Matrix Metalloproteinase Inhibition Modulates Postoperative Scarring after Experimental Glaucoma Filtration Surgery

Tina T. L. Wong, Anna L. Mead, and Peng T. Khaw

Purpose. To determine whether postoperative application of a broad-spectrum matrix metalloproteinase (MMP) inhibitor, GM6001 (ilomastat), reduces scarring after glaucoma filtration surgery.

Methods. In a randomized, prospective, masked-observer study, 40 New Zealand White rabbits underwent modified glaucoma filtration surgery. The animals were randomly allocated to receive postoperative subconjunctival injections of either phosphate-buffered saline (PBS) or 100 μM ilomastat for 10 days. The animals were killed on days 7, 14, 21, and 30. Clinical characteristics, which included bleb morphology and intraocular pressure, were recorded. Tissue sections were immunohistochemically stained for α smooth muscle actin (αSMA) and extracellular matrix components in the two groups.

Results. Surgical outcome was significantly prolonged in the ilomastat-treated group compared with the vehicle-treated group (P < 0.001). At day 30, all the blebs had survived except two in the ilomastat-treated group, whereas no blebs survived to day 30 with vehicle treatment (n = 11). The intraocular pressure remained significantly lower throughout the course of the experiment in the ilomastat group compared with the vehicle group (P < 0.0017). Histologically, less scar tissue was observed at the sclerostomy site with inhibition of MMP, compared with vehicle treatment.

Conclusions. The data presented suggest that the healing response after surgery can be modulated by inhibiting the effects of MMPs. Inhibition of MMP significantly improved surgical outcome by reducing the amount of scar tissue produced. By targeting the actions of these proteolytic enzymes, a more controlled and physiological method of modulating scarring may be achieved. (Invest Ophthalmol Vis Sci. 2003;44:1097–1103) DOI:10.1167/iovs.02-0366

The primary determinant of long-term success after glaucoma filtration surgery is the wound-healing response. The cellular events during wound healing play prominent roles in the outcome of filtration surgery. Since the introduction of the antiglaucoma agents 5-fluorouracil and mitomycin-C (MMC), the outcome of glaucoma filtration surgery has been considerably improved. Despite this, their use has been associated with potentially blinding complications, such as hypotony and endophthalmitis through the development of thin-walled, cystic blebs. This suggests that inhibition of cell proliferation is insufficient and does not prevent the cells from performing other physiological functions. Therefore agents with more specific physiological actions with less cytotoxicity are needed.

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes that are essential in many physiological processes, such as embryogenesis, development, and wound healing. Dysregulated MMP activity has long been implicated in diseases associated with uncontrolled proteolysis of connective tissue matrices, such as arthritis, tumorogenesis, tissue ulceration, and atherosclerosis. The use of synthetic MMP inhibitors (MMPIs), such as ilomastat, in ocular disorders has been shown to reduce tissue damage. Recent work in our laboratory has demonstrated that these agents have a significant inhibitory effect on a variety of fibroblast-mediated functions, such as collagen contraction, cell migration, and collagen production, at concentrations not associated with cellular toxicity. In view of this, we investigated the effect of ilomastat on scarring after experimental filtration surgery.

We performed a prospective, randomized, masked-observer study. The purposes of this study were to investigate the effects of subconjunctival ilomastat in an animal model of aggressive subconjunctival scarring and to assess the safety and tolerance of the inhibitor in vivo by subconjunctival administration in the rabbit.

Methods

An established rabbit model of glaucoma filtration surgery, previously devised by our group, was used to investigate the effects of an MMPI on the wound-healing events after surgery. All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Surgical Procedure

