Otago Glaucoma Surgery Outcome Study: Further Histology and Immunohistochemistry of Molteno Implant Blebs

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PURPOSE. To describe findings of light microscopic examination of Molteno implant bleb capsules.

METHODS. Histological and immunohistochemical features of bleb capsules including distribution of apoptotic cells and cell fragments were examined in 11 eyes 0.2 to 30.4 years after Molteno implants.

RESULTS. In the superficial layer of capsules, high proportions of cells showed cytological features of apoptosis, with a higher proportion of cells showing specific immunohistochemical features of apoptosis with a mean of 22% (range, 3%–40%) of cells staining positively for Fas ligand. This demonstrated that cells migrating into the superficial capsule were replaced over approximately 1 to 6 days. In the deeper layer a mean of 8% (range, 3%–38%) of apoptosing cells stained positively for Fas ligand. These lower proportions of positively staining cells and cell fragments in the deeper layers, and the presence of occasional positively staining cells on the inner surface of capsules, demonstrated continuous migration of cells into the deeper layers associated with breakdown of connective tissue matrix and the release of numerous membrane-bound vesicles.

CONCLUSIONS. These findings demonstrated unexpectedly rapid turnover of cells in the superficial layer of the bleb capsule, where most cells were efficiently phagocytosed by nearby monocytic cells, macrophages, and histiocytes; the remaining cells migrating into the deeper layers completed apoptosis and disintegrated with release of collagenolytic enzymes and Fas ligand positive presumed “death messengers” that were carried toward the superficial layers by the aqueous.

Keywords: Molteno implants, glaucoma drainage implants, bleb structure, apoptosis

Prospective clinicopathological studies of bleb capsule formation around Molteno implants from the Otago Glaucoma Surgery Outcome Study provide detailed descriptions of histological, immunohistochemical, and ultrastructural features of bleb capsules at different times after operation. They demonstrate that the normal lifecycle of capsules in both primary and secondary glaucoma includes continual superficial layer renewal balanced by deeper layer degeneration associated with apoptosis and breakdown of tissue matrix components that becomes more marked over time.¹⁻⁶

On the basis of these clinical and histological observations, we hypothesized:

1. Drainage of aqueous into well-vascularized subconjunctival connective tissue (mesodermal tissue) dilutes the interstitial tissue fluid and acts as a “noxious” stimulus causing vasoconstriction of mesodermal cells. Activated fibroblasts synthesize collagen, producing a barrier to the passage of the aqueous. This process continues for as long as cells in close proximity to patent blood vessels (<50 µm) are exposed to aqueous.⁷
2. Once sufficient fibrosis has occurred to resist the passage of aqueous, the intraocular pressure (IOP) rises and exceeds the capillary pressure (15–25 mm Hg) in the deeper part of the bleb capsule. This zone effectively becomes avascular (>50 µm from the nearest patent blood vessel), changing the tissue environment, as aqueous displaces the interstitial tissue fluid. Under these conditions, cells deplete nutrients and are exposed to proapoptotic factors that include low protein concentration and hypoxia. The local effects of apoptosis include breakdown of the deeper layers of the collagen barrier and transport of death messengers with the flow of aqueous to the superficial fibroproliferative portion of the capsule, where they destroy activated cells and inhibit the fibroproliferative response. Decreasing numbers of cells migrate into the capsule, where they are activated and undergo apoptosis for the rest of the patient’s life.

This communication reports results of immunohistochemical staining of Molteno implant bleb capsules to identify the extracellular proapoptotic death messenger Fas ligand (also known as CD95L), and the death-inducing signaling complex composed of Fas-associated death domain (FADD) and caspase 8, which are constituents of the extrinsic death receptor pathway of apoptosis, together with caspase 3, a marker of the mitochondrial (intrinsic) pathway of apoptosis.
Otago Glaucoma Surgery Outcome Study

METHODS

Eleven specimens of bleb and overlying connective tissue were examined by light microscopy. All eyes had a Molteno implant inserted 0.2 to 30.4 years previously. 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. Ten cases identified as New Zealand European and one as Pacific peoples.

