A SEQUENTIAL, MULTIPLE-TREATMENT, TARGETED APPROACH TO REDUCE WOUND HEALING AND FAILURE OF GLAUCOMA FILTRATION SURGERY IN A RABBIT MODEL (AN AMERICAN OPHTHALMOLOGICAL SOCIETY THESIS)

BY Mark Brian Sherwood FRCS, FRCP, FRCOphth

ABSTRACT

Purpose: The purpose of this study was to evaluate the concept of targeting mediators of the scarring process at multiple points across the course of bleb failure, in order to prolong bleb survival.

Methods: There were three linked parts to the experiment. In the first part, a cannula glaucoma filtration surgery (GFS) was performed on 32 New Zealand White (NZW) rabbits, and bleb survival was assessed for six different regimens plus controls by grading bleb height and width. For the second part of the study, the same GFS surgery was performed on an additional 10 NZW rabbits. Two additional filtering blebs were treated with balanced saline solution (BSS), two received mitomycin-C (MMC) (0.4 mg/mL), and for the remaining six, a sequential regimen was given consisting of 200 mmol/L mannose-6-phosphate (M-6-P) solution at the time of surgery, followed by subconjunctival injections of antibody to connective tissue growth factor at days 2 and 4, and Ilomastat, a broad-spectrum matrix metalloproteinase inhibitor, at days 7, 12, and 20 postoperatively. Bleb survival was again assessed. In the final part of the experiment, blebs treated with either BSS, MMC, or the above sequential multitre- atment regimen were examined histologically at 14 days postoperatively in three additional NZW rabbits.

Results: All six individual therapies selected resulted in some improvement of bleb survival compared to BSS control. Blebs treated with the new sequential, multitreatment protocol survived an average of 29 days (regression slope, \(P < .0001\) compared to control), those receiving BSS an average of 17 days, and those treated with MMC (0.4 mg/mL) an average of 36 days. The sequential, multitreatment regimen was significantly superior to any of the six monotherapies for time to zero analysis (flattening) of the bleb (\(P < .002\)). Histologic examination of the bleb tissues showed a markedly less epithelial thinning, subepithelial collagen thinning, and goblet cell loss in the multitreatment group, when compared with the MMC blebs.

Conclusions: In a rabbit model of GFS, a sequential, targeted, multitreatment approach prolonged bleb survival compared to BSS controls and decreased bleb tissue morphological changes when compared to those treated with MMC. It is not known whether these findings can be reproduced in humans, and further work is needed to determine an optimum regimen and timing of therapeutic delivery.

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INTRODUCTION

Worldwide, it is estimated that 65 million people are affected by glaucoma, which remains a leading cause of blindness.\(^1\)\(^-\)\(^4\) Primary open-angle glaucoma, the most common cause and presentation of the disease, is estimated to have an incidence of 2.4 million new cases per year.\(^6\)

There are many risk factors for glaucoma, including intraocular pressure (IOP), older age, black race,\(^7\)\(^-\)\(^12\) family history, genetic predisposition,\(^13\)\(^-\)\(^17\) and thin central corneal thickness.\(^18\)\(^-\)\(^20\) Considering that at present the only treatable risk factor is IOP, the goal of glaucoma therapy is to lower it to safe levels for the optic nerve.\(^21\)\(^-\)\(^22\) This can be achieved with medical therapy (eye drops or systemic medications), laser surgery, or incisional surgery. Of these options, glaucoma filtering surgery (GFS) has been demonstrated to produce the largest and most sustained decrease in IOP.\(^23\)\(^-\)\(^26\)

There is growing recognition that many patients with glaucoma require low-normal IOPs to prevent progression of visual field loss.\(^23\)\(^27\)\(^-\)\(^33\)

GFS is generally performed when medical therapy fails to adequately control IOP. Excessive subconjunctival scarring following GFS is responsible for failure of the surgery in the majority of cases.\(^34\)\(^-\)\(^42\) There is a huge interest in developing a new drug or treatment modality that would be able to minimize fibrosis and provide better outcome with GFS.

