Trabeculectomy has become the standard drainage operation for primary glaucoma since its introduction in 1968.1 Like earlier drainage operations, trabeculectomy depends on conjunctival connective tissue responding to the presence of aqueous with a period of mild self-limited inflammation and fibrosis. This reaction must be sufficient to heal the tissues and deposit enough collagen within the forming bleb to raise the IOP to low normal levels. Failure of trabeculectomy to lower the IOP is usually due to excessive bleb fibrosis.

Apart from routine postoperative management of topical steroids and cycloplegic drops, various approaches have been suggested to prevent excessive bleb fibrosis, including application of cytostatic agents at operation or during the postoperative period;2 and systemic antiinflammatory fibrosis suppression of oral prednisone, colchicine, and nonsteroidal antiinflammatory agent for 4 to 6 weeks during the postoperative period.3–5

Prospective clinicopathologic studies of bleb capsule formation around Molteno implants from the Otago Glaucoma Surgery Outcome Study provide detailed descriptions of histologic, immunohistochemical, and ultrastructural features of bleb capsules at different times after operation. They demonstrate that the normal cell processes in capsules in both primary and secondary glaucoma includes continual outer surface renewal balanced by inner surface degeneration associated with apoptosis and breakdown of tissue matrix components, which becomes more marked over time.6–10

This communication reports the findings of light microscopic examination of blebs formed following trabeculectomy operations, including types of cells with their patterns of apoptosis and distribution of normal and altered extracellular matrix components, and correlates these findings with those from capsules formed around Molteno implants.

METHODS

Ocular Specimens

Six specimens of bleb and overlying connective tissue were examined by light microscopy. All eyes had a trabeculectomy without cytostatic agents 6.0 to 25.9 years previously and had functioned well with IOP control of 7 to 14 mm Hg at final follow up. Details of specimens are shown in Table 1. Ethnicity data was retrieved from Dunedin Hospital records. In New Zealand, ethnicity is a measure of self-perceived cultural affiliation.11 All six cases identified as either New Zealand European or Other European.
Microscopic Examination of Trabeculectomy Blebs

Informed consent for donation of eyes for research purposes was obtained from patients before their death. This study adhered to the tenets of the Declaration of Helsinki.

**Surgical Technique**

All operations were performed by specialist ophthalmologists or supervised residents in training. The surgical technique for trabeculectomy closely followed that described by Cairns involving an incision through conjunctiva and Tenon’s tissue 7 mm back from the limbus, raising of a limbus based flap of Tenon’s tissue and conjunctiva, and formation of 4 × 4 mm² half thickness scleral flap hinged in the peripheral cornea. After excision of a square block of limbal tissues and performing a peripheral iridectomy the flap was sutured and Tenon’s tissue and conjunctiva carefully closed in separate layers using 8.0 silk. All operations were concluded by subconjunctival injection of cefalosporin, gentamycin, and methyl prednisolone acetate. The postoperative course was smooth in all cases and all cases developed diffusely spreading blebs without areas of conjunctival thinning, which functioned well until death.

**Collection of Specimens**

All six eyes were enucleated 1 to 3 hours after death. Immediately after enucleation eyes were injected with formal saline (a 10% solution of 37% formaldehyde in PBS) using a 30-gauge needle inserted across the limbus in order to slightly distend the blebs and then placed in formal saline. After 72 hours, the eyes were bisected in the horizontal plane and placed in 70% ethanol for processing. After excision of a square block of limbal tissues and performing a peripheral iridectomy the flap was sutured and Tenon’s tissue and conjunctiva carefully closed in separate layers using 8.0 silk. All operations were concluded by subconjunctival injection of cefalosporin, gentamycin, and methyl prednisolone acetate. The postoperative course was smooth in all cases and all cases developed diffusely spreading blebs without areas of conjunctival thinning, which functioned well until death.

