Meibomian Gland Dysfunction

II. The Role of Keratinization in a Rabbit Model of MGD

James V. Jester,* Nicholas Nicolaides,† Illdiko Kiss-Palvolgyi‡ and Ronald E. Smith‡

Meibomian gland dysfunction (MGD) was induced in 34 albino rabbits by the twice-daily topical application of 2% epinephrine over a period of 6 months to 1 year. Seven age-matched control rabbits, not receiving epinephrine, were followed up for a similar period. All lids were evaluated pre- and post-treatment by gross clinical examination and by transillumination biomicroscopy and photography. Of the 68 rabbit lids evaluated, 56% developed signs of MGD, which ranged from plugging of the meibomian gland orifice and presence of microcysts (subclinical lesions; 30.9% of the lids) to opacification and enlargement of the glands with increasing severity (clinical lesions; 25.0% of the lids). The remaining lids (44%) remained normal. MGD did not develop in the seven control rabbits. After the development of MGD, lids were evaluated by immunofluorescent microscopy, SDS-PAGE and Western blotting using mouse monoclonal antibodies to keratin proteins. Development and progression of MGD in the rabbit appears to correlate with increasing stratification and keratinization of the meibomian gland duct epithelium. In the early stages of MGD, focal areas of epithelial hyperkeratinization were identified by immunohistochemical staining using AE2 monoclonal antibody, specific for the 56.5 kD and 65–67 kD keratin protein marker for keratinized epidermis. As the severity of MGD progressed there was progressive increase in the AE2 staining of the duct epithelium. SDS-PAGE and immunoblotting of proteins from meibomian gland excreta in chronic MGD showed a progressive increase in both the 56.5 kD and 65–67 kD keratinization protein markers during development of MGD. We conclude that hyperkeratinization of the duct epithelium leading to plugging and dilation of the meibomian gland underlies the development of MGD following topical epinephrine treatment.

Recent studies suggest that keratinization of the meibomian gland may be important in the development of meibomian gland dysfunction (MGD) as it relates to lid margin disease. Gutgesell et al, in a histopathologic study of lid specimens from chronic blepharitis patients, identified areas of keratinization within the meibomian gland. Furthermore, abnormal keratinization of the meibomian gland has been shown in various animal models of MGD, including the rabbit epinephrine-induced MGD,2,3 the primate polychlorinated biphenyl-induced MGD,2,3 the primate polychlorinated biphenyl-induced MGD2,3 and the rhino mouse genetic MGD models. Both chronic epinephrine and acute polychlorinated biphenyl exposure appear to result in dilation of the meibomian glands, inspissation of excreta and keratinization of the meibomian gland duct.2,4 In the rhino mouse, abnormal keratinization appears to lead to plugging of the meibomian gland orifice and marked atrophy of the entire meibomian gland.7 Although keratinization appears to be a prominent feature of human and animal MGD, the effect of keratinization on meibomian gland function and its relation to lid margin disease has yet to be determined.

In an earlier report we evaluated the expression of keratin proteins as a differentiation marker for the duct epithelium in order to clearly establish the keratinizing nature of the meibomian gland.6 Previous studies have shown that keratin proteins, important cytoskeletal proteins of epithelial cells, are regulated by growth, development, differentiation and the cell environment.7–9 Furthermore, fully differentiated epithelial cells are known to express unique keratin proteins that may be considered molecular markers for specific differentiation pathways.10,11 Keratinized epithelial cells are known to express an acidic, 56.5 kD and a basic, 65–67 kD keratin protein pair. Ex-
pression of these keratins by epithelial cells has been shown to indicate the commitment of the epithelium to the process of keratinization. Our evaluation of human and rabbit meibomian glands using monoclonal antibodies AE1, AE2 and AE3, previously characterized by Sun et al., indicate that meibomian glands express an acidic, 56.5 kD (AE1-positive), and a basic, 65-67 kD (AE3-positive) keratin protein. Furthermore, suprabasal meibomian gland duct epithelial cells in the anterior and posterior portion of the gland immunostain with the monoclonal antibody AE2, which is specific for keratinized epidermis. Overall, these data suggest that the duct epithelial cells are committed to the process of keratinization.

