Expression of Connective Tissue Growth Factor after Glaucoma Filtration Surgery in a Rabbit Model

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PURPOSE. Connective tissue growth factor (CTGF) appears to play a significant role in mediating fibrosis in several tissues. To gain further understanding of the role of CTGF in the scar formation that occurs after glaucoma filtering surgery (GFS), experiments were performed in a rabbit model.

METHODS. Three experiments were performed: (1) CTGF and transforming growth factor (TGF)-β expression were measured quantitatively after GFS, using ELISA. (2) After GFS conjunctival bleb tissues were immunostained for the presence of CTGF and TGF-β. (3) Exogenous CTGF was injected into mitomycin-C (MMC)-treated filtering blebs and the scaring response compared to TGF-β and physiological saline–injected blebs.

RESULTS. CTGF and TGF-β were expressed maximally by day 5 after surgery and were both shown to be present in the bleb tissues after GFS. The addition of exogenous CTGF and TGF-β increased the rate of failure of GFS blebs.

CONCLUSIONS. These data support the hypothesis that CTGF plays an important role in scarring and wound contraction after GFS. Inhibition of CTGF synthesis or its action may help prevent bleb failure and improve long-term GFS outcomes.

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laucoma filtering surgery is commonly performed when medication fails to control IOP adequately. Excessive subconjunctival scarring after GFS is responsible for failure of the surgery in most cases.1–3 The processes involved in the scarring response are heavily influenced by growth factors, including the TGF-β family. This is present in three isoforms (β1, β2, and β3), of which TGF-β2 is predominant in the eye.4–9 As well as stimulating the formation of scar tissue, these factors mediate subsequent wound contractions.10 Cell migration induced by TGF-β has been demonstrated in several cell types, including neutrophils and peripheral monocytes.11,12 TGF-β has also been implicated as a potent stimulant of the scarring process in the eye.5–12 TGF-β inhibition, using antisense oligonucleotide or antibody, has been shown to reduce scarring after GFS in both animals and humans.16,17

In GFS, the presence of aqueous at the wound site, as well as the breakdown of the blood–aqueous barrier and initiation of the inflammatory and clotting cascade may influence the amount of TGF-β secreted and its degree of activation.18–21

Connective tissue growth factor (CTGF) is a secreted peptide that was originally discovered in human umbilical vein endothelial cell conditioned medium and has been implicated in multiple cellular events, including angiogenesis, skeletogenesis, and wound healing.22 It has been found to act as a mitogen in fibroblast cell cultures and to cause significant upregulation of components of the extra cellular matrix, such as collagen, integrin, and fibronectin.23–25 The actions of CTGF have been clearly distinguished from those of TGF-β by showing that CTGF alone does not induce anchorage-independent growth of fibroblasts.26

TGF-β1 has been shown to produce a five- to sixfold increase in CTGF expression in cultured fibroblasts.27 The mechanisms of CTGF induction involve Smads, Ras/MEK/ERK, protein kinase C, and fibroblast-enriched factors.28 CTGF has been shown to be a downstream mediator of TGF-β.29–31

We undertook a series of experiments, using an established model of GFS in the rabbit eye in which surgery rapidly fails secondary to aggressive subconjunctival scarring.32 In the first experiment, both CTGF and TGF-β expression were measured quantitatively after GFS, using an ELISA technique. In the second experiment, CTGF and TGF-β were immunolocalized in the conjunctival and Tenon’s tissues after GFS. In the third experiment, exogenous CTGF was injected into mitomycin-C (MMC)-treated blebs and the results compared with TGF-β and physiological saline (balanced salt solution [BSS]; Santen Pharmaceuticals, Osaka, Japan)–injected blebs, to demonstrate its effect on bleb survival.

METHODS

Glaucoma Filtering Surgery

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Florida Animal Care and Use Committee.

New Zealand albino male rabbits weighing approximately 2 to 4 lb were used. The animals were anesthetized with a combination of ketamine (50 mg/kg; Ketaset) and xylazine (10 mg/kg; Xylaject; both from Phoenix Pharmaceuticals, St. Joseph, MO) administered by intramuscular injection. Topical anesthesia in the form of 0.1% proparacaine eye drops was also administered.

Two types of GFSs were performed (by the same surgeon) in the rabbit eyes. In both, a partial-thickness corneal traction suture was placed in the superior cornea and used to rotate the eye inferiorly. A limbus-based conjunctival flap was fashioned in the superior lateral limbus and a viscoelastic material (10 mg/mL,
Healon; Pharmacia & Upjohn, Uppsala, Sweden) was injected to maintain the anterior chamber.

In method one, performed in the right eye, the anterior chamber was entered using a Beaver blade (Becton Dickinson & Co., Franklin Lakes, NJ), and the sclerostomy completed using a 1.5-mm Kelly’s Descemet punch. A peripheral iridectomy was then performed (Fig. 1A).

