Aberrant Wound-Healing Response in Mitomycin C–Treated Leaking Blebs

A Histopathologic Study

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Objective: To characterize histopathologic features of leaking mitomycin C–treated blebs and aberrant wound healing that may lead to persistent conjunctival thinning and leakage.

Methods: Forty mitomycin C–treated filtering blebs excised for persistent leaks from 40 patients were examined histopathologically using hematoxylin-eosin, periodic acid–Schiff, Masson trichrome, and Alcian blue histochemical stains.

Results: Ninety percent of the leaking blebs contained epithelial-stromal domes with areas of acellular stroma covered by attenuated epithelium. Seventy-five percent of the blebs demonstrated varying degrees of fibrovascular repair growing from the bleb margin, either underneath or interdigitating with the acellular zone. A novel observation in 65% of specimens was Alcian blue–positive myxoid stroma at the interface between the fibrovascular proliferation and the epithelial-stromal dome. The association between the presence of fibrovascular proliferation and Alcian blue–staining myxoid stroma was significant by Fisher exact test ($P=.002$).

Conclusions: A desirable filtration bleb requires a sufficient reparative fibrovascular response to maintain an epithelial-stromal barrier to prevent leakage. Fibroblasts must lay down a continuous collagen-rich fibrous layer, rather than merely myxoid stroma, beneath the conjunctival epithelium to promote bleb stability. Surgical techniques and postsurgical care should aim to attain this desired outcome.

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METHODS

Forty MMC-treated leaking blebs, excised from 40 patients during routine bleb revisions, were examined. The average patient age of 51.4 years, male patient percentage of 55%, and minimal time from MMC-treated trabeculectomy to bleb excision of 1.8 years (range, 1.8-3.0 years) were all similar to those of previously reported clinical studies. The specific site of bleb leakage was not always available from the clinical history but single or multiple leaks were noted in all cases that persisted despite conservative management. The leakage was not always at the dome of the bleb. The study was approved by the institutional review board at the University of Michigan and the ethics committee at Sankara Nethralaya, Chennai, India.

All specimens were processed after sectioning through the center of the blebs to include a complete cross-section. The histopathologic features of the tissues stained with hematoxylin-eosin were studied by light microscopy and epithelial basement membranes delineated using periodic acid–Schiff reagent. Collagen and glycosaminoglycans in adjacent bleb tissue sections were identified using Masson trichrome and Alcian blue (pH 2.5) staining, respectively. In addition, 14 archival conjunctival specimens from the bulbar conjunctiva were studied as controls, using the same histochemical stains.

The presence and absence of fibroblast proliferation as well as results of staining with Masson trichrome and Alcian blue were recorded for each specimen. The Fisher exact test (GraphPad Software Inc, La Jolla, California) was used to test for the association between deposition of glycosaminoglycans and fibroblast proliferation.

RESULTS

In hematoxylin-eosin–stained sections, the leaking blebs demonstrated several characteristic epithelial and stromal changes. As previously reported, the vast majority of blebs (36 of 40 [90%]) demonstrated regions of severe epithelial attenuation, often reduced to a monolayer of cells, overlying poorly formed basement membrane and damaged conjunctival substantia propria in the domes of the blebs. In these areas, presumably corresponding to sites of clinical leakage, the damaged substantia propria formed attenuated, acellular subepithelial bands (Figure 1) that stained positively for collagen with Masson trichrome, but minimally for glycosaminoglycans with Alcian blue (Figure 2) and to a degree not greater than that present in normal bulbar conjunctiva (not shown). These acellular zones were periodic acid–Schiff–negative but variable degrees of epithelial basement membrane deposition were noted in these regions (Figure 3). Basement membrane formation was more evident in areas where greater surface maturation of the epithelium was present. In most specimens, the

Figure 1. Photomicrograph of bleb with hypertrophic scar (S) at its margin. Underlying the markedly attenuated epithelium are distinct zones, including thick acellular collagen (*), spaces lined by myxoid fibrillary material, and reparative fibrovascular tissue that extends toward the center (F; arrow) (hematoxylin-eosin, original magnification × 80). Inset shows the zones at higher magnification (original magnification × 200).

