Prolonged Antiscarring Effects of Ilomastat and MMC after Experimental Glaucoma Filtration Surgery

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PURPOSE. To determine the long-term antiscarring effect of ilomastat treatment after experimental glaucoma filtration surgery (GFS).

METHODS. In a randomized, prospective, masked-observer study, 21 New Zealand White rabbits underwent modified GFS. The animals were allocated to receive either intraoperative mitomycin-C (MMC) at a concentration of 0.2 mg/mL or postoperative subconjunctival injections of either ilomastat or phosphate-buffered saline (PBS). Fifteen injections were given to each animal during the study period. The animals were killed 60 days after surgery. Bleb morphology and intraocular pressures were recorded. Tissue sections were immunohistochemically stained for signs of scarring.

RESULTS. Ilomastat significantly improved surgical outcome compared with vehicle (P < 0.0163) and length of bleb survival was similar to the MMC group. The mean day of failure was 46.2 (range, 42–60) with ilomastat, 51.3 (range, 49–60) with MMC, and 16 (range, 15–21) with vehicle. IOP maintenance with ilomastat was similar to that in the MMC group. Histologically, minimal scar tissue was seen with MMC and ilomastat. MMC-treated tissue demonstrated subconjunctival hypocellularity associated with peripheral fibrosis. Ilomastat resulted in normal-appearing conjunctival morphology.

CONCLUSIONS. Ilomastat successfully prolongs bleb survival. MMP inhibition may provide an additional, potentially safer method of controlling intraocular pressure, thus preventing failure of glaucoma surgery, and may also act as a potential adjuvant treatment when MMC alone is inadequate. (Invest Ophthalmol Vis Sci. 2005;46:2018–2022) DOI:10.1167/iovs.04-0820

Successful glaucoma filtration surgery is dependent on postoperative healing. Wound contraction and scarring are the prime causes of blockage of aqueous flow at the sclerostomy. Excessive subconjunctival scar contracture, which can lead to inadequate intraocular pressure control, is the principal cause of failed surgical failure.1,2

After surgical trauma, a tightly controlled sequence of cellular events occurs. The Tenon’s fibroblast is the effector cell responsible for most aspects of healing. Activated fibroblasts proliferate and migrate to the wound site and remodel the extracellular matrix (ECM). These processes are mediated by matrix metalloproteinases (MMPs), a family of proteolytic enzymes.3,4 These proteins have been grouped according their preferred substrate, sequence homology, extracellular release as proteozymes, and inhibition by their natural inhibitors, tissue inhibitors of MMPs (TIMPs).5

Cell migration is facilitated by the release of MMPs that proteolytically break down the surrounding ECM, thus creating a path for cell movement. Furthermore, collagen and ECM production by fibroblasts is remodeled with continuous synthesis and breakdown of the matrix. The amount of ECM turnover at the wound depends on the extent of MMP activity.6,7 MMP-1 and TIMP-1 have been immunohistochemically identified in healing subconjunctival tissues, whereas these proteins were absent in the conjunctival epithelium of normal tissue specimens.8

Cell-mediated collagen gel contraction in vitro has been shown to be inhibited by ilomastat, a broad-spectrum MMP inhibitor, without evidence of cellular toxicity.9 Furthermore, the use of ilomastat has been demonstrated to improve surgical outcome significantly, by reducing the amount of scar tissue produced after experimental glaucoma filtration surgery.10 More recently, we have reported a reduction in collagen synthesis by Tenon’s fibroblasts in vitro with ilomastat treatment. The inhibitory effect was reversed on removal of the MMP inhibitor (Wong TTL, unpublished data, 2003–4; Wong et al. IOVS 2003;45:ARVO E-Abstract 987).

In this study, we investigated whether ilomastat could effectively modulate the continuing postoperative wound-healing process in an established model of aggressive scarring.

