The Eyelid Margin

A Transitional Zone for 2 Epithelial Phenotypes

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Objective: To investigate the differentiation profile of the epithelium of the eyelid margin in the primate.

Methods: The expression of cytokeratins (CKs) CK1/10, CK4, CK14, CK5/8, and CK19; involucrin; connexin 43; filaggrin; and muscarinic receptor subtypes (m1-m5) on eyelid tissues from adult Macaca fascicularis (n = 3) was studied by immunohistochemistry.

Results: Cytokeratin 1/10, CK4, CK19, filaggrin, and connexin 43 expression varied across the epithelium of the mucocutaneous junction (MCJ). At the MCJ, CK4-positive cells overlapped basal CK14-expressing cells. The meibomian gland duct and the MCJ revealed layers of CK14-positive cells. Cytokeratin 5/8 was expressed in the basal layer of the epidermis and conjunctiva, while involucrin-positive cells were superficial. The m2 was expressed throughout the conjunctiva; m3 was found in the basal cells of the skin, conjunctiva, and alveolar epithelial cells of the meibomian glands; and m4 was expressed in the suprabasal layers of the skin.

Conclusions: The eyelid margin epithelium is formed from 2 different epithelial cell subpopulations with specific but overlapping distributions. The meibomian gland duct or MCJ may be a site of conjunctival progenitor cells.

Clinical Relevance: This study provides a basis for understanding eyelid pathological conditions and eventually for developing methods for cellular reconstruction of the eyelid margin using expanded progenitor cells.

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The Eye lids and Lips are unique boundary tissues separating mucous membranes from the keratinized skin epithelium. Along the eyelid, the keratinized epithelium of the skin meets the tarsal nonkeratinized conjunctival epithelium at the mucocutaneous junction (MCJ) just posteriorly to the ducts of the meibomian glands. These tissue boundaries formed during development, separate dissimilar epithelial tissues, act as a barrier between these epithelial cells and mediate the interactions of the cells. Clinical observations suggest that the breakdown of tissue boundaries can lead to a dysfunctional interchange of dissimilar epithelia. In the cicatricial complications of Stevens-Johnson syndrome, the palpebral conjunctiva of the eyelid becomes keratinized.1,2 Conversely, the skin of the eyelid can take on a mucosal membrane phenotype following the breakdown of the MCJ in Stevens-Johnson syndrome or after extensive burns to the face and eyelids.3,4

A complex regulation must exist between these adjacent epithelial surfaces to ensure the stability of the overall morphology of the tissue boundary so that the MCJ has definable characteristics. However, it is not known how these 2 cell types interact to maintain a properly positioned tissue boundary that will prevent the skin epithelium overgrowing onto the conjunctiva or conjunctivalization of the skin. Elucidating the tissue architecture at the MCJ in terms of the cytokeratin (CK) profiles of the interacting epithelial surfaces would provide fundamental knowledge about the lineage of the interacting cells. This can also act as a template for understanding alterations in CK profiles in pathological conditions affecting the MCJ.

Keratin proteins compose the predominant cytoskeletal or structural component of epithelial cells. These proteins are critical for the maintenance of cell morphology and tissue stability, cell-to-cell communication, intracellular signal transduction, cell motility, and proliferation.3 In vivo, a basic keratin usually heterodimerizes or “pairs” with a particular acidic keratin.4 The expression of keratin pairs is tissue specific, differentiation dependent, and developmentally regulated.5 For example, CK1/CK10 is the major differentiation-specific keratin pair expressed by suprabasal epidermal epithelial cells, whereas the CK5/CK14 keratin pair has been implicated in the transition from suprabasal to basal cells in the epidermis.
Cytokeratin 4 and CK19 are expressed on the conjunctival epithelium. Cytokeratin 19 is also found in the bulge region of the outer root sheath of the hair follicle. Basal keratinocytes are undifferentiated and still capable of proliferation, and this cell compartment includes epidermal stem cells as well as transit-amplifying cells. These cells all express CK5/CK14 as their major, primary keratins. Cytokeratin 5/CK14 expression is down-regulated during differentiation and switched to CK1/CK10 in the skin epidermis.