Figure 2. Photomicrographs of attenuated dome of a mitomycin C–treated bleb. A, Photomicrograph showing bleb in an area with attenuated epithelium and mostly acellular stroma (hematoxylin-eosin). B, Shown is an acellular subepithelial band that stains positively with Masson trichrome. C, The acellular stroma stains faintly with Alcian blue (all, original magnification × 200).
areas of damaged substantia propria demonstrated subtle changes suggestive of attempted repair and were surrounded by areas of glycosaminoglycan-rich myxoid stroma (Figure 4). We did not attempt to correlate the time between attempted clinical leak repair and bleb excision to the degree of these subtle changes, because attempts at leak repair were often poorly documented and all blebs were excised at least 1.8 years after the initial MMC-aided procedure.

Of the 90% of blebs that showed severe epithelial attenuation, 10 of 36 (27%) exhibited a total lack of healing response in the bleb domes. In these specimens, attenuated epithelium, fragmented basement membrane, and acellular stroma composed the entire domes of the blebs, indicating that the entire bleb dome was prone to leakage. Fibrovascular proliferation was absent except at the bleb margins, where hypertrophic scars were present as previously described. A few of these blebs exhibited Alcian blue–positive myxoid material near the bleb margins.

Most blebs (30 of 40 [75%]) (Table) demonstrated areas of repair that appeared as tonguelike projections of fibrovascular tissue growing from the periphery along the deep surface of and interdigitating into the acellular stroma of the attenuated regions (Figures 1 and 2). In zones adjacent to or surrounding the acellular band of substantia propria, varying degrees of myxoid, basophilic (bluish) tissue containing vacuolated spaces surrounded by fibrillary material were noted (Figure 4). The myxoid areas were positive with Alcian blue, but did not stain with Masson trichome. The Alcian blue staining extended into the fibrovascular projections (Figure 3). The pattern and degree of Alcian blue staining was substantially different than that seen in normal control conjunctiva in which faint Alcian blue staining was noted in a diffuse pattern throughout the stroma (not shown). The conjunctival epithelium overlying these areas was attenuated but stratified with more orderly maturation. Fibrovascular proliferation and glycosaminoglycan-rich myxoid stroma were present in 26 of 40 specimens (65%), and both were absent in 7 of 40 (17.5%) (Table); their simultaneous presence and absence (82.5%) in these particular areas correlated significantly by Fisher exact test ($P = .002$).

In blebs exhibiting proliferation and maturing fibrovascular scars away from the bleb margin, the acellular subepithelial zone was still visualized, but appeared thinned or fragmented. Alcian blue–positive myxoid stroma was no longer visible where fibrovascular tissue was mature, and neither it nor the damaged stromal remnants exhibited Alcian blue staining. The epithelium overlying these areas appeared normal in thickness and stratification (Figure 5). However, as previously reported, few goblet cells were noted when compared with the control tissue. Care was taken to distinguish maturing areas of fibrovascular scarring adjacent to epithelial and stromal thinning in the domes of the blebs from hypertrophic scars, sometimes containing chronic inflammatory infiltrates, at the margins of the bleb, as has been previously described.

In this large series of MMC-treated blebs, we describe several significant histopathologic changes in the tissue. The thin bleb domes showed an attenuated epithelium overlying an acellular zone of damaged stroma virtually devoid of fibroblasts. The acellular zones likely represented remnants of extracellular matrix of MMC-damaged substantia propria that were pushed against the attenuated epithelium by aqueous in the bleb. Though these bands morphologically resembled thickened basement membrane, they were consistently periodic acid–Schiff–negative and often discontinuous with the periodic acid–Schiff–positive epithelial basement membrane. The poor reparative response in these areas was either due to paucity of fibroblasts or fibroblasts too damaged to secrete

**Figure 3.** Photomicrographs showing relationships of epithelium, basement membrane, and acellular stroma. A, Photomicrograph showing moderately mature epithelium with continuous, moderately periodic acid–Schiff–positive basement membrane (arrows) with underlying periodic acid–Schiff–negative acellular material (*) extending into deep stroma (original magnification × 200). B, Photomicrograph showing attenuated epithelium with thick continuous basement membrane (arrows) and a thick band of periodic acid–Schiff–negative acellular material (original magnification × 400).
sufficient extracellular matrix to provide tensile strength to the bleb dome and prevent bleb leaks.

