Cytokeratin Expression in Normal Human Bulbar Conjunctiva Obtained by Impression Cytology

Kathleen L. Krenzer*† and Thomas F. Freddo*‡

Purpose. To document the cytokeratin expression patterns in the normal human conjunctival epithelium obtained directly from patients using impression cytology.

Methods. Impression cytology specimens were obtained from normal volunteers using pure nitrocellulose membranes rather than cellulose acetate. The 31 volunteers of both sexes ranged in age from 18 to 79 years. Impression cytology specimens were analyzed for individual cytokeratins by either immunocytochemistry or electrophoresis with immunoblotting using a defined panel of monoclonal antibodies.

Results. Using the corroborative methods of immunocytochemistry and electrophoresis with immunoblotting, cytokeratins characteristic of nonkeratinized, stratified (K4 and K13), simple (K8 and K19), and glandular epithelia (K7) were present in the superficial layer(s) of normal human conjunctiva. Cytokeratins typical of keratinized epithelia (K1, K2, and K10) and the keratinization-related proteins filaggrin and involucrin were not expressed in normal conjunctival epithelium.

Conclusions. The normal human conjunctiva demonstrates a unique cytokeratin expression pattern containing cytokeratins characteristic of nonkeratinized, stratified epithelia, as well as others more typical of a simple differentiation pattern, a glandular differentiation pattern, or both. These findings provide a foundation for examining changes in the cytokeratin expression pattern in diseased human conjunctival epithelium using impression cytology.


The cytokeratins (CKs) are a heterogeneous class of approximately 30 structurally related polypeptides (Mr 40,000 to 70,000) that comprise the intermediate filament system characteristic of epithelial cells. Based on isoelectric charge, the CKs are subcategorized into neutral-basic (type 1) or acidic (type 2) groups. In vivo, the CK filament system is composed of types 1 and 2 obligate heterodimers that exist as specific pairs. Defined subsets of individual CK pairs are characteristically expressed based on epithelial cell and tissue type, level of differentiation, or disease state (for example, squamous metaplasia). Because of this differential expression, CKs have been studied extensively in many epithelia in developmental, normal, and disease states.

Among the mucosae, however, the human conjunctival epithelium has received comparatively little attention. A few investigators have examined CK expression in cultured human conjunctival epithelium, but the obvious limitation of these studies is that protein expression often can be altered in vitro. A few other investigators have examined human conjunctival CK expression patterns in tissues obtained from post mortem or enucleated eyes. These reports generally used broad-reacting α-CK markers and a limited number of specific markers, and/or they focused on the cornea-specific CKs—most of which were restricted to the perilimbal region (probably a unique subpopulation of cells). The result has been that the expression pattern of the CKs in the normal human bulbar conjunctiva has not been examined systematically.

Squamous metaplasia secondary to alterations in the microenvironment is associated with changes in CK expression in several mucosae. These include cervical, tracheal, oral, and esophageal mucosae. Histologically, squamous metaplasia is known to develop in conjunctiva with various ocular surface

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disorders (for example, dry eye conditions), and the conjunctiva most likely undergoes changes in CK expression similar to those of other mucosae. To probe for such changes in the human conjunctiva, it became clear that it was important to be able to assess CK expression from clinical specimens in the least invasive manner possible. Conjunctival impression cytology, a simple, noninvasive technique for obtaining a clinical sample of the conjunctival epithelium, offered promise in this regard.

Several investigators have used impression cytology for the documentation of ocular surface changes using general, nonspecific laboratory stains, such as periodic acid–Schiff and Papanicolaou, for evaluation. The subjectivity and the intralaboratory and interlaboratory variabilities inherent in interpreting any cytology specimen prepared in this manner remain as limitations. We have developed a protocol that allows sensitive and specific immunohistochemical stains to be performed on impression cytology specimens (ICSs). Additionally, we have developed a method to extract proteins from ICSs and to perform immunoblot analysis.