**Histologic and Immunohistochemical Tissue Staining**

Sections for routine histologic examination were stained with hematoxylin and cosin, and Giemsa stains. SMA, CD34, CD45, CD68, CD138, Proliferating Cell Nuclear Antigen (PCNA), and Ki67. Sections were dewaxed in xylene three times and rehydrated through a graded series of ethanol, then placed in 3% hydrogen peroxide for 10 minutes and rinsed in distilled water. Heat retrieval of antigen was used to stain for CD45 (leucocyte common antigen), CD68, smooth muscle actin (SMA), CD138 (plasma cell marker), and Ki67 (actively cycling cells) by autoclaving the specimen in citrate buffer (pH 6) for 20 minutes to 121°C. Enzyme retrieval from sections stained for CD34 required covering the section with Proteinase-K (Dako S3030; Dako Australia Company Pty Ltd., Campbellfield, Victoria, Australia) for 10 minutes. Sections were then placed in Tris buffer. A protein blocking agent (normal human serum) was added for 10 minutes and then tapped off. Antiserum appropriate to each immunohistochemical marker was used: (SMA, Dako M0851; Dako Australia Company Pty Ltd.; CD138, cell marque CM943-2 clone B-A38; Cell Marque Corporation, Rocklin, CA; Ki67, Dako M7240 clone MIB1; Dako Australia Company Pty Ltd.; CD34, Novo NCL-END; Leica Biosystems Melbourne Pty Ltd., Mount Waverley, Victoria, Australia; CD45, Dako M0701; Dako Australia Company Pty Ltd.; CD68, Dako M0876; Dako Australia Company Pty Ltd.; and the marker visualised with Dako LSAB2-HRP and Dako diamino benzidine chromogen (K3466; Dako Australia Company Pty Ltd.), counterstained with Mayer’s haematoxyl (Amber Scientific Pty Ltd., Midvale, Western Australia, Australia) and mounted in Depex (Southern Biological Knoxfield, Victoria, Australia).

PCNA stained sections were exposed to antigen unmasking by heating to boiling in 0.01 M citrate buffer (pH 6) for 20 minutes in a Sharp 1000W microwave oven (Sharp New Zealand, Penrose, Auckland, New Zealand) before cooling in solution for 15 minutes. Nonspecific binding was blocked with 5% (vol/vol) goat serum before applying anti-PCNA (Novacapra; Leica Microsystems GmbH, Wetzlar, Germany) at a 1:150 dilution and incubating overnight at 4°C. Biotinylated anti-rabbit IgG was applied and amplified with streptavidin-biotinylated horseradish peroxidase complex. Signals were developed for visualization with amino ethyl carbazole (AEC; Sigma-Aldrich, Auckland, New Zealand) and Mayer’s haematoxylin #2 (Amber Scientific Pty Ltd.), and mounted with Depex.

PCNA stained sections were exposed to antigen unmasking by heating to boiling in 0.01 M citrate buffer (pH 6) for 20 minutes in a Sharp 1000W microwave oven (Sharp New Zealand, Penrose, Auckland, New Zealand) before cooling in solution for 15 minutes. Nonspecific binding was blocked with 5% (vol/vol) goat serum before applying anti-PCNA (Novacapra; Leica Microsystems GmbH, Wetzlar, Germany) at a 1:150 dilution and incubating overnight at 4°C. Biotinylated anti-rabbit IgG was applied and amplified with streptavidin-biotinylated horseradish peroxidase complex. Signals were developed for visualization with amino ethyl carbazole (AEC; Sigma-Aldrich, Auckland, New Zealand) counterstained with Gill’s haematoxylin #2 (Amber Scientific Pty Ltd.), and mounted with Depex.

Negative and positive controls were sections of human tonsil and negative controls were processed without exposure to the primary antibody.

**Caspase 3, Fas, and Fas Ligand.** Sections were dewaxed in xylene three times and rehydrated through a graded series of ethanol: 100% ethanol twice, and then once each in 95% ethanol, 70% ethanol, and then distilled water. They were then placed in a sequence of: methanolic hydrogen peroxide for 10 minutes, washed with tap water, rinsed with distilled water, and washed twice in 2 minutes change of PBS. Heat retrieval of antigen was performed by incubating in citrate buffer (pH 6.0) for 20 minutes at 95°C in Milestone KOS microwave (Milestone Srl, Sorisole, Italy) histostation followed by rinsing in PBS, and washing twice for 2 minutes in PBS. This was followed by protein blocking using 10% BSA for 30 minutes. Primary antibodies were applied as follows: caspase 3, 30 minutes; fas ligand, 20 minutes; and fas, 60 minutes. Negative controls were treated with 2% BSA for the same times. Sections were then rinsed in PBS before secondary staining by EDL (Dako EnVision+ Dual Link System-HRP) for 30 minutes, after which sections were rinsed in PBS followed by two 2 minute washes.