In an effort to determine the role of keratinization in the pathogenesis of MGD we have evaluated the expression of keratin proteins in the epinephrine-induced rabbit MGD model. We present, herein, the changes in duct epithelial keratinization during the development of MGD as identified by immunofluorescent microscopy and SDS-PAGE using mouse monoclonal antibodies to keratin proteins. These data support our hypothesis that hyperkeratinization of the meibomian gland plays an important role in the development of MGD. Based on these data we present a unifying theory relating meibomian gland hyperkeratinization to meibomian gland dysfunction as it relates to lid margin disease.

**Materials and Methods**

**Meibomian Gland Dysfunction Model**

A total of 41 albino rabbits were used in the epinephrine-induced MGD study. All animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research. Thirty-four rabbits in three separate groups received 2% topical epinephrine hydrochloride treatment twice daily for a period of 6 months to 1 year. Seven age-matched rabbits served as controls and were followed up for the spontaneous development of MGD.

All rabbits were evaluated at bimonthly intervals by transillumination biomicroscopy according to techniques previously described. The presence of MGD was graded by transillumination biomicroscopy based on the presence of orifice plugging (Grade 1), development of microcysts (Grade 2), hypotransillumination of the meibomian gland duct (Grade 3), dilation of the meibomian gland (Grade 4), and swelling with gross enlargement of the meibomian gland (Grade 5). At the end of the observation period, animals were sacrificed and lid samples were obtained for either: (1) immunofluorescent; or (2) biochemical studies.

<table>
<thead>
<tr>
<th>Clinical grade</th>
<th>Biomicroscopy grade*</th>
<th>Treatment group</th>
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<tr>
<td>Normal</td>
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<td>Control</td>
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<td>Subclinical MGD</td>
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<tr>
<td>MGD</td>
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<td>Grade 5</td>
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Number of rabbits: 7

| Number of rabbits | 34 |

### Table 1. Development of meibomian gland dysfunction

**Immunofluorescent studies:** At the time of sacrifice, all lids were immediately resected, placed in OCT compound, frozen in liquid nitrogen and stored in an ultralow freezer (−80°C). Fresh frozen cryostat sections (8 μm) were evaluated by indirect immunofluorescence using mouse monoclonal antibodies AE1, AE2 and AE3, according to techniques previously described.

**Biochemical studies:** Protein samples from the meibomian glands of normal and MGD lids were obtained by hard expression of the lids using hemostats. The expressed material was collected using a clean spatula and the samples from individual lids placed in cold (4°C) buffer containing 25 mM Tris-HCl, pH 7.4, containing 1 mM EGTA, 1 mM EDTA, and protease inhibitors, including 1 mM PMSF, 5 μg/ml antipain and 5 μg/ml pepstatin. Samples from individual lids were processed and run individually on 12.5% SDS-PAGE gels according to techniques previously described.

### Results

**Development of MGD**

Daily application of topical epinephrine for 6 months to 1 year resulted in the slow, progressive development of MGD in 56% of the rabbit lids evaluated by transillumination biomicroscopy (Table 1, Fig. 1). The first change noted by biomicroscopy was the development of hypotransillumination defects within the anterior portion of the meibomian gland, suggesting obstruction and plugging of the orifice (Fig. 1B, Grade 1). Progression of MGD was marked by the development of discrete hypotransillumination defects (microcysts) deep within the meibomian gland (Fig. 1C, Grade 2). Occasionally, microcysts could be detected without the presence of an anterior transillumination defect, suggesting that plugging of
the orifice may not be necessary for the development of microcysts. Continued progression was marked by the extension of the transillumination defect to include the entire central portion of the gland (Fig. 1D, Grade 3). In some cases, lesions continued to enlarge with time, completely obscuring normal structural details of the gland (Fig. 1E, Grade 4). After 1 year of treatment, affected lids were grossly swollen by abnormal, enlarged and distended meibomian glands (Fig. 1F, Grade 5).

In the majority of rabbits, clinical examination without transillumination biomicroscopy revealed injection of the palpebral conjunctiva and signs of conjunctivitis with normal-appearing meibomian glands. By 6 months to 1 year, clinical evidence of MGD was noted only in 25% of the lids, representing those cases with advanced Grade 3–5 MGD (Table 1, Fig. 1). An additional 30.9% of the lids showing subclinical changes (Grades 1 and 2) were identified by transillumination biomicroscopy that went undetected by direct clinical examination.