The goal of the current study was to use this new protocol to document the CK expression pattern in normal human conjunctival epithelium, focusing specifically on CK expression that might be expected to change in ocular surface diseases. Two other proteins involved in the keratinization process, filaggrin and involucrin, were studied for expression. Impression cytology specimens from normal volunteers were examined using a panel of commercially available monoclonal antibodies to selected CKs, filaggrin, and involucrin. Cytokeratin fractions were extracted from ICSs to perform sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analyses using a similar panel of CK markers. With these corroborative methods, we have documented the CK expression pattern in the normal human conjunctival epithelium.

**MATERIALS AND METHODS**

**Specimen Procurement**

Institutional review board-approved informed consent was obtained from each normal volunteer, and research was carried out according to the tenets of the Declaration of Helsinki. Criteria for participation included persons of either gender, 18 years or older, with no known external ocular disorders, no dry eye complaints, and no ocular surgery within the previous 6 months. Volunteers were enrolled if they had normal results of slit lamp evaluation and Schirmer’s test (with anesthetic) value >15 mm of wetting in 5 minutes. Impression cytology specimens were obtained as described by Nelson. In preliminary studies, staining ICSs with a standard avidin–biotin protocol using the conventional cellulose acetate filter membrane (Millipore, Bedford, MA) resulted in high levels of background staining that rendered interpretation impossible. Attempts to minimize this background by using different membrane materials were unsuccessful. To surmount this difficulty, a protocol was developed that involved dissolving the filter membrane, effectively transferring the specimen to a poly-L-lysine-coated glass slide. The best results were obtained using a pure nitrocellulose membrane (FMC Bioproducts, Rockland, ME) with acetone and cellulase as the solvents. When comparing the pure nitrocellulose to cellulose acetate, we found no apparent difference in the ability of the membranes to obtain quality specimens.

To obtain specimens, the ocular surface was anesthetized with proparacaine HCl 0.5%. After waiting 2 to 3 minutes, a 6 mm disc of pure nitrocellulose filter membrane was placed on the bulbar conjunctiva approximately 2 to 3 mm from the limbus. Uniform pressure was applied using an ophthalmodynamometer (Bailliart-type) with 40 g of pressure for 15 seconds. The specimen was peeled gently from the conjunctival surface and prepared for laboratory analyses. Four ICSs were obtained from each eye, one from each of the bulbar quadrants. For SDS–PAGE and immunoblot analyses, ICSs were obtained in an identical fashion.

**Immunocytochemistry**

For immunocytochemistry, ICSs were fixed with Spraycyte fixative (Clay–Adams, Parsippany, NJ). Each ICS was halved, and each half was placed specimen-side down on a separate poly-L-lysine-coated glass slide. After the ICS was dried completely, a drop of acetone was added to begin the dissolution of the filter matrix and to promote adherence of the cells to the slide. The ICS was again allowed to dry completely, then transported to the laboratory. Because each ICS was halved, eight halves from each eye were available for analysis. Monoclonal antibody treatment assignment was determined from a matrix designed to ensure that data representative of each marker were obtained from each quadrant.

Before immunocytochemical staining, each slide was placed in acetone for 1 hour with continuous agitation to dissolve the bulk of the filter membrane. After a 5-minute wash in tap water, the ICS was subjected to cellulase digestion (Sigma, St. Louis, MO) for 2 hours at 37°C (10 U/ml in 0.1 M acetate buffer, pH 5) to remove residual membrane material. The specimen was washed in tap water, and endogenous peroxidase activity was quenched with 3% hydrogen peroxide in 70% methanol for 5 minutes. After blocking nonspecific binding sites with a 4% solution of low-fat milk, the specimen was treated using a

* The blocking agent, primary antibodies, and secondary antibodies were diluted in a phosphate-buffered saline (pH 7.6)–0.05% Tween 20 (Fisher Scientific, Pittsburgh, PA) solution.
TABLE 1. Monoclonal Antibodies and Sources