In method two, performed in the left eye, a scleral tract was fashioned by tunneling a beveled 22-gauge intravenous cannula (Inspyte; Becton Dickinson Vascular Access, Sandy, UT) through the sclera, beginning behind the limbus and continuing until the cannula was visible in the anterior chamber. The cannula needle was then withdrawn and the cannula advanced beyond the pupillary margin to prevent iris blockage of the tube. The cannula was trimmed at its scleral end so that it protruded approximately 1 mm from the insertion point and was secured to the sclera using an encircling 10-0 nylon suture (Ethicon Inc., Somerville, NJ; Fig. 1B).

In both procedures the Tenon’s capsule was closed with a continuous locking suture of 8-0 absorbable suture material (Vicryl; Ethicon Inc.) attached to a BV needle and the conjunctiva closed with a continuous, nonlocking suture of the same material. A single application of combined neomycin and dexamethasone ointment was instilled at the end of surgery.

The rationale behind performing two different types of sclerostomy was to evaluate whether the presence of the foreign material within the wound influences the scarring process.

**Protein Sample Collection and Extraction**

In the first part of the study, the CTGF and TGF-β ELISA experiment was repeated twice in sets of eight rabbits, ensuring two animals per time point studied. Both eyes of two rabbits were used as the control. The other 14 rabbits underwent a full-thickness sclerostomy procedure in the right eye, and in the left eye a 22-gauge intravenous cannula was inserted through a scleral tunnel into the anterior chamber, as described in the two surgical methods.

Tissue was harvested from the bleb area before surgery in the control rabbits, and on days 1, 3, 5, 7, 10, 14, and 21 (by which time all blebs had failed) after surgery, in the remaining rabbits. Sclerostomy was performed in the right eye, and in the left eye a 22-gauge intravenous cannula attached to a BV needle and the conjunctiva closed with a continuous, nonlocking suture of the same material. A single application of combined neomycin and dexamethasone ointment was instilled at the end of surgery.

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CTGF ELISA

CTGF was measured in tissue extracts by sandwich ELISA with biotinylated and nonbiotinylated affinity-purified goat polyclonal antibodies to human CTGF. Briefly, a flat-bottomed ELISA plate (96-well Costar; Corning, Corning, NY) was coated with 50 µL of goat anti-human CTGF antibody (which recognizes predominately epitopes in the N-terminal half of the CTGF molecule) at a concentration of 10 µg/mL in PBS and 0.02% sodium azide for 1 hour at 37°C. The wells were washed four times and incubated with 500 µL of blocking buffer (PBS, 0.02% sodium azide, and 1% bovine serum albumin) for 1 hour at room temperature. The wells were washed again four times, and 50 µL of recombinant human CTGF standard or sample was added and incubated at room temperature for 1 hour. After the wells were washed, 50 µL of biotinylated goat anti-human CTGF was added at a concentration of 2 µg/mL and incubated at room temperature in the dark. Fifty microliters of alkaline phosphatase-conjugated streptavidin (Zymed, San Francisco, CA) was added at a 1:1000 dilution and incubated at room temperature for 1 hour after washing. The wells were washed again and incubated with 100 µL of alkaline phosphatase substrate solution (1 mg/mL p-nitrophenyl phosphate [Sigma-Aldrich, St. Louis, MO] in sodium carbonate, bicarbonate buffer, 0.02% sodium azide [pH 9.6]) until the reaction developed. Absorbance readings were obtained at 405 nm by microplate reader (Molecular Devices, Sunnyvale, CA).

The values for CTGF concentration were normalized for total protein content in the sample using bicinchoninic acid protein assay reagent (Pierce Biotechnology, Rockford, IL).

**TGF-β2 ELISA**

The amount of active TGF-β2 in the same samples was measured using a commercial ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

Repeating the experiment twice in two sets of eight rabbits each provided data from two animals per time point studied. The CTGF and TGF-β2 concentrations obtained at each time point were averaged to yield a mean level of CTGF and TGF-β2 present in the tissues at days 1, 3, 5, 7, 10, and 21 after surgery.

**Tissue Processing for Immunohistochemistry in Bleb Sections**

In the second part of the experiment, the right eyes of seven additional rabbits underwent GFS with a cannula used as described earlier. The left eyes were the control. In addition, one rabbit that did not undergo surgery was used as a control animal. The eyes were harvested at days 0, 3, 5, and 7 after surgery, perfused in situ with 4% formaldehyde for 3 minutes, dissected en bloc, fixed in 4% formaldehyde overnight, and transferred to 70% ethanol. The eyes were then processed for paraffin embedding and 4- to 6-µm-thick sections were cut. The paraffin-embedded sections were rehydrated through xylene, a graded series of alcohol, followed by distilled water.

