseases.^{15,16,22} Our present study shows that VEGF induces TGF-β1 protein production in a dose-dependent manner, and inhibition of VEGF reduces TGF-β1 levels. By inhibiting VEGF, subconjunctival fibrosis was decreased after trabeculectomy. Down-regulation of VEGF may have reduced TGF-β1 expression in the subconjunctival tissue and, in turn, attenuated myofibroblast transformation. The interaction between VEGF and TGF-β1 in fibrosis is poorly understood;

however, this interaction has recently been reported to be mediated by the phosphoinositide 3-kinase/Akt and mitogen-activated protein kinase pathways.^{16,22}

In the eyes of patients with glaucoma, it is reported that both VEGF and TGF-β1 are elevated compared with controls.^{23,24} Our previous study shows that VEGF is elevated in Tenon's tissue, the site of glaucoma surgery, and that this is related to the success of glaucoma surgery.²⁵ Inhibition of VEGF is a wound-modulating method that is of potential use as an adjunct in glaucoma surgery. Use of antimitotic agents, such as mitomycin C and 5-fluorouracil, is the only wound-healing modulation that is applicable. Application of these agents at the subconjunctival tissue causes widespread nonselective cell death and apoptosis of fibroblasts around the trabeculectomy bleb. Therefore, use of these agents is associated with severe complications.^{18,26} Several animal experiments and clinical studies have compared the scar formation after glaucoma surgery with the inhibition of VEGF.^{12,27–29} Anti-VEGF agents, such as bevacizumab, have shown antifibrotic effects via the inhibition of fibroblast proliferation and the induction of fibroblast cell death *in vitro*.³⁰ In addition, our results suggest that VEGF inhibition may play a role in the reduction of scar formation by decreasing myofibroblast

transformation, via either epithelial-mesenchymal transition or fibroblast-to-myofibroblast transdifferentiation.

In summary, our findings suggest that VEGF has potential effects on the TGF- β 1/Smad/Snail pathway involved in myofibroblast transformation. This gives an experimental basis for the use of anti-VEGF agents in glaucoma surgery. Further studies to modulate myofibroblast transformation may have potential clinical uses for improving the outcome of glaucoma surgery.

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