Antimetabolites, predominantly 5-fluorouracil (5-FU) and mitomycin-C (MMC), are commonly used to reduce the formation of scar tissue at the site of GFS.\(^36\)\(^-\)\(^42\)\(^-\)\(^47\) These antimetabolites have been shown to be beneficial in preventing scarring and enhancing the long-term success of GFS, but they are relatively nonspecific and may be associated with an increased incidence of severe and potentially blinding complications.\(^48\)\(^-\)\(^61\) Some of the factors that mediate the bleb-scarring process have recently been identified, including transforming growth factor \(\beta\) \(2\) (TGF-\(\beta2\)),\(^62\)\(^-\)\(^63\) the predominant form in the eye, and its downstream mediator connective tissue growth factor (CTGF).\(^64\)

It is possible to neutralize TGF-\(\beta\) using some agents, including TGF-\(\beta\) antibody CAT-152 (Cambridge Antibody Technology, Cambridge, United Kingdom), specific to the active form of human TGF-\(\beta2\). Another way to neutralize TGF-\(\beta\) is to block gene expression of a growth factor or its receptor. This can be achieved using antisense oligonucleotide, a sequence of DNA complementary to the gene sequence of the growth factor.\(^62\)\(^-\)\(^65\) Antisense oligonucleotide binds to TGF-\(\beta\) mRNA and prevents protein synthesis by inhibiting transcription. A clinical trial using a human monoclonal antibody to TGF-\(\beta2\) reported initial promising results.\(^66\)

From the Department of Ophthalmology, College of Medicine, University of Florida, Gainesville.
CTGF is a secreted peptide that has been implicated in multiple cellular events, including angiogenesis, skeletogenesis, and wound healing. 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. Gene regulation of CTGF is also a target for antifibrotic therapy.

A rat model of GFS was recently used to investigate postoperative changes in gene expression in bleb tissues and confirmed highly significant up-regulation of certain growth factors (TGF-β1, 2, 3 and CTGF), various structural proteins, and matrix metalloproteinase enzymes (MMPs) 2, 3, and 9. Highest levels of MMPs were expressed during the later part of the wound-healing cycle. Highest levels of TGF-β2 and CTGF were noted at day 5, which is consistent with previous enzyme-linked immunosorbent assay work in the rabbit model. MMPs are a family of proteolytic enzymes that are essential in the wound-healing process. In addition to degradation of the extracellular matrix, these enzymes are also believed to be involved in wound contraction. Wound healing following GFS is a complex process involving multiple pathways and mediators. Until now, only broad-spectrum antifibrotic agents or therapies targeting single pathways have been investigated. The purpose of this study was to examine the concept of treating multiple factors at several different time points following GFS, in order to maximally prolong bleb survival while at the same time minimizing the long-term tissue-damaging side effects of the currently used antimetabolites.

METHODS

All animal experiments were conducted in accordance with the ARVO statement governing the use of animals in ophthalmic research and were approved by the University of Florida’s Institutional Animal Care and Use Committee. New Zealand White, male rabbits weighing approximately 2 to 4 lb were used in the study. Surgery was performed on the left eye only of each animal, and the same surgeon performed all surgeries.

ANESTHESIA

All animals were anesthetized using a combination of ketamine (Ketaject; Phoenix Pharmaceuticals, St Joseph, Missouri) (50 mg/kg) and xylazine (Xylaject; Phoenix Pharmaceuticals) (10 mg/kg) administered by intramuscular injection. Additional topical anesthetic in the form of 0.1% proparacaine eye drops (Bausch & Lomb, Tampa, Florida) was also administered.

GLAUCOMA FILTRATION SURGERY

The technique used has been described previously. Briefly, the eyelids were retracted using an eyelid speculum. A partial-thickness, A single application of combined neomycin and dexamethasone ointment was instilled at the end of surgery. Surgery was performed on the left eye only of each animal, and the same surgeon performed all surgeries.