The animals were anesthetized with a combination of ketamine (Ketaset; Fort Dodge Animal Health, Southampton, UK) and medetomidine HCl (Domitor; Pfizer Animal Health, Sandwich, UK). Briefly, a partial thickness 8-0 silk cornea traction suture (Ethicon, Edinburgh, Scotland, UK) was placed superiorly, and the eye pulled down. A fornix-based conjunctival flap was raised, after which a blunt dissection of the subconjunctival space was performed of approximately 5 mm along the limbus and 8 mm posteriorly. A microvitrector (MVR) blade was used to make a partial-thickness scleral incision 3 to 4 mm behind the limbus, and a scleral tunnel to the corneal stroma was fashioned. A 22-gauge, 25-mm intravenous cannula (Ventron 2; Beckton Dickinson, Oxford, UK) was passed through a scleral tunnel anteriorly until the cannula needle was visible in the clear cornea. Entry into the anterior chamber was made with a cannular needle, which was then withdrawn as the cannula was advanced to the midpupillary area. The cannula was trimmed and beveled at its scleral end so that it protruded 1 mm from the insertion point, and a 100-4 nylon suture was placed to fix the tube to the scleral surface. The conjunctival incision was closed with two interrupted sutures and a central, mattress-type 100 nylon suture attached to a needle (B/V 100-4; Ethicon) to give a watertight closure. One drop each of guttae

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chloramphenicol and Betnesol-N (Glaxo Wellcome, Uxbridge, UK) ointment was instilled at the end of surgery. The surgery was executed by the same masked individual. Only the left eye was operated on, and the surgical procedure was performed at the same site, superiorly and temporally, in each animal.

Animals

Forty female New Zealand White rabbits (2–2.4 kg, 12–14 weeks old; Charles River UK Ltd., Margate, UK) were acclimatized for 5 days before the experiments started.

Treatment Regimen

Twenty-two rabbits underwent filtration surgery to the left eye only with random allocation to one of two treatment groups: subconjunctival injection of 0.1 mL of either 100 μM ilomastat or PBS as the control, immediately after surgery and on each of days 1 through 9 after surgery. The rationale for this dose regimen was to attempt to achieve local availability of the inhibitor at the critical time of postoperative wound repair and remodeling, as observed in other studies.17–18

The animals were examined before receiving the injections at set intervals for 30 days after surgery. Clinically, bleb appearance, size, height, and surrounding vascularity were recorded in addition to the intraocular pressure (IOP), anterior chamber depth, and epithelial toxicity.

The animals were killed on day 30. Both eyes were enucleated and histologically analyzed. The nonsurgical right eyes served as the normal control for histologic comparison. This first group of 22 animals thus served to characterize the histology at day 30. To complete the histologic comparison, the experiment was repeated with a second group of 18 rabbits (9 per treatment group), which were killed for histology analysis on days 7 (n = 6), 14 (n = 6), and 21 (n = 6).

Preparation of Drugs and Administration

Ilomastat was dissolved in 100% DMSO (Sigma, Poole, UK) at 10 mM and then diluted to 100 μM with PBS. Previous cell culture experiments at this concentration of DMSO showed no toxicity (Occleston NL, Khaw PT, unpublished data, 1996). For the vehicle control, the same volume of DMSO was diluted with PBS (vol/vol). The rabbits in the current study was to achieve the maximum inhibitory effect without cellular toxicity. After topical anesthesia of 0.5% proxymetacaine HCL eye drops, ilomastat or vehicle control was administered subconjunctivally. A 29.5-gauge needle was placed on 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 a masked individual. No other agents were given simultaneously.

Baseline Examinations

At the start of the study, the baseline recordings included measurement of IOP and the appearance of the superior bulbar conjunctiva. IOPs were recorded in both eyes with a handheld tonometer (Tono-pen; Mentor, Norwell, MA), after topical instillation of 0.5% proxymetacaine HCL.

All the assessments were made by an observer who was masked to the treatment received by each rabbit. Postoperative observations were performed daily from day 1 to day 4 and then at regular periods until death at day 30. Bleb survival was used as the primary efficacy endpoint. A bleb had not survived, and therefore had 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

Observations of the bleb’s appearance, size, and vascularity and the anterior chamber’s depth were graded and documented as described. Width and length were measured by using calipers to delineate the margins of the bleb, and height was graded semiquantitatively by slit lamp examination (0, flat; 1, shallow/forme<1 mm; 2, elevated <2 mm; 3, high >2 mm). These measurements in millimeters were used as a guide to the grading of the blebs. Bleb vascularity was graded (0, avascular; 1, normal vascularity; 2, hyperemic; 3, very hyperemic) and its locality noted (top, nasal, temporal). Anterior chamber inflammation was assessed by slit lamp examination (0, no inflammation; 1, cells present; 2, fibrin formation; 3, hypopyon present), and anterior chamber depth was recorded as deep, shallow, or flat. Failure of IOP was defined as an indefinite return to baseline or an increase over baseline IOP.