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>CK (Moll Number)</th>
<th>Source</th>
<th>ICC</th>
<th>Immunoblot</th>
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</tr>
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CK = cytokeratin; NA = not applicable.
* Boehringer-Mannheim, Inc.

standard avidin–biotin protocol. Each ICS was incubated in an experimentally determined dilution of primary antibody (Table 1) overnight at 4°C. It was incubated sequentially in biotinylated—secondary antibody (1:200; Vector, Burlingame, CA), streptavidin—biotin complex (1:500; Dakopatts, Santa Barbara, CA) and dianaminobenzidine (0.6 mg/ml; Sigma) with thorough phosphate-buffered saline (PBS)–Tween-20 washes between each incubation. The specimen was counterstained in Mayer’s hematoxylin (Sigma), dehydrated, and mounted with Permount (Fisher, Fairlawn, NJ). For each run, ethanol-fixed, paraffin-embedded tissues with known reactivity for each antibody were included as positive controls, and one ICS served as a negative control (primary antibody exclusion). Impression cytology specimens were examined with a Leitz–Wetzlar photomicroscope (Ernst Leitz [Canada] Midland, Ontario, Canada). The ICSs were evaluated for the presence or absence of staining. An ICS was considered positive if unequivocal cytoplasmic staining was detected in two or more cells and if patterns of staining were noted. Results were analyzed for gender, age, and quadrant differences.

Immunofluorescence

To localize further the K7 (α-CK RCK105) staining, confocal imaging was used. Four ICSs were fixed with Spray-cyte (Clay–Adams), and each ICS was placed in a 0.5 ml Eppendorf tube. The ICSs were rehydrated in PBS. Nonspecific background staining was blocked with 4% low-fat milk. Primary antibody (α-CK RCK105) was added, and the ICSs were incubated overnight at 4°C. The next day, the ICSs were incubated sequentially in fluorescein isothiocyanate-conjugated horse antimouse immunoglobulin G (1:50; Vector, Burlingame, CA) and propidium iodide (Sigma) with thorough washes in PBS between each incubation. After the final wash, each ICS was placed cell-side up on a glass slide and was mounted with antifade media (Molecular BioProducts, Eugene, OR). One specimen served as the negative control (primary antibody exclusion), and a section of urinary bladder epithelium served as a positive control.² ICSs were examined with a Leica (Leica, Deerfield, IL) confocal microscope.

Extraction

For SDS–PAGE and immunoblot analyses, the four ICSs from one eye of each normal volunteer were pooled to ensure that there would be sufficient material for analysis. Proteins were extracted using a modification of the protocol described by Meiklejohn et al., which renders a protein fraction enriched in cyto-keratins. The soluble protein fraction was extracted in 1 ml of buffer A (10 mM Tris HCl, pH 7.2, 1.5 M KCl, 0.5% Triton X-100, 5 mM EDTA, and 0.4 mM phenylmethylsulfonyl fluoride [PMSF]). The ICSs

CK = cytokeratin; NA = not applicable.
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TABLE 2. Results of Cytokeratin Staining in Impression Cytology Specimens

<table>
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<th>N</th>
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<td>11/11</td>
<td>9/9</td>
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<td>Glandular CK</td>
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<td>α-CK RCK 105†</td>
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<td>6/11</td>
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<td>2/15</td>
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CK = cytokeratin.
* Uniform cytoplasmic staining.
† Staining concentrated around goblet cells.
‡ Patchy staining of single or small patches of cells.

RESULTS

Immunocytochemistry
ICSs for immunocytochemical analysis were obtained from 31 volunteers (12 men, 19 women) ranging in age from 18 to 79 years. At least one man and one woman were included for each decade of age. The findings of the immunocytochemical studies are summarized in Table 2.