MATERIALS AND METHODS

A prospective, randomized, controlled, masked-observer study was performed in an established rabbit model of glaucoma filtration surgery previously devised by our group.11 Twenty-one female New Zealand White rabbits (2–2.4 kg, 12–14 weeks old; Charles River, Ltd., Margate, UK) were acclimatized for 5 days before the start of the experiments. The animals underwent modified glaucoma filtration surgery as previously described10 and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Treatment Regimen

Filtration surgery was performed on the left eye only. The animals were randomly allocated to one of three treatment regimens: intraoperative MMC (current gold standard; AAH Pharmaceuticals Ltd., Coventry, UK) or postoperative subconjunctival injection of 0.1 mL of 100 μM ilomastat (Calbiochem-Novabiochem, Nottingham, UK) or phosphate buffered saline (PBS) as the vehicle control (n = 7). Previous cell culture experiments using the same concentration of ilomastat showed no cellular toxicity while demonstrating maximum inhibition to cell migration and matrix degradation.9 Furthermore, the volume of 0.1 mL was chosen because this is the volume of 5-fluorouracil normally administered subconjunctivally to patients in the clinical setting. We wanted to replicate this clinical setting as much as possible in our study.

In the MMC-treated group, a sponge measuring 4 × 1 mm (Weck, Research Triangle Park, NC) was cut and soaked in 0.2 mg/mL MMC. The sponge pieces were placed between the conjunctiva and sclera.
over the determined filtration site for 3 minutes. The area was then thoroughly irrigated with 30 mL of physiologic saline (Balanced Salt Solution; Alcon Laboratories, Inc., Fort Worth, TX). Subconjunctival injections into the bleb were administered daily for the first 9 days, followed by twice weekly injections for 2 weeks, then weekly injections for a further 2 weeks. The rationale for this dose regimen was to achieve local availability of the inhibitor at the critical time of postoperative wound repair, as observed in other studies, and to maintain its inhibitory effect at the later stages of remodeling.12,13 A 29.5-gauge needle was placed at the same site in each eye, at the nasal margin of the superior rectus muscle, so that a visible bleb was formed on the supranasal quadrant of each eye. The injections were administered at the same site each time by an individual masked to the drug being given. No other drugs were given at the same time. The animals received their injections after the clinical assessment. Animals from all treatment groups were examined at specified intervals for 60 days after surgery. Clinically, bleb appearance and intraocular pressure measurements were recorded. The PBS and ilomastat groups received the injections after the clinical assessment. The animals were killed on day 60. Both eyes were enucleated and histologically analyzed. The nonsurgical right eyes served as normal control animals were killed on day 60. Both eyes were enucleated and histologically analyzed. The nonsurgical right eyes served as normal control.

Clinical Assessment
Baseline clinical examinations were performed that included measuring intraocular pressure and recording the appearance of the superior bulbar conjunctiva. Intraocular pressures were recorded in both eyes with a handheld tonometer (Tonopen; Mentor, Norwell, MA). This was performed after topical instillation of 0.5% proxymetacaine HCl.

Bleb survival was used as the primary efficacy endpoint. A bleb was judged to have failed if a flat, vascularized, scarred bleb, associated with a deep anterior chamber, was observed on examination with a handheld slit lamp.

Postoperative Clinical Evaluation
Bleb appearance was graded and documented as previously described.10 The presence of lower-than-baseline intraocular pressure is an important indicator of effective filtration and bleb function. Failure of intraocular pressure was defined as a return to baseline or an increase in intraocular pressure from the baseline measurement for the entire duration of the 60-day study.

Histologic Evaluation
After death on day 60, both eyes were enucleated, fixed in 4% paraformaldehyde (pH 7.4) and embedded in paraffin before 5-μm sections were cut. The sections were stained for the presence of inflammation (hematoxylin and eosin), scar tissue formation (picrosirius red, for collagen), and α-smooth muscle actin by immunohistochemistry (myofibroblast phenotype identification). All the surgical eyes were compared with the contralateral, nonsurgical eyes. Semiquantitative grading of these parameters by two observers masked to the treatment received by each animal was performed, by using a modified grading system originally described by Shah et al.,14 who devised a histologic grading scale that ranged from −4 to +4 according to staining intensity. We simplified this system to provide a grading scale of 0 to +4.10,15

Statistical Evaluation
Statistical analysis was performed to determine the differences between the three treatment groups. Survival analysis was performed for bleb failure and intraocular pressure using the Kaplan-Meier log rank test. The bleb area was analyzed using a generalized linear model (GLM; SPSS 8.0; SPSS, Chicago, IL) repeated-measures procedure to compare the two groups. Histologic analysis was performed with a graphic display of mean values for the two groups and 95% confidence intervals.

