A New Epithelial Cell Type in the Human Cornea

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Purpose. To study the expression of intermediate filaments in the human cornea.

Methods. Light and electron microscopic and immunohistochemical studies were performed on 20 corneas from subjects of various ages.

Results. A hitherto unrecognized epithelial cell population emerged from the immunohistochemical studies. Epithelial cells were invariably present in the superior cornea, whereas the nasal, temporal, and inferior segments almost lacked these cells. They were situated at the transition between peripheral cornea and limbus, and occurred as small groups in the basal epithelium. On electron microscopy, they were recognized by their marginated nuclear chromatin, large nucleoli, prominent bundles of intermediate filaments, and numerous hemidesmosomes and desmosomes. Immunohistochemistry on frozen sections revealed a unique intermediate filament make-up: ie, strong co-expression of vimentin and cytokeratin 19; other intermediate filaments, including cytokeratins 3, 4, 6, 7, 8, 10, 13, and 18 were negative. Finally, the cells lacked ultrastructural and immunohistochemical features of melanocytes, neuroendocrine cells, Langerhans’ cells, and leukocytes.

Conclusions. A new epithelial cell type in the human cornea is described with characteristic morphologic and immunohistochemical features. According to their particular segmental distribution, restricted localization at the junction between cornea and limbus, and expression of an “early” intermediate filament profile, it is tempting to speculate that they represent stem cells of the human cornea. Further studies are aimed to characterize their phenotype and function more extensively. Invest Ophthalmol Vis Sci 1993; 34:1983–1990.

 Intermediate filaments (IF; diameter between 7 and 11 nm) can be divided into five major subclasses on the basis of chemical composition, immunoreactivity, and cell type of origin.1 Although their function is not fully defined, they are believed to play a role in differentiation or functional specialization state, histogenesis, intracellular transduction of signals, and malignant transformation.2,3

The five subclasses of the IF1-47 include the cytokeratins (CK), that occur in various epithelia; the vimentin filaments, found in mesenchymal cells; desmin, present in muscle fibers; neurofilaments, typical of neuronal cells; and glial fibrillary acid protein, found in glial cells.

The CK are the largest and most complex group within the intermediate filament family and are characteristic of epithelial cells. Using two-dimensional gel electrophoresis, Moll et al5 identified 19 different cytokeratin polypeptides. These can be divided into an acidic (type I) and a (neutral to) basic (type II) subfamily.5,8 CK are usually expressed as polypeptide pairs consisting of type I and type II keratins.8 All human epithelia can be classified according to their CK content.5 Using two-dimensional gel electrophoresis, corneal epithelial cells have been found to express CK5, along with CK pair 3/12, which appears to be specific for the corneal-type of differentiation.5,8,9,10,12 However, as such biochemical techniques might fail to de-
tect CK polypeptides that are expressed in only a sub-
population of cells, the application of immunohisto-
chemical techniques could be useful to obtain a more
accurate picture of CK distribution, and to gain more
insight into the differentiation stage of some epithelial
cells (for example, see reference 13).

In this study, we investigated the expression of
various CK and vimentin in the human corneal and
limbal epithelium, using in situ immunohistochemical
techniques on frozen sections and a panel of monoclo-
onal antibodies. Previous studies\textsuperscript{14-21} raised the possibility
that in fixed material CK are antigenically altered
with less labeling affinity for the antibody, so unfixed
frozen material was used. Moreover, superior, infe-
rior, nasal, and temporal regions of human corneal
and limbal epithelium were studied separately to de-
termine whether topographic differences could be
seen. This report focuses on a hitherto unrecognized
epithelial cell population that emerged from our im-
munohistochemical studies. The light and electron mi-
croscopic and immunohistochemical characteristics of
this new epithelial cell type in the human cornea are
discussed in detail.

MATERIALS AND METHODS

The research followed the tenets of the Declaration of
Helsinki and was approved by the institutional human
experimentation committee.

Ten pairs of adult human eyes (age range, 50–80
yr) were obtained postmortem by enucleation. The
cornea and adjacent sclera were excised from the
whole globe. From each pair, one cornea was cut hori-
zontally into two equal parts to include nasal and tem-
poral regions in the tissue sections, and the other cor-
nea was cut vertically into two equal parts to include
superior and inferior regions. The corneal halves were
then snap-frozen in liquid nitrogen-cooled isopen-
tane, and stored at $-75 ^\circ\text{C}$ until studied further.

