Otago Glaucoma Surgery Outcome Study: The Pattern of Expression of MMPs and TIMPs in Bleb Capsules Surrounding Molteno Implants

Peter McCluskey,[1,2] *Anthony Molteno,*[3] *Denis Wakefield,*[2] *and Nick Di Girolamo*[2]

Purpose. To determine whether MMPs and TIMPs are present in the bleb wall of Molteno implants.

Methods. An observational case series consisting of ocular specimens from 10 human eyes obtained postmortem from patients who had undergone placement of Molteno implants for glaucoma. Immunohistochemistry was performed on the specimens to determine the distribution of MMP1, -2, and -3 and TIMP1, -2, and -3 in each bleb. The immunohistochemical staining was correlated with the histologic features observed in hematoxylin and eosin (H&E)–stained sections of the bleb wall.

Results. The specimens demonstrated extensive cytoplasmic staining for MMP1, -2, and -3 in fibroblasts within the bleb wall and in degenerating collagen near inner bleb wall fibroblasts undergoing apoptosis. In addition, there was staining for the presence of TIMP2 but not -1 or -3.

Conclusions. MMPs are present in the bleb walls of Molteno implants. TIMP2 is expressed in most bleb capsules. The observations from this study support the hypothesis that bleb capsules undergo a cycle of collagen breakdown and renewal throughout the life of the bleb as members of the MMP family were localized in the bleb wall. (*Invest Ophthalmol Vis Sci.* 2009;50:2161–2164) DOI:10.1167/iovs.08-2063

Glaucoma surgery has been performed for many years and has evolved greatly over the past 50 years. In patients with refractory glaucoma and high-risk glaucoma, a common surgical option is to use a glaucoma drainage device such as a Molteno drainage implant to improve the likelihood of a successful outcome after glaucoma surgery.[1] Through the Otago Glaucoma Surgery Outcome Study, it has been possible to acquire postmortem ocular specimens that have had Molteno drainage implants inserted and have been followed up clinically over a period of many years.[2]

This study has resulted in several important observations regarding the histologic features of glaucoma filtration blebs and has led to the development of a hypothesis to explain these observations.[3] In summary, the various histologic and immunohistochemical studies of filtration blebs have demonstrated that there are two histologic zones within a filtration bleb—an outer fibroproliferative zone characterized by collagen synthesis, numerous fibroblasts, tissue macrophages and capillaries, and an inner fibrodegenerative zone characterized by cells undergoing apoptosis surrounded by a network of denatured collagen.

Our hypothesis to explain these observations is that, after surgery, a healing response characterized by granulation tissue and inflammation results in the formation of a well-vascularized, fibroblast-rich, thick, collagenous bleb wall. Aqueous accumulating in the bleb cavity generates a resistance to aqueous inflow into the bleb, which leads to elevation of intra-bleb pressure. Once the pressure in the bleb is high enough, there is a disturbance of capillary blood flow in the inner zone of the developing bleb wall that results in local tissue hypoxia, which then triggers apoptosis of the inner fibroblasts and breakdown of the surrounding collagen. There appears to be a cycle of collagen synthesis and breakdown in bleb walls with formation of new collagen from fibroblasts in the outer wall and breakdown of the collagen and apoptosis of the resident fibroblasts once they become the inner layers of the bleb.[2] A balance is struck between the opposing forces of local tissue hypoxia related to the aqueous, and the healing response in the outer layers of the bleb, which leads to the development of a well-functioning, thin-walled filtration bleb.[3] The present study has added weight to this hypothesis by exploring the expression of MMPs and TIMPs in the wall of these blebs.