**CTGF Immunostaining**

The bleb section slides were blocked for 30 minutes at room temperature with blocking buffer (10% horse serum, Tris-buffered saline 2% milk powder, 0.1% saponin, 0.01% HEPES solution). Each processing step was followed by a wash cycle with TBS three times. The blocked slides were incubated at room temperature for 1 hour with goat anti-human CTGF antibody at a dilution of 1:20 (in blocking buffer) followed by a wash cycle. This was followed by incubation at room temperature with the secondary antibody, a biotinylated-rabbit-anti-goat IgG (Vector Laboratories Inc., Burlingame, CA) at a dilution of 1:200 diluted in blocking buffer followed by another wash cycle. Next, the sections were incubated for 45 minutes at room temperature with an ABC-AP kit (Vectastain; Vector Laboratories Inc., Burlingame, CA) and diluted as per the manufacturer’s recommendations in blocking buffer followed by a wash cycle. Finally, the sections were incubated with red substrate (Vector Red; Vector Laboratories Inc.) in 500 mM Tris-0.1% saponin at a pH of 8.5 for 30 minutes. The stained slides were processed for viewing by rehydration through graded dilutions of ethanol and mounted (Permount; Fisher Scientific, Fair Lawn, NJ).

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933227/)
TGFβ2 Immunostaining

After initial tissue processing as described, slides were blocked for 30 minutes at room temperature with 1% horse serum in PBS. After being washed three times with PBS, sections were incubated with goat anti-human TGF-β2 solution (1:60 dilution in 1% horse serum in PBS) overnight at 4°C. Slides were again washed three times with PBS, before incubation with biotinylated horse anti-mouse IgG at room temperature for 1 hour, according to the manufacturer’s instructions (5002 Mouse IgG kit; Vector Laboratories, Inc.). Slides were washed again three times with PBS before being incubated with streptavidin ABC-AP (diluted in 1% horse serum in PBS) for 45 minutes at room temperature. Slides were washed again three times with PBS and incubated with red substrate (SK-5100 Alkaline Phosphatase substrate kit, Vector Red; Vector Laboratories) for 30 minutes at room temperature in the dark, until a reaction developed. Finally, slides were washed twice with PBS, dehydrated through 50%, 75%, 95%, and 100% ethanol and xylene, covered, and mounted (Permount; Fisher Scientific).

Mounted CTGF- and TGF-β2-stained sections were photographed with bright-field illumination at 200× magnification. Photographs were taken at a constant exposure (430 ms) using a Peltier-cooled digital camera (Olympus, Tokyo, Japan).

Addition of Exogenous CTGF to the Site of GFS

In the final experiment, six rabbits underwent GFS in the right eye by the implantation of a 22-gauge intravenous catheter, as described in

![Graph A: CTGF Expression](image)

![Graph B: TGF-β2 Expression](image)

**Figure 2.** (A) CTGF and (B) TGF-β2 expression. The increase in the level of TGF-β2 preceding that of CTGF is supportive of a role for TGF-β2 as an inducer of CTGF.
method two. In this experiment the Tenon’s capsule and scleral tissue at the site of the limbal based flap were exposed for 5 minutes to a 0.4 mg/mL solution of mitomycin C (Novartis, East Hanover, NJ) to delay wound healing and extend the survival of the bleb. Eight days after surgery, when inflammation had subsided and blebs were relatively stable, two of the blebs were injected with 5 μg of recombinant human CTGF in 0.2 mL of physiological saline (BSS, Santen Pharmaceuticals); two blebs with 5 μg of recombinant human TGF-β2 in 0.2 mL of saline solution and two blebs with 0.2 mL of saline solution alone as a control. Bleb survival was assessed for a further 21 days by measuring intraocular pressure, estimating bleb height and by measuring bleb width and depth to calculate the area of the bleb and evaluating the anterior chamber depth subjectively as shallow, normal, or deep. Rabbits were killed on day 21 after injection.

RESULTS

CTGF ELISA

The amount of CTGF expression at the bleb site after the two different types of GFS performed in the sclerostomy (right) and cannulated (left) eyes is shown in Figure 2A. CTGF levels decreased at day 1 and then rapidly peaked by day 5 (approximately a two- to threefold increase, \( P < 0.01 \)) after which levels rapidly decreased, returning to baseline by day 21 after both types of surgery. The amount of TGF-β2 expression at the bleb site in the same rabbit tissues is shown in Figure 2B.

TGF-β2 levels began to rise within 1 day after surgery and also reached a peak by days 5 to 7 after surgery (also approximately a two- to threefold increase, \( P < 0.01 \)), after which they progressively decreased. Baseline levels are reached by day 21 after cannula surgery but not after sclerostomy surgery (\( P < 0.05 \)).

