Potential Role of Tissue Transglutaminase in Glaucoma Filtering Surgery

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PURPOSE. Scarring of the filtering bleb site is the main cause of failure in glaucoma filtration surgery. In the present study, the role of tissue transglutaminase (tTgase) in the accumulation of extracellular matrix (ECM) proteins in these scars was investigated. Transglutaminases are enzymes capable of cross-linking ECM proteins to proteolysis-resistant complexes.

METHODS. Expression of tTgase, its reaction product ε-(γ-glutamyl)-lysine, and fibronectin and their colocalization were investigated immunohistochemically in failed blebs and in an in vitro trabeculectomy model. Failed blebs were analyzed by RT-PCR for the presence of tTgase mRNA. Human Tenon fibroblasts (HTFs) were treated with transforming growth factor-β2 (TGF-β2). The effect was studied with immunohistochemistry, Northern blot analysis, and Western blot analysis. Tgase activity was assayed by incorporation of biotinylated cadaverine into fibronectin.

RESULTS. Expression of tTgase and ε-(γ-glutamyl)-lysine was present in all failed blebs. Staining was most prominent at the rim of the Tenon cyst. In the in vitro trabeculectomy model, tTgase and ε-(γ-glutamyl)-lysine were barely present at the incision side of the flap but were sparsely increased by TGF-β2 treatment. Enzyme and its reaction product were localized with fibronectin. Cultured HTFs contained a basal level of tTgase mRNA. After treatment with TGF-β2, expression and activity of tTgase significantly increased.

CONCLUSIONS. The findings demonstrated that tTgase is present and functionally active in failed blebs. Expression and activity of tTgase appeared to be stimulated by TGF-β2, a growth factor known to be increased in primary open angle glaucoma. Intervention at this pathway might open a new approach to prevent scarring after glaucoma filtration surgery. (Invest Ophthalmol Vis Sci. 2006;47:3835–3845) DOI:10.1167/iows.05-0960

The major determinant of the long-term outcome of glaucoma surgery is the wound-healing response. Excessive postoperative scarring at the level of the conjunctiva and sclerostomy sites is associated with poor postoperative pressure control.1–4 The precise pathogenic mechanism involved in scar tissue formation after glaucoma surgery still is not completely understood. However, human Tenon fibroblasts (HTFs) from the subconjunctival space are known to be the central player in the wound repair and scarring processes after filtering glaucoma surgery.5 By proliferation, migration, production, and subsequent contraction of extracellular matrix (ECM) components, HTFs form the scar tissue responsible for filtering failure of the bleb.5,6

Previous studies regarding scar formation in skin and in proliferative vitreoretinopathy demonstrated transglutaminase-induced irreversible cross-links of ECM that cannot be digested by any known enzyme.7–12 In keeping with this and the finding that tissue transglutaminase (tTgase) is basally expressed in the conjunctiva and sclera,11,12 excessive accumulation of ECM in bleb scars could result from the formation of irreversible ECM cross-links in the fibrocellular tissue.

Transglutaminases are calcium-dependent enzymes that catalyze the posttranslational modification of proteins through an acyl transfer reaction between the γ-carboxamidogroup of a peptide-bound glutaminyl residue and various amines. Covalent cross-linking using ε-(γ-glutamyl)-lysine bonds is stable and resistant to enzymatic, chemical, and mechanical disruption.13 Endopeptidases capable of hydrolyzing the ε-(γ-glutamyl)-lysine cross-links formed by transglutaminases have not been described in vertebrates, and even lysosomes do not contain enzymes capable of splitting the ε-(γ-glutamyl)-lysine bonds.14–16 Tissue transglutaminase (tTgase, type 2) is the most widespread member of this family and is present in many different cell types and tissues, with diverse functions.17–19 The enzyme plays a role in programmed cell death10 and cell adhesion20 and in normal and abnormal wound healing.21 tTgase controls interaction between the cell and its ECM through the cross-linking of proteins such as fibronectin,22 and collagen type 3.23 Fibronectin has been shown to be increased in the ECM of scar tissue,24 and collagen type 3 is widely recognized as the collagen in new scar tissue.25 Both proteins play a major role in the healing process after glaucoma filtering surgery,26–27 and are produced by HTFs in vitro.28