Matrix metalloproteinases (MMPs) are a family of more than 20 cloned and characterized endopeptidases that are counterregulated by four naturally occurring tissue inhibitors of MMPs (TIMPs). MMPs are recognized as the predominant mediators of collagen synthesis and breakdown both physiologically and pathologically.[4] The expression of MMPs and TIMPs is tightly regulated physiologically, and TIMPs are typically expressed at levels comparable to those of MMPs, as they are a critical regulator of MMP activity.[4] Although MMPs are traditionally renowned for their ability to model and remodel the extracellular matrix under physiological and pathologic conditions, recent studies have highlighted their ability to indirectly modulate an inflammatory response by processing cytokines, chemokines and their receptors.[5] TIMP proteins have the specific ability to bind to and suppress the enzymatic activity displayed by MMPs. Paradoxically however TIMP-2 is a member of a cell-membrane–associated trimolecular complex which is involved in the activation of MMP-2 with documented growth-promoting activity.[6,7] Additional evidence suggests that TIMP-2 may be involved in apoptotic mechanisms[8]

Materials and Methods

Pathologic Material

Ten postmortem eyes with capsules and overlying connective tissue that had been drained by Molteno implants for a period of 2 months up

From the [1]Department of Ophthalmology, Liverpool Hospital, Sydney, Australia; the [2]Laboratory of Ocular Immunology, School of Medical Sciences, University of New South Wales, Sydney, Australia; and the [3]Department of Ophthalmology, University of Otago, Dunedin, New Zealand.

Supported by research grants from the Ophthalmic Research Institute of Australia and the Healthcare Otago Charitable Trust.

Submitted for publication March 21, 2008; revised November 26 and December 9, 2008; accepted March 11, 2009.

Disclosure: **P. McCluskey**, None; **A. Molteno**, None; **D. Wakefield**, None; **N. Di Girolamo**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Peter McCluskey, Department of Ophthalmology, Liverpool Hospital, Locked Bag 7103, Liverpool BC NSW 1871, Australia; peter.mccluskey2@sswahs.nsw.gov.au.

TABLE 1. Clinical Features of the Bleb Specimens from Patients with Glaucoma

Patient	Glaucoma Diagnosis	Age at Operation (y)/Sex	Final IOP	Ocular Medications at Final Follow-up	Age of Bleb
1	Neovascular	32/M	6	Timolol Betamethasone	2 months*
2	Ghost cell	70/M	13	—	3.7 years
3	PXE	68/M	18	—	7.5 years
4	POAG	93/F	13		12.5 years
5	PXE	69/M	14	—	12.6 years
6	Trauma	63/M	6	Acetazolamide	13.4 years
7	PXE	81/F	7	—	15.8 years
8	POAG	70/F	16	—	19.2 years
9	PXE	65/F	12	Propine	20.0 years
10	Trauma	35/M	17	—	22.9 years

* This patient was taking an oral non-steroid anti-inflammatory drug and colchicine. No other patient was taking any systemic anti-inflammatory medication

to 22.9 years were examined after histologic and immunohistochemical staining. The clinical details are summarized in Table 1.

Tissue Fixation

On enucleation, 1 to 4 hours after death, all eyes were injected with formol saline using a 30-gauge needle inserted across the limbus to distend the bleb capsules, after which they were placed in 10% neutral buffered formalin (37% formaldehyde in phosphate buffered saline) for 3 hours, microwaved for 20 minutes at 50°C, then placed in 70% alcohol to harden further. The lateral half of the bleb capsule and adjacent tissue was excised to allow removal of the episcleral plate of the implant before standard paraffin processing. The excised half of the bleb capsule was embedded separately and oriented to allow for the cutting of serial sections that were initially tangential to the surface of the bleb capsule but subsequently became less oblique as the plane of section extended to the margin of the capsule where they were almost perpendicular to the wall of the bleb. The portion of capsule remaining on the eye was oriented to allow the cutting of sections perpendicular to the capsule. After the portions were embedded in paraffin, 5-μm serial sections were cut and mounted on glass slides.

Tissue Specimens

Ten formalin-fixed, paraffin-embedded, specimens from the medial half of the bleb capsule were obtained from the archival tissue collection at the Department of Ophthalmology, University of Otago. Informed consent had been obtained and experimental protocols approved by the University of Otago Human Research Ethics Committee and the University of NSW Human Research Ethics Committee. Experiments were performed in accordance with the tenets of the World Medical Association's Declaration of Helsinki.