Only the animals in the initial experiment killed on day 30 (n = 22) were used to analyze survival. The animals in the subsequent experi-

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932918/ on 06/24/2017)
TABLE 1. Comparison between Treatments of Failure to Maintain IOP

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Failure to maintain IOP was defined as an indefinite return to or an increase from the baseline reading. This table compares the time when failure occurred in the two treatment groups in the initial 30-day study (n = 22). Levels of failed IOPs are indicated in bold.

Hyd fuchsin (for oxytalan), Gomori’s trichrome and picrosiris red (for collagen), and α smooth muscle actin (αSMA), using a primary antibody to αSMA (monoclonal mouse anti-human smooth muscle actin antibody, Clone 1A4; Dako, High Wycombe, UK) and a biotinylated secondary antibody (rabbit anti-mouse; Dako) and detected with a streptavidin avidin-biotin complex–horseradish peroxidase kit (Dako). All the surgically treated eyes were compared with the control, nonsurgical eyes. Semi-quantitative grading of the histology by two masked observers was performed by using a modified version of the grading system originally described by Shah et al. In brief, histologic sections representing the same anatomic area from the 40 eyes were assessed by two masked observers. The unknown sections were compared with a standard template, which comprised five histologic slides. Each slide in the template represented a point on the grading system. The grades were as follows: 0, normal conjunctival tissue; 1, 1% to 25% more than normal; 2, 26% to 50% more than normal; 3, 51% to 75% more than normal; and 4, 76% to 100% more than normal. A template for each histologic parameter was used for the grading. Mean scores were calculated for each treatment group per time point. Reproducibility of the masked readers was assessed before grading the slides. No significant between-reader grading differences were found (data not shown). The nonsurgical eyes were also stained and used as a reference for the appearance of normal conjunctival tissue.

Statistical Evaluation

Statistical analysis was performed to determine the differences between the two treatment groups. Survival analysis was performed for bleb failure and IOP, by using the Kaplan-Meier log rank test. Area, height, and conjunctival vascularity of the bleb were analyzed with a generalized linear model (GLM; SPSS, ver. 8.0; SPSS, Chicago, IL) repeated-measures procedure to compare the two groups. Histologic analysis was performed by using graphic display of mean results in the two groups with 95% confidence intervals.

RESULTS

Clinical Findings

Bleb Morphology. A bleb was deemed not to have survived, and therefore the surgery to have failed, if a flat, vascularized, and scarred bleb associated with a deep anterior chamber was observed by slit lamp examination. At day 28, all 11
(100%) of the vehicle-treated group showed failure compared with 3 (27%) of 11 in the ilomastat-treated group. The mean day of failure in the vehicle group was 18.5 (range, 14–24) and 26.5 (range 24–28) in the ilomastat-treated group.

Comparison of bleb survival between the two groups demonstrated a significant difference when analyzed by Kaplan-Meier curve (log rank, \( P < 0.001 \); Fig. 1A). It is of particular note that throughout the period of the study, most of the ilomastat-treated blebs remained elevated and diffuse, with only mild conjunctival injection.

Failure to maintain IOP was defined as an indefinite return to or an increase from baseline IOP. Figure 1B illustrates maintenance of IOP between the two groups, with the ilomastat-treated group demonstrating significant IOP maintenance \( (P = 0.0017) \). Only 1 animal in the ilomastat group demonstrated a persistent increase in IOP above baseline IOP from day 24. Table 1 illustrates the time after surgery when failure to maintain IOP occurred in the two treatment groups. The mean day when the IOP returned to or was higher than baseline IOP indefinitely was 15.9 (range, 7–24) in the vehicle-treated group and 26 (range, 24–28) in the ilomastat-treated group.