The present study was designed to investigate the possible role of irreversible ε-(γ-glutamyl)-lysine cross-link of ECM by tTgase in scar tissue of failed blebs. In addition to conducting immunohistochemistry and molecular biology study on the scar tissue of failed blebs and on an in vitro trabeculectomy model, we investigated the capacity of HTFs to synthesize tTgase. HTF cell culture is a well-accepted in vitro model for wound repair and scarring processes that occur in the anterior segment of the eye.5 In vitro HTFs have been shown to be capable of synthesizing ECM components29 and degrading enzymes such as matrix metalloproteinases.29–31

Given that TGF-β2 is one of the most potent stimulators of scarring in the eye and that it is involved in the pathogenesis of conjunctival scarring and wound healing after filtration surgery,22–25 we investigated the influence of TGF-β2 on tTgase synthesis of cultured HTFs. The activity of extracellular tTgase was demonstrated by its ability to cross-link fibronectin, an
ECM component that takes part in the initial healing process after glaucoma filtering surgery.26,27

**MATERIALS AND METHODS**

**Tissue Samples**

Eight samples of scar tissue of failed blebs (Tenon cysts) were obtained from patients undergoing trabeculectomy revision at the Department of Ophthalmology of the Ludwig-Maximilians-University Munich (Table 1). Methods for securing human tissue were humane, included proper consent and approval, and complied with the Declaration of Helsinki. Operations were performed by different surgeons using the same technique. Conjunctiva and Tenon cyst were carefully separated by subconjunctival injection of phosphate-buffered saline (PBS; pH 7.4). After opening the Tenon cyst at its border area, the whole cyst over the trabeculectomy flap and adjacent scar tissue were excised en bloc and put into PBS (pH 7.4) intraoperatively. Afterward Tenon cysts were either snap-frozen in liquid nitrogen for mRNA extraction or mounted in OCT mounting medium (Merck, Darmstadt, Germany) and then stored in liquid nitrogen for cryostat sectioning.

**In Vitro Trabeculectomy Model**

Six eyes from donors between 35 and 65 years of age were obtained from the Eye Bank of the Ludwig-Maximilians-University, Munich. Only eyes from donors who gave specific consent for research purposes and with contraindication for transplantation according to the guidelines of the German Federal Physicians’ Association were included.38 All eyes were preserved in a moist chamber at 4°C within 45 hours after death. All experiments were performed in accordance with the Declaration of Helsinki. Whole eyes were thoroughly cleansed in 0.9% NaCl solution, immersed in 5% polyvinyl pyrrolidone iodine, and rinsed again in the sodium-chloride solution.

After intravitreal injection of 0.9% NaCl to normalize the intraocular pressure, estimated by palpation to be between 15 and 20 mm Hg, conventional trabeculectomy26,39 was performed in vitro (Fig. 1A). Afterward corneoscleral disks, including the trabeculectomy side, were excised and put into 50-mL tissue culture flasks (Primaria Tissue Culture Flask, Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) fixed on a cornea disk holder40 (Figs. 1B, 1C). Organ culture medium consisted of Eagle minimum essential medium (MEM) with Earle salts and 25 mM HEPES buffer (Gibco Life Technologies, Paisley, Scotland, UK), containing 2% fetal calf serum (FCS; Biochrom, Berlin, Germany), 2 mM glutamine (Gibco), 100 U/mL penicillin, 0.1 mg/mL streptomycin (Gibco), and 0.25 µg/mL amphotericin B (Gibco). After corneal transplantation, the residual corneoscleral ring with the in vitro trabeculectomies was kept in organ culture for another 2 months. Three of the six trabeculectomy samples now were also treated with 2 ng/mL TGF-β2 (R&D Systems, Wiesbaden, Germany), the maximal total concentration previously found in the aqueous humor of patients with

**TABLE 1. Clinically Relevant Data of Patients Who Underwent Trabeculectomy Revision with Tenon Cyst Excision**

<table>
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<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Diagnosis</th>
<th>Antimetabolites</th>
<th>Time after TET</th>
<th>Local Therapy, y</th>
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<td>2</td>
<td>68</td>
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<td>1 y</td>
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<td>3 mo</td>
<td>5</td>
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<td>2 y</td>
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<td>59</td>
<td>POAG</td>
<td>No</td>
<td>7 wk</td>
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</table>

TET, trabeculectomy; PEX, pseudoexfoliation glaucoma; POAG, primary open-angle glaucoma.

**FIGURE 1. In vitro trabeculectomy.**

(A) Conventional trabeculectomy was performed in vitro on a human donor eye before preparation of the corneoscleral disk. (B) Corneoscleral disks, including the trabeculectomy, were excised and put into a culture flask before preparation of the corneoscleral disk. (C) Magnification of the trabeculectomy in organ culture.
different types of glaucoma. Corneas were stored under submerged organ culture conditions in a standard incubator at 37°C and 5% CO₂, and the medium was changed every 3 days as described previously.