RESULTS
Clinical Findings
The bleb failed at a mean of 16 days (range, 15–21) with vehicle, 46.2 days (range, 42–60) with ilomastat, and 51.3 days (range, 49–60) with MMC (Table 1). A significant difference in bleb survival was observed between groups (log rank; $P = \text{significant}$).

**Table 1.** Time at Which the Blebs Failed after Surgery in Each Treatment Group

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<tr>
<th>Treatment</th>
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The Mean day of failure was 16 for vehicle, 51.3 for MMC, and 46.2 for Ilomastat. $n = 7$ in all groups.

**Figure 1.** (A) Kaplan-Meier survival curve showing bleb survival. On day 21, all the vehicle-treated blebs had failed in seven (100%) of seven animals, compared with no failures with ilomastat or MMC treatment. Four (57%) of seven MMC-treated blebs and two (29%) of seven ilomastat-treated blebs survived to the end of the study. Thin dashed line, vehicle; solid line, MMC; bold dashed line, ilomastat (log rank $P = 0.0163$). (B) Kaplan-Meier survival curve showing IOP maintenance. Lower-than-baseline IOP was maintained consistently longer with MMC than with vehicle or ilomastat (log rank $P = 0.033$).

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All seven blebs (100%) of the vehicle-treated animals failed to survive beyond 21 days, compared with no failures with ilomastat or MMC treatment. With MMC, four (57%) of seven blebs survived to day 60. Ilomastat treatment resulted in survival of two (29%) of seven blebs at the end of the study.

Lower-than-baseline intraocular pressure was maintained consistently longer after surgery in the MMC group than in the vehicle or ilomastat groups (Fig. 1B). However, intraocular pressure survival was significantly prolonged with ilomastat compared with vehicle ($P < 0.033$). The mean day when the intraocular pressure returned to or was consistently higher than baseline measurement was day 23 (range, 14–28 days) with vehicle, day 46.7 (range, 35–56 days) with ilomastat, and day 49 with MMC, suggesting a decrease in aqueous outflow from the anterior chamber into the subconjunctival space, which would be seen clinically as a flat bleb, although intraocular pressure does not always correlate with bleb appearance.

MMC treatment was associated with typical avascular blebs surrounded by local conjunctival hyperemia. Treatment with ilomastat, however, resulted in a diffusely elevated bleb and normal-appearing conjunctiva (Fig. 2).

### Histopathologic Features

MMC treatment resulted in a thickened conjunctival epithelium with cells sloughing from the surface. In addition, extensive disruption of the epithelium was noted. In contrast, ilomastat and vehicle treatments resulted in an intact, healthy-looking conjunctival epithelium (Fig. 3).

Total scar formation, judged by staining with picrosirius red, was significantly reduced by ilomastat ($P = 0.01$). MMC treatment revealed loose connective tissue in the subconjunctival space. However, a dense layer of scar tissue was observed at the periphery. Ilomastat reduced the population of cells expressing $\alpha$-smooth muscle actin, indicating fewer myofibroblasts. MMC treatment revealed multiple $\alpha$-smooth muscle actin-staining cells at the edge of the bleb surrounded by scar tissue (Fig. 4).

### DISCUSSION

The use of antimetabolites to modulate postoperative scarring after trabeculectomy has been one of the most important developments in the surgical management of glaucoma. Results of landmark clinical trials have suggested that an intraocular pressure in the low teens is associated with less progression of glaucoma.16,17 These low intraocular pressure levels are most easily achieved with the use of strong antiscarring agents such as MMC. Antimetabolite treatment can result in the production of thin, leaky blebs.18,19 Therefore, alternative antiscarring agents that can achieve an efficacy similar to that of MMC but without extensive tissue damage are needed.