EVALUATION OF POTENTIAL TREATMENTS

In part 1 of the experiment, a number of agents that target previously identified mediators of the scarring process were individually evaluated to determine their potential efficacy. Compounds active against CTGF, TGF-β2, and MMPs were selected.

With the exception of MMC, which was given as a single, 5-minute, intraoperative treatment, the other compounds were injected aseptically into the bleb area at time of surgery and again 5 days postoperatively. Five days was selected as the time point for the second injection based upon the known expression patterns of TGF-β2 and CTGF in rabbit bleb tissues, and in the case of the MMP inhibitor, to provide uniformity across treatment groups. This treatment regimen may not be the ideal protocol for each of these individual agents but was selected in order to provide comparable efficacy data.

The antiscarring compounds were prepared as follows:

1. Control. Physiological saline solution (Santen Pharmaceuticals, Napa, California).
2. Mitomycin-C. A solution of MMC, 0.4 mg/mL, (Novartis, East Hanover, New Jersey) was prepared and applied using a section of sponge (Microsponge; Alcon, Fort Worth, Texas) soaked in the solution.
3. CTGF and TGF-β2 Antisense. Twenty-mer CTGF and TGF-β2 antisense oligodeoxynucleotides with homology to the rabbit sequences were synthesized as previously described. Briefly, human, mouse, and rat mRNA genes were analyzed for unique, nonrepetitive, 20-mer nucleotide sequences with high GC contents that would minimize self-hybridization and provide stability of the oligodeoxynucleotide mRNA complex. After testing their ability to reduce mRNA levels using a cell
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culture screening assay, a CTGF oligodeoxynucleotide sequence and a TGF-β2 oligodeoxynucleotide sequence were selected. The oligonucleotides were suspended in phosphate-buffered saline at a final concentration of 10 µM and filter sterilized through a Millex GP syringe-driven filter unit (Millipore Corp, Bedford, Massachusetts) before injection.

4. **CTGF and TGF-β2 Antibody:** A specific goat anti-human CTGF polyclonal antibody, which recognizes predominantly epitopes on the N-terminal half of the protein, was used at a concentration of 5 µg per 100 µL injection. A specific goat anti-porcine TGF-β2 polyclonal antibody (R&D Systems, Minneapolis, Minnesota) was also used at a concentration of 5 µg per 100 µL injection. Both antibodies were diluted in phosphate-buffered saline containing 0.1% bovine serum albumin and filter sterilized through a Millex GP syringe driven filter unit before injection.

5. **D-Mannose-6-Phosphate (D-6-P).** This product (Sigma, St Louis, Missouri) was reconstituted in NaCl to a concentration of 200 mmol/L and sterilized through a Millex GP syringe-driven filter unit.

6. **Ilomastat (GM6001).** N-[(2R0)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (Chemicon, Temecula, California) was reconstituted from 1 mg/mL (2.5 nmol/L in dimethyl sulfoxide) to 100 µmol/L in NaCl and sterilized through a Millex GP syringe-driven filter unit. To facilitate the postoperative, subconjunctival injection on day 5, animals were anesthetized using a combination of ketamine (50 mg/kg) and xylazine (10 mg/kg) administered by intramuscular injection. Topical anesthetic in the form of 0.1% proparacaine eye drops was also administered. Eyelids were retracted with a speculum, the conjunctivae were tented using a pair of nontoothed Bishop-Harmon forceps, and a 0.1-mL injection was given using a 30-gauge needle attached to a 1-mL syringe.