There are various other structural proteins that define either the epithelial cell source or cell differentiation status. Filaggrin, a protein synthesized in the cells of the stratum granulosum, is important in the formation of cytokeratin bundles in cells of the stratum corneum of the keratinized epithelium. Connexin 43 is the major constitutive protein of gap junctions, which are involved in keratinocyte differentiation. Involucrin is a critical component of the cornified envelope, a protein sheath that coats the inner side of the keratinocyte cell membrane during terminal differentiation. It acts as a binding site for lipids that, during terminal differentiation, are extruded to form a water-insoluble barrier.

The conjunctiva is innervated by sensory, sympathetic, and parasympathetic nerves. Acetylcholine released from parasympathetic nerves stimulates a G protein–coupled membrane receptor found in the epithelial cells of the conjunctiva and goblet cells; activation of the muscarinic receptor will stimulate goblet cell secretion. The receptor is also expressed in the skin epidermis and is involved in modulation of keratinocyte proliferation, migration, cell differentiation, and cell-to-cell contact.

We studied the expression of cytokeratins, involucrin, connexin 43, filaggrin, and muscarinic receptors in the normal eyelid margin of the adult primate. An understanding of the dynamics of the interacting 2-cell phenotypes at this region could provide a basis for new approach to MCJ pathological conditions.

METHODS

MATERIALS

Mouse antihuman CK4, CK1/10, and filaggrin antibodies were purchased from Acris Antibodies GmbH (Hiddenhausen, Germany). Mouse antihuman CK14, CK3/8, and CK19
antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Mouse antihuman involucrin antibody was purchased from Sigma-Aldrich (St Louis, Mo). Mouse antihuman connexin 43 antibody was purchased from BD Transduction Laboratories (Lexington, Ky). Rabbit polyclonal antibodies against muscarinic receptor subtypes m1 through m5 were obtained from Research and Diagnostic Antibodies (Berkeley, Calif). Fluorescein isothiocyanate–conjugated antimouse and antirabbit secondary antibodies were purchased from Chemicon International (Temecula, Calif). Mounting media VectaShield containing DAPI (4,6-diamidino-2-phenylindole) was purchased from Vector Laboratories (Burlingame, Calif).

Eyelid tissues obtained from adult *Macaca fascicularis* (*n* = 3), killed for other purposes, were frozen in OCT Compound (Pelco International, Redding, Calif) and stored at −80°C. All experimental procedures conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

**IMMUNOHISTOCHEMISTRY**

Monkey eyelid tissues were cut at 6 µm and placed on polylysine-coated glass slides. The sections were fixed with 4% paraformaldehyde for 10 minutes, blocked with 4% goat serum in phosphate buffer solution (pH 7.4) for 30 minutes, and incubated with one of the following antibodies diluted in PBS with 4% goat serum at room temperature for 2 hours: CK1/10, 1:100; CK4, 1:10; CK19, 1:100; CK14, 1:100; CK5/8, 1:100; involucrin, 1:100; connexin 43, 1:100; and muscarinic receptor subtypes m1 through m5, 1:500. After washing with PBS, the sections were incubated with fluorescein isothiocyanate–conjugated proper secondary antibody for 1 hour at room temperature. Slides were mounted with VectaShield with DAPI as a counterstain. For negative controls, primary antibodies were omitted. The slides were examined with a Zeiss Axioplan 2 fluorescence microscope (Zeiss, Oberkochen, Germany).

**RESULTS**

**EYELID STRUCTURE**

Inspection of hematoxylin-eosin–stained sections showed that, as expected, eyelid structure was similar between monkey and human. Keratinized stratified squamous epithelial cells of the eyelid skin change to nonkeratinized cuboidal epithelial cells of the palpebral conjunctiva at the MCJ. The MCJ was located just posteriorly to the meibomian gland orifices (Figure 1A).