Immunohistochemical Analysis

Tissue sections were cut (4 μm) and processed for immunohistochemical assessment as previously described.[9–11] In brief, sections were deparaffinized in xylene, immersed through graded ethanol, hydrated in H_2O, and equilibrated in 0.05 M Tris-buffered saline (TBS; pH 7.6). Endogenous peroxidase activity was quenched by immersing slides in 0.3% (final) H_2O_2 in 100% methanol for 5 minutes. Sections were then washed with TBS before incubating with 20% goat serum in 2% bovine serum albumin (BSA) made in TBS. Sections were incubated overnight at 4°C in a moist chamber with preoptimized dilutions (~100 ng/mL final) of commercially available mouse monoclonal antibodies directed against human MMPs and TIMPs (Table 2). Control reactions consisted of sections incubated with an appropriate isotype control antibody (either IgG_1 or IgG_{2a}; Dako Cytomation) or in the absence of a primary antibody. Sections were extensively washed in TBS before the addition of a biotinylated goat anti-mouse secondary antibody (Table 2) for 30 minutes, The sections were washed before the addition of HRP-conjugated streptavidin (Dako Cytomation) and were incubated for 1 hour before the addition of 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich, St. Louis, MO), counterstained with hematoxylin, and mounted (Ultra-Mount; Laboratory Vision Corp, Fremont, CA). Immunoreactivity for each antigen was either positive (present) or negative (absent) and staining intensity was scored as +/−, no significant staining; +, mild staining, few scattered positive cells; ++, moderate staining, several positively stained cells; and +++, intense staining, many positively stained cells. The results were summarized in Table 3. We have successfully used a similar semiquantitative analysis in other studies.[11,12] Direct staining comparison for each antigen was possible as diseased tissue was assessed in the one experimental run. Heat, pressure, or enzyme-assisted antigen retrieval was not necessary. Images were

TABLE 2. Antibodies Used for Immunohistochemistry

Antibody	Source	Catalog No.	Clone #	Dilution
Primary antibody				
MMP-1	Calbiochem	IM35L	41-1E5	1:600
MMP-2	Calbiochem	IM33	42-5D11	1:100
MMP-3	Calbiochem	IM36L	55-2A4	1:100
TIMP-1	Calbiochem	IM41L	147-6D11	1:60
TIMP-2	Chemicon	MAB3310	67-4H11	1:200
TIMP-3	Calbiochem	IM43L	136-13H4	1:200
Mouse IgG_1	Dako	X0931		1:100
Mouse IgG_{2a}	Dako	X0943		1:600
Secondary antibody				
Goat α-mouse^{biotin}	Dako	E0433		1:200

TIMP antibodies were raised in mouse and directed against human proteins.