**EVALUATION OF SEQUENTIAL MULTIPLE TREATMENT**

In part 2 of the study, the left eyes of six rabbits were injected with a sequential protocol of compounds selected on the basis of the results of part 1 of the experiment. This multitreatment protocol consisted of a single dose of 200 mmol/L M-6-P, which was applied to the bleb tissues during surgery, before the anterior chamber was entered, 0.1 mL of CTGF antibody, which was injected subconjunctivally on days 2 and 4 postoperatively, and 0.1 mL of Ilomastat injected on days 7, 12, and 20 postoperatively. As a negative control, two additional randomly selected rabbits were treated at the same five postoperative time points following surgery using a similar volume of balanced saline solution (BSS). As a positive control, two additional randomly selected rabbits were treated at the time of surgery with a solution of 0.4 mg/mL MMC using a section of soaked Microsponge, applied to the exposed bleb area for 5 minutes.

**SURVIVAL ANALYSIS**

All glaucoma filtering blebs were evaluated postoperatively each day until failure. This was accomplished by measuring the length and width of the bleb using a caliper and thus calculating its area as previously described. All bleb areas were recorded as percentages of their maximum size, to eliminate variation resulting from individual differences in initial bleb area following surgery. Additionally, the anterior chamber of each animal was evaluated daily at the slit lamp for evidence of inflammation, hemorrhage, or shallowing; the eye was stained with fluorescein to look for evidence of corneal epithelial toxicity or bleb leak; and conscious IOPs were measured (average of three consecutive readings) using a handheld tonometer (Tonopen; Mentor, Santa Barbara, California).

**STATISTICAL ANALYSIS**

To compare the rate of bleb failure of the MMC and sequential multitreatment groups with the control group, a one-way analysis of variance of the time to bleb failure was conducted. The groups were significantly different ($F = 250.87, df = 8,31, \ P < .0001$). The mean time to bleb failure of the multitreatment group was 29.2 days. This mean was compared to the mean time to bleb failure for each of the other groups. The bleb survival time in the multitreatment group was significantly greater than the single-therapy groups or BSS control, but significantly less than the MMC positive control (Table 1).

**TABLE 1. BLEB SURVIVAL TIME OF THE SEQUENTIAL MULTITREATMENT GROUP COMPARED TO THE SIX INDIVIDUAL SINGLE-THERAPY GROUPS FROM PART 1 OF THE STUDY, AND TO CONTROL GROUPS**

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MEAN DIFFERENCE</th>
<th>SE</th>
<th>T VALUE</th>
<th>P VALUE</th>
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<tr>
<td>Negative control (BSS)</td>
<td>−12.2</td>
<td>0.54</td>
<td>−22.8</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>CTGF antisense</td>
<td>−4.70</td>
<td>0.59</td>
<td>−7.9</td>
<td>&lt;.0001</td>
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<tr>
<td>CTGF antibody</td>
<td>−3.45</td>
<td>0.59</td>
<td>−5.8</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>TGF-β2 antisense</td>
<td>−8.45</td>
<td>0.59</td>
<td>−14.2</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>TGF-β2 antibody</td>
<td>−8.95</td>
<td>0.59</td>
<td>−15.1</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>M-6-P</td>
<td>−9.95</td>
<td>0.59</td>
<td>−16.8</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Ilomastat</td>
<td>−2.20</td>
<td>0.65</td>
<td>−3.4</td>
<td>.0019</td>
</tr>
<tr>
<td>Mitomycin-C</td>
<td>7.30</td>
<td>0.54</td>
<td>13.6</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

BSS = balanced saline solution; M-6-P = mannose-6-phosphate; SE = standard error of the mean.
HISTOLOGY
In the part 3 of the experiment, similar GFS was performed on three additional rabbits to generate tissues for histological evaluation. One rabbit received the sequential multitreatment protocol, one received injections of BSS only (negative control), and one received an intraoperative application of MMC (positive control).