The eyelid skin epidermis was composed of 4 layers: the basal layer, the several-cell-thickness malpighian layer, the granular layer, and the most superficial keratin layer. Numerous hair follicles were part of the normal tissue structure (Figure 1B). Meibomian glands were prominent in the substance of the tarsus (Figure 1C). There were 10 to 15 alveolar structures per meibomian gland. Each gland contained a central duct whose orifice was located at the lid margin. The palpebral conjunctiva changed from the squamous epithelium at the MCJ to the columnar epithelium toward the fornix. It appeared from hematoxylin-eosin–stained sections and sections observed under phase contrast that goblet cells increased in number from the MCJ toward the fornix (Figure 1D).

**DIFFERENTIAL EXPRESSION OF EPITHELIAL CELL MARKERS AT THE EYELID MARGIN**

Cytokeratin 4, which differentiates conjunctiva epithelial cells from those of the cornea or skin, displayed a characteristic distribution. Positive staining for CK4 was seen along the superficial layers of the palpebral conjunctiva up to the MCJ (Figure 2A). However, staining extended to involve the full thickness of the conjunctival

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epithelium toward the fornix (Figure 2B and C). The basal layers of the conjunctival epithelium at the MCJ were not found to express CK4. Neither the skin epidermis nor cells of the meibomian gland expressed CK4. The difference in expression of CK4 at the MCJ indicates that the MCJ is a transition zone between 2 types of epithelial cells of different lineages.

Cytokeratin 1/10 was expressed in the suprabasal layers of the skin epidermis up to the MCJ as well as the superficial ductal epithelium of the meibomian gland (Figure 3A, B, and C). The conjunctival epithelium and basal layers of the skin epithelium were not found to express CK1/10 (Figure 3D). Similar to CK1/10 expression, filaggrin staining was positive in the superficial layers of the skin epidermis up to the MCJ along with the meibomian gland ductal epithelium (Figure 4A, B, and C).

Cytokeratin 19 was not expressed at the MCJ (Figure 5A); however, 1 to 2 mm posterior to the MCJ, all conjunctival epithelial cells were found to express CK19 (Figure 5B). The skin epithelium anterior to the MCJ did not stain positively for CK19 (Figure 5C).

In contrast, CK14 was expressed in cells of the basal epithelial layer of the epidermis (including the hair follicles), conjunctiva, and meibomian glands (both the duct and acini) (Figure 6). Within the skin epidermis, CK14-positive staining was confined to the basal layer (Figure 6A). However, the location of positive-staining cells was not consistent. At the MCJ and ductal epithelium of the meibomian gland near the orifice, CK14-positive staining was seen in 3 to 4 basal layers (Figure 6B). From the MCJ to the fornix, CK14-positive staining gradually reduced from 3 to 4 basal layers to just a single basal layer; eventually, CK14 expression was only found intermittently in basal cells (Figure 6C and D). Cytokeratin 5/8 staining was positive in the basal conjunctival epithelial layer and goblet cells (Figure 7A), as well as the basal layer of the skin epidermis (Figure 7B).

Involucrin, a marker for mature epithelial cells, was seen in the superficial epithelial layers at the MCJ (Figure 8A). Expression extended from the MCJ to both the conjunctiva and skin epithelia and was maintained in the superficial cell layers (Figure 8B and C).

Figure 3. Cytokeratin 1/10 (CK1/10) expression on the monkey eyelid. CK1/10 was expressed in the suprabasal layers of the skin epidermis (A) (original magnification ×100) up to the mucocutaneous junction (B) (original magnification ×100) and the superficial ductal epithelium of the meibomian gland (C) (original magnification ×200). The palpebral conjunctiva failed to stain positively for CK1/10 (D) (original magnification ×100).
Connexin 43, a marker for epithelial cell membrane junctions, was abundantly expressed in the suprabasal epithelial cells at the MCJ and skin (Figure 8D). Its expression was reduced in the granular layers and absent in the stratum corneum. Basal cells at the MCJ did not express connexin 43. The palpebral conjunctiva had little expression of connexin 43.