Other areas of the specimens illustrated how the fibroblasts in the substantia propria attempt to repair MMC-treated areas by secreting myxoid extracellular matrix similar to that produced during early wound healing.3,36 Areas adjacent to the leaking blebs, along the conjunctival substantia propria, contained varying deposits of glycosaminoglycan-rich, Alcian blue–positive myxoid extracellular matrix that were highly associated with the presence of fibroblast proliferation ($P = .002$). Bands of hypocellular, trichrome-stained collagen still lined the subepithelial area, suggesting that the MMC-damaged fibroblasts fail to secrete sufficient collagen to provide ten-

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**Figure 4.** Photomicrograph showing patterns of subepithelial fibrous proliferation. The thick subepithelial acellular band (A-F) shows varying degrees of Masson trichrome staining (C and D). Vacuolated empty spaces (*) in adjacent areas (A and B) stain strongly with Alcian blue (E and F, arrowheads) and negatively with Masson trichrome (C and D). These spaces are adjacent to the band of acellular fibrous tissue. The overlying epithelium appears attenuated yet stratified and preserved (all, original magnification $\times 200$).
sile strength to the bleb dome and prevent leaks. In areas of the bleb away from the margin that showed scar maturation, the Alcian blue–positive myxoid matrix was not visible and the overlying epithelium had regained normal thickness and stratification.

Another proposed cause of bleb leaks is that the attenuated epithelium is a dysfunctional barrier to leakage. Previous studies showed that leaking, MMC-treated blebs have thinned conjunctival epithelium and decreased the number of goblet cells.12,19,22 Our specimens also exhibited these features, but the epithelium was only attenuated over areas with a poor reparative fibrovascular response. In areas exhibiting collagen-rich fibrovascular repair, the epithelium was thickened. Because the tensile strength of the bleb does not lie in the epithelium but in the fibrous tissue of the substantia propria, leaks are likely due to the lack of functionally normal fibroblasts that secrete collagen beneath the epithelium. The lack of appropriate stroma also may impair growth factor signaling, leading to epithelial attenuation. We hypothesize that a threshold number of functioning fibroblasts is necessary to produce fibrovascular support for the bleb epithelium, creating an aqueous–tear film barrier that prevents bleb leakage.

Processes that occur during wound healing after surgical trauma are likely to occur following a trabeculectomy.3 Initially leukocytes and plasma proteins enter the wound from surrounding vessels, while injured tissue secretes factors to form a fibrin-fibronectin matrix.23,24 Various inflammatory cytokines stimulate angiogenesis, which begins with the appearance of capillary buds emerging from vessels at the wound margin. Fibroblasts proliferate from the angiogenic response and are the principal cells responsible for creating extracellular matrix, including glycosaminoglycans. This process produces the granulation tissue that begins to fill in the wound 1 week after injury. The fibroblasts, some of which differentiate into myofibroblasts, produce type III collagen, reinforcing the granulation tissue. Myofibroblasts express contractile proteins that assist in contracting the wound and facilitating reepithelialization of the wound surface. Subsequently, the fibroblasts remodel the wound by degrading the type III collagen with metalloproteinases and replace it with denser type I collagen. Myofibroblast surface integrins connect to the collagen bundles and compact them by generating contractile forces.22 The type I collagen molecules are then cross-linked by lysyl oxidase to produce a mature scar with increased tensile strength.24

Table. Contingency Table of Fibrosis and Myxoid Stroma in 40 Excised Leaking MMC Filtering Blebs

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<tr>
<th>Myxoid Stroma</th>
<th>Fibrosis, No. (%)</th>
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<tr>
<td>With</td>
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<td>With</td>
<td>26 (65)</td>
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<td>Without</td>
<td>4 (10)</td>
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Table: The association between the presence and absence of fibrosis with the presence and absence of myxoid stroma was statistically significant (P=.002) by 2-tail Fisher exact test.