MGD Keratinization

In a previous report we have shown that epinephrine-induced meibomian gland dysfunction results in the formation of what appear to be keratic cysts with dilation of the meibomian gland duct, retention of squamous epithelial cells and loss or atrophy of meibomian gland acini with minimal inflammation (Fig. 2A). Epithelium lining the central duct (L) showed increased stratification with the development of a stratum granulosum containing keratohyaline granules (arrow). Epithelium lining the short ductules leading to acini also appeared more prominent and there was an increase in the number of basal epithelial cells lining the acini (arrowheads). A characteristic feature of clinical MGD (Grade 3–5) is the presence of an epithelial plug at the orifice of the meibomian gland (Fig. 2B). Epithelial cells within these plugs, as well as those comprising the duct epithelium at the orifice, show intense staining with the AE2 mouse monoclonal antibody, greater than that seen in the epidermis (Fig. 2C). Since AE2 is a marker for keratinization, intense staining suggests that the duct epithelium surrounding the meibomian gland orifice is hyperkeratinized.

In clinical MGD, the meibomian gland showed a markedly thickened duct epithelium with retention of squamous epithelial debris within the lumen of the duct. In all cases evaluated, both the duct epithelium and the squamous debris showed marked staining with AE2 antibody (Fig. 3A). AE2 staining was also localized to suprabasal cells within the acini adjacent to the duct (Fig. 3A, arrow). Although in the normal meibomian gland, basal acinar cells may stain with the AE2 antibody, suprabasal staining in MGD suggests extension of the keratinization processes into the acinus.

In subclinical MGD, AE2 staining of Grade 1 MGD showed strong staining of the epithelium at the orifice, the lining of the central duct and the lining of the ductules leading to individual acini (Fig. 3B); however, normal meibomian gland orifice, central duct and ductule epithelium also stains with this antibody, albeit less intensely (not shown). Although biomicroscopy suggests the presence of enhanced keratinization at the orifice in Grade 1 MGD, the extensive keratinization normally present in this area limited our ability to detect any increased staining. Similarly, an increase in AE2 staining of the ductule epithelium could not be determined in this study. AE2 staining of Grade 2 MGD lesions characteristically identified areas of duct epithelial hyperkeratinization with thickening of the duct epithelium, as previously shown.3 For the most part, the location of epithelial hyperkeratinization correlated with the presence of microcysts as seen by biomicroscopy.

Localization of AE1 and AE3 Keratin Proteins

Alterations in the localization of AE1-recognized acidic keratin proteins and in AE3-recognized basic keratin proteins were seen in clinically evident (Grade 3–5) MGD. AE1 antibody, which normally stains the cortex of developing rabbit acinar cells, showed a decrease in the number of acinar cells staining with this antibody (Fig. 4A, B). Basal acinar cells as well as the next two to three layers of cells above the basal cells (Fig. 4B, arrows) did not stain at all with the AE1 antibody. This suggests a change in the expression of keratin protein during acinar cell development that may underlie the loss or atrophy of the acini in clinical MGD. AE1 staining of the duct...
Fig. 2. Light and immunofluorescent micrographs of clinical MGD in the rabbit lid. (A) Meibomian glands appear to develop markedly dilated glands containing desquamated cellular debris within the lumen of the central duct (L). Epithelium lining the central duct appears to show increased stratification and development of a stratum granulosum containing keratohyaline granules (arrow), which are not seen in normal meibomian glands. Epithelium lining the short ductules leading to individual acini appear more prominent while the basal epithelial cells lining the acini appear increased in number (arrowheads) (hematoxylin and eosin, X400). (B) The orifice of the meibomian gland appears hyperkeratinized and is filled with squamous cells leading to plugging the orifice (hematoxylin and eosin, X300). (C) AE2 staining of a Grade 3 lesion shows marked fluorescence of cells at the orifice of the meibomian gland (X300).

epithelium remained limited to the superficial epithelial cells.

AE3 antibody, which normally stains all duct epithelial cells and basal acinar cells, shows increased staining of the duct epithelium as well as increased staining of the basal acinar cells (Fig. 5). In some areas, the AE3 antibody appears to stain acinar cells above the basal cell layer.