For immunohistochemistry, 5 $\mu$m serially cut fro-
zened cryostat sections were dried overnight at room
temperature, fixed in absolute acetone for 10 min,
and stained with a three-step avidin/biotin peroxi-
dase-labeled complex procedure. Rehydrated slides
were incubated for 30 min with the mouse monoclonal
antibodies listed in Table 1. The secondary and ter-
tiary antibodies consisted of biotin-conjugated rabbit
antimouse immunoglobulin and the avidin/biotin per-
oxidase-labeled complex, respectively, which were
both purchased from Dakopatts A/S (Copenhagen,
Denmark). All incubations were carried out for 30 min
at room temperature, then washed in three changes of
phosphate-buffered saline at pH 7.2 for 15 min. The
reaction product was visualized by incubation for 10
min in 0.05 M acetate buffer at pH 4.9, containing
0.05% 3-amino-9-ethyl-carbazole and 0.01% $\text{H}_2\text{O}_2$,
resulting in bright-red immunoreactive sites. The
slides were faintly counterstained with Harris’ hema-

\begin{table}[h]
\centering
\caption{Monoclonal Antibodies Used in This Study}
\begin{tabular}{lll}
\hline
Antibody (Clone) & Specificity & Source* \\
\hline
Intermediate filaments & & \\
6B10 & CK4 & Organon \\
LP34 & CKs 6 and 18 & Dakopatts \\
RCK 105 & CK7 & Organon \\
M20 & CK8 & Organon \\
RKSE 60 & CK10 & Organon \\
IC7 & CK13 & Organon \\
RPN 1165 & CK19 & Amersham \\
AE5 & CK5 & Lorei and Paesel \\
K 4.62 & CK19 & Sigma \\
PK-V & Vimentin (58-kDa protein) & Labsystems \\
RPN 1102 & Vimentin (54-kDa protein) & Amersham \\
Neuroendocrine markers & & \\
LK2H10 & Chromogranin A & Biotest \\
SY38 & Synaptophysin & Sigma \\
Other markers & & \\
HMB45 & Melanoma cells & Enzo Diagnostics \\
OKT6 & Langerhans’ cells & Ortho Pharmaceutical Systems \\
Dako-LC & Leukocytes & Dakopatts \\
Anti-S-100 protein (polyclonal) & Melanocytes, Langerhans’ cells & Dakopatts \\
\hline
\end{tabular}

* Location of manufacturers: Organon Teknika NV, Turnhout, Belgium; Dakopatts A/S, Glostrup,
Denmark; Amersham Corporation, Amersham Buck, England; Sigma Chemical Co., St. Louis, MO;
Labsystems BV, Waddinxveen, The Netherlands; Lorei and Paesel GmbH, Frankfurt, Germany;
Biotest Seral, Brussels, Belgium; Enzo Diagnostics, New York, NY; Ortho Pharmaceutical Systems,
Raritan, NJ.
\end{table}
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Hematoxylin. S-100 protein was detected with a three-step unlabeled peroxidase-antiperoxidase method using polyclonal rabbit antiserum S-100-antibody. This was followed by swine-antirabbit immunoglobulin and rabbit-peroxidase-antiperoxidase complex, both purchased from Dakopatts A/S. Controls, which were invariably negative, consisted of omission of primary or secondary antibody and use of chromogen alone.

For electron microscopy small parts of peripheral corneal and limbal regions from the superior corneal segment were fixed in glutaraldehyde, postfixed in O₂O₄ and embedded in epon. The fragments were taken from two persons: one eye, enucleated for malignant melanoma in a 65-yr-old woman who had not undergone previous radiotherapy, and one eye, enucleated for retinoblastoma in a 2-yr-old girl.

RESULTS

Observations on hematoxylin and eosin-stained sections of corneal tissue clearly demonstrated the central, peripheral, and limbal corneal regions. The junction between corneal and limbal epithelium was determined by the termination of Bowman's membrane and the appearance of underlying stromal blood vessels.