**Table 1.** Clinical Features of Bleb Specimens

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Glaucoma</th>
<th>Age at Operation, y, Sex</th>
<th>AIFS</th>
<th>Final IOP, mm Hg</th>
<th>Hypotensive Medication at Final Follow-up</th>
<th>Age of Bleb, y</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PXF</td>
<td>79, M</td>
<td>Yes</td>
<td>7</td>
<td>-</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
<td>POAG</td>
<td>83, F</td>
<td>No</td>
<td>14</td>
<td>Latanaprost</td>
<td>6.4</td>
</tr>
<tr>
<td>3</td>
<td>POAG</td>
<td>84, M</td>
<td>No</td>
<td>14</td>
<td>-</td>
<td>9.8</td>
</tr>
<tr>
<td>4</td>
<td>POAG</td>
<td>79, M</td>
<td>No</td>
<td>7</td>
<td>-</td>
<td>16.6</td>
</tr>
<tr>
<td>5</td>
<td>POAG</td>
<td>74, F</td>
<td>No</td>
<td>10</td>
<td>-</td>
<td>25.1</td>
</tr>
<tr>
<td>6</td>
<td>PXF</td>
<td>65, F</td>
<td>No</td>
<td>10</td>
<td>-</td>
<td>25.9</td>
</tr>
</tbody>
</table>

AIFS, anti-inflammatory fibrosis suppression1–3; PXF, pseudoexfoliative glaucoma.
in PBS. Sections stained by diaminobenzidine (DAB) were stained for 5 minutes after which they were washed in distilled water followed by tap water, before counter-staining with haematoxylin (1 dip), after which they were washed in tap water and differentiated in Scott’s tap water (Amber Scientific Pty Ltd.) for 30 seconds, and washed again in tap water. Sections were dehydrated through 100% ethanol, cleared in xylene, mounted in Entellan (Merck Group, Darmstadt, Germany), and cover slipped. The antibodies (ThermoFisher Scientific, Auckland, New Zealand) were caspase 3 (CPP32): mouse monoclonal antibody; CD95 (fas): mouse monoclonal antibody; and fas ligand: rabbit polyclonal antibody.

Negative and positive controls were sections of human tonsil, appendix, and lymph node. Negative controls were processed without exposure to the primary antibody.

**Slide Examination**

Stained sections were examined and photographed using brightfield and polarized light techniques (Leitz Orthoplan; Ernst Leitz Wetzlar GmbH, Wetzlar, Germany).