Biochemical Identification of Keratin Proteins

Keratin proteins isolated from the excreta of normal and MGD rabbit meibomian glands were compared with rabbit ear epidermal proteins to identify the presence of 65-67 kD and 56.5 kD keratin protein markers for keratinized epithelia (Fig. 6). Although the presence of 56.5 kD and 65-67 kD keratin
proteins is detected in the whole meibomian gland from the normal rabbit, the analysis of samples from excreta routinely fails to detect the presence of keratinization markers (Fig. 6, lane 2). A 56.5 kD and 65–67 kD keratin protein was not detected in excreta from epinephrine-treated lids having normal meibomian glands as identified by biomicroscopy (Fig. 6, lane 3). On the other hand, meibomian gland excreta obtained from both subclinical (Grade 1 and 2) and clinical (Grade 3–5) MGD, blotted onto nitrocellulose and stained with AE1 and AE3 antibodies, contained a 56.5 kD, AE1-positive and a 65–67 kD, AE3-positive keratin protein, characteristic of keratinized epithelia (Fig. 6, lanes 4 and 5, respectively). Additionally, 56 kD and 58 kD, AE3-positive and a 50 kD, AE1-positive keratin proteins were resolved. The expression of the 50 kD and 58 kD keratin proteins is consistent for stratified epithelia. Although a
Fig. 4. (A) AE1 staining of normal meibomian gland shows staining of superficial duct epithelium within the lumen (L) of the duct and staining of all acinar cells (×189). (B) Central acinar cells in Grade 4 MGD stain with AE2 antibody; however, staining of basal acinar cells is not detected (arrows denote area of basal acinar cells). AE1 continues to stain only the superficial epithelial cells lining the lumen (L) of the duct similar to normal (×189).

Discussion

This study establishes that at least one of the earliest changes in epinephrine-induced MGD is full keratinization or hyperkeratinization of the meibomian gland duct epithelium. In the normal meibomian gland, keratinization can be identified histologically by the presence of keratohyaline granules and biochemically by the detection of a 56.5 kD and 65-67 kD AEl-positive keratin protein was not resolved, the 56 kD AE3-positive keratin may be related to the 56 kD and 48 kD keratin protein markers for hyperproliferation. Additional study using two-dimensional electrophoresis is necessary to characterize more completely these proteins. The 56 kD and 48 kD keratins would be expected if the duct epithelium were hyperproliferative, as may occur during hyperkeratinization.
Fig. 5. (A) AE3 staining of normal meibomian gland shows staining of basal acinar cells as well as duct epithelium (X189). In Grade 4 MGD, AE3 appears to stain more intensely the basal acinar cells as well as the duct epithelium (X189).

kD keratin protein. We have previously shown that the normal meibomian gland duct epithelium expresses a 56.5 kD and 65-67 kD keratin protein, indicating a commitment of the duct epithelium to the process of keratinization. However, the histologic scarcity of keratohyaline granules within the deeper portions of the meibomian gland suggests that the normal meibomian gland epithelium may not be fully keratinized.

In epinephrine-induced MGD the first changes that can be detected clinically are hypotransillumination defects within focal areas of the meibomian gland, including the orifice (Grade 1 MGD) and deeper portions of the gland (Grade 2 MGD). These defects appear to be related to the development of fully keratinized duct epithelium within areas of the meibomian gland duct. Using a monoclonal antibody (AE2) that recognizes the 56.5 kD and 65-67
kD keratin protein markers for keratinization,11,13 Grade 1 and 2 MGD show increased immunofluorescent staining of the duct and ductule epithelium that line the meibomian gland. As shown in an earlier report,2 the histologic identification of keratohyaline granules within the duct epithelium lining Grade 2 MGD microcysts indicates that these areas are fully keratinized.

The mechanism by which epinephrine induces hyperkeratinization of the meibomian gland duct epithelium is not known. However, SDS-PAGE and immunoblotting of keratin proteins from MGD excreta identified the presence of a 56 kD, AE3-positive keratin protein that is not present in protein extracts from normal meibomian glands or their excreta. Recent studies have linked the expression of a 48 kD, AE1-positive and 56 kD, AE3-positive keratin protein pair to epithelial hyperproliferation. As reported by Sun et al, epithelial cells taken from various proliferative skin disorders including callous, keratoacanthoma, actinic keratoses and carcinoma, characteristically express the 48 kD and 56 kD keratin protein markers for hyperproliferation. Although the 46 kD, AE1-positive keratin pair was not identified, our finding of the 56 kD, AE3-positive keratin protein suggests that epinephrine may induce duct epithelial hyperproliferation. Further study using two-dimensional gel electrophoresis is necessary to clearly characterize the presence of the 46 kD and 56 kD keratins, and additional studies using 3H-thymidine are necessary to clarify the relationship between hyperproliferation and hyperkeratinization in this model.