The results of the immunohistochemical staining revealed a particular distribution of CK19 and vimentin on the corneal and limbal epithelium. CK19 showed a heterogeneous distribution pattern throughout the corneal epithelium. The central cornea was entirely negative for CK19, whereas the peripheral cornea displayed a mosaic-like pattern of irregularly distributed immunoreactive cells. In the limbus, the basal epithelial cells were uniformly positive for CK19, whereas the suprabasal cell compartment exhibited a patchy, heterogeneous staining pattern. In both peripheral and limbal cornea, the most superficial cell layer often displayed a rather uniform positivity for CK19. In the transition zone between peripheral cornea and limbus, a distinct and readily identifiable subset of basal epithelial cells, still belonging to the peripheral cornea proper, demonstrated intense immunoreactivity for CK19 (Figs. 1 and 2). These CK19-positive cells were present in the superior cornea of all eyes examined, whereas their occurrence in the inferior parts was much more variable (present in 4 of 10 cases); the nasal and temporal regions were generally devoid of these cells. In the superior cornea, they occurred as one or more compact clusters, composed of 5 to 10 cells, that frequently assumed an elongated, columnar shape (Fig. 2A). In four cases, smaller clusters and singular cells with similar histologic and immunohistochemical features were found among the basal cells of the midperipheral and central cornea. Retrospective examination of hematoxylin and eosin-stained sections from the superior cornea revealed that in comparison to adjacent basal epithelial cells, these clusters were composed of smaller cells with crowded nuclei and prominent nucleoli. Electron microscopy, performed in two cases, revealed their dense margination of nuclear chromatin, large nucleoli, very prominent bundles of IF and large numbers of hemidesmosomes and desmosomes (Figs. 3 and 4). No age-related changes in the form and structure of these cells could be observed. Both on light and electron microscopy, these small and dark basally located cells could be distinguished from the surrounding larger, pale epithelial cells (Figs. 1C, 3, and 4).

When serial sections were stained with anti-vimentin, mouse monoclonal antibodies, no immunoreactivity could be observed in the limbal and corneal epithelium, except on scattered Langerhans' cells in the limbus and on small clusters of cells (Figs. 1 and 2) at the transition zone between peripheral and limbal cornea.

FIGURE 1. Serial frozen sections of superior limbal and corneal epithelium, stained with (A) CK19, (B) vimentin, and (C) hematoxylin and eosin. (A) CK19 is distributed in a mosaic-like pattern in the peripheral cornea and limbus. Basal cell layer in the limbus, and clusters of basally located cells at the junction between peripheral cornea and limbus (arrowheads), are strongly stained. Arrow indicates termination of Bowman's membrane. (B) Vimentin-reactivity is restricted to the same clusters of basal epithelial cells in the peripheral cornea (arrowheads) and limbus. Vascular endothelium in the limbus and keratocytes are also immunoreactive. (C) On hematoxylin and eosin-staining, the clusters of basally located epithelial cells can be discerned (arrowheads). Three-step avidin/biotin peroxidase-labeled complex-technique for (A) CK19, and (B) vimentin, counterstained with Harris' hematoxylin; (C) hematoxylin & eosin.

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FIGURE 2. Serial frozen sections of superior limbus and cornea, stained with (A) CK19 and (B) vimentin. Clusters of basally located cells (arrowheads) are distinctly vimentin-positive (B) and CK19-positive (A). Their elongated, columnar shape can be observed. Arrow indicates termination of Bowman's membrane. Three-step avidin/biotin peroxidase-labeled complex technique for (A) CK19 and (B) vimentin, counterstained with Harris’ hematoxylin.

Comparison of CK19 and vimentin staining in consecutive serial sections confirmed the co-expression of both types of intermediate filaments on the same clusters of cells.

Further studies on the phenotype of these cells at the transition between peripheral cornea and limbus revealed that they were negative for synaptophysin and chromogranin A, two markers of neuroendocrine cells. Similarly, no immunoreactivity was observed on these clusters of cells for Leucocyte-Common-Antigen (Dako-LC, Dakopatts A/S, Glostrup, Denmark) used as marker for leukocytes, OKT6 (marker for Langerhans' cells), and for HMB 45 and S-100 protein (marker for melanocytes).