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

Pathological Material

Eleven capsules with overlying and adjacent connective tissue, of which eight postmortem cases included conjunctiva, from functioning Molteno implants in noninflamed eyes obtained between 0.2 to 30.4 years after operation were subject to histological, cytological, and immunohistochemical studies. Specimens were obtained between 0.5 and 23 hours after death (eight cases); at enucleation (two cases); and one at operation when the first Molteno implant was removed and replaced by a second 6 months after operation. The underlying etiology and implant type are described in Table 1. No eyes had received antimetabolites and four cases received the synergistic anti-inflammatory regimen of prednisone, a nonsteroidal anti-inflammatory agent, and colchicine. Ten eyes had good IOP control until shortly before death, enucleation, or implant removal (mean 11.9 mm Hg; range, 6.5–21 mm Hg). In the remaining case (Table 1, case 8) the IOP was not measured in the comfortable blind eye for 6 years before death.

Surgical Technique

The surgical techniques used for immediate and delayed aqueous drainage have been described.12–17

Fixation and Processing of Tissue

Capsules and whole eyes were fixed in 10% neutral buffered formalin for 3 hours, microwaved for 20 minutes at 50°C, then placed in 70% alcohol to further harden. 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 the 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 embedding in paraffin, 5-μm serial sections were cut and mounted on glass slides.

Histological and Immunohistochemical Staining of Tissue

Stains included hematoxylin and eosin, anti-Fas ligand, anti-FADD, anti-caspase 8, and anti-caspase 3.

Immunohistochemistry

All sections were dewaxed in xylene and rehydrated through a graded series of ethanol followed by immersion in methanolic hydrogen peroxide for 10 minutes before being washed in PBS three times for 2 minutes each. Heat retrieval in a microwave histostation (Milestone KOS; Milestone Srl, Sorisole, Italy) was carried out in citrate buffer solution at pH 6.0 for 20 minutes, then cooled for 20 minutes and rinsed in PBS three times for 2 minutes each. Protein blocking was carried out in 10% bovine serum albumin (BSA; Life Technologies, Frederick, MD, USA) for 30 minutes, and was followed by exposure to primary antibodies at room temperature using the following dilutions: Fas ligand (ready to use antibody for 20 minutes); caspase 3 (ready to use antibody for 30 minutes); FADD (1:200 dilution for 60 minutes); and caspase 8 (1:50 dilution for 60 minutes).

The positive and negative control slides consisted of sections of tonsil for Fas ligand, placenta for FADD, and appendix for caspases 3 and 8. Negative controls were placed in 2% BSA for 60 minutes.

All slides were then rinsed in PBS three times for 2 minutes each before exposing slides to the secondary antibody (Envision Dual Link, K406189-2; Dako, Carpinteria, CA, USA) for 30 minutes. Slides were rinsed again in PBS three times for 2 minutes each before being exposed to 3,3′ diaminobenzidine ([DAB] cm957040 Abacus ALS; Cell Marque Corp., Rocklin, CA, USA) for 5 minutes and being washed in distilled water and then tap water and being exposed to hematoxylin for 2 minutes. Slides were then washed well in tap water for 2 minutes, Scott’s tap water for 1 minute, and tap water for 2 minutes. Slides were then dehydrated in a series of four changes of 100% alcohol for 30 seconds, 30 seconds, 1 minute, and 1 minute, followed by four changes of xylene for 2 minutes.

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TABLE 1. Clinical Features of Specimens