Pan-epithelial Markers
α-CK AE 1,3 is a cocktail that detects a broad array of cytokeratins and, therefore, is considered a pan-epithelial marker. Positivity was seen in all specimens.
FIGURE 1. Immunocytochemical staining of impression cytology specimens from normal human conjunctival epithelium (diaminobenzidine with hematoxylin counterstain). (A) α-CK 1C7 (K13); (B) α-CK NCL 5D3 (K8, 18, 19); (C) α-CK RCK 105 (K7); (D) α-CK RKSE 60 (K10); (E) α-filaggrin; (F) α-involucrin.

demonstrating a uniform intracytoplasmic distribution (not shown).

Cytokeratin Markers of Nonkeratinized, Stratified Epithelia

α-CK 8.12 (K13 and K16) and α-CK 1C7 (K13) detect an overlapping cytokeratin spectrum. Both markers demonstrated reactivity in all specimens. Uniform cytoplasmic staining was noted in the epithelial cells; however, no staining was detected in the goblet cells (Fig. 1a; α-CK 1C7).

Cytokeratin Markers of Simple Epithelia

α-CK 4.62 (K19) and α-CK NCL 5D3 (K8, K18, and K19) detect an overlapping set of cytokeratins that are characteristic of simple epithelia. Most specimens were positive, demonstrating a diffuse cytoplasmic staining pattern (Fig. 1b, α-CK NCL 5D3).
Cytokeratin Marker of Glandular Epithelia

α-CK RCK 105 (K7), when positive, demonstrated a remarkable distribution of reactivity concentrated primarily in the immediate vicinity of the goblet cells (Fig. 1c). At the light microscopic level, we were unable to determine whether goblet cells, epithelial cells, or both reacted with this marker. However, subsequent analysis with immunofluorescence confocal microscopy demonstrated that this marker reacted only in the goblet cells (Fig. 2A), and no staining was present in the surrounding epithelial cells (Fig. 2B).

Cytokeratin Markers of Keratinization

α-CK AE 2, α-CK 8.60, and α-CK RKSE 60, considered markers for keratinized epithelium, were negative in virtually all the specimens (Fig. 1d, α-CK RKSE 60). In specimens in which positivity was noted, only a few scattered groups of cells were reactive, and expression was slightly more common in the nasal and temporal quadrants.

Other Markers of Keratinization

α-filaggrin and α-involucrin are two proteins involved in the formation of the cornified envelope during the keratinization process. These two markers were generally negative (Figs. 1e, 1f). In the few positive specimens, as with α-CK AE 2 and α-CK 8.60, only a few scattered cells were reactive. Again, positivity was detected in the nasal and temporal quadrants more commonly than in the superior and inferior quadrants. For all runs, positive and negative controls reacted appropriately. No age or gender differences in staining were noted.

Electrophoresis and Immunoblot Analyses

ICSs for SDS-PAGE analysis were obtained from six volunteers ranging in age from 23 to 61 years (three men, three women) (Fig. 3). Within the reported cytokeratin molecular range of 40 to 70 kDa, three major bands—41, 54, and 59 kDa—were common to all specimens. Based on previously reported molecular weights, these roughly correlate with K19, K7, K8, or K13, and K4 or K5, respectively. Two other bands were variably present in the 42 and 62 kDa region. The latter most closely correlates with K5, a cornea-specific keratin that has been reported to be in the conjunctival epithelium, whereas the former does not correlate with any reported cytokeratin molecular weight.

Specimens from four of the normal volunteers were used for immunoblot analysis, and all demonstrated similar staining patterns. For each marker, the results from one of the volunteers is shown in Figure 4. Between the two pan-epithelial markers, α-CK 8.13 and α-CK AE 1.3, four major bands in the cytokeratin molecular range were detected (Fig. 4, lanes C and D). Probing with α-CK RKSE 60, which reacts with K10, a CK characteristic of keratinized epithelium, revealed no reactivity (Fig. 4, lane E).

Treatment with α-CK 1C7, a marker that detects K13, revealed strong reactivity with the 54 kDa band (Fig. 4, lane F). Similarly, reaction with the monoclonal antibody that is specific for K13's basic partner, K4, revealed strong reactivity with the 58 kDa band (Fig. 4, lane G). This marker, however, also demonstrated weak cross-reactivity with the 54 kDa band.