**Immunohistochemistry of Tissue Sections**

Immunohistochemical double staining for extracellular Tgase and fibronectin and combined staining of e-(γ-glutamyl)-lysine and fibronectin were performed in sections obtained from scarred blebs (Tenon cysts) and from the in vitro trabeculectomy model. Cryostat sectioning of unfixed scar tissue with a thickness of 8 μm was performed. After washing in Tris-buffered saline (TBS, pH 7.2–7.4) and preincubation with TBS containing 5% bovine serum albumin (BSA; Roche, Mannheim, Germany) to minimize nonspecific binding, the sections were incubated for 2 hours at room temperature (RT) with mouse anti–tissue-transglutaminase (Cub7402; Quartett, Berlin, Germany), mouse anti-e-(γ-glutamyl)-lysine (CovalAb Oullins, Cedex, France), and rabbit anti-fibronectin (Sigma, Deisenhofen, Germany). All antibodies were diluted 1:100 (1 μg/100 μL) in TBS containing 5% BSA. After washing in TBS, the sections were incubated with Cy-2–conjugated goat anti–mouse IgG (Dianova, Hamburg, Germany) and Cy-3–conjugated swine anti–rabbit IgG (Dianova) diluted 1:100 (1 μg/100 μL) in blocking buffer for 2 hours at RT.

Control sections were incubated with TBS-BSA without primary antibody or with a combination of 1:100 (1 μg/100 μL) diluted primary antibody plus a fivefold (5 μg/100 μL) weight excess of guinea pig Tgase (Sigma). A fluorescence microscope (Leica, Bensheim, Germany) was used for visualization of the stained sections. For standard morphology, either hematoxylin/eosin or phase-contrast microscopy imaging of the tissue sections was performed.

**RNA Isolation and RT-Polymerase Chain Reaction of Scarred Blebs and Normal Conjunctiva**

Total mRNA of five scarred blebs was extracted using a micro RNA kit (peqGOLD RNAPure; Peqlab, Erlangen, Germany). After confirming the structural integrity of the total RNA samples of each failed bleb (Tenon cyst) by electrophoresis on 1% agarose gels and subsequent staining with 0.5 μmol/mL ethidium bromide, RNA samples were treated with 3 U RQ RNase-free DNase (Promega, Madison, WI) for 35 minutes at 37°C to remove traces of contaminating genomic DNA. The content of RNA was measured by photometric measurement, and the RNA concentration was adjusted. Using Moloney murine leukemia virus (MMLV) reverse transcriptase and oligo(dT)-17 primer (Gibco), first-strand complementary DNA (cDNA) was prepared from total RNA. Quality of RNA and cDNA synthesis was proven by amplification of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The PCR of the same quantity of total cDNA was performed in a total volume of 50 μL with 1 U native Taq polymerase (Eppendorf, Hamburg, Germany). PCR was started with a hot start: 10 minutes for 94°C to denature DNA, followed by 36 cycles of 1-minute melting at 94°C, 1 minute annealing at the respective annealing temperature, and 2 minutes extension at 72°C in a thermocycler (Mastercycler Gradient; Eppendorf). After the last cycle, the polymerization step was extended for another 10 minutes to complete all strands. Each PCR was repeated at least twice.

Primers used were as follows: Tgase forward, 5′-CAGACACGCAACCTTCCTGAGG3′; Tgase reverse, 5′-TGGACTCCGTAAGCAGAACAG-3′ positions, 416 to 852; product size, 437 bp; annealing temperature, 59.7°C (Metabion); fibronectin forward, 5′-ATTGGTGCAACGACATTG3′; fibronectin reverse, 5′-AAGTCTCAGGTCTCTGCCGAAC3′; positions, 3752 to 4288; product size, 537 bp; annealing temperature, 56.8°C (MWG-Biotech, Ebersberg, Germany).

The specificity of the PCR product was analyzed by automated DNA sequencing (Sequiseve, Vaterstetten, Germany). PCR performed on each sample of RNA that had not been reverse transcribed to cDNA was used as negative control and showed no amplified product.