Apoptotic cells were identified using the criteria of Kerr et al. who describe a sequence including initial condensation of nucleus and cytoplasm, with nuclear fragmentation and separation of protuberances that form on cell surfaces and phagocytosis of cell remnants by adjacent cells. In addition there is formation of lucent cytoplasmic vacuoles and dense masses of nuclear material in some cases, while others are composed only of condensed cytoplasmic elements. Apoptotic bodies frequently occur as clusters in the intercellular space from which the smaller bodies tend to disperse from their site of origin.
Cell migration in a 25.1-year-old bleb, the same bleb as in Figures 1, 3, and 4 (Table 1, case 5). (A) Tangential section of bleb showing distribution of alpha smooth muscle actin+ myofibroblasts with most intact cells concentrated in (a) superficial conjunctiva and (b) deep conjunctiva; and (c) Tenon’s capsule and (d) superficial scleral flap contained stained bundles of collagen fibers and few cells, and occasional collagen fibers crossing (e) the intrascleral cleft. Stain: anti-SMA. (B) Tangential section of bleb showing (a) superficial conjunctiva and (b) deep conjunctiva. Arrows indicate positively staining cytoplasm of spindle cells with weak staining of surrounding collagen and ground substance. Stain: anti-SMA. (C) Tangential section of bleb showing SMA+ collagen bundles and ground substance in (c) Tenon’s capsule and (d) superficial scleral flap. The arrow indicates fragmented irregular collagen bundles with some staining in (e) the intrascleral cleft. Stain: anti-SMA. (D) Tangential section of bleb showing distribution of CD34+ bone marrow derived angiogenic cells concentrated in (a) superficial conjunctiva, (b) deep conjunctiva, and (c) Tenon’s capsule, and not present in (d) the superficial scleral flap, (e) the intrascleral cleft, or (f) the deep scleral layer. Stain: anti-CD34. (E) Tangential section of bleb showing CD34+ bone marrow derived angiogenic cells in (a) superficial conjunctiva and (b) deep conjunctiva. Stain: anti-CD34. (F) Tangential section of bleb showing CD34+ bone marrow derived angiogenic cells and tissue fibrils in (c) Tenon’s capsule, and not present in (d) the superficial scleral flap. Stain: anti-CD34. (G) Tangential section of bleb showing CD68+ macrophage cells in (a) superficial conjunctiva, (b) deep conjunctiva, and (c) Tenon’s capsule. Note relatively few CD68+ cells in (d) superficial scleral layer. Stain: anti-CD68. (H) Tangential section of
bleb with arrows indicating CD68+ cells in (a) superficial conjunctiva and (b) deep conjunctiva. Stain: anti-CD68. (I) Tangential section of bleb with the upper arrow indicating numerous CD68+ cells in (c) Tenon’s capsule and lower arrow indicating occasional cells in (d) the superficial scleral flap. Stain: anti-CD68. (J) Tangential section of bleb with the upper arrow indicating numerous CD138+ plasma cells in (a) superficial conjunctiva, and very few CD138+ plasma cells in (b) deep conjunctiva and (c) Tenon’s capsule. The lower arrow indicates CD138+ staining of collagen and ground substance in (d) superficial scleral flap. Note positively staining conjunctival cell membranes. Stain: anti-CD138. (K) Tangential section of bleb showing CD138+ plasma cells in (a) superficial conjunctiva, and (b) deep conjunctiva. Note positively staining conjunctival cell membranes. Stain: anti-CD138. (L) Tangential section of bleb showing CD138+ plasma cells in (c) Tenon’s capsule; and CD138+ staining of collagen and ground substance in (d) superficial scleral flap. Stain: anti-CD138. All brightfield. Magnification: ×40 (A, D, G, J); ×160 (B, C, E, F, H, I, K, L). Scale bars: 400 μm (A, D, G, J); 100 μm (B, C, E, F, H, I, K, L).

Cell density, cell types, and the number of apoptotic cells were determined by examining sections using a graticule having 100 33/33 μm squares using a ×40 objective and presumed apoptotic cells were examined individually using a ×100 oil immersion objective.

RESULTS

Histologic Structure of Blebs

The histologic features of all specimens were very similar. The internal opening in the chamber angle communicated directly into an approximately 4 mm² intrascleral cleft. Irregular channels around the edges of the superficial scleral flap communicated with overlying edematous conjunctiva. Tissues lining the intrascleral drainage channels showed fragmentation, disorientation, and disappearance of collagen fibers, and few swollen, distorted, and disintegrating cells. These changes were more marked and extensive along “aqueous drainage paths” extending through the superficial scleral flap into the overlying connective tissue of the conjunctiva (Fig. 1).

Structure of Blebs

The superficial layers of blebs comprised of edematous connective tissue with irregularly branching, interconnected, fluid filled channels. Evenly spaced capillaries formed a network 50 to 150 μm below the surface. The average distance between capillaries of approximately 100 μm corresponded to an overall mean density of 27 capillaries and 569 cells/0.33 mm². The deeper layers of conjunctiva were less vascular and cellular with an average mean density of nine capillaries and 228 cells/0.33 mm². The next layer consisting of Tenon’s capsule and episcleral blood vessels was slightly denser and contained a mean of 17 blood vessels and 527 cells/0.33 mm². However, these blood vessels were more unevenly distributed and included arterioles and venules with relatively few capillaries. Some capillaries showed abnormally thick endothelial linings. The deepest layer, the scleral flap, showed occasional, apparently empty blood vessels, and small numbers of degenerate cells concentrated in the vicinity of blood vessels to give overall densities of means of three vessels and 121 cells/0.33 mm². The surrounding tissue matrix showed marked fragmentation of collagen fibers with occasional irregular deposits of amorphous material (Figs. 1–3).

Cytology of Blebs

Most cells in the superficial conjunctiva showed features of normal connective tissue histiocytes. Approximately 25% of cells close to the conjunctival epithelium showed features suggestive of plasma cells. A small proportion of cells showed palely staining nuclei while occasional cells showed darkly staining condensed nuclei with very occasional cells (approximately 1%) showing characteristic features of apoptosis. Cells in the deeper layers of the conjunctiva showed similar changes while those in Tenon’s capsule showed more marked changes with a higher proportion showing hyperchromic nuclei, chromatin condensation, and blebbing, while some apoptotic bodies were observed being phagocytosed (Figs. 1E, 1F).