**Figure 4.** Filaggrin expression on the monkey eyelid. Filaggrin was expressed in the superficial layers of the skin epidermis (A) (original magnification ×100) up to the mucocutaneous junction (B) (original magnification ×100) and the meibomian gland ductal epithelium (C) (original magnification ×200).

**Figure 5.** Cytokeratin 19 (CK19) expression on the monkey eyelid. A, CK19 was not expressed at the mucocutaneous junction (original magnification ×200). B, CK19 was expressed in the full-thickness palpebral conjunctival epithelium about 1 to 2 mm posterior to the mucocutaneous junction (original magnification ×100). C, CK19 was not expressed in the skin epidermis (original magnification ×100).

**Differential Expression of Muscarinic Receptor Subtypes**

The m2 subtype was expressed throughout the layers of the conjunctival epithelium, including goblet cells, (Figure 9A) but was not detected in the skin epidermis. The m3 receptor staining was more specific for the...
basal layer of the palpebral conjunctival and alveolar epithelial cells of the meibomian glands (Figure 9B and C). Goblet cells were weakly stained with the antibody to the m3 receptor. The m4 subtype showed strong staining in the suprabasal layers of the skin epithelium and the duct of the meibomian gland (Figure 9D and E); however, the m4 subtype showed strong staining only occasionally on goblet cells of the palpebral conjunctiva (Figure 9F). Neither m1- nor m5-positive staining was detected in the skin epidermis or the conjunctiva (data not shown).

Expression of the proteins characterizing these epithelia is summarized in the Table. From our results, we conclude that the eyelid margin supports 2 types of epithelial cells that have specific distributions and different phenotypes. The MCJ is the site where these epithelial cell phenotypes interact.

**COMMENT**

This study has characterized some of the heterogenic protein expression in the epithelium at the eyelid margin where keratinized stratified squamous epithelial cells originating from the skin merge and interact with cuboidal epithelial cells of the palpebral conjunctiva. There are clinical situations that indicate that the breakdown in the regulation of these 2 epithelial cell phenotypes develops into diseases. This study has provided a basic description of epithelial cell interactions at the MCJ, and at the same time, it suggests the importance of a more detailed analysis of the progenitor cells supplying this site.

The conjunctival-specific differentiation marker CK4 was expressed in the superficial layers of the palpebral conjunctival epithelium. The epidermal-specific differentiation marker CK1/10 was expressed in the suprabasal layers of the skin. Cytokeratin 19 was expressed on the full-thickness conjunctival epithelium but not at the MCJ. Basal layers of both the skin and conjunctiva failed to express the differentiation markers (CK1/10 and CK4) but expressed CK14, which indicates that the basal cells are relatively primitive. There was a greater number of CK14-positive cells at the superior part of the meibomian gland duct and the MCJ compared with the basal layer of the skin and conjunctiva. It has been shown that skin epidermal stem cells are lo-
cated at the basal layer of the epidermis (unipotent stem cells) and in the bulge of hair follicles (multipotent stem cells).\textsuperscript{22-24}  

The location of conjunctival stem cells is controversial. Specific markers for ocular epithelial stem cells have not been found.\textsuperscript{25} Some studies have suggested that in mouse

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**Figure 7.** Cytokeratin 5/8 (CK5/8) expression on the eyelid (original magnification $\times 100$). CK5/8 was expressed in the basal layer of the conjunctival epithelium as well as goblet cells (A), while in the skin, staining was primarily in the basal epithelial layer (B).

**Figure 8.** Involucrin and connexin 43 expression on the eyelid (original magnification $\times 200$). Involucrin was seen in the superficial epithelial layers at the mucocutaneous junction (MCJ) (A), conjunctiva (B), and skin epithelium (C). Connexin 43 was abundantly expressed in the suprabasal epithelial cells of the skin at the MCJ (D). Its expression was reduced in the granular layers and was absent in the stratum corneum. Basal cells at the MCJ did not express connexin 43. The palpebral conjunctiva had little expression of connexin 43.
and rabbit conjunctiva, stem cells were essentially concentrated in the fornix; however, in cultivation of human conjunctival cells, the bulbar and fornical conjunctiva epithelia had similar proliferative capacity. Wirtschafter et al showed that cells at the MCJ rarely went through mitosis and suggested that the MCJ was the source of conjunctival stem cells and that their progeny migrated toward the fornix. Pe’er et al found similar findings. Our results showed that the ductal epithelium near the meibomian gland orifices and the MCJ serve as a reservoir for CK14-positive cells and that CK14-expressing cells decreased in number along the palpebral conjunctiva. This supports the idea that progeny cells of the meibomian gland duct or MCJ migrate toward the fornix.