Levels of CTGF measured in tissues from an area 180° away from the filtering bleb demonstrated no induced peak. Likewise, the levels of TGF-β2 measured 180° away from the filtering bleb, showed no induced peak. There was no statistically significant difference in the amount of CTGF expressed 180° from the surgical site in either type of surgery and likewise no significant difference in the amount of TGF-β2 expressed 180° from the surgical site, in either type of surgery (\( P > 0.05 \)). These values have been combined and represented by single lines in Figures 2A and 2B.

CTGF Immunostaining of Bleb Tissues

CTGF and TGF-β2 were localized by immunofluorescence in paraffin-embedded sections of rabbit eyes extracted at days 0 (control), 3, 5, and 7 after surgery (Fig. 3, panels A and B). CTGF and TGF-β2 immunostaining were noted in the conjunctival, corneal, uveal, and scleral tissues. Staining in the bleb tissue was most pronounced at day 5 after surgery for the CTGF and at days 5 and 7 for the TGF-β2. These findings mirror the peak expression determined by ELISA in the first part of the experiment.

Exogenous CTGF Injection

MMC-treated blebs receiving an injection of 0.2 mL saline solution (BSS; Santen Pharmaceuticals) on day 8 after cannula GFS contracted to 50% of their original area by an average of 13 days after injection (Fig. 4A). In contrast, blebs treated with CTGF failed more rapidly, reaching 50% of their original size by an average of 6 days after injection. CTGF-treated blebs were more vascular in appearance for the first 2 to 3 days after injection and failed completely by an average of 16 days after injection, whereas all the saline solution-treated blebs continued to survive at 21 days after injection. Similar MMC-treated blebs injected with TGF-β2 also failed completely by day 16 but decreased to 50% of their original area even earlier, by day 3. Differences in IOP were not statistically significant (Fig. 4B).

DISCUSSION

The scarring that occurs at the wound site after GFS generally leads to subsequent bleb failure. Currently, limiting the formation of scar tissue at the site of GFS is heavily reliant on the use of antimetabolites; primarily mitomycin-C and 5-fluorouracil (5FU).33,34 Although these antimetabolites have been shown to be beneficial in preventing postsurgical scarring and improving glaucoma surgical outcome, they are relatively nonspecific and may be associated with an increased incidence of severe and potentially blinding complications, including hypotony-macularopathy, bleb leaks, bleb infections, and endophthalmitis.32-45

An ideal therapy to prolong bleb survival and improve long-term surgical outcomes would be both safe and more specific. Manipulation of the growth factors that regulate the
scarring the process or the genes that control them offer a potential target.

Recent work by Cordiero et al.\textsuperscript{16,17} using a novel, recombinant human monoclonal antibody to TGF-\textbeta-2 showed improved bleb survival in high-risk patients. Elevated CTGF protein and mRNA levels have been demonstrated in sclerotic skin,\textsuperscript{46} atherosclerotic blood vessels,\textsuperscript{47} specimens of inflammatory bowel disease,\textsuperscript{48} and corneal scar tissue\textsuperscript{49} when compared with normal tissue.

We have shown that CTGF and TGF-\textbeta-2 are both present and induced in rabbit bleb tissues after GFS, reaching peak concentrations by 5 days after surgery. The levels of both CTGF and TGF-\textbeta-2 induced by the sclerostomy procedure were greater than with the cannula procedure. The reason for this is unknown but one possibility is that the sclerostomy procedure is more traumatic to the ocular tissues and therefore provokes a greater inflammatory response than the cannula procedure. The levels of both factors induced at the site of sclerostomy surgery were statistically significant (\(P < 0.01\)) when compared to those induced at the cannula site. Further, the levels of both CTGF and TGF-\textbeta-2 induced by the cannulation surgery were not statistically significantly when compared with levels in nonsurgical tissues.

This is the first study to show CTGF production in a rabbit model of GFS and the first to demonstrate the changes in level of expression over time of TGF-\textbeta-2 in the bleb tissues. The effect is a localized response to tissue injury, with no peak in tissues 180° away from the bleb area, suggesting that only a localized treatment may be necessary to control bleb scarring.

\textbf{FIGURE 4.} (A) Saline solution–, CTGF–, and TGF-\textbeta-2–injected blebs and (B) bleb survival.
Khaw et al.\(^5\) have shown that TGF-\(\beta\) injected into a rabbit bleb induces scarring and early failure. We have demonstrated that the addition of exogenous CTGF also increases the rate of filtering bleb failure in a rabbit model—perhaps with a slower onset but with failure at a similar time point. We interpret these data to support the hypothesis that CTGF and TGF-\(\beta\) play a role in the scarring process, which results in the failure of GFS. It may be most desirable to inhibit this response at the level of the signaling events immediately proximal to the unwanted effects (namely scarring and contracture), thus minimizing any side effects. CTGF and its activation genes may provide another target for limiting the failure of glaucoma filtering surgery.

References