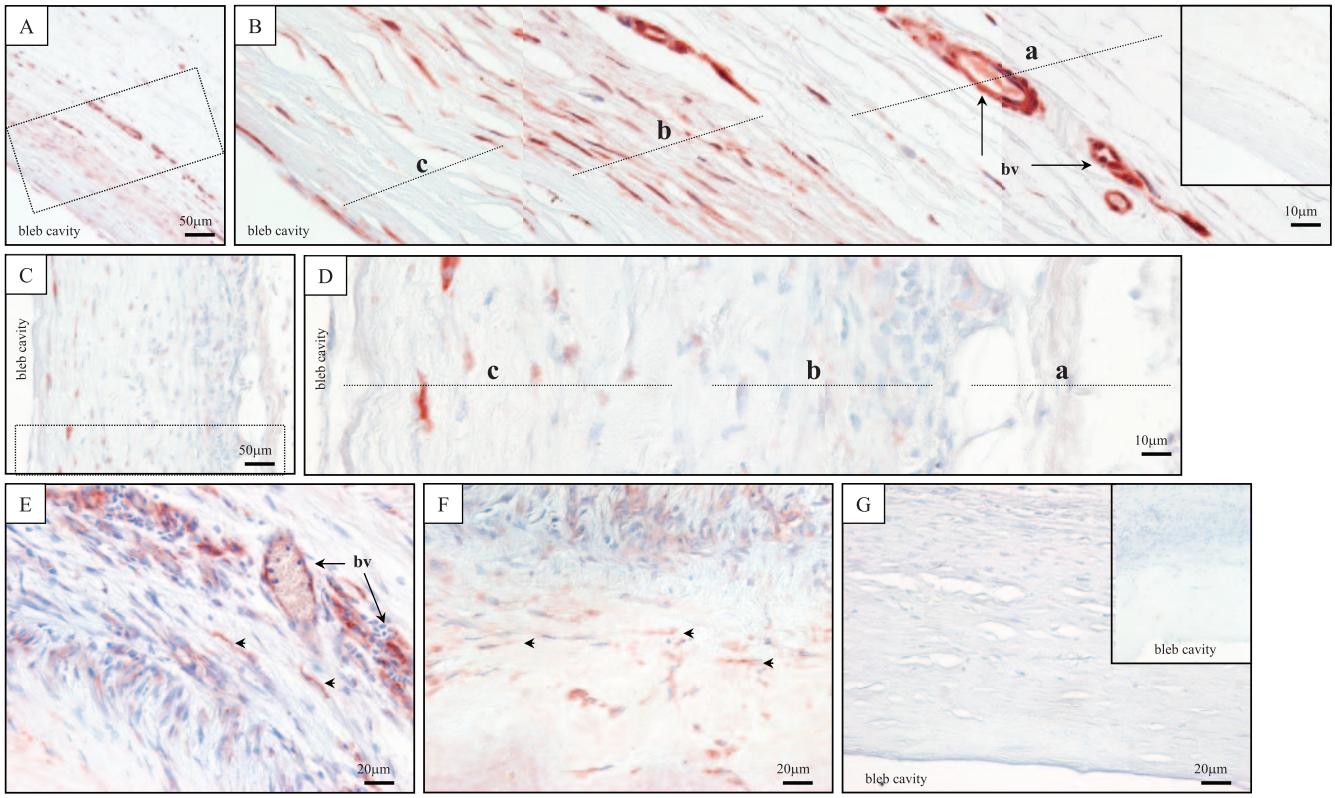

FIGURE 1. MMP and TIMP expression in bleb capsules. Molteno bleb capsules were immunohistochemically assessed after staining for MMP-1 (**A**, **B**), TIMP-2 (**C**, **D**), TIMP-1 (**B**, *inset*), MMP-2 (**E**), MMP-3 (**F**), and TIMP-3 (**G**). The regions encompassed by the *rectangle* in (**A**) and (**B**) are magnified in (**B**) and (**D**), respectively. Immunoreactivity was identified by *red* staining, and hematoxylin established the nuclear counterstain. Sections incubated with an isotype control antibody (IgG2a) showed no reactivity (**G**, *inset*). *Red* cell–associated staining indicated the specific immunoreactivity of the respective antigens, and hematoxylin counterstain distinguished the cell nuclei (*blue*). (**B**, **E**, *arrows*) Blood vessels (bv); (**E**, **F**, *arrowheads*) immunoreactivity in fibroblast-like cells. (**B**) and (**D**) are composed multiple contiguous images. Original magnification: (**A**; **B**, *inset*; **C**; **G**, *inset*) ×200; (**B**, **D**) ×1000 oil immersion; (**E**–**G**) ×400.

taken with a digital camera mounted on a microscope (BX51 DP70; Olympus, Tokyo, Japan). The original images were processed with image-analysis software (Photoshop 7; Adobe Systems, San Jose, CA).

RESULTS

In this study, we wanted to establish the spatial distribution of several MMPs and their counterregulatory molecules the TIMPs, as we hypothesize that an imbalance between these two families of proteins may be a mechanism involved in developing the characteristic histologic features of Molteno bleb capsules. Therefore, our histologic study focused predominantly on the connective tissue layer that comprises the bleb capsule and the cells within the wall. Previous studies have documented the distinct extracellular matrix reaction and the cellular component of bleb capsules.[3] The bleb capsule consists of three major layers, as detailed in Figures 1A and 1B. There is an outermost layer (a) of Tenon's connective tissue and congested blood vessels, a middle fibroproliferative layer (b), and an inner fibrodegenerative layer (c).[3]

Sections through extracted bleb capsules demonstrated intense immunoreactivity for MMP-1 (Figs. 1A, 1B). Figures 1C and 1D demonstrated TIMP-2 immunoreactivity in the bleb wall. In 4 of 10 specimens TIMP-2 expression was intense, and in a further two specimens was expressed at low levels, whereas TIMPs-1 and -3 were rarely represented (Table 3). The immunoreactivity of MMP-2 (Fig. 1E), MMP-3 (Fig. 1F), and TIMP-1 and -3 (Fig. 1B, inset; 1G) is less intense. Staining was scored from an average of five high-powered fields in the bleb wall opposite the Molteno plate and in fibroblast-like cells. Controls included omitting the primary antibody (data not shown) or adding an appropriate isotype control antibody (Fig. 1G, inset). In both cases no immunoreactivity was noted. Pterygium tissue was used as a positive control[10] (data not shown).