Cells in control sections of inferior conjunctiva appeared normal with no features suggestive of apoptosis (Fig. 1D).

**Figure 3.** Distribution of CD45+ cells of the lymphocyte series in a 25.1-year-old bleb, the same bleb as in Figures 1, 2, and 4 (Table 1, case 5). (A) Tangential section of bleb showing absence of CD45+ cells of the lymphocytic and myeloid series in (a) superficial conjunctiva, (b) deep conjunctiva, (c) Tenon’s capsule, and (d) superficial scleral flap. Stain: anti-CD45. (B) Tangential section of bleb with arrow indicating one of very few CD45+ cells in (a) superficial conjunctiva and (b) deep conjunctiva. Stain: anti-CD45. (C) Tangential section of bleb showing very few CD45+ cells in (c) Tenon’s capsule and (d) superficial scleral flap. Stain: anti-CD45. All brightfield. Magnification: ×40 (A); ×160 (B, C). Scale bars: 400 μm (A); 100 μm (B, C).
Immunohistochemical Staining

Immunohistochemical staining demonstrated numerous myofibroblasts (SMA+) bone marrow derived angiogenic cells (CD34+), macrophages (CD68+), and plasma cells (CD138+) in the superficial conjunctiva and control sections of inferior conjunctiva. Staining for circulating lymphocytes (CD45) proved negative (Figs. 2, 3). The proportion of PCNA+ cells in blebs varied between 50% to 90% compared with 30% to 60% in control conjunctiva. Staining for Ki67 gave negative results in both bleb and control conjunctiva (Fig. 4).

Cell Migration

There was SMA+ staining of the cytoplasm of myofibroblasts without staining surrounding collagen and ground substance in the superficial layers of the bleb. However, SMA+ cells decreased in the deeper layers of the conjunctiva, Tenon's capsule, and the scleral flap while large deposits of SMA+- bundles of collagen fibers and amorphous material accumulated in these layers. CD138+ plasma cells formed a distinct layer close to the conjunctival epithelium. These cells decreased through the deeper layers of the bleb while increasing amounts of CD138+ amorphous material and bundles of collagen fibers accumulated in the deeper layers of the scleral flap (Fig. 2).

Immunostaining for Apoptotic Cells

Caspase 3 immunostaining showed approximately 8% of cells in the superficial conjunctival layer were apoptotic, with approximately 3% in the deep conjunctiva and approximately 16% in the deeper layers of Tenon's capsule around the episcleral plexus. Scanty caspase 3+ cell remnants were observed in the superficial scleral flap. Fas showed moderately positive staining of cytoplasm of apparently normal and shrunken cells concentrated around blood vessels in the superficial and deep
conjunctiva and Tenon’s capsule while small numbers of positively staining cell fragments were present in the deeper layers of the superficial scleral flap (Fig. 5).

Staining for Fas ligand identified occasional fas ligand+ cells and cell fragments in the scleral flap, while larger numbers were concentrated around blood vessels of the episcleral plexus and in Tenon’s capsule, and the greatest numbers were present in the superficial layer of conjunctiva. Endothelial cells in deeper blood vessels showed diffuse weakly positive staining for fas ligand and the endothelial layer of most of these vessels appeared slightly thickened (Fig. 5).

The results are summarized in Tables 2 through 5.