The effect of keratinization or hyperkeratinization of the meibomian gland duct epithelium on gland function is not known but may involve blockage or stenosis of the meibomian gland duct or orifice through various mechanisms. Plugging of the meibomian gland orifice has been demonstrated by scanning electron microscopy in both the rhino mouse and the epinephrine-induced rabbit MGD model.3,5 Initially we postulated that hyperkeratinization leading to thickening of the epithelium might constrict the orifice of the gland and result in stenosis and blockage.5 Although we were unable to identify any increased thickness of the duct epithelium by histologic or immunofluorescent techniques, increased keratinization at the meibomian gland orifice would appear to be a likely explanation, based on the translumination results.

Alternatively, development of a fully keratinized duct epithelium may result in increased desquamation of squamous cells into the lumen of the duct, leading to blockage of the meibomian gland orifice. Within the normal meibomian gland, few squamous cells are detected within the duct lumen, whereas during the development of MGD there is a distinct increase. Furthermore, evaluation of proteins from meibomian gland excreta indicates that MGD is associated with a marked increase in the presence of keratin proteins characteristic of keratinizing epithelium. In the normal meibomian glands the 56.5 kD and 65-67 kD keratin proteins are only detected in whole meibomian glands and not within the excreta.6 During development of MGD, 56.5 kD and 65-67 kD keratins are detected in increasing amounts beginning at Grade 2 MGD. The appearance of these proteins within the excreta during the early stages of MGD clearly indicates and supports the histologic and immunofluorescent observation that there is an increased contribution of duct epithelial cells within the meibomian gland excreta. This may have two effects: first, squamous cells may not pass through the meibomian gland orifice and mechanically block excretion. Second, the increased contribution of epithelial cells and cellular debris to the meibomian gland excreta may result in thickening or inspissation of excreta and inhibit flow of excreta from the meibomian glands.

Finally, increased keratinization of meibomian glands may affect acinar cell differentiation and, hence, gland function. During the development of MGD, basal acinar cells appear to stain more intensely with AE3 and less intensely with AE1 com-
pared to the normal meibomian gland. This pattern is similar to that seen in the duct epithelium where all epithelial cells stain with the AE3 antibody and only the superficial cells stain with the AE1 antibody. In addition, extension of AE2 staining into the suprabasal acinar cells in MGD also suggests that the acinus may be replaced by keratinizing duct epithelial cells. Although these immunofluorescent results do not establish a shift of the acinar cell toward epithelial differentiation, they suggest that during the development of MGD there is a change in the phenotypic expression of keratin proteins that may underlie a change in acinar cell differentiation. These changes may be the result of multiple factors, including hyperproliferation, hyperkeratinization, gland obstruction and de novo squamous metaplasia. Further study is necessary to evaluate clearly acinar cell differentiation during development of MGD and show how this may affect meibomian gland function.

Although keratinization of the meibomian gland clearly plays a role in the epinephrine-induced rabbit MGD model, the relationship of keratinization to human MGD and blepharitis remains to be determined. Previous studies by Ohnishi indicate that blepharitis associated with human polychlorinated biphenyl poisoning may be related to hyperkeratinization of the meibomian gland, as shown in a primate model. More recently, Gutgesell et al has identified areas of hyperkeratinization within meibomian glands taken from patients with blepharitis. Clearly, keratinization or hyperkeratinization of the meibomian gland duct may occur in human meibomian glands. We therefore propose that under conditions which lead to increased keratinization of the meibomian gland, blockage of the meibomian glands occurs.

In conclusion, our data support the hypothesis that meibomian gland keratinization or hyperkeratinization plays an important role in the pathogenesis of MGD. Furthermore, we propose that keratinization of the meibomian glands results in blockage of the gland, leading to gland dysfunction by decreasing the flow of excreta or lipid from the meibomian gland.

Key words: meibomian gland dysfunction, keratinization, chronic blepharitis, keratin proteins

References