For semiquantitative PCR, the number of cycles was optimized by checking amplification after each cycle from cycles 25 to 36 for Tgase and from 20 to 34 for GAPDH. This showed that the 29th cycle was in the geometric phase for Tgase and GAPDH. PCR amplification products were separated by agarose gel electrophoresis and stained with ethidium bromide for visualization (LAS-1000 imager workstation; RayTest, Pforzheim, Germany). In addition, band intensity was measured (LAS-1000 Imager workstation; RayTest). Quantification was performed with the AIDA software package (RayTest). The final amount of PCR product was expressed as the ratio of the Tgase gene amplified to that of the GAPDH gene. Experiments were repeated three times.

**Human Tenon Fibroblast Isolation and Cell Culture Conditions**

Human Tenon samples were obtained from tissue explants of three white patients (ages 40, 50, and 60 years) who underwent routine buckling surgery and did not have any topical eye treatment before-hand. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the procedure. HTFs were cultured as previously described and were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 50 mg/mL gentamicin, and 0.25 mg/mL amphotericin B (all from Gibco Life Technologies) at 37°C with 5% CO₂ in air.

For growth factor experiments, second- to fifth-passage HTFs were grown to confluence. HTFs were then washed three times with TBS, pH 7.2 to 7.4, incubated overnight in serum-free medium, and subsequently incubated in serum-free DMEM supplemented with 2.0 ng/mL TGF-β2 (R&D Systems) for 24 hours. To prevent the adhesion of TGF-β2, BSA (Roche) was added during the activation process. Controls were incubated under identical conditions without growth factor in the medium. The guidelines of the Declaration of Helsinki were followed, and institutional human experimentation committee approval was granted.

**Immunohistochemistry of Cell Cultures**

For the detection of extracellular Tgase, confluent HTFs grown in four-well plastic chamber slides were incubated for 2.5 hours with serum-free DMEM (Biochrom) containing 0.75 μg/mL monoclonal antibody to Tgase (Quartett). Cells were then washed in PBS and fixed in 4% paraformaldehyde in PBS. After blocking in 3% BSA, the cells were incubated for 15 hours at 4°C with rabbit anti–fibronectin antibody (Sigma) diluted 1:100 in TBS containing 3% BSA. Samples were then washed with PBS and incubated with goat anti–mouse IgG Cy-2 (Dianova) and swine anti–rabbit IgG Cy-3 (Dianova) diluted 1:100 in blocking buffer for 2 hours at RT.

**Northern Blot Analysis**

Total RNA was isolated from confluent HTF cultures in 10 cm Petri dishes using the guanidinium thiocyanate-phenol-chloroform extraction method (RNA isolation kit; Stratagene, Heidelberg, Germany). Total RNA (3 μg per lane) was denatured and size-fractionated by gel electrophoresis in 1% agarose gels containing 2.2 M formaldehyde. The RNA was then vacuum blotted onto a nylon membrane (Roche) and cross-linked (1600 μf; Stratallinker; Stratagene). To assess the amount and quality of the RNA, the membrane was stained with methylene blue and images were taken (LAS-1000 Imager; RayTest). Prehybridization, hybridization, and chemiluminescence detection of the digoxigenin riboprobe were performed as described previously. In brief, after hybridization, the membrane was washed twice with 2 × SSC, 0.1% sodium dodecyl sulfate (SDS) at RT, followed by two washes in 0.1 × SSC, 0.1% SDS for 15 minutes at 68°C. After hybridization and posthybridization washes, the membrane was washed for 5 minutes in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5; 0.3% Tween 20)
and incubated for 60 minutes in blocking solution. Blocking solution contained 100 mM maleic acid (pH 7.5), 150 mM NaCl, and 1% blocking reagent (Roche). Alkaline phosphatase–conjugated sheep anti-digoxigenin IgG (Roche, Mannheim, Germany) was diluted 1:10,000 in blocking solution and used to incubate the membrane for 30 minutes. The membrane was then washed four times (15 minutes each time) in washing buffer and was equilibrated in detection buffer (100 mM Tris-HCl; 100 mM NaCl; pH 9.5) for 10 minutes. For chemiluminescence detection, a 1:100 dilution (CDP-star; Roche) was made in detection buffer and was used to incubate the filter for 5 minutes at RT. After air drying the semi-dry membrane was sealed in a plastic bag.

Chemiluminescence was detected (LAS-1000 Imager workstation; RayTest) with exposure times ranging from 10 minutes to 1 hour. Chemiluminescence signal quantification was performed with a software package (AIDA; RayTest). All experiments were repeated at least three times with HTF cells from different cell lines.