<table>
<thead>
<tr>
<th>Case</th>
<th>Specimen Obtained</th>
<th>Type of Glaucoma</th>
<th>Age at Operation, y, Sex</th>
<th>Type of Molteno Implant</th>
<th>AIFS</th>
<th>Final IOP, mm Hg</th>
<th>Hypotensive Medication At Final Follow-up</th>
<th>Length of Follow-up, y</th>
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<tbody>
<tr>
<td>1</td>
<td>Post mortem</td>
<td>Neovascular</td>
<td>32, M</td>
<td>M3 250</td>
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<td>6</td>
<td>Alphagan</td>
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<td>2</td>
<td>At operation</td>
<td>POAG</td>
<td>68, F</td>
<td>M5 175</td>
<td>No</td>
<td>21</td>
<td>-</td>
<td>0.5</td>
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<tr>
<td>3</td>
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<td>Neovascular</td>
<td>76, M</td>
<td>M3 175</td>
<td>No</td>
<td>11</td>
<td>Acetazolamide, timolol</td>
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<tr>
<td>4</td>
<td>Post mortem</td>
<td>PXE</td>
<td>81, M</td>
<td>M3 175</td>
<td>No</td>
<td>8.5</td>
<td>-</td>
<td>4.3</td>
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<tr>
<td>5</td>
<td>Post mortem</td>
<td>POAG</td>
<td>95, F</td>
<td>M3 175</td>
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<td>15</td>
<td>Timolol</td>
<td>6.8</td>
</tr>
<tr>
<td>6</td>
<td>Enucleation</td>
<td>POAG</td>
<td>74, F</td>
<td>2-plate</td>
<td>No</td>
<td>7</td>
<td>Alphagan</td>
<td>8.3</td>
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<td>Post mortem</td>
<td>Traumatic</td>
<td>74, F</td>
<td>1-plate</td>
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<td>12</td>
<td>-</td>
<td>8.8</td>
</tr>
<tr>
<td>8</td>
<td>Post mortem</td>
<td>POAG</td>
<td>79, F</td>
<td>M3 175</td>
<td>No</td>
<td>14</td>
<td>Alphagan</td>
<td>8.8</td>
</tr>
<tr>
<td>9</td>
<td>Post mortem</td>
<td>POAG</td>
<td>80, F</td>
<td>2-plate</td>
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<td>-</td>
<td>15.9</td>
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<td>2-plate</td>
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<td>Juvenile</td>
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<td>Yes</td>
<td>14</td>
<td>Timolol</td>
<td>30.4</td>
</tr>
</tbody>
</table>

AIFS, anti-inflammatory fibrosis suppression; POAG, primary open-angle glaucoma; PXE, pseudoexfoliative glaucoma.
each and were finally mounted in entellan with a glass cover slip.

Specific antibodies used were anti-Fas ligand antibody (RB-9029-R7; Thermo Fisher Scientific, Inc., Rockville, MD, USA); anti-FADD antibody (ab24533; Abcam, Cambridge, MA, USA); anti-caspase 8 (ab4052; Abcam); and anti-caspase 3 (CPP32) antibody (MS-1123-R7; Thermo Fisher Scientific, Inc.).

**Slide Examination**

Stained sections were examined and photographed by light microscopy using bright field, polarized light, and phase contrast (Orthoplan, Leitz Wetzlar, Germany).

**Figure 1.** Histological structure of 0.2-year-old bleb, the same bleb shown in Figures 3 and 4 (Tables 1, 2, 4, case 1). Scale bars: 1000 μm (A); 95 μm (B–E); 37.5 μm (F G). (A) Vertical section of bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer of capsule with dilated capillaries. (c) Deeper fibrodegenerative layer with decreasing numbers of mesodermal cells. (d) The cavity. (e) Region of aqueous exclusion by contact between margin of implant and inner surface of Tenon’s capsule. (f) Region of immediate aqueous drainage showing primary bleb capsule. (g) Region of delayed aqueous drainage showing secondary bleb capsule. All figures are identically oriented and labeled. Stain, hematoxylin and eosin; bright field. (B) Vertical section of same bleb. (a) Tenon’s capsule. (e) Region of aqueous exclusion by contact between margin of implant and inner surface of Tenon’s capsule. Arrows indicate approximately 50-μm-thick layer of condensed connective tissue. Stain, hematoxylin and eosin; bright field. (C) Vertical section of same bleb covering region of immediate aqueous drainage. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer. (c) Deeper fibrodegenerative layer with decreasing numbers of mesodermal cells. (d) Cavity. Stain, hematoxylin and eosin; bright field. (D) Vertical section of same bleb covering region of delayed aqueous drainage. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer. (c) Deeper fibrodegenerative layer with decreasing numbers of mesodermal cells. Arrows indicate approximately 50-μm-thick layer of condensed connective tissue. Stain, hematoxylin and eosin; bright field. (E) Oblique section of same bleb covering region of immediate aqueous drainage. (b) Superficial fibroproliferative layer of capsule. (c) Deeper fibrodegenerative layer with decreasing numbers of mesodermal cells. Arrows indicate apoptotic cells. Stain, hematoxylin and eosin; bright field. (F) Oblique section of same bleb covering region of immediate aqueous drainage. (b) Superficial fibroproliferative layer of capsule. Arrows indicate condensed apoptotic cells with surrounding clear zones (tingible bodies). Note swollen faintly staining cells toward the deeper layers. Stain, hematoxylin and eosin; bright field. (G) Oblique section of same bleb covering region of immediate aqueous drainage. (c) Deeper fibrodegenerative layer. (d) Cavity. Arrows indicate swollen disorganized tissue fibrils, clear zones surrounding apoptosing cells and clear zones remaining after cell death. Stain, hematoxylin and eosin; bright field.