In addition, the staining pattern of some other CK was tested. It appeared that CK 6, 7, 8, 10, and 18 were unreactive with the corneal and limbal epithelium. CK4 stained only the upper third of limbal, peripheral, and central corneal epithelium. CK13 was not present in the central cornea. It stained the superficial cell layer of the limbal and peripheral corneal epithelium, however. The clusters at the transition between peripheral cornea and limbus were negative for CK 4, 6, 7, 8, 10, 13, and 18.

Additionally, and like the limbal basal cells, they were negative for AE5, a mouse monoclonal antibody directed against CK3. The remaining corneal epithelial cells appeared to be positive for AE5.

DISCUSSION

The human cornea is lined by a noncornifying stratified squamous epithelium. Thoft and Friend\(^22\) proposed an "X,Y,Z hypothesis of corneal epithelial maintenance" in which the desquamated cells (Z component) are continuously replaced not only by the basal cells (X) that divide but also by cells that migrate in from the periphery (Y). The source of the Y-compartment is believed to be the stem cells located in the basal epithelial layer of the limbus. Several data support this evidence. Cotsarelis et al\(^23\) demonstrated the existence of a population of limbal basal cells that are normally slow to cycle, but can be preferentially stimulated to proliferate by a tumor promotor or by the physical removal of central corneal epithelium. Schermer et al\(^12\) showed that in cultured rabbit corneal epithelial cells CK3 (64 kD) and CK12 (55 kD) are characteristic of suprabasal cell layers and suggested that these two keratins may be regarded as molecular markers for an advanced stage of corneal epithelial differentiation. When the mouse monoclonal antibody AE5 against CK3 (64 kD) was used on frozen sections, they found that CK3 is located suprabasally (as expected) in limbal epithelium, whereas it can be detected in the entire thickness of central corneal epithelium. Their results suggest that, as far as keratin expression is concerned, limbal basal cells are less differentiated than corneal epithelial cells and might represent an early, stem cell compartment. Similar data concerning the human cornea were obtained by Rodrigues et al.\(^9\) Finally, Ebato et al\(^24,25\) showed that under explant culture conditions, human limbal epithelial cells grew much better than peripheral and central corneal epithelial cells. All these data have led to the current view that limbal basal cells may correspond to the stem cells of the cornea.

During our study on the expression of intermediate filaments in the human cornea, a hitherto unrecognized epithelial cell population engaged our attention. Morphologically, these cells appeared as clusters of basally located cells, situated at the transition zone between peripheral cornea and limbus, but still belonging to the peripheral cornea proper. On hematoxylin and eosin stained sections, they were considerably smaller than surrounding epithelial cells and their crowded nuclei contained prominent nucleoli. They frequently had an elongated and columnar shape. On electron microscopy, they were easily recognized by their margined nuclear chromatin, large nucleoli, very prominent bundles of IF and large numbers of hemidesmosomes and desmosomes. These cells corresponded to epithelial cells because they lacked ultrastructural and immunohistochemical features of melanocytes, neuroendocrine cells, Langerhans' cells, and...
leukocytes. Immunohistochemistry on frozen sections revealed an "early" IF profile on these cells, that is, strong co-expression of CK19 and vimentin. CK19 exhibited a mosaic-like pattern in the peripheral cornea and additionally a strong and homogeneous reaction pattern on the small clusters of cells and on the limbal basal cell layer. This finding is analogous to most other types of nonkeratinizing stratified squamous epithelia, for example, exocervix, vagina, tongue, oral mucosa, and esophagus epithelium, where CK19 is expressed by the regeneratory basal layer. The staining pattern of CK19 in the human cornea therefore provides some evidence that not only the limbal basal cells, but also the described clusters of peripheral basal cells may represent potential stem cells. The mosaic-like pattern observed in the suprabasal compartment of limbus and peripheral cornea might indicate retention of proliferative (stem cell) potential by CK19-positive suprabasal cells. Similarly, the occasional presence of similar CK19-positive cells among the basal cells in the middle part of the human cornea (4 cases of 10) could suggest a delay in commitment to terminal differentiation of cells migrating toward the central cornea. These data corroborate the X and Y movement of the “X,Y,Z hypothesis” of Thoft and Friend.