Western Blot of tTgase

HTFs grown in 35-mm tissue culture dishes were washed twice with PBS, collected, and lysed in NP-40 (150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40) cell lysis buffer. Samples for gel analysis were boiled for 5 minutes, and protein content was measured with the use of BCA protein assay reagent (Pierce, Rockford, IL). Proteins were loaded (2 μg/lane) and separated by electrophoresis with the use of 5% SDS-PAGE stacking gel and 8% SDS-PAGE separating gel.11 After gel electrophoresis, the proteins were transferred with semi-dry blotting onto a PVDF membrane (Roche). The membrane was incubated with PBS containing 0.1% Tween 20 (PBST, pH 7.2) and 5% BSA for 1 hour. The primary antibody (tTgase 1:2000, Cub7402; Quartett) was then added and allowed to react overnight at RT. After the membrane was washed three times in PBST, it was incubated with an alkaline phosphatase–conjugated swine-anti–mouse antibody (Dianova; diluted 1:20,000) for 30 minutes. The membrane was then washed four times (15 minutes each) in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% Tween 20). The membrane was equilibrated in detection buffer (100 mM Tris-HCl; 100 mM NaCl; pH 9.5) for 10 minutes. Visualization of the alkaline phosphatase was achieved using chemiluminescence. CDP-star (Roche) was diluted 1:100 in detection buffer, and the filter was incubated for 5 minutes at RT. After air drying, the semi-dry membrane was sealed in a plastic bag. Chemiluminescence was detected (LAS-1000 Imager workstation; RayTest) with exposure times from 1 minute to 5 minutes. Quantification of chemiluminescence was performed with software (AIDA; RayTest). All experiments were repeated at least three times using HTF cells derived from different donors.

tTgase Activity of Cultured HTFs

tTgase activity was measured by the incorporation of biotinylated cadaverine into fibronectin.26 For this assay, 96-well plates were precoated with plasma fibronectin (5 μg/mL; Sigma) and were incubated overnight at 4°C. Twenty-four hours before seeding, HTFs were treated with 2.0 ng/mL TGF-β2 (R&D Systems). Untreated and cytokine-treated HTFs were then plated at a density of 2 × 105 cells/mL in 100 μL serum-free DMEM (Biochrom) in the presence of 0.1 mM biotinylated cadaverine (Mobi-Tec, Göttingen, Germany). HTFs were allowed to incubate on the fibronectin-coated plates for different time periods (0, 5, 10, 20, 40, 60, 90, or 120 minutes) at 37°C and were then washed twice with PBS containing 3 mM EDTA. As a negative control, fibronectin-coated 96-well plates were incubated with 100 μL serum-free DMEM containing 0.1 mM biotinylated cadaverine alone. The detergent solution (100 μL) consisting of 0.1% deoxycholate in PBS containing 3 mM EDTA was then added to each well and was incubated with gentle shaking for 20 minutes. The supernatant was discarded, and the remaining fibronectin layer was washed three times with 0.1 M Tris-HCl, pH 7.4. Reaction was then blocked with 3% BSA in Tris-HCl buffer for 30 minutes at 37°C and was washed three times with Tris-HCl buffer. The incorporated biotinylated cadaverine was revealed with a 1:5000 dilution of peroxidase conjugate (Extravidin; Sigma) incubated for 1 hour at 37°C. After three washes with Tris-HCl, the fibronectin layer was incubated for 20 minutes at RT in 200 μL substrate solution (one tablet of tetramethylbenzidine was dissolved in 1 mL dimethylsulfoxide and added to 9 mL 0.05 M phosphate-citrate buffer, pH 5.0; 2 μL fresh 30% hydrogen peroxide was added per 10 mL substrate buffer solution). Color development was stopped by adding 50 μL stop solution (25% H2SO4) to each well. Optical density was determined with the use of an ELISA reader (Molecular Devices, Garching, Germany) set at 450 nm. Experiments were performed nine times.

Statistical Analysis

Statistical analysis was based on Wilcoxon matched-pair signed-rank test, and P ≤ 0.05 was considered statistically significant.

RESULTS

Scar Tissue of Failed Blebs

Tgase mRNA and Protein in Failed Blebs. Eight samples of failed blebs were obtained from patients undergoing trabeculectomy revision. Six (75%) of those patients had primary open angle glaucoma, and the others had pseudoxfoliation glaucoma and congenital glaucoma, respectively (Table 1). Mean age was 59.6 (±12.3) years, mean follow-up time after trabeculectomy was 11.5 (±7.1) months, and mean duration of local therapy was 12 (±6.3) years.