α-CK 19, a marker for simple epithelium, showed specificity for the 41 kDa band (Fig. 4, lane H). Another marker for simple epithelium, α-CK 8, demonstrated reactivity in the 54 kDa region (Fig. 4, lane I). An antibody against K18, the acidic pair of K8,
FIGURE 3. Silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cytokeratin (CK) extracts from impression cytology specimens. For each subject, four impression cytology specimens were obtained and pooled. The CK fraction was extracted from six persons, separated using a minigel electrophoresis apparatus, and revealed by a silver stain. (a) Molecular weight standards; (b) 28-year-old woman; (c) 36-year-old woman; (d) 61-year-old man; (e) 57-year-old woman; (f) 22-year-old man; (g) 30-year-old man.

demonstrated reactivity in the 56 kDa region (Fig. 4, lane J). However, the reported molecular weight of K18 is in the 45 kDa range.2

To assess whether this was a case of cross-reactivity or aberrant migration, this marker for K18 was tested against a cytokeratin extract from liver, a tissue known to contain K18 (Fig. 5, lane D).2 The marker strongly detected a band at 45 kDa. In an adjacent lane, this marker again reacted with the 56 kDa region in the cytokeratin extract from impression cytology specimens (Fig. 5, lane E). There was no reactivity in the 45 kDa region, indicating that, at least in its native form, K18 does not exist in the superficial layers of the normal human conjunctiva.

Reaction with α-CK RK5 105, the marker for K7, revealed weak positivity in the 54 kDa (Fig. 4, lane K) region, correlating with the molecular weight for that CK reported by Moll et al.2 The α-CK CKB1 antibody (K14) did not react with any of the bands (Fig. 4, lane L). The K5/K14 heterodimer is considered a marker for the basal layers of various epithelia. As impression cytology obtains cells from the superficial strata, the absence of stain for K14 in the immunoblot analysis is considered a basal distribution.

DISCUSSION

The current study documents unique mergers of existing methods to obtain basic information of clinical relevance on the biology of human conjunctiva. Specifically, immunocytochemical and immunoblot methods were modified to document the normal pattern of cytokeratin expression in the superficial strata of the normal human bulbar conjunctival epithelium by using impression cytology,20 permitting clinically relevant interpretation of the results. We chose to examine CKs because their differential expression may give clinically useful information on the state of the surface of the eye.

Immunoblot analysis served to clarify and/or con-
Cytokeratins in Normal Human Conjunctiva

97.4 42.7 40.0 21.5

FIGURE 5. Immunoblot analysis of K18 with liver extract control. The cytokeratin (CK) fractions from liver, known to contain K18, and from conjunctiva were analyzed concurrently for K18 using an α-CK K18 marker. Molecular weight standards (a), liver extract (b), and conjunctival extract (c) stained with India ink. Liver extract (d) and conjunctival extract (e) immunoblotted for K18.

firm the findings of immunocytochemistry. Because the ICSs were pooled, no conclusions could be made beyond noting the presence or absence of a particular CK. We have not confirmed whether immunoblot analysis could be performed on a single ICS, but clearly this would open several new avenues for investigation.

On the basis of the nonkeratinized, stratified architecture of the conjunctival epithelium, it was reasonable to expect a CK expression pattern similar to that reported in other nonkeratinized, stratified mucosae, including cervical,17–19 tracheal20 oral,21 and esophageal22–24 epithelia. Indeed, the presence of the K4/K13 pair, characteristic for this pattern of epithelial differentiation, correlates well with reports in other tissues and electrophoretic data in the conjunctiva from the perilimbal area.18

The general absence of the keratinization-related CK peptides, K1, K2, K10 and/or K11, filaggrin24,41 and involucrin,24,41 is consistent with the nonkeratinized, stratified differentiation pattern in the conjunctiva. Our observation of patchy positive areas with these markers has been reported in other mucous membranes24,41,44,53. It has been suggested that the sporadic expression of these proteins in nonkeratinized epithelia is the result of focal inflammatory responses to environmental stresses.53 Consistent with this theory, expression of these proteins was observed more commonly in the exposed nasal and temporal quadrants.

