Cell Lineage and the Differentiation of Corneal Epithelial Cells

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Purpose. Studies were designed to determine whether cell division and cell differentiation are linked directly in the corneal epithelium. To obtain these data, corneal basal epithelial cells were labeled during DNA synthesis, and the resultant daughter cells were followed for as long as 2 weeks.

Methods. Adult rats were injected with 5-bromo-deoxyuridine (BrdU), a thymidine analog, and were killed 6 hours to 14 days later. Corneas were fixed and permeabilized, and BrdU-labeled nuclei were detected with a monoclonal antibody to BrdU and a fluorescent-labeled secondary antibody. Fluorescent nuclei were visualized in three dimensions in corneal whole mounts using a laser scanning confocal microscope. Optical sections were collected and displayed as serial images, three-dimensional anaglyphs, color-encoded projections, or three-dimensional reconstructions. Data were confirmed using 3 H-thymidine autoradiography of epithelia sectioned parallel to the corneal surface.

Results. Cells synthesizing DNA at the time of injection incorporated BrdU into their DNA. Pairs of labeled nuclei were produced by the division of cells that had been labeled with BrdU. These daughter cells remained in the basal layer of the epithelium for a variable period of time. Some daughter cells continued to divide, producing clusters of labeled basal cells. When labeled daughter cells left the basal layer and began the process of terminal differentiation, they nearly always did so together. The synchronous differentiation of daughter cells was evident from the pairs of labeled nuclei seen throughout the depth of the epithelium from 2 to 14 days after labeling.

Conclusions. Cell division and differentiation are not linked directly in the corneal epithelium. After cell division, daughter cells either remain in the basal layer, where they may undergo additional rounds of cell division, or both cells differentiate synchronously. When the daughter cells of a mitosis differentiate, the time between the previous cell division and differentiation is highly variable. This suggests that the coordination of cell division and differentiation in the corneal epithelium involves a complex regulatory network. Investigative Ophthalmol Vis Sci. 1996;37:1815–1825.
ply stated that the preservation of cell numbers in the corneal epithelium results from the addition of new cells to the basal layer by cell division (X) and by the migration of new cells from the periphery (Y). The accumulation of these new cells is balanced by the loss of cells from the corneal surface (Z). That is, in the normal cornea, \( X + Y = Z \). Thus, epithelial defects were caused by decreased cell migration and/or proliferation, or by an increased rate of desquamation.5

In the past decade, a great deal has been learned about the proliferation and differentiation of corneal epithelial cells. We now know of several growth factors that stimulate corneal epithelial proliferation or wound healing.3–12 However, because many of these studies have been conducted using cultured corneal epithelial cells or they have been conducted in animals with wounded corneas, we do not know which of these growth factors is important in the normal cornea in vivo. There is now considerable evidence that new basal epithelial cells enter the cornea from a slowly proliferating stem cell population residing in the corneal limbus.13–16 In addition, the migration of basal epithelial cells from the peripheral region of the cornea toward the center has been observed directly in the living human eye.17 Finally, information is accumulating concerning the events that occur during the maturation of corneal epithelial cells.18–20 The end product of this maturation is the terminally differentiated epithelial cells that are desquamated from the surface of the cornea.21

What is lacking now, as it was when Thoft and Friend formulated the X, Y, Z hypothesis in 1983, is an understanding of the control points in these processes. We do not know what regulates the number of basal cells entering the cornea from the limbal stem cell pool, what controls the proliferation of basal cells in vivo, or what controls the terminal differentiation and desquamation of epithelial cells. Information about these control points is critical to an understanding of homeostasis in the corneal epithelium.

The aim of our work has been to define better the events that occur when a cell becomes committed to leave the proliferating pool in the basal layer and to begin the process of terminal differentiation. We examined the kinetics with which cells differentiated by "pulse-labeling" proliferating basal epithelial cells in vivo with the thymidine analog, 5-bromo-deoxyuridine (BrdU). BrdU is incorporated into the DNA of cells preparing for mitosis. Soon after they incorporate BrdU, these cells divide to form two BrdU-labeled daughter cells. BrdU-labeled cells were identified among the majority of unlabeled cells using monoclonal antibodies to BrdU and fluorescent-labeled secondary antibodies. We followed the fate of labeled cells in corneal wholemounts at successive times after labeling using three-dimensional confocal microscopy. This method permitted us to locate labeled cells in the full thickness of the epithelium. These data were confirmed by \(^{3}\text{H}\)-thymidine autoradiography.