With the RT-PCR technique, we could demonstrate the presence of tTgase mRNA in all investigated scar tissue samples of failed blebs (Fig. 2). The actual size of the PCR product of tTgase was found to be close to the theoretically expected value (on the basis of the primer position 758 bp) and showed the expected sequence (data not shown). Semiquantitative RT-PCR analysis revealed expression of tTgase in scar tissue of failed blebs three times higher than in normal conjunctiva (Fig. 2). Results of PCR performed on negative controls, when the
reverse transcriptase step was omitted, were negative (data not shown).

Immunohistochemical staining with antibodies against tTgase revealed specific staining in all sections of scar tissue samples of failed blebs (Tenon cysts) studied (Fig. 3A). Staining was present throughout the ECM of the entire scar but was more intense at the border of the Tenon cyst (Fig. 3A). Double staining (Fig. 3C) revealed colocalization (yellow staining) of tTgase and fibronectin (Fig. 3B). Colocalization of tTgase and fibronectin was visible over the entire tissue sample (Fig. 3C). The staining pattern of the tTgase-catalyzed reaction product $\varepsilon$-(γ-glutamyl)-lysine bond (Fig. 3D) was similar to the staining pattern of the enzyme. Double staining (Fig. 3F) for fibronectin and $\varepsilon$-(γ-glutamyl)-lysine bonds also showed almost complete colocalization (Fig. 3F). All control sections incubated without the primary antibody or with a combination of primary antibody and fivefold excess of tTgase were unstained (data not shown).

**In Vitro Trabeculectomy Model.** To further confirm the hypothesis that tTgase influences extracellular matrix formation after trabeculectomy, we established an in vitro trabeculectomy model. The expression of tTgase and its reaction product, $\varepsilon$-(γ-glutamyl)-lysine, and the influence of TGF-β2, a growth factor known to play a major role in wound healing reaction after trabeculectomy, have been studied. Although immunohistochemistry of in vitro trabeculectomy sections revealed only moderate staining for tTgase at the interface of the trabeculectomy flap (Fig. 4A), treatment of the
in vitro trabeculectomy with TGF-β2 led to higher intensity of tTgase staining (Fig. 4E). Staining with antibodies against the tTgase-catalyzed reaction product ε-(γ-glutamyl)-lysine bond showed almost the same patterns. Although little specific staining for ε-(γ-glutamyl)-lysine bond was seen in untreated in vitro trabeculectomy samples (Fig. 4I), TGF-β2 treatment led to a distinct increase in ε-(γ-glutamyl)-lysine staining (Fig. 4M). Double staining of parallel sections with FN and ε-(γ-glutamyl)-lysine reveals broad colocalization (yellow) (Fig. 4K, O). Phase-contrast images of the corresponding cryosections. Original magnifications, ×100. (P) Illustration demonstrating a cross-section of a trabeculectomy flap with a box marking the region of magnification as shown in the (immuno) histologic sections (A-O). Representative immunohistochemical images of cryosections obtained from six patients with failed blebs are shown.

**Cell Culture of Human Tenon Fibroblasts**

**Induction of Fibronectin and tTgase mRNA and Protein by TGF-β2.** Northern blot analysis of untreated HTFs showed a single faint band of 7.7 kb after hybridization with an antisense fibronectin RNA probe (Fig. 5A) and of 3.5 kb after hybridization with an antisense tTgase RNA probe, respectively.
Tissue Transglutaminase after Filtering Surgery

FIGURE 5. TGF-β2 induces fibronectin expression in human Tenon fibroblasts. (A) Representative Northern blot analysis of fibronectin (FN) mRNA expression in confluent HTF cells 24 hours after treatment with 2 ng/mL TGF-β2. Levels of FN mRNA in TGF-β2-treated HTFs were 4.0 (±0.8)-fold higher than those detected in the untreated control cells. (B) Methylene blue staining of the 28S and 18S rRNA bands to demonstrate relative integrity and loading of RNA. (C) Representative Western blot analysis of FN in HTF cell culture treated as described for Northern blot analysis. The number below each band shows the chemiluminescence measurement (2.5 ±0.5 ma). All experiments were repeated four times with HTF cells from different cell lines. Co, control; RDI, relative densitometric intensity (normalized to values of untreated controls [=1.0]; differences in 28S rRNA were considered for Northern blot quantification. For Western blot, equal amounts were loaded per lane.