Apoptotic cells were identified by their morphology in oblique sections stained by hematoxylin and eosin. The criteria used for their identification were those of Kerr et al. The first stage involved marked condensation of the nucleus and cytoplasm, nuclear fragmentation and separation of protuberances that formed on cell surfaces, while the second stage involved phagocytosis by adjacent cells, and the formation of lucent cytoplasmic vacuoles and dense masses of nuclear material or condensed cytoplasmic elements.

Cell density and the proportion of apoptotic cells in capsules were determined by examining obliquely cut sections with a graticule having 53 × 53-μm squares (n = 100) and placing it over the normally stained outer fibroproliferative and...
Table 2. Thickness and Cell Density of Fibroproliferative and Fibrodegenerative Layers of Bleb Capsules

<table>
<thead>
<tr>
<th>Case</th>
<th>Vertical Thickness of Capsule, μm</th>
<th>Number of Cells/0.055 mm²</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Superficial Fibroproliferative</td>
<td>Deeper Fibrodegenerative</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>45</td>
<td>80</td>
<td>104</td>
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<td>45</td>
<td>90</td>
<td>105</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>85</td>
<td>110</td>
</tr>
<tr>
<td>11</td>
<td>40</td>
<td>125</td>
<td>153</td>
</tr>
</tbody>
</table>

Figure 2. Histological structure of 30.4-year-old bleb (Table 1, case 11). Scale bars: 75 μm (A, B), 15 μm (C-G). (A) Oblique section of bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer of capsule with cluster of apoptotic cells. (c) Deeper fibrodegenerative layer. (d) Cavity. Arrow indicates swollen apoptotic cells. Stain: hematoxylin and eosin; bright field. (B) Oblique section of same bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer with cluster of apoptotic cells. (c) Deeper fibrodegenerative layer with occasional scattered cells on the inner surface. (d) Cavity. Note birefringence in layers (a) and (b) and decreasing in (c). Stain: hematoxylin and eosin; polarized light. (C) Oblique section of same bleb. (b) Superficial fibroproliferative layer of capsule. Note cluster of blebbing apoptotic cells. Stain: hematoxylin and eosin; bright field. (D) Oblique section of same bleb. (c) Deeper fibrodegenerative layer with variably swollen disintegrating apoptotic cells. (d) Cavity. Stain: hematoxylin and eosin; bright field. (E) Oblique section of same bleb. (b) Superficial fibroproliferative layer with cluster of apoptotic cells. Stain: hematoxylin and eosin; positive phase contrast. (F) Oblique section of same bleb. (c) Deeper fibrodegenerative layer. Arrows indicate disintegrating cell and numerous membrane-bound vesicles. Stain: hematoxylin and eosin; positive phase contrast. (G) Oblique section of same bleb. (c) Deeper fibrodegenerative layer with apoptotic cells. (d) Cavity. Stain: hematoxylin and eosin; positive phase contrast.
poorly stained inner fibrodegenerative layers of the capsules. Cells were counted in 6 to 27 successive 106 × 530-µm strips using ×25 and ×100 objectives until a minimum of 400 cells had been counted. The proportion of apoptotic cells was calculated by averaging counts for each capsule layer.