Even in MMC-treated leaking blebs excised years after treatment, normal wound healing was evident in areas where epithelium with normal thickness overlay a collagenous fibrovascular layer that maintained aqueous inside the bleb.11 However, in areas adjacent to
those of attenuation and leakage, we often saw an excess of myxoid material with a dearth of collagen. Other studies have also shown an excess of Alcian blue–stained extracellular matrix material beneath the bleb epithelium.\textsuperscript{2,25,26} Mietz and colleagues\textsuperscript{2} also found that the scar tissue over the scleral flap in MMC-treated blebs was hypocellular and hypovascular with randomly oriented collagen fibers, suggesting that the tissue was arrested in the early phases of wound healing after drug application. They also observed absence of smooth muscle actin in the fibroblasts, indicating that they had not differentiated into myofibroblasts. Based on our collective results and these previous findings, we interpret that after MMC treatment, the fibroblasts undergo a compensatory response, attempting to heal the wound, though they are too few in number or too damaged to effectively produce scar tissue. They continuously secrete the myxoid matrix that usually occurs early in wound healing, attempting to close the wound with mechanisms used to produce granulation tissue, as they cannot effectively produce sufficient collagen. It is also possible that this healing process is reinitiated by the bleb leak itself, but this remains speculative because we did not have tissue from nonleaking MMC-treated blebs for comparison.

Hypotony maculopathy after MMC trabeculectomy has increased, with reported frequencies ranging from 2% to 14% in some case series.\textsuperscript{9,27} Hypotony maculopathy is reported even without a detectable bleb leak.\textsuperscript{9,12} Hypotony maculopathy is proposed to be the result of ciliary body toxicity,\textsuperscript{21,28} leading to decreased production of aqueous, or of the effect of MMC on bleb structure, which allows seepage of aqueous across the conjunctiva of the bleb into the tear film.\textsuperscript{12} The formation of an insufficient extracellular matrix in MMC-treated blebs may also explain why hypotony maculopathy occurs frequently and can even occur without a detectable bleb leak. It may be that aqueous is more easily filtered through the loose myxoid stroma\textsuperscript{29} of the bleb wall and into the tear film. The bleb leaks subclinically and appears Seidel-negative, while it is nonetheless seeping aqueous through microscopic areas of attenuated epithelium with underlying acellular or loose myxoid areas that lack sufficient fibrovascular or collagen support.

In conclusion, intraoperative MMC may deplete and damage fibroblasts, which then secrete a modified, myxoid extracellular matrix in leaking blebs. This matrix along with subepithelial acellular collagenous tissue does not provide adequate subepithelial support for a strong bleb dome. In contrast, functional, nonleaking blebs likely require epithelial domes reinforced by either a layer of undamaged substantia propria or mild subepithelial fibrosis derived from the bleb margin. The fibroblasts that cause bleb failure due to fibrosis are likely to be eclipteral in origin,\textsuperscript{5,30} since subepithelial fibrosis in functioning blebs exists without causing bleb fibrosis. Hwang and Kee\textsuperscript{31} developed a rabbit model of bleb leaks, in which the conjunctiva was repeatedly injected with MMC to show that cultured autologous fibroblasts are effective in treating bleb leaks. The successful treatment of leaking bleb domes by repopulating them with autologous fibroblasts supports the idea that a fibrovascular response from the substantia propria may be crucial in creating a nonleaking bleb. Thus, alternative surgical protocols that primarily treat episcleral tissue with MMC while minimizing substantia propria exposure and toxicity should be considered to decrease the risk of MMC-related bleb leaks while achieving the desired outcomes of low intraocular pressure and leak-free bleb morphology.

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REFERENCES


In 1795, Dr Isaac Thompson concocted an eye water of zinc sulfate, saffron, camphor, and rose water. It was sold as late as 1939. This is 1 of a series of 32 medical trade cards advertising the product from 1875 through 1895.

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