One eye from each of the three treatment groups was harvested at day 14 following surgery. In addition, an eye that had undergone no surgery was also examined histologically. The eyes were perfused in situ with 4% formaldehyde for 3 minutes, before being dissected en bloc, fixed in 4% formaldehyde overnight, and transferred to 70% ethanol. The eyes were then processed for paraffin embedding and 4μ- to 6μ-thick sections made. Sections were stained with standard hematoxylin-eosin for cellularity (including fibroblasts), Gomori’s trichrome for collagen density and architecture, periodic acid–Schiff (PAS) for goblet cell identification, and Verhoeff stain for elastic fiber presence. All evaluations were performed by a qualified, ocular histopathologist, masked to treatment group and with the normal, nonoperated eye used as a control standard.

PHOTOGRAPHY
Mounted, stained sections were photographed using bright field illumination at ×40 magnification. Photographs were taken at a constant exposure (430 ms) using a Peltier-cooled Olympus digital camera.

RESULTS
No bleb leaks or corneal epithelial staining was noted during the postoperative period. One of the animals treated with Ilomastat in part 1 of the study and one of the animals receiving multitreatment in part 2 of the study exhibited tube advancement into the anterior chamber (at postoperative days 16 and 10, respectively). Data for these two animals were censored beyond these time points.

Figure 1 shows the results of treatment using individual agents. In keeping with the author’s previous experience with this rabbit model, the BSS-treated (negative control) blebs survived an average of 17 days and the MMC-treated (positive control) blebs an average of 36 days. The two rabbits that received BSS and the two that received MMC in part 2 of the experiment had a similar time to zero bleb survival as the four in part 1 of the study, and their results have been combined (n = 6 for each). All of the individual experimental therapies, which were given at the time of surgery and at 5 days postoperatively, enhanced bleb survival, with the TGF-β2 antibody and antisense and the M-6-P improving bleb survival a little to approximately 22 days and the CTGF antibody and antisense and Ilomastat improving bleb survival to approximately 26 days.

Figure 2 shows the results of treatment using the sequential multitreatment approach. Multiple-treated blebs survived an average of 29 days, those treated with BSS an average of 17 days, and those treated with MMC an average of 36 days. Table 2 shows the results of the (SAS) statistical analysis, indicating that the slopes of the curves for both MMC and multitreatment groups in Figure 2 are significantly different from the control group (P < .0001). The clinical appearance of these blebs is shown in Figure 3.

Comparison of the bleb survival time (time to zero analysis) between the sequential multitreatment group and each of the six
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Individual therapies in part 1 of the study showed a significantly longer bleb survival for the multitreatment group ($P < .002$) (Table 1).

**FIGURE 2**

Bleb survival for sequential multitreatment (MULT) regimen (D-mannose-6-phosphate at surgery, CTGF antibody injections on days 2 and 4, and Ilomastat on days 7, 12, and 20). Blebs receiving MULT survived an average of 29 days. Those treated with balanced saline solution, the negative control (CONT), survived an average of 17 days, and those treated with mitomycin-C (MMC) survived an average of 36 days.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>INTERCEPT</th>
<th>SLOPE</th>
<th>LOWER CI</th>
<th>UPPER CI</th>
<th>$P$ VALUE FOR DIFFERENT FROM CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSS control</td>
<td>118.7</td>
<td>−6.86</td>
<td>−7.22</td>
<td>−6.5</td>
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</tr>
<tr>
<td>MMC</td>
<td>110.3</td>
<td>−3.25</td>
<td>−3.35</td>
<td>−3.13</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Multi-Rx</td>
<td>104.3</td>
<td>−3.67</td>
<td>−3.84</td>
<td>−3.5</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

CI = confidence interval.
HISTOLOGY

Nonoperated (Normal Rabbit Conjunctiva)

Hematoxylin-eosin staining sections from the nonoperated eye showed a normal, bulbar conjunctiva consisting of columnar epithelium, which was approximately three to five cell layers thick (Figure 4A). PAS staining indicated a relatively high density of epithelial goblet cells, which decreased in density toward the limbus (Figure 4B). Trichrome staining revealed loosely arranged, subepithelial connective tissue consisting of small collagen fibrils, which was more compacted immediately beneath the epithelium (Figure 4C). Verhoeff staining showed the presence of occasional, scattered, fine, elastic fibers in the subepithelial connective tissue (Figure 4D).