A shallow anterior chamber was an indicator of effective aqueous flow from the anterior chamber into the subconjunctival space. The anterior chamber’s depth remained significantly shallower with ilomastat treatment (Fig. 2A; \( P = 0.002 \)) compared with vehicle treatment.

Mean bleb area in relation to time after surgery revealed a significant difference between the two groups (Fig. 2B; \( P < 0.001 \), between-subjects analysis). In the first week after surgery, the bleb’s size was not markedly different between the

**Figure 3.** Treatment with ilomastat improved surgical outcome with no apparent signs of toxicity and was well tolerated. The conjunctival blebs in the ilomastat-treated group remained diffusely elevated, even 30 days after surgery (A). This contrasted with the flat, scarred, vascularized blebs that were observed with vehicle treatment (B). *Arrows:* edges of the blebs.

**Figure 4.** Picrosirius red staining revealed that the deposition of total scar tissue and densely packed collagen fibers in the subconjunctival space, stained green/yellow, in the vehicle-treated eyes were significantly greater in the vehicle-treated (A) than in the ilomastat-treated eyes (B) and the no-treatment control (right eye; C) at day 30. Normal scleral collagen (S) was also stained. The deposition of elastic and collagen fibers, as demonstrated by aldehyde fuchsin and Gomoris trichrome staining, respectively, was more abundant in the vehicle-treated eyes (D, G) than in the ilomastat-treated group (E, H) and the untreated control (F, I). Significantly fewer cells were found to express \( \alpha \)SMA in the ilomastat-treated animals (K) than in the vehicle-treated (J) and untreated control (L). *Thin arrows:* area of increased expression of \( \alpha \)SMA in (J), which contrasts with no expression in the same representative areas in (K) and (L). *Thick arrows:* normal \( \alpha \)SMA expression by the ciliary muscle cells. b, subconjunctival space; S, sclera; and t, tube. Magnification, \( \times 10 \).
two groups. However from day 10, the area was noted to reduce in size in the vehicle compared with the ilomastat-treated group. Figure 5 illustrates clinically the bleb’s morphology between the two treated groups on day 30. A scarred, flat bleb had developed by day 21 in the vehicle-treated group, whereas with ilomastat treatment, the bleb remained diffusely elevated with minimal conjunctival injection at day 30.

Throughout the study period, local tissue reaction to treatment was assessed by conjunctival vascularity. No significant differences were found between the two groups (superior vascularity, $P = 0.324$; nasal, $P = 0.654$; temporal, $P = 0.693$). Furthermore, there were no cases of epithelial defects recorded in either treatment group.

Histopathological Features. At day 30, significant reduction in scar tissue was noted at the surgical site ($P < 0.01$, Figs. 4A–C). The presence of newly laid extracellular matrix was present to a greater degree in the vehicle-treated group ($P < 0.05$, Figs. 4D–I). Total cellularity remained increased in vehicle-treated compared with ilomastat-treated eyes ($P < 0.01$). In addition, ilomastat treatment resulted in a reduction in expression of $\alpha$SMA by cells, suggesting the presence of fewer myofibroblasts ($P < 0.01$, Figs. 4J–L). In all cases, the ilomastat-treated eyes showed very similar conjunctival morphology to the contralateral, control eyes. The conjunctival histologic changes observed on days 7, 14, 21, and 30 are summarized in Figure 5.

**DISCUSSION**

MMC is highly effective in preventing postoperative scarring in high-risk patients undergoing conventional glaucoma filtration surgery. It works, however, by causing widespread cell death and apoptosis, which in turn may lead to sight-threatening complications. Hence, there is a need to develop a more physiological agent that can act more specifically on the wound healing process.

It is well established that MMPs are essential for extracellular matrix degradation and wound healing throughout the body. MMPs play a central role in collagen contraction and matrix reorganization. Moreover, they have been found in normal ocular tissues and their overexpression is associated with excessive scarring in the eye. Our group and others have demonstrated that raised levels of MMP are associated with more aggressive scarring in the eye, such as occurs in proliferative vitreoretinopathy.