To validate that this increase in mRNA transcription translates into increased protein synthesis, whole cellular protein extracts were analyzed by Western blotting (Figs. 5C, 6C). Immunoblot analysis revealed a single band at the molecular mass of approximately 240 kDa for fibronectin and of approximately 80 kDa for tTgase, respectively. Treatment with TGF-β2 led to an increase of fibronectin by a factor of 2 to 3 (2.5 ± 0.5; P < 0.05) and of tTgase by a factor of 4 to 5 (4.95 ± 0.52; P < 0.05) than those detected in the untreated control cells. Representative blots of experiments that were repeated at least three times are shown in Figures 5C and 6C.

Extracellular tTgase Activity. To demonstrate that tTgase is functionally active and further influence by TGF-β2, assay screening for the incorporation of biotinylated cadaverine into fibronectin by tTgase activity was performed. Basal incorporation of biotinylated cadaverine into fibronectin was found after 10 to 120 minutes (Fig. 7). This incorporation increased markedly, when HTFs were pretreated for 24 hours with 2.0 ng/mL TGF-β2 before seeding (Fig. 7).

Colocalization of tTgase and Fibronectin. To demonstrate extracellular tTgase immunohistochemically, anti-tTgase antibody was added to the culture medium of unfixed HTFs. Only weak staining of tTgase developed in untreated HTFs (Fig. 8A). Treatment with TGF-β2 increased the amount of extracellular tTgase (Fig. 8D). Double staining of fibronectin (Figs. 8B, 8E) and tTgase revealed partial colocalization of extracellular tTgase and fibronectin (Figs. 8C, 8F).

FIGURE 6. TGF-β2 induces tTgase expression in human tenon fibroblasts. (A) Representative Northern blot analysis of tTgase mRNA in confluent HTF cells 24 hours after treatment with 2 ng/mL TGF-β2. Levels of tTgase mRNA in TGF-β2-treated HTFs were 4.95 (±0.52)-fold higher than those detected in the untreated control cells. (B) Methylene blue staining of the 28S and 18S rRNA bands to demonstrate relative integrity and loading of RNA. (C) Representative Western blot analysis of tTgase in HTF cell culture treated as described for Northern blot analysis. The number below each band shows the chemiluminescence measurement (4.53 ±0.99 ma). All experiments were repeated four times with HTF cells from different cell lines. Co indicates control; RDI, relative densitometric intensity (normalized to values of untreated controls [=1.0]; differences in 28S rRNA were considered for Northern blot quantification. For Western blot, equal amounts were loaded per lane.)

DISCUSSION

To the best of our knowledge, this is the first study to demonstrate the presence of tTgase and its end product, ε-(γ-glutamyl)-lysine, in scarred tissue of failed trabeculectomy blebs and in an in vitro trabeculectomy model. Irrespective of patient age, mean follow-up time after trabeculectomy, and duration of
local therapy, all eight samples of scar tissue of failed blebs showed comparable protein expression and staining patterns. Although a relatively low number of probes have been tested, reproducibility of the findings in all immunohistochemistry and molecular biology experiments in this study proves the validity of the results and justifies the relatively small number of samples. The almost identical staining pattern of tTgase and $\epsilon$-(γ-glutamyl)lysine in scarred blebs of failed trabeculectomies and the incorporation of biotinylated cadaverine into fibronectin indicated that the tTgase, at least in vitro, was functionally active. However, with respect to the patient samples, tTgase activity was only correlative. In addition to fibronectin, tTgase is known to cross-link other ECM components such as vitronectin, laminin, nidogen, and collagen type III, anticipating an important role of tTgase in ECM remodeling after glaucoma filtering surgery.

Regulatory elements of the tTgase gene appear to be responsive to several cytokines. Of all the growth factors involved in the wound-healing cascade, TGF-β has been shown to be one of the most potent stimulators of scarring in the eye and is involved in the pathogenesis of cataract, proliferative vitreoretinopathy, and conjunctival scarring. TGF-β, the most predominant isoform of the three mammalian isoforms in the eye, is the most potent growth factor in the aqueous humor, stimulating conjunctival fibroblast function and HTF activity. Elevated levels of this isoform are found in the

**FIGURE 7.** Cell-mediated incorporation of biotinylated cadaverine into fibronectin by tTgase. Basal incorporation of biotinylated cadaverine into fibronectin was found after 10 to 120 minutes in untreated (Co.) HTFs. This incorporation increased markedly, when HTFs were pretreated with 2.0 ng/mL TGF-β2 for 24 hours before seeding. Data are expressed as the mean results ± SEM of nine experiments with three different HTF cell cultures.