**MATERIALS AND METHODS**

All methods conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats weighing 300 to 350 g each were injected intraperitoneally with BrdU (200 mg/kg; 20 mg/ml, Sigma, St. Louis, MO) in sterile Ham's F-10 tissue culture medium between 10 and 11 AM and were killed by pentobarbital overdose 6 hours, or 2, 3, 4, 8, or 14 days later. Because BrdU photosensitizes DNA, rats were maintained in dim light and were protected further from excessive light exposure by partial covering of their cages with an aluminum foil tent. Previous studies showed that, with this dose of BrdU, blood levels peaked before 2 hours after injection and declined substantially by 6 hours.22 Whole corneas with small amounts of scleral ring were dissected and fixed overnight at 4°C in 10% neutral-buffered formalin. In preparation for BrdU antibody staining, corneas were treated with 2 N HCl and 0.5% Triton X-100 for 30 minutes, followed by an additional 30 minutes in 2 N HCl–Triton X-100 containing pepsin (1 mg/ml; Sigma). Tissues were washed and stored at 4°C in phosphate-buffered saline (PBS) containing 0.02% sodium azide. Whole corneas were reacted with 200 \( \mu \)l of a monoclonal antibody to BrdU (clone B44; Becton Dickinson, San Jose, CA) at a dilution of 1:20 in 1% bovine serum albumin, 0.5% Tween-20 in PBS overnight at 37°C; washed extensively in several changes of PBS with gentle agitation; exposed to rhodamine-labeled, Fc-specific, goat anti-mouse IgG F(ab')2 fragment at a 1:20 dilution in PBS–bovine serum albumin–Tween-20 (#T1659; Sigma) overnight at 37°C; washed extensively in PBS–Tween-20; and stored at 4°C in PBS–azide. These relatively high antibody concentrations were used to assure that sufficient reagent penetrated the tissue. In subsequent experiments not included in this study, lower antibody concentrations (1:100) were equally effective in labeling BrdU-containing nuclei. In our first studies, the nasal pole of each cornea was marked with a suture to permit identification of the nasal–temporal and dorsal ventral axes of the cornea. No differences in cell behavior were observed in the quadrants of the peripheral cornea. Therefore, corneas were not marked in later experiments.

Corneas were incised with four radial scalp cut and flattened on microscope slides. Tissues were mounted in PBS using two layers of double-sided cellophane tape as spacers between the slide and the coverslip and examined with a Bio-Rad MRC 600 (Bio-Rad, Sunnyvale, CA) or a Zeiss LSM 410 (Carl Zeiss, Thornwood, NY) using excitation and barrier filters appropriate for rhodamine fluorescence. Epithelia
were sampled by collecting serial images, spaced 2 μm apart, through the full thickness of the epithelium at five locations—the center of the cornea and in each quadrant of the peripheral cornea. Each image was Kalman averaged at least eight times during collection. The serial optical sections (Z-sections) were processed using the software supplied with the confocal microscopes and viewed as three-dimensional anaglyphs. These images showed the position of all BrdU-labeled nuclei in the region of the corneal epithelium under analysis. Using this method, labeled nuclei appeared to be suspended within the epithelial volume, against a low level of background fluorescence. The location of each labeled nucleus in the epithelium, with respect to the basal layer, could be observed readily. Serial sections also were viewed as projections, with the depth of an object in the epithelium represented by a color scale. Epithelial volumes also were viewed as serial reconstructions. Serial optical sections were collected at 0.18 μm intervals with the x and y resolution adjusted to assure that volume elements (voxels) were cubic and were volume rendered using Voxel View software (Vital Images, Fairfield, IA) running on a Silicon Graphics (Mountain View, CA) Iris Indigo workstation.