Ilomastat is a synthetic peptidyl hydroxamic inhibitor of MMPs that has been shown to reduce cell migration and matrix contraction without evidence of cellular toxicity in an RPE-populated model of tissue contraction. The use of another MMP inhibitor, prinomastat (AG3340) has also demonstrated a significant reduction in scarring and fibrosis in an experimental model of proliferative vitreoretinopathy.

In this study, inhibition of MMP was highly effective in reducing total scar tissue formation in our animal model. The presence of less subconjunctival scarring resulted in prolonged bleb survival. Furthermore, the MMPI was safe and well tolerated. It is important to mention that the maintenance of bleb survival using this model is usually only achieved with high-dose MMC. Moreover, we noted that ilomastat did not give rise to extensive cell death, which can sometimes occur with MMC. Thus, the mechanism by which ilomastat reduces postoperative scarring appears to be associated with less cell death. Our cell culture data showing that inhibition of MMP reduces collagen production by fibroblasts may be important in relation to the finding of reduced scar formation.

We found that the bleb’s area remained consistently greater in the ilomastat group than in the vehicle group from day 10. It appears that subconjunctival injections of ilomastat in the period immediately after surgery diminished the rate of postoperative healing, which is particularly aggressive in the rabbit. Histologic data support this hypothesis. Ilomastat treatment resulted in the deposition of less scar tissue, and cellularity was reduced compared with vehicle-treated eyes. MMPs are known to facilitate cell migration. Cells secrete MMPs to degrade the surrounding matrix components, and this enables cells to migrate through the extracellular matrix after injury. If proteolytically...
sis is inhibited, cell migration may be reduced and lead to a less-pronounced increase in the cellularity normally seen in the postoperative period.37

There were fewer myofibroblasts detected in the subconjunctival tissue with ilomastat treatment. The myofibroblast phenotype, which is characterized by the expression of αSMA, is present in some normal tissues and more commonly in scar tissue.50,59 Myofibroblasts are implicated in the development of fibrocontractive disorders and scar contraction after surgery.40,41

In an animal model of filtration surgery, it has been demonstrated that obstruction of the sclerostomy site from excessive matrix deposition and contraction was initially due to the fibroblasts migrating from the subconjunctival connective tissue. However, in the later phase of wound healing, the fibroblasts were histologically observed to be replaced by myofibroblasts.18 Our results suggest that inhibition of MMP can reduce the myofibroblast population at the sclerostomy site essentially by reducing the number of migrating fibroblasts.

The composition of the aqueous may also have an impact on the degree and nature of all the stages of subconjunctival healing. After surgery, the wound site is bathed in circulating aqueous humor, which contains elevated levels of cytokines and growth factors. MMPs have been identified in normal aqueous humor, and elevated levels of active MMP-2, -3, and -9 have been detected during inflammatory states in the anterior chamber.42 The cellular origin of MMPs in the aqueous is uncertain, but they are likely to be derived from stimulated inflammatory cells and surrounding fibroblasts of resident tissues in the anterior segment. MMPs have been reported to activate transforming growth factor-β,43,44 which could further enhance the wound-healing response.

Our study is preliminary and is limited by the following considerations. First, the high frequency of subconjunctival injections administered to the animals is a drawback. We acknowledge that this would be impractical in the clinical setting and that a better delivery system is needed. Second, the optimal dosage and concentration of ilomastat in this model have yet to be established. Thus, to ensure that the maximum therapeutic effect of the MMPi was achieved, the dosage regimen described herein was used. Currently, we are conducting a similar study to address concerns about dosage. Third, no direct comparisons were made with the effects of MMC, the current gold standard. Again, to investigate this we are currently conducting a study to compare the surgical outcome between ilomastat and MMC, using the model described in this study. Finally we inserted a cannula into the anterior chamber in preference to the full-thickness procedure, because the former method is less operator-dependent and allows greater consistency in postoperative assessments.

In conclusion, our results indicate that inhibition of MMP can effectively reduce subconjunctival scarring after experimental glaucoma filtration surgery. Ilomastat has been shown to be safe and well tolerated in this model. Further work is needed to determine whether the inhibition of MMP activity can lead to the development of an alternative, more physiologically relevant inhibitor of postoperative scarring.

References