**FIGURE 8.** TGF-β2 increases tTgase expression in human Tenon fibroblasts. Confluent human Tenon fibroblast cell cultures were stained for tTgase (A, D) and fibronectin (B, E). Staining was performed in cells after treatment with 2.0 ng/mL TGF-β2 for 24 hours (D–F) and in untreated controls (A–C). Extracellular staining for tTgase was less pronounced in the untreated (A) than in the TGF-β2–treated (D) cells. Double staining revealed a partial colocalization (yellow-stained strands) of extracellular tTgase and fibronectin (Figs. 7C, 7F). Original magnifications, ×200.
aqueous humor of glaucomatous eyes compared with normal eyes.5,4–58 In a previous study, Picht et al.8 demonstrated a tendency for favorable bleb development in patients with normal TGF-β2 levels, indicating that there might be some relationship between bleb formation and TGF-β2 levels, especially for patients with primary open angle glaucoma. Analogous to this previous study, most (75%) failed blebs in this study were obtained from patients with primary open angle glaucoma (Table 1). Furthermore, 10-fold higher concentrations of TGF-β2 were found in the tear fluid.59 Given that TGF-β2 plays a major role in the pathogenesis of glaucoma and conjunctival scarring treatment of cultured scleral fibroblasts or in vitro trabeculectomy with TGF-β2 seems to be an ideal model to mimic the in vivo condition after glaucoma surgery.

In our study cultured HTFs and in vitro trabeculectomies, when exposed to TGF-β2, showed increased Tgase expression and enhanced cross-linking of the ECM component fibronectin through ε-(γ-glycyl)-lysine bonds. These results suggest that TGF-β2, in addition to stimulating proliferating HTPs to produce numerous ECM components, induces irreversible cross-links of these ECM components, thereby forming the fibrocellular scar tissue of failed blebs. In keeping with this hypothesis in a recent experimental animal study,60 postoperative use of the TGF-β antibody CAT-152 significantly improved outcomes after surgery and reduced subconjunctival scarring.

In the present study we used a human in vitro trabeculectomy model to study conjunctival scarring after trabeculectomy. The major drawback of this in vitro approach compared with in vivo animal models was the lack of blood supply, which might have significantly contributed to the inflammatory response. However, as evidenced by several previous studies, findings obtained from animal models do not necessarily translate into the human in vivo condition. For instance, Cordeiro et al.33 demonstrated successful inhibition of conjunctival scarring in a rabbit animal model of aggressive conjunctival scarring with significant improvement of glaucoma filtering surgery by the use of recombinant neutralizing antibodies to human TGF-β2 (rhAnti-TGF-β2 mAb). However, in vivo application of rhAnti-TGF-β2 mAb could not confirm the data of the animal model (Khaw PT, et al. JOVS 2005;46:ARVO E-Abstract 87). In view of these experiences, we do think the application of a human in vitro trabeculectomy model is an appropriate approach to study conjunctival scarring after trabeculectomy. However, despite the apparent limitations of animal models, future studies in animals will be needed to confirm the present findings in vivo conditions.

Current established concepts of therapy to prevent the onset of increased fibrocellular scarring after trabeculectomy, such as intraoperative administration of the antiprofylactic agents 5-fluorouracil (5-FU) and mitomycin C (MMC), have increased the success rate of filtration surgery, and these techniques have been accepted in clinical practice.45–68 However, despite the use of these antiprofylactic agents, failure can occur later in the postoperative period,56–66,69–70 and the administration of MMC and 5-FU, which induce widespread cell death and apoptosis, can result in corneal erosions, cystic avascular blebs, chronic hypotony with maculopathy, and endophthalmitis.65,68,71–73

Therefore, more physiologic antiscarring agents are needed for the postoperative prevention of bleb failure and increased intraocular pressure. Although fully speculative at this point, the inhibition of Tgase might offer a future therapeutic approach to prevent irreversible ECM cross-linking of TGF-β-activated HTPs. Possible agents are competitive inhibitors of transglutaminase such as putrescine, which decreases wound breaking strength in incisional skin wounds by topical application,8 and spermidine, which decreases the healing of gastric and duodenal stress ulcers by inhibiting transglutaminase.74

Furthermore, transglutaminase-mediated effects can be inhibited by specific antibodies,75 and some pharmaceutical compounds even inhibit transglutaminase not only competitively but irreversibly.76 The use of such components may prevent increased scar tissue formation after trabeculectomy and therefore offer a new, specific therapeutic strategy to prevent bleb failure after filtering glaucoma surgery.

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