**RESULTS**

**Histological Structure of Connective Tissue and Conjunctiva**

The overlying connective tissue was edematous but otherwise normal while the inner surface of Tenon’s capsule in direct contact with the implant showed a moderate fibroblastic response forming an avascular collagenous layer approximately 50-µm thick. The inner surface of Tenon’s tissue exposed to aqueous formed a well defined bleb capsule with a superficial relatively cellular and moderately vascular connective tissue layer merging more deeply with an avascular less cellular layer adjacent to the aqueous cavity. The thickness and cellularity of this layer was greater in the primary bleb capsule while the secondary bleb capsule was thinner and less cellular (Fig. 1).

**Histological Structure of Bleb Walls**

The wall of the bleb capsule, when cut perpendicular to its surface, consisted of a superficial relatively cellular and moderately vascular connective tissue layer (the fibroproliferative layer), merging more deeply with an avascular, relatively acellular layer of bleb wall adjacent to the aqueous cavity (the fibrodegenerative layer). Oblique planes of sectioning showed more detail. Fibroblasts exhibited an altered appearance in the deeper aspects of the bleb walls toward the aqueous cavity. Fibroblast nuclei and cytoplasm became enlarged, vacuolated, and fragmented with occasional replacement by clumps of cellular fragments. These cells were often surrounded by numerous tiny membrane-bound vesicles and minute basophilic particles that could not easily be recognized in perpendicular sections but were well seen in oblique sections using phase contrast microscopy (Figs. 2C–G).

**Cellular Immunohistochemistry**

**Distribution of Labeled Cells**

Immunohistochemically labeled cells and cell fragments were concentrated in the superficial fibroproliferative layers of the bleb capsules. The proportions of labeled cells and cell fragments fell progressively toward the internal surface of the fibrodegenerative layer of capsules, and were higher during the earlier postoperative period (10–24 weeks) and thereafter remained relatively stable for the duration of follow-up (Table 3). Anti–Fas ligand antibody stained the cytoplasm of a mean of 22% (range, 3%–40%) of cells in the superficial fibroproliferative layer and a mean of 8% (range, 3%–58%) of the deeper fibrodegenerative layer of bleb capsules (Table 4). Positively staining cells were concentrated in the periocular spaces of capillary blood vessels and venules (Figs. 3–5A, 5B). In addition, there was nonspecific diffuse staining of capillary endothelial cells (as well as plasma and erythrocytes where present) of vessels on the superficial surface of capsules through which aqueous was draining. Intense diffuse staining of collagen fibers was present in the innermost layer of the 10-week-old bleb (Table 1, case 1; Figs. 3B–G). In older capsules, this diffuse staining was noted in both layers of the capsules (Figs. 4B, 4F, 4G). In one case (Table 1, case 1), where part of the capsule had remained in contact with the plate so that aqueous was not passing through, the capsule showed complete absence of positive staining cells, capillary endothelia, blood plasma, and collagen (Fig. 3A).

**Morphology of Cells**

Perpendicular sections showed little detail while tangential and oblique sections showed more detail. Fibroblasts exhibited an altered appearance in the deeper aspects of the bleb walls toward the aqueous cavity. Fibroblast nuclei and cytoplasm became enlarged, vacuolated, and fragmented with occasional replacement by clumps of cellular fragments. These cells were often surrounded by numerous tiny membrane-bound vesicles and minute basophilic particles that could not easily be recognized in perpendicular sections but were well seen in oblique sections using phase contrast microscopy (Figs. 2C–G).

**Membrane-Bound Vesicles**

Disintegration of cells in the fibrodegenerative layers of the bleb capsule produced large numbers of variably basophilic membrane-bound vesicles ranging in size from a diameter of 4 µm to the limit of optical resolution (0.4 µm). These vesicles were most numerous in the deeper layers of maximal cellular degeneration with smaller numbers in the more superficial layers of the capsule (Fig. 2F).
TABLE 4. Distribution of Morphologically Identified Apoptotic and Immunohistochemically Positive Cells in Both Layers of Capsules

<table>
<thead>
<tr>
<th>Case</th>
<th>Superficial Fibroproliferative Layer</th>
<th>Deeper Fibrodegenerative Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proportion of Morphologically Identified Apoptosing Cells, %</td>
<td>Proportion of Fas Ligand+ Cells, %</td>
</tr>
<tr>
<td>1</td>
<td>4.5</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
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<td>3</td>
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<tr>
<td>9</td>
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<td>12</td>
</tr>
<tr>
<td>Mean</td>
<td>4.0</td>
<td>22</td>
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</table>