Though considered a marker for simple epithelia, K19 has been noted in other human nonkeratinized, stratified epithelia, including esophageal,25 corneal,13,16 and limbal and perilimbal epithelia.13,14 However, the presence of K8 was surprising because this CK, along with K12 and K19, is found in abundance in the simple epithelia and is not considered typical of stratified epithelia. K8 pairs with K18 in vivo,26 but a lack of detectable K18 suggests that K8 may be pairing with K196 in the conjunctival epithelium.

The additional presence of K7 in the goblet cells (GCs) of the human conjunctiva also was unexpected because this CK is characteristic of glandular epithelia (for example, apocrine glands).2 This finding is reconcilable if one considers that the GC is a unicellular gland and that it may share features with multicellular glands. K7 has been noted in the GC-containing pseudostratified epithelium of the trachea.2 However, the GC-containing mucosa of the intestinal tract did not contain K7 by two-dimensional gel electrophoresis.2 This suggests that perhaps the GCs of the conjunctiva are related more closely to the GCs in the respiratory epithelium than those in the intestine. A similar pattern of staining for K7 was noted in the conjunctival GCs of rabbits.55 This specific distribution suggests that K7 may play a role in GC function. Perhaps the structure of the K7 filament is more malleable, permitting deformation of the cell during extrusion of mucous. Alternatively, perhaps the K7 filament actually interacts with contractile elements, facilitating extrusion of the mucous granules. Regardless, the specific staining of GCs for K7 may be valuable for study of non-mucous-containing GCs.

Despite the inability to detect K14, it is most likely present in the conjunctival epithelium because this CK, along with K5, is a marker of the basal layer in stratified epithelium, and both have been demonstrated by two-dimensional gel electrophoresis of full-thickness perilimbal epithelium.19 The conjunctiva is often involved in patients with epidermolysis bullosa simplex, which appears to be caused by point mutations in the K14 gene.60,61 Therefore, the K5/K14 pair is most likely present in the basal cells of the conjunctival epithelium.

This pattern of CK expression indicates that the human conjunctiva is a composite epithelium having characteristics in common with simple and glandular epithelia, yet demonstrating a nonkeratinized, stratified architecture. The conjunctival epithelium is the only stratified epithelium that contains GCs. Other stratified mucous membranes acquire their mucous from submucosal glands. Conversely, all other GC containing epithelia have either a simple or a pseudostra-
tified architecture. The unusual CK expression pattern in the conjunctiva reflects its unique morphology among mucous membranes.

As with other mucosae, the conjunctival epithelium can undergo changes in its state of differentiation, an example of which is the squamous metaplasia that accompanies the ocular surfacing disorders.25–29

In other stratified mucosae, squamous metaplasia is accompanied by a change in the CK expression pattern.17,20,21,41,53,58–60 We think that similar changes in the expression of these proteins may take place in the conjunctival epithelium as a result of the various dry eye disorders.

Impression cytology offers a simple and relatively noninvasive method for acquiring sequential samples of the surface conjunctival epithelium directly from patients.30 The novel combination of impression cytology and immunocytochemistry permits the simultaneous examination of ICSs for immunoreactivity and morphologic detail, facilitating discrimination of staining patterns. Using this method, objective data can be generated about the ocular surface of the living human eye. Having established the CK expression pattern in the normal human conjunctiva using this method, it should be possible to investigate whether changes in CK expression occur in the squamous metaplasia that accompanies various ocular surfacing disorders.

Key Words
conjunctiva, cytokeratin, human, impression cytology, immunocytochemistry

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References

Cytokeratins in Normal Human Conjunctiva