BSS-Treated (Negative Control) Eye

Hematoxylin-eosin staining of sections from the negative control showed a thicker, bulbar conjunctiva consisting of a more stratified epithelium. The subepithelial connective tissue contained numerous fibroblasts as well as scattered inflammatory cells, including lymphocytes, macrophages, and occasional neutrophils (Figure 5A). PAS staining indicated the presence of epithelial goblet cells, although fewer than in the unoperated eye (Figure 5B). Trichrome staining revealed significantly more densely packed subepithelial collagen, with less distinction between the immediate subepithelial layer and the rest of the underlying connective tissue (Figure 5C). Verhoeff staining showed an increased density of elastic fibers throughout the connective tissue (which were also thicker), relative to the nonoperated eye (Figure 5D).

FIGURE 3
Progression of clinical bleb failure. Representative photographic examples of clinical blebs for balanced saline solution (BSS), the negative control; mitomycin-C (MMC) (0.4 mg/mL), the positive control; and the sequential multitreatment group are shown at 1, 2, and 3 weeks postoperatively.

FIGURE 4

**FIGURE 5**

**MMC-Treated (Positive Control) Eye**
Hematoxylin-eosin staining of sections from the positive control eye showed a bulbar conjunctiva consisting of stratified epithelium, which was sharply demarcated from the adjacent tissues and markedly reduced in thickness, consisting of only a single layer over the major portion of the bleb area. Occasional inflammatory cells (lymphocytes and plasma cells), as well as rare fibroblasts, were noted in the subepithelial connective tissue (Figure 6A). PAS staining indicated an absence of epithelial goblet cells over the bleb area (Figure 6B). Trichrome staining revealed a significant reduction in the density of subepithelial collagen, with no distinction between the immediate subepithelial layer and the rest of the underlying connective tissue but an increase in density at the bleb margins (Figure 6C). Verhoeff staining showed an increased density of elastic fibers immediately beneath the conjunctival epithelium (Figure 6D).

**FIGURE 6**

**Sequential, Multiple Therapy Eye**
Hematoxylin-eosin staining of sections from the multitreated eye showed a bulbar conjunctiva consisting of stratified to columnar epithelium, which was similar in structure and thickness to that of the nonoperated eye. Numerous fibroblasts, as well as occasional inflammatory cells (predominantly lymphocytes and macrophages), were noted in the subepithelial connective tissue (Figure 7A). PAS staining indicated an increased density of goblet cells relative to the positive control (MMC) (Figure 7B). Trichrome staining revealed an even, moderate density of subepithelial collagen, with no distinction between the immediate subepithelial layer and the
rest of the underlying connective tissue (Figure 7B). Verhoeff staining showed occasional elastic fibers scattered throughout the connective tissue with a density similar to the nonoperated eye (Figure 7D).

**FIGURE 7**

Histology of sequential multitreatment bleb site rabbit conjunctival and Tenon’s capsule tissues. A, Hematoxylin-eosin stain for cellularity, including fibroblast. B, Periodic acid–Schiff stain for goblet cell identification. C, Gamoris trichrome for collagen density and architecture. D, Verhoeff stain for elastic fiber presence

**DISCUSSION**

It is hypothesized that ocular wound healing, similar to healing in other tissues, occurs in several overlapping phases.90-92 These include hemostasis, inflammation, fibroblast migration, matrix production, and, finally, remodeling and contracture.