14% (range, 4%–30%) in the deeper fibrodegenerative layer of capsules (Table 4). In addition, there was weak nonspecific diffuse staining of capillary endothelial cells and blood vessel contents (Table 1, case 1; Table 3; Figs. 3B–E, 4C, 4H, 4I). In addition, there was weak diffuse staining of collagen fibers in the same pattern as the Fas ligand and FADD distributions (Table 3; Figs. 3D, 4H, 4I, 4L, 5C, 5D).

Anti-caspase 8 staining stained a mean of 28% (range, 12%–48%) of cells in the superficial fibroproliferative and a mean of 17% (range, 2%–43%) of cells in the deeper fibrodegenerative layer of capsules (Table 4). In addition, anti-caspase 8 staining stained the capillary blood vessel wall weakly with no staining of blood vessel contents, and there was diffuse staining of collagen fibers in the same pattern as the Fas ligand and FADD distributions (Table 4; Figs. 3F, 4D, 4J, 4K, 5E, 5F).

Anti-caspase 3 staining stained the cytoplasm of a mean of 13% (range, 7%–22%) of cells in the superficial fibroproliferative layer, but there was no staining of capillary endothelia or blood vessel contents, and a mean of 6% (range, 2%–16%) of cells in the deeper fibrodegenerative layer of capsules with weak diffuse staining of collagen fibers in the same pattern as the Fas ligand and FADD distributions (Table 4; Figs. 3G, 4E, 4I, 4M, 5G, 5H).

**DISCUSSION**

This study demonstrated continuing migration of a population of mesodermal cells from capillaries in the superficial layers of capsules toward the inner surface. During migration, an increasing proportion of cells became apoptotic and released collagenolytic enzymes and minute membrane-bound vesicles. These vesicles were carried by the flow of aqueous toward the superficial capillaries where they suppressed inflammation and fibrosis by inducing apoptosis via the extrinsic pathway.18–22

Apoptosis is an energy-dependent process in which mouse liver cells digest themselves over 6 to 12 hours.23 Apoptosis over a 12-hour period implies that a group of cells of which approximately 20% stain positive for an apoptotic marker would be completely replaced in ~6 days. However, it seems likely that connective tissue cells are metabolically less active in the relatively inert vascular conjunctival tissues and much less active in the avascular deeper layers of the capsule where they are exposed to the low oxygen and protein levels of aqueous which may delay apoptosis.

**Evidence for Cell Migration From Superficial Blood Vessels Through the Capsule Layers**

Varying proportions of cells in the superficial capsule showed typical histological features of apoptosis and stained positive for Fas ligand, FADD, caspase 8, and caspase 3 (Table 3), and demonstrated that these cells were replaced over a period of approximately 1 to 6 days. This rapid turnover was consistent with circulating monocylic cells continuously migrating from the lumen of capillary vessels into perivascular spaces and adjacent tissues where they encountered aqueous containing Fas ligand–positive death messengers formed in the deeper layers of the bleb.3,6

Caspase 3 positive staining indicates that the apoptosis is irreversible and caspase 3 positive cells secrete lysyl-phosphatidylcholine, a potent chemotactant, and express phosphatidylserine, an “eat-me” signal on the outer layer of the cell membrane. These changes induce prompt phagocytosis and disposal of apoptotic cells by macrophages and histiocytes, thereby preserving the integrity and function of the tissue in the well oxygenated superficial layers of the capsule.22,24–27
Evidence for Cell Migration From the Superficial to Deep Layers of the Bleb

Migration of cells was demonstrated by progressively decreasing numbers of intact cells and cell fragments on passing from the superficial to the deeper layer of capsules together with nonspecific staining of ground substance and collagen toward the inner surface. While phagocytosis of apoptosing cells was observed in the superficial and intermediate layers of the earlier blebs it did not occur in the deeper layer where apoptotic cells disintegrated into numerous membrane bound vesicles (presumed death messengers; Figs. 4G–M). The persistence of positively staining apoptotic cells in the deeper layer and/or on the inner surface of blebs likewise demonstrated the continuous migration of cells toward the inner surface of capsules (Figs. 2D, 2F, 4G, 4I, 4K, 4M, 5B, 5D, 5F, 5H).