DISCUSSION

Some evidence has been provided by a rat model of failing glaucoma blebs that MMPs and TIMPs are upregulated at the

TABLE 3. Immunohistochemical Staining Results of Bleb Specimens for MMPs and TIMPS

Patient	MMP-1	MMP-2	MMP-3	TIMP-1	TIMP-2	TIMP-3
1	−	+	−	−	−	−
2	+++	++	++	++	−	−
3	+++	++	+++	−	++	−
4	+++	+	+	−	−	−
5	+++	+++	+++	+/−	+++	+
6	+++	++	++	+/−	+++	−
7	++	+++	+++	−	+/−	−
8	−	+/−	+	−	+++	−
9	++	−	−	−	−	−
10	++	++	+	−	+/−	−

−, no staining; +/−, no significant staining; +, mild staining, few scattered positive cells; ++, moderate staining, several positively stained cells; +++, intense staining, many positively stained cells.

mRNA level in the short term.[13] The results of the present study clearly show the involvement of MMPs and TIMPs in the collagen turnover characteristic of Molteno bleb walls. There is abundant expression of MMP-1, -2, and -3 in the inner zone of the bleb wall and in the cytoplasm and cells in the bleb wall. Figures 1A and 1B detail the inner and outer layers of the bleb wall and the expression of MMP1. In addition, TIMP-2 was expressed in the inner region of the bleb walls, but TIMP-1 and -3 were expressed only minimally.

In the Molteno bleb walls in this study, there is abundant expression of MMP-1, -2, and -3 and barely detectable expression of TIMP-1 and -3. This pattern of expression of MMPs and TIMPs has been observed in the sclera from patients with scleritis and in other pathologic processes characterized by collagen breakdown.[14–16] This pattern of MMP and TIMP expression may represent a dysregulation of the balance between MMP and TIMP expression and is thought to be a critical pathway mediating collagen breakdown. Based on the observations in this study, it is tempting to speculate that this same dysregulation occurs in Molteno filtration blebs and is an important mechanism that maintains functioning of the bleb after Molteno surgery. Specimens in this study were obtained from patients many years after insertion of a Molteno glaucoma drainage device and a similar pattern of MMP and TIMP expression was demonstrated in all specimens irrespective of the time point sampled.

The expression pattern of TIMP-2 is different to TIMP-1 and -3. TIMP-2 is part of the trimolecular complex that is involved in activating MMP-2. It is also recognized to be involved in the initiation of apoptosis in cells.[8] TIMP-2 expression was variable in the specimens in this study, abundant in some specimens and present in the majority of specimens. We hypothesize that the presence of TIMP-2 is related not only to its role in activating the MMP-2 but may also be involved in the triggering of apoptosis in the inner zone of the blebs.

It is likely that there is a critical and complex interaction between the aqueous and the bleb wall. Aqueous contains numerous potential regulatory factors, such as TGF-β, which is a known regulator of MMPs and a potent proapoptotic factor and is therefore likely to promote the degenerative changes seen in the inner zone of the bleb wall. In addition, there are significant differences in the protein content of aqueous secreted at different pressure levels and time points after glaucoma drainage surgery.[17] Different protein profiles of aqueous may contain MMP activators and/or inhibitors and greatly alter the likelihood of bleb survival. As well, aqueous contains low concentrations of oxygen that may further increase local tissue hypoxia and thus promote apoptosis and collagen breakdown.[18]

This study provides evidence to support our hypothesis that the maintenance of Molteno bleb wall function involves collagen breakdown mediated by MMPs. The lack of expression of TIMP-1 and -3 and the expression pattern of TIMP-2 provide further support for the notion that there may be a relative overexpression of MMPs compared with TIMPs and that TIMP-2 may be involved in apoptosis generation in bleb walls.