After hemostasis, an inflammatory phase occurs, characterized by the influx of neutrophils and monocytes, followed by lymphocytes and macrophages and the release of inflammatory mediators and growth factors.93-97 Angiogenesis and fibroblast migration then occur,93-95 followed by extracellular matrix deposition, fibroblast-mediated wound contracture, and tissue remodeling.116,117 Scarring can be reduced and the long-term success of GFS enhanced by treatment with 5-FU118-122 and MMC.123-126 Primarily because of the long-term side effects of the currently used antimetabolite agents, there has been interest in identifying other approaches to preventing the formation of scar tissue following GFS.

In the initial part of this study, agents were selected for pilot investigation that could potentially target mediators of the various phases noted above. Antibody to TGF-β2, antisense to TGF-β2, antibody to CTGF, antisense to CTGF, M-6-P, and a broad-spectrum MMP inhibitor (Ilomastat) were individually tested. All six compounds prolonged bleb survival in the rabbit model compared to BSS control, although none were as effective as MMC (0.4 mg/mL). Overall, antibody and antisense to CTGF and the MMP inhibitor Ilomastat appeared to be slightly more effective than the other three agents and resulted in a similar length of bleb survival of 25 to 26 days.

Many fields in medicine, most notably the use of chemotherapy in cancer, have found that therapies targeting more than one area are required to decrease toxicity and maximize efficacy of therapy.127-130 In ocular wound healing, topical corticosteroids131-134 have been used to decrease early postoperative inflammation for many years, and either single antimetabolite agents135-139 or, more recently, antibody to TGF-β2140-142 has been used to further modify the wound-healing response. A rat model of GFS was recently developed, and using microarray analysis, changes in the expression of numerous genes postoperatively were demonstrated. Some, such as the growth factors TGF-β2 and CTGF, reached maximal levels at day 5 following surgery and then fell, whereas others, such as the MMPs, were stable early on and then rose and remained high in the later stages of bleb scarring.143

Following surgery (or wounding), it is theorized that TGF-β is released in two phases, initially predominantly from degranulating platelets and later from attracted activated inflammatory cells, mainly monocytes and macrophages.144 TGF-β is released from platelets in its latent form, and activation of latent TGF-β occurs by cleavage of part of the molecule.137 The latent TGF-β requires binding to the large M-6-P/insulin-like growth factor-II (IGF-II) receptor for this activation to occur.145-147 TGF-β activation in cultured endothelial and smooth muscle cells can be disrupted by the addition of M-6-P or by antibodies directed against the M-6-P/IGF-II receptor in a dose-dependent manner.148

The production of TGF-β by inflammatory cells is thought to contribute to the postoperative peak noted around days 5 to 7 following GFS in both rabbit and rat models.57,64 Neutralizing antibodies have the ability to bind to and inhibit the action of target antigens. Cambridge Antibody Technology (CAT, Cambridge, United Kingdom) has produced a human monoclonal antibody that is specific to human TGF-β2 (CAT-152)148-151. After modified glaucoma surgery in 48 rabbits, seven postoperative subconjunctival injections of CAT-152 (1 mg/mL) significantly improved surgical outcome and reduced subconjunctival scarring compared with 5-FU (50 mg/mL) or no treatment. Median bleb survival was 23.5 days in the CAT-152 group, 20 days with 5-FU, and 16 days for the control treatment group.141 A phase I/II two-center study of this antibody in glaucoma filtration surgery has shown no significant side effects or inflammatory reaction and possibly some benefit compared with placebo in a masked randomized clinical study.152
A human phase I/II clinical trial examining CAT-152 in primary glaucoma filtration surgery showed improved efficacy compared to no antifibrosis treatment, but a recent report of the 1-year result of the first European phase II/III study (CAT-152-0102) with 344 patients did not support this.

CTGF has been shown to be a downstream mediator of TGF-β. CTGF acts as a mitogen in fibroblast cell cultures and up-regulates components of the extracellular matrix, such as collagen, integrin, and fibronectin. In a rabbit model of GFS, injected exogenous CTGF was shown to increase the rate of bleb failure. Additionally, in a rat model, CTGF showed the highest percentage of up-regulation following GFS. The addition of CTGF antisense oligonucleotides (ASOs) or CTGF-neutralizing antibody blocked more than 85% of the increased collagen synthesis induced by TGF-β1 in human corneal fibroblast cultures and rat corneas after photorefractive keratectomy. However, neither ASOs nor neutralizing antibody totally suppressed the effect of TGF-β.