### Table 2. Distribution of Cell Types in Blebs and Control Conjunctiva of All Six Blebs

<table>
<thead>
<tr>
<th></th>
<th>Bleb</th>
<th>Control Conjunctiva</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myofibroblasts, SMA+</td>
<td>++++</td>
<td>+ (in blood vessel walls)</td>
</tr>
<tr>
<td>Bone marrow derived angiongenic cells, CD34+</td>
<td>++++</td>
<td>++ (in blood vessel lumens)</td>
</tr>
<tr>
<td>Lymphocytes, CD45+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Macrophages, CD68+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Plasma cells, CD138+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Mast cells, Giemsa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Eosinophils, haematoxylin and eosin</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Figure 5.** Distribution of caspase 3+, fas+ and fas ligand+ cells and material in a 6.0-year-old bleb (Table 1, case 1). (A) Tangential section of bleb showing (a) superficial conjunctiva, (b) deep conjunctiva, and (c) Tenon’s capsule. Stain: haematoxylin and eosin. (B) Tangential section of bleb with arrows indicating caspase 3+ cells in (a) superficial conjunctiva, and fewer cells in (b) the deep conjunctiva, and intermediate numbers of cells in (c) Tenon’s capsule. Stain: anticaspase 3. (C) Tangential section of bleb with arrow indicating a cluster of caspase 3+ apoptotic cells in (a) superficial conjunctiva. Stain: anticaspase 3. (D) Tangential section of bleb with arrows indicating fas+ cells in (a) superficial conjunctiva, (b) deep conjunctiva, and (c) Tenon’s capsule. Stain: antifas. (E) Tangential section of bleb with arrow indicating fas+ cells in (a) superficial conjunctiva. Stain: antifas. (F) Tangential section of bleb with arrows indicating numerous fas ligand+ cells in (a) superficial conjunctiva, fewer cells in (b) the deep conjunctiva, and numerous swollen vacuolated positive staining cells in (c) Tenon’s capsule. Stain: antifas ligand. (G) Tangential section of bleb with arrows indicating fas ligand+ cells, cell fragments, and membrane bound vesicles in (a) superficial conjunctiva. The right arrow indicates a group of positive cells and the left arrow indicates cell fragments and membrane bound vesicles. Stain: antifas ligand. All brightfield. Magnification: ×160 (A); ×200 (B, D, F); ×1000 (C, E, G). Scale bars: 100 μm (A); 80 μm (B, D, F); 15 μm (C, E, G).
DISCUSSION

This study supported the authors’ hypothesis that there was continued migration of mesodermal cells from superficial capillary blood vessels into the conjunctival connective tissue. These cells then migrated into the deeper layers of Tenon’s capsule and the scleral flap. During migration an increasing proportion of cells became apoptotic and released collagenolytic enzymes and minute membrane bound vesicles, which acted as proapoptotic death messengers.17 These vesicles were carried by the flow of aqueous toward the superficial layers where they suppressed inflammation and fibrosis by inducing apoptosis in metabolically active cells via the extrinsic pathway.13–15

Apoptosis is an energy dependent process, in which mouse liver cells digest themselves over approximately 6 to 12 hours.16 Apoptosis over approximately 12 hours implies all cells in the bleb would be replaced in approximately 6 days. However, it seems likely the cells are metabolically less active in the low protein environment of the bleb and would undergo apoptosis much less rapidly. We therefore estimated that the cells in these relatively less vascular blebs would turnover within weeks during the early postoperative period and months in longstanding blebs. More work in this field is needed.

Evidence for Cell Migration From Superficial Blood Vessels

The observation of approximately 8% of apoptotic cells in the superficial layers implied that this cell population was continually replaced by migration of monocytes from capillary blood vessels into the perivascular tissues. These cells then differentiated into myofibroblasts, bone marrow derived angiogenic cells, macrophages, and plasma cells.

Evidence for Cell Migration From the Superficial to Deep Layers of the Bleb

Migration of cells was demonstrated by decreasing numbers of cells in the deeper layers of blebs and increasing proportions of apoptotic cells and cell fragments in the deeper layers. Additional evidence was provided by immunohistochemical staining of cell types. SMA+ myofibroblasts were concentrated in the superficial conjunctival layers and decreased markedly in the deeper layers. There were no SMA+ collagen fiber bundles in the superficial conjunctiva, a few in the scleral flap and many in the deeper layers of Tenon’s capsule. CD34+ bone marrow derived angiogenic cells formed blood vessel walls in the superficial conjunctiva. There was widespread deposition of CD34+ staining material on connective tissue fibers in the conjunctiva and Tenon’s capsule but not in the scleral flap. CD68+ macrophages were found throughout the bleb with decreasing numbers in the deeper layers; but there was no significant staining of tissue matrix components. CD138+ plasma cells formed a thin layer immediately beneath the conjunctival epithelium and were virtually absent from the deeper layers. However, CD138+ bundles of collagen fibers and ground substance formed a distinct layer in the scleral flap. CD138 (syndecan 1), a transmembrane protein expressed in the walls of plasma and epithelial cells, has a strong affinity for epithelial cell membranes including conjunctiva, as well as fibronectin and thrombospondin, which are normal components of the extracellular connective tissue matrix found in significantly increased amounts in the inner fibrodegenerative layers of capsules formed around Molteno implants.8 It seems likely that the distribution of SMA+ and CD138+ cells, ground substance and collagen bundles is consistent with migration, apoptosis, and staining of collagen fibers in the deeper layers. Further investigation is required.