In contrast to the limbal basal cells, which were vimentin-negative, the described clusters of peripheral basal cells exhibited strong immunoreactivity for vimentin. Staining for vimentin in the adult human corneal epithelium has not been reported previously, with the exception of a report by Kasper et al. They mentioned the presence of a few predominantly basally located vimentin-positive epithelial cells situated at the transition between peripheral and limbal cornea in “some” of their examined tissues (probably superior regions).

Comparison of serially cut immunostained sections confirmed the co-expression of CK19 and vimentin on the same clusters of cells. Co-expression of two different IF in some normal cells has already been reported. In nearly all these instances, vimentin is one of the filament types expressed, and its presence in normal epithelial cells is thought to be related to proliferation. Van Muijen et al. found that in primary cultures of keratinocytes, marked expression of vimentin is found in the most proliferative compartment, whereas it is absent in differentiating keratinocytes. In addition, co-expression of CK and vimentin may be regarded as a transient event during development. Indeed, when comparing fetal and adult human tissues, co-expression of cytokeratins and vi-
mentin is more frequently observed in fetal tissues than in adult specimens.\textsuperscript{32} As such, vimentin could be a marker of less mature epithelial cells and its expression could be down-regulated in more differentiated cells.\textsuperscript{33} Previous experimental and immunohistochemical data have yielded circumstantial evidence that corneal stem cells are localized in the limbus.\textsuperscript{9,12,24,25} The clusters of cells observed in the current study may either represent stem cells, migrating from the limbus toward the central cornea that acquire, for an as yet unknown reason, vimentin IF. Or, they may correspond to a second stem cell compartment, whose co-expression of CK19 and vimentin could be regarded as a transient event during development\textsuperscript{32,37,38,39} or be related to their high proliferative potential.\textsuperscript{33,34,35,36}

Indeed, stem cells are known to be slow cycling, but have a great proliferative potential.\textsuperscript{33}

Analyzing superior, inferior, nasal, and temporal corneal segments, we provided some additional information about a possible regional heterogeneity in limbal and corneal epithelia. Indeed, the clusters of cells were invariably present in the superior part of the cornea. They were much more variably observed in the inferior part, whereas the nasal and temporal segments lacked them. These observations, anticipating that these cells belong to a stem cell compartment, could be in agreement with previous results describing limbal characteristics to be more pronounced in the superior cornea than in the other regions. Indeed, Wiley et al\textsuperscript{40} analyzed limbal epithelial staining characteristics by using mouse monoclonal antibody AE5 against CK3 (64 kD)\textsuperscript{12} and AE1, an antibody against a subset of acidic keratins.\textsuperscript{41,42} AE5 is known to be present in the entire corneal epithelium and only in the suprabasal layer in the limbus. AE1 has a staining pattern somewhat complementary to that produced by AE5: it does not react with the basal cells of the central cornea but reacts with the apical cells. In the limbus it is present on the basal and suprabasal cells. Wiley et al\textsuperscript{40} found limbal epithelial staining characteristics (basal cells AE5-negative but AE1-positive) to extend more deeply into the peripheral cornea proper in the superior and inferior regions and only marginally in the medial and lateral regions. The presence of these AE5-negative (and AE1-positive) basal cells in the peripheral cornea may correspond to the described subsets of cells exhibiting co-expression of CK19 and vimentin. Indeed, frozen sections stained with mouse monoclonal antibody AE5 directed against CK3 revealed no immunoreactivity on the described subsets of cells in the peripheral cornea and on the limbal basal cells. Similarly, immunolocalization of the monoclonal antibody 4G10.3, characterized by Zieske and Bukusoglu,\textsuperscript{43,44} against a 50 kD protein present in limbal basal cells, extends beyond the origin of Bowman’s membrane. Clinically, this suggests that the superior cornea might be endowed with more stem cells than other corneal regions. This has important implications in processes of wound healing and reepithelialization and suggests that diseases or processes affecting the superior corneal region would be particularly harmful.

In conclusion, we have described a new epithelial cell type in the human cornea with characteristic morphologic and immunohistochemical features. According to their particular distribution, their restricted localization at the junction between basal peripheral and limbal cornea and their expression of an ‘‘early’’ IF-profile (co-expression of CK19 and vimentin and negativity for AE5), we suggest that they may have stem cell characteristics. Further studies will be carried out to characterize their phenotype and function in more detail.
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Key Words
human cornea, stem cell, cytokeratin, vimentin, intermediate filaments

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References