Reorganization of fibrillar collagen and other matrix proteins, together with contraction, occurs in the later stages of wound healing. This function is mediated by a family of structurally related proteolytic enzymes, the MMPs. Their function is to degrade extracellular matrix, and more than 20 different MMPs have been identified. Most MMPs are not expressed in normal tissues but are transcribed in response to stimuli such as inflammatory cytokines and growth factors by multiple cell types, including macrophages, fibroblasts, and neutrophils. Using gene array analysis in the rat model, a more than fivefold increase in the expression of MMPs 2, 3, and 9 following GFS was observed, and these were maximally expressed in the later phase of wound healing, between days 5 and 12 postoperatively. Scott and coworkers showed that collagen contraction can be inhibited using MMP inhibitors.

Iломастат is a broad-spectrum MMP inhibitor that has shown activity in a number of biologic systems, including animal wound-healing models. Wong and coworkers have shown an improved duration of bleb survival in the rabbit model of GFS using Ilomastat (GM6001) alone, but in these experiments 10 subconjunctival injections of Ilomastat were given. As noted by the investigators, for many patients, this intensity of postoperative care may be difficult to achieve in clinical practice.

In a subsequent experiment using Ilomastat, the same investigators showed that Ilomastat successfully prolongs bleb survival, giving 15 injections during the study period. Blebs started to fail, on average, 12 days after the last subconjunctival injection, with total failure occurring at 46.2 days.

In the first part of the study reported here, Ilomastat was delivered only twice, on postoperative days 0 and 5, and although it was better than BSS control, it was not as effective as the multitherapy regimen at this reduced dosage. The sequential regimen in this study was based on the hypothesis that the healing process for glaucoma blebs involves multiple pathways and processes. Hemostasis was achieved at surgery, and corticosteroids were given at the end of surgery to decrease inflammation during the initial phase of wound healing. Based on the data above, a sequential protocol was devised and designed to target existing latent TGF-β in the initial phase following surgery by using M-6-P. Just before its peak expression, CTGF was then targeted using CTGF antibody, as this was shown to be slightly more effective for prolonging bleb function than the TGF-β2 antibody or antisense. Finally, Ilomastat was used to reduce MMP activity in the later part of wound healing, during the peak of the tissue remodeling phase. As shown in Figure 2, this sequential multiple therapy protocol enhanced bleb survival significantly beyond that of any of the individual therapies to over 29 days, using a total of five postoperative subconjunctival injections. The MMC (0.4 mg/mL) blebs survived longer, to a mean of 36 days, but of key importance, they clinically appeared more avascular initially and histologically showed significant thinning and alteration of the conjunctival tissue morphology.

CONCLUSIONS

An ideal therapy to prolong bleb survival and improve long-term surgical outcomes following GFS should be both safe and specific. The conventional approach in the search for alternatives to the nonspecific antimetabolites currently in use has focused on the identification and treatment of individual mediators of the bleb scarring process. The process of bleb failure is complex, involving multiple families of mediators, including inflammatory mediators, growth factors, structural proteins, and MMPs.

This study has demonstrated that the sequential use of multiple agents to target different modulators of wound healing prolongs bleb survival in this aggressively scarring animal model of GFS and histologically appears to result in less thinning and other changes in the bleb tissues. To the author’s knowledge, this is the first study investigating a multiple sequential treatment approach to enhancing bleb survival in an animal model of GFS. Future studies to determine the most effective combination of agents and to optimize the timing and method of their delivery may improve efficacy further while maintaining the histological integrity of the tissues.

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REFERENCES


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