The results of this study, despite the small number of animals in each treatment group, demonstrate that postoperative...
application of ilomastat may modify the contraction and remodeling of subconjunctival tissue. More important, the application of ilomastat in this animal model apparently did not lead to the conjunctival damage normally associated with MMC use. Cell culture studies conducted in our laboratory showed no change in the proliferative capacity of fibroblasts exposed to high concentrations of ilomastat (100 μM) compared with serum-treated cells.9

We have previously demonstrated that MMC treatment induces an increase in apoptosis gene expression by Tenon’s fibroblasts, which results in a reduction in viable fibroblasts in culture.20,21 Furthermore, it has been reported that the application of high-dose MMC (0.4 mg/mL) in this model results in a greater incidence of complications associated with antimetabolite use, which include endophthalmitis, conjunctival epithelial defects, corneal neovascularization, and bleb leaks.22 Therefore, we used 0.2 mg/mL MMC, which is the most common concentration used clinically at our center.

 Conjunctival epithelial damage is a recognized complication associated with the clinical use of MMC and is characterized by nonhealing leaking blebs and the risk of infection and hypotony.23 MMC treatment resulted in abnormal, avascular, hypoxic subconjunctival tissue, which was associated with a border of scar tissue containing multiple myofibroblasts. Our histologic findings in the rabbit are similar to those reported by Nuys et al.24 in human specimens. They reported on seven MMC-treated blebs. All the blebs displayed extensive cell loss in the subconjunctival and conjunctival layers at the site of the scleral flap. Histologically, they found that MMC treatment resulted in well-formed bleb cavities with peripheral fibrosis and inflammation. The features described are typical of the ring of scar tissue, for which we have coined the term “ring of steel,” seen clinically after the use of MMC. Our group has demonstrated the importance of the size of the treatment area on final bleb morphology.11 A large treatment area has been shown to produce more diffuse, noncylindrical blebs and subsequently has significantly lowered sight-threatening bleb-related complications.25 However, although adopting a wider area of application of MMC dramatically reduces the bleb complication rate, its antiproliferative and apoptotic effects still result in cell death and tissue damage.

Although tissue appears to be a static entity, continuous baseline degradation and synthesis are occurring. Fibroblasts are central to these crucial biological events that preserve healthy tissue. Certainly, irreversible local tissue damage with a loss in tissue integrity has been reported after short exposure to MMC.22,26 Thus, the preservation of viable fibroblasts in the subconjunctival tissue seems to be important for the maintenance of normal tissue morphology and strength. Witte et al.27 have reported that the inhibition of MMP activity with ilomastat during the acute wound-healing phase after dorsal skin incisions in the rat enhances wound strength. Their study provides evidence that controlling MMP activity in fibroblasts may play an important role in the development of a structurally healthy wound.

Ilomastat-treated blebs started to fail, on average, 12 days after the last subconjunctival injection, with total failure occurring at 46.2 days. This important observation confirms that the effect of the inhibitor is reversible and supports the findings from previous in vitro studies.9,28 The administration of multiple injections in a clinical setting does pose some difficulties. The optimum dosage and delivery of the drug have yet to be determined. We are currently developing a slow-release formulation of ilomastat. However, no overt toxicity with frequent injections of ilomastat has been reported. More tantalizing, it raises the possibility of postoperative injections that could be given only after sufficient healing has occurred, to keep the intraocular pressure at a level in the low teens. This treatment strategy would minimize the chance of hypotony and provide with an efficacy close to MMC without the tissue damage and complications.

In this study, MMP inhibition with a prolonged regimen of ilomastat injections continued to maintain bleb function in an aggressive model of scarring. Ilomastat provided better tissue preservation and retention of epithelial cellularity than did MMC. We propose that MMP inhibition may provide an additional, potentially safer method of controlling intraocular pressure and preventing the failure of glaucoma surgery. Further studies are needed to determine the potential use of ilomastat in humans, either alone or in combination with other agents.

References