Interactions Between Apoptosing Cells and Collagen Fibers in Blebs

Hematoxylin and eosin stained sections demonstrated interaction between apoptotic cells and connective tissue matrix. The fibroproliferative layer contained cells predominantly normal in appearance. Morphological changes of apoptosis were seen in

Figure 3. Immunohistochemical staining of 0.2-year-old bleb capsule, the same bleb shown in Figures 1 and 4 (Tables 1, 2, and 4, case 1). Scale bars: 95 μm (A–G). (A) Vertical section of bleb. (c) Region of aqueous exclusion by contact between margin of implant and inner surface of Tenon’s capsule. Note absence of reaction to material of the implant. Arrows indicate minimal staining of blood vessels. Stain: anti-Fas ligand; bright field. (B) Vertical section of same bleb showing area of primary bleb capsule. (a) Tenon’s capsule with positively staining blood vessels, (b) Superficial fibroproliferative layer with strongly staining dilated capillaries, (c) Deeper fibrodegenerative layer with positively staining ground substance and collagen fibrils. (d) Cavity. Stain: anti-Fas ligand; bright field. (C) Vertical section of same bleb showing delayed aqueous drainage in the secondary bleb capsule. (a) Tenon’s capsule with positively staining blood vessels. (b) Superficial fibroproliferative layer with positively staining capillaries. (c) Deeper fibrodegenerative layer with positively staining ground substance and collagen fibrils. (d) Cavity. Note fewer capillaries in (b) and fewer cells in (c) compared with (B), and stronger staining of ground substance and collagen on the inner surface compared with (B). Stain: anti-Fas ligand; bright field. (D) Oblique section of same bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer. (c) Deeper fibrodegenerative layer. (d) Cavity. Note positively staining cells, blood vessel walls, and ground substance in (b) superficial fibroproliferative layer. Note absence of blood vessels and presence of positively staining cells, ground substance, and collagen fibrils in the (c) deeper fibrodegenerative layer and on the inner surface of the bleb capsule. Stain: anti-Fas ligand; bright field. (E) Oblique section of the same bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer. (c) Deeper fibrodegenerative layer with positively staining cytoplasm of cells, and markedly swollen cells. (d) Cavity. Stain: anti-FADD; bright field. (F) Oblique section of the same bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer. (c) Deeper fibrodegenerative layer. (d) Cavity. Note positively staining cells and ground substance in all layers. Stain: anti-caspase 8; bright field. (G) Oblique section of the same bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer showing positively staining cells. (c) Weakly staining deeper fibrodegenerative layer. (d) Cavity. Stain: anti-caspase 3; bright field.
FIGURE 4. Immunohistochemical staining of a 0.5-year-old bleb, the same bleb shown in Figures 1 and 3 (Tables 1, 2, and 4, case 2). Scale bars: 75 μm (A–E); 15 μm (F–M). (A) Oblique section of bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer. (c) Deeper fibrodegenerative layer. (d) Cavity. Arrow indicates congested blood vessels in (a) and (b). Stain: hematoxylin and eosin; bright field. (B) Oblique section of same bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer. (c) Deeper fibrodegenerative layer with positively staining cells concentrated around blood vessels and occasional positive cells in the deeper layers. (d) Cavity. Arrow indicates positively staining material in blood vessels. Stain: anti-Fas ligand; bright field. (C) Oblique section of same bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer with arrow indicating positively staining cells around blood vessels and decreasing numbers in the deeper layers and weak staining of contents of blood vessels. (c) Deeper fibrodegenerative layer. (d) Cavity. Stain: anti-FADD; bright field. (D) Oblique section of same bleb. (a) Tenon’s capsule. (b) Superficial fibroproliferative layer with arrow indicating positively staining cells around blood vessels and no staining of contents of blood vessels. (c) Deeper fibrodegenerative layer. (d) Cavity. Stain: anti-caspase 8; bright field. (E) Oblique section of same bleb showing (a) Tenon’s capsule. (b) Superficial fibroproliferative layer with arrow indicating positively staining cells around blood vessels and no staining of contents of blood vessels. (c) Deeper fibrodegenerative layer. (d) Cavity. Stain: anti-caspase 7; bright field. (F) Oblique section of same bleb. (b) Superficial fibroproliferative layer with arrows indicating positively staining cells and cell remnants. Stain: anti-Fas ligand; bright field. (G) Oblique section of same bleb. (d) Cavity. Stain: anti-FADD; bright field. (H) Oblique section of same bleb. (b) Superficial fibroproliferative layer with arrows indicating positively staining cells and cell remnants. Stain: anti-FADD; bright field. (I) Oblique section of same bleb. (c) Deeper fibrodegenerative layer with arrows indicating positively staining cells and cell remnants. Stain: anti-caspase 8; bright field. (J) Oblique section of same bleb. (b) Superficial fibroproliferative layer with arrows indicating positively staining cells and cell remnants. Stain: anti-caspase 8; bright field. (K) Oblique section of same bleb. (c) Deeper fibrodegenerative layer with arrows indicating positively staining cells and cell remnants. Stain: anti-caspase 8; bright field. (L) Oblique section of same bleb. (b) Superficial fibroproliferative layer with arrows indicating positively staining cells and cell remnants. Stain: anti-caspase 3; bright field. (M) Oblique section of same bleb. (c) Deeper fibrodegenerative layer with arrows indicating positively staining cells and cell remnants, and (d) cavity. Stain: anti-caspase 3; bright field.
4% to 22% of cells: nuclear chromatin condensation with nuclear and cytoplasmic blebbing, and formation of free cytoplasmic vesicles, while phagocytosis of apoptotic cell remnants was commonly observed in the outer fibroproliferative layer. In the deeper fibrodegenerative layer, most cells (approximately 90%) showed swelling, distortion, and blebbing, before cells disintegrated with the formation of numerous membrane-bound vesicles. Diffuse staining of ground substance and collagen fibers increased in intensity toward the deeper layers of the bleb. Polarizing light microscopy demonstrated birefringent collagen fibrils of normal appearance in the superficial fibroproliferative layer and loss of birefringence of collagen fibrils in the deeper fibrodegenerative layer.