**Figure 9.** Muscarinic receptor expression on the palpebral conjunctiva. The m2 subtype was expressed in the full-thickness conjunctival epithelium (A) (original magnification ×100). Positive staining for m3 was more specific for the basal layer of the palpebral conjunctiva (B) (original magnification ×100) and alveolar epithelial cells of the meibomian glands (C) (original magnification ×100). The m4 subtype showed strong staining in the suprabasal layers of the skin epithelium (D) (original magnification ×100) up to the mucocutaneous junction (E) (original magnification ×100); however, the m4 subtype was expressed only occasionally on goblet cells of the palpebral conjunctiva (F) (original magnification ×200).
The meibomian gland duct shares structural similarity with the hair follicle. Meibomian glands can transform into a hair follicle, which is seen clinically as an atopic production of vibsirenae (distichiasis).32-34 Thus, meibomian glands must contain multipotent cells. Previous research on the pancreas, prostate, and mammary gland has shown that the ductal epithelium serves as a pool of progenitor cells.35-37 Recently, Lavker et al38 suggested that the meibomian gland duct is a major site of epithelial stem cells that could give rise to both MCJ and palpebral conjunctival epithelium. However, whether meibomian gland ductal cells have the ability of self-renewal and long-term tissue reconstitution, a property common to all stem cells,22,39 remains to be determined. Meanwhile, cell signaling events between the epithelium and mesenchymal layers must play a major role in the coordination of the fundamental aspects of epithelial proliferation and differentiation at the MCJ.40,41

Protein markers have been found to be associated with stages of cell differentiation. In this study, filaggrin and involucrin were expressed in the superficial layers of the skin epidermis, indicating that the superficial layers are terminally differentiated keratinocytes. Consistent with the findings of other groups,42 connexin 43 was not seen in the basal layer of the epidermis, suggesting that those cells are less differentiated. Similarly, in limbal tissue, connexin 43 staining was absent in basal cells.43

We found that receptors m2 through m4 were expressed on monkey conjunctival epithelium and m3 and m4 were expressed on the skin epidermis. We did not find m1 expression in conjunctival cells, but m1 was reported in human conjunctiva.18 We did not find the m1 or m5 receptors in epidermal keratinocytes, but m1 and m5 expression was reported in human epidermis.44 The disparity in results may be due to species differences.

In conclusion, the eyelid margin mediates the interactions of 2 types of epithelial cells that have specific distributions and different phenotypes. Our results provide a basis for future studies on the morphology and protein expression profile changes at the eyelid margin in pathological clinical scenarios (eg, dry eye syndrome and meibomian gland and MCJ carcinomas).

The distribution pattern of cells with specific phenotypes supports the hypothesis that the lid margin may be a source of progenitor cells for the conjunctival epithelium. However, further studies will be needed to investigate the self-renewal and long-term tissue reconstitution ability of meibomian gland ductal cells.

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Table. Expression of Differentiation Markers in the Eyelid Margin

<table>
<thead>
<tr>
<th>Protein</th>
<th>Skin Epidermis</th>
<th>Muco-cutaneous Junction</th>
<th>Palpebral Conjunctiva</th>
</tr>
</thead>
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<tr>
<td>Cytokeratin 4</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Cytokeratin 1/10</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Filaggrin</td>
<td>−</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Involutrin</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
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<tr>
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<td>−</td>
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<tr>
<td>Muscarinic receptor 4</td>
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Abbreviations: ++, strongly positive; +, moderately positive; +, positive; −, negative.

REFERENCES