Evidence for Increased Proportion of Apoptotic Cells and Associated Breakdown of Collagen Fibers in Deeper Layers of Blebs

Haematoxylin and eosin stained sections demonstrated interaction between apoptotic cells and connective tissue matrix. The most superficial conjunctival layer contained cells that were predominantly normal in appearance. Approximately 1% of cells showed nuclear chromatin condensation with nuclear and cytoplasmic blebbing and formation of free cytoplasmic vesicles, while polarizing light microscopy demonstrated birefringent collagen fibrils of normal appearance. In deeper conjunctival layers, Tenon’s capsule and the scleral flap, an increasing proportion of cells showed changes of swelling, nuclear condensation, distortion, fragmentation, and formation of apoptotic bodies together with loss of birefringence, fragmentation, and disappearance of collagen fibrils.

Numerous caspase 3+ cells were observed in the conjunctiva and Tenon’s capsule showing apparently normal cell

Table 4. Blood Vessel and Cell Density and Degree of Collagen Breakdown in Different Layers of All Six Blebs

<table>
<thead>
<tr>
<th></th>
<th>Blood Vessel Numbers Per 0.33 mm², Mean (Range)</th>
<th>Cell Numbers Per 0.33 mm², Mean (Range)</th>
<th>Collagen Breakdown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial conjunctiva</td>
<td>27 (10–44)</td>
<td>569 (222–794)</td>
<td>++*</td>
</tr>
<tr>
<td>Deep conjunctiva</td>
<td>9 (5–13)</td>
<td>228 (149–300)</td>
<td>+++</td>
</tr>
<tr>
<td>Tenon’s/episcleral vessels</td>
<td>17 (12–22)</td>
<td>527 (429–628)</td>
<td>++++</td>
</tr>
<tr>
<td>Scleral flap</td>
<td>3 (2–4)</td>
<td>121 (85–234)</td>
<td>+++++</td>
</tr>
<tr>
<td>Deep scleral bed</td>
<td>3 (1–5)</td>
<td>54 (31–68)</td>
<td></td>
</tr>
</tbody>
</table>

* Case 2 had thin oedematous conjunctival epithelium, which was intact but with more marked collagen loss in the superficial and deep conjunctival layers compared with the other cases.
morphology, shrunken condensed cells with reduced cytoplasm, swollen vacuolated cells, distorted cell remnants, and cell fragments.

Staining for fas, the cell membrane receptor for fas ligand, identified positive cells of normal appearance in moderate numbers throughout the bleb.

Staining for fas ligand stained the cytoplasm of numerous cells and free fragments of cytoplasm and membrane bound vesicles in the more superficial layers of Tenon’s capsule and conjunctiva. This suggested that it identified cells expressing fas ligand on their surface before they disintegrated to release fas ligand- fragments of cytoplasm, which acted as proapoptotic death messengers that were transported by aqueous and eventually phagocytosed by metabolically active cells in the superficial conjunctiva, in which they induced apoptosis via the extrinsic apoptotic pathway.17

**CONCLUSIONS**

These findings supported the hypothesis that the complex cycle of cell activation, migration, apoptosis, and death messenger formation demonstrated in bleb capsules around Molteno implants also occurred in trabeculectomy blebs. They showed that long term drainage of aqueous from within the eye into the extraocular connective tissue depended on a dynamic balance between an inflammatory fibroproliferative response and an antiinflammatory apoptotic fibrodegenerative response to aqueous. The details and relative importance of the mechanisms driving these two processes are not yet completely understood. However, recent advances in cell biology suggest that improved methods of minimising the fibroproliferative and enhancing the apoptotic fibrodegenerative responses of connective tissue to aqueous during the postoperative period will be developed in the future.

**Acknowledgments**

The authors thank the patients and their families who kindly donated the eyes and the ophthalmologists who provided follow up data.

Supported by a grant from the Healthcare Otago Charitable Trust (THB).

Disclosure: **A.C.B. Molteno**, Molteno Ophthalmics (S), P. T.H. Bevin, None; **A.G. Dempster**, None; **M. Sarris**, None; **P. McCluskey**, None

**References**