Staining for Fas ligand stained the cytoplasm of numerous cells and free fragments of cytoplasm and membrane-bound vesicles in the deeper layers of the bleb capsule. This suggested 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 toward the superficial fibroproliferative layer where they were phagocytosed by metabolically active cells to induce apoptosis via the extrinsic apoptotic pathway.24,25

We were surprised at the findings of unexpected ongoing activity of cells, particularly in the deeper fibrodegenerative layers of the blebs, and that there was not a more marked difference between the blebs over time. However, the cells...
involved in this process were a small metabolically active subset of the capsule connective tissue cell population.²

This study depended on patients donating their eyes or tissues for research purposes before death or reoperation. Thus limitations of the study included the limited number of individuals and their families willing to donate eyes or tissue; the varying etiology of the glaucoma; different styles of Molteno implants; and operative and postoperative management, with specimens obtained at varying intervals after death. By its very nature, observations were limited to a single snapshot per specimen obtained at a single time point after operation. Because of the small numbers and variability of the cases, statistical analysis was not considered appropriate. However, the significance of the study was the demonstration that morphologically and immunohistochemically identified apoptotic cells were present in oblique sections of all capsules at all times after operation. This has not been demonstrated before. In the current state of knowledge, we have no convincing alternative explanation for these findings.

CONCLUSIONS

These findings demonstrated unexpectedly rapid turnover of cells in the superficial layer of the bleb capsule where most cells were efficiently phagocytosed by nearby monocytic cells, macrophages and histiocytes; while cells migrating into the deeper layers completed apoptosis and disintegrated with the release of collagenolytic enzymes and Fas ligand–positive presumed death messengers that were carried toward the superficial layers by aqueous.

Recent advances in our understanding of cell biology offer hope that improved methods for minimizing the fibroproliferative and enhancing the apoptotic fibrodegenerative responses of connective tissue to aqueous will be developed in the foreseeable future.

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