Acknowledgments

The authors thank the families of the patients who kindly donated the eyes.

References

1. Sung VCT, Barton K. Management of inflammatory glaucomas. *Curr Opin Ophthalmol.* 2004;15:136–140.
2. Molteno AC, Fucik M, Dempster AG, Bevin TH. Otago glaucoma surgery outcome study: factors controlling capsule fibrosis around Molteno implants with histopathological correlation. *Ophthalmology.* 2003;110:2198–2206.
3. Molteno AC, Suter AJ, Fenwick M, Bevin TH, Dempster AG. Otago glaucoma surgery outcome study: cytology and immunohistochemical staining of bleb capsules around Molteno implants. *Invest Ophthalmol Vis Sci.* 2006;47:1975–1981.
4. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res.* 2006;69:562–573.
5. Opdenakker G, Van den Steen PE, Van Damme J. Gelatinase B: a tuner and amplifier of immune functions. *Trends Immunol.* 2001; 22:571–579.
6. Elliot S, Catanuto P, Stetler-Stevenson W, Cousins SW. Retinal pigment epithelium protection from oxidant-mediated loss of MMP-2 activation requires both MMP-14 and TIMP-2. *Invest Ophthalmol Vis Sci.* 2006;47:1696–1702.
7. Hayakawa T, Yamashita K, Ohuchi E, Shinagawa A. Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J Cell Sci.* 1994;107:2373–2379.
8. Lim MS, Guedez L, Stetler-Stevenson WG, Stetler-Stevenson M. Tissue inhibiter of matrix metalloproteinase-2 induces apoptosis in human T lymphocytes. *Ann NY Acad Sci.* 1999;878:522–523.
9. Di Girolamo N, McCluskey PJ, Lloyd A, Coroneo M, Wakefield D. Expression of MMPs and TIMPs in human pterygia and cultured pterygium epithelial cells. *Invest Ophthalmol Vis Sci.* 2000;41: 671–679.
10. Di Girolamo N, Wakefield D, Coroneo MT. Differential expression of matrix metalloproteinases and their inhibitors at the advancing pterygium head. *Invest Ophthalmol Vis Sci.* 2000;41:4142–4149.
11. Di Girolamo N, Coroneo MT, Wakefield D. Active matrilysin/MMP-7 in human pterygia: potential role in angiogenesis. *Invest Ophthalmol Vis Sci.* 2001;42:1963–1968.
12. Hettiaratchi A, Hawkins NJ, Ward RL, Hunt JE, Wakefield D, Di Girolamo N. The collagenase (MMP-1) gene promoter polymorphism −1607/2G is associated with favorable prognosis in patients with colorectal cancer. *Br J Cancer.* 2007;96:783–792.
13. Esson DW, Popp MP, Liu L, Schultz GS, Sherwood MB. Microarray analysis of the failure of filtering blebs in a rat model of glaucoma filtering surgery. *Invest Ophthalmol Vis Sci.* 2004;45:4450–4462.
14. DiGirolamo N, Lloyd A, McCluskey PJ, Filipic M, Wakefield D. Increased expression of matrix metalloproteinases in vivo in scleritis tissue and in vitro in cultured human scleral fibroblasts. *Am J Pathol.* 1997;150:653–666.
15. Wu W, Billinghurst RC, Pidoux I, et al. Sites of collagenase cleavage and denaturation of type II collagen in aging and osteoarthritic articular cartilage and their relationship to the distribution of matrix metalloproteinase 1 and matrix metalloproteinase 13. *Arthritis Rheum.* 2002;46:2087–2094.
16. Garcia-Touchard A, Henry TD, Sangiorgi G. Extracellular proteases in atherosclerosis and restenosis. *Arterioscler Thromb Vasc Biol.* 2005;25:1119–1127.
17. Forrester JV, Dick AD, McMenamin P, Lee WR. The aqueous. In: *The Eye*: *Basic Sciences in Clinical Practice.* London: WB Saunders; 1996:P169.
18. Duke-Elder S, Gloster J. The physiology of the eye. In: Duke-Elder S, ed. *System of Ophthalmology*. Vol 4. London; Henry Kimpton; 1968:3–432.