**Counting Cell Pairs**

Our data indicated that, in many cases, the two daughter cells of a mitosis began the process of terminal differentiation at the same time and that they differentiated synchronously as a cell pair. To determine the extent of synchrony between daughter cells, we tabulated the number of pairs of labeled nuclei present in epithelial volumes at 2, 3, or 4 days after labeling. In these analyses, single labeled nuclei at the edge of a volume were not counted because it is possible that they were paired with a nucleus that was out of the field of view. Single labeled nuclei at the surface of the epithelium were counted as single cells, even though it is possible that the other member of a pair recently had been desquamated. Cells were scored as members of a cell pair when their nuclei lay within two nuclear diameters of each other and when the staining properties of their nuclei were similar (see Results and Table 1). The frequency of cell pairs was not tabulated at 8 and 14 days after labeling because successive rounds of cell division in the basal layer made it difficult to identify cell pairs in this layer. However, cell pairs were present throughout the more superficial layers of the epithelium at these later times.

**Labeling Corneas With 3H-Thymidine**

Five microliters of 3H-thymidine (30 Ci/mmol; DuPont NEN, Boston, MA) were placed on the conjunctiva beneath the lower lid of each eye using a microliter pipette.24 Rats were killed 3 days later as described above. Corneas were incised with eight radial scalpels, flattened between two thin sponges, fixed in neutral-buffered formalin, and embedded in paraffin by standard procedures. Serial 5 μm sections were cut parallel to the surface of the cornea, collected onto glass slides, deparaffinized, and dipped into NTB-2 autoradiography emulsion (Eastman Kodak, Rochester, NY). Slides were exposed for 1 to 2 weeks before they were developed. Sections were lightly stained with cresyl violet, coverslipped, and photographed. Attempts to align serial sections to perform three-dimensional reconstructions were unsuccessful because of the distortion that occurred during sectioning.

**RESULTS**

The position of labeled nuclei in the different layers of the epithelium was determined by reference to several landmarks. Background fluorescence decreased sharply when the plane of optical section passed from Bowman’s membrane into the epithelium. The basal epithelial cells located at this boundary could be identified by their small, regular size, as revealed by the low level of background fluorescence. As sections passed through more superficial layers of the cornea, the flatter, broader wing cells occupied two or three layers above the basal cells. Near the epithelial surface, the flattened superficial cells could be seen. These cells often had the highest background levels of staining, possibly because of the higher concentration of keratins in their cytoplasm. Background fluorescence again dropped as the plane of focus passed from the superficial epithelial cells to the surrounding medium. For purposes of this article, a cell was considered to have begun the process of terminal differentiation when it left the basal layer (detached from the basal lamina) and began to enter the wing cell layer.

Six hours after BrdU injection, labeled nuclei were located in the basal layer of the epithelium. Some cells were dividing, and BrdU-labeled chromosomes...
FIGURE 1. Confocal fluorescence micrograph of a late anaphase mitotic figure in the basal epithelium of the rat cornea. The chromosomes are stained with 5-bromo-deoxyuridine (BrdU). The boundary of the enlarged mitotic cell is outlined by background fluorescence. The rat from which this cornea was optioned was injected with BrdU 6 hours earlier. The fixed cornea was immunostained for BrdU as described in Materials and Methods. Bar = 10 μm.

Nuclei with these distinctive patterns of labeling could easily be recognized in the corneal epithelium. Adjacent nuclei were only scored as members of a cell pair when both cell had the same labeling pattern.

Two days after BrdU labeling, nearly all basal cells with labeled nuclei were members of cell pairs. Figure 3 shows a single optical section through the basal cell layer. In this section, most of the cells are in pairs. The few single nuclei seen in this section were shown to be members of a pair by moving the focal plane up or down slightly from the one shown (data not shown). Few labeled mitotic figures were seen in the basal layer at this time. Labeled nuclei were present in the wing cell layers but not in the most superficial layers of the epithelium (data not shown). Cell pairing can be appreciated more in the three-dimensional images in Figures 4 and 5 and in the serial optical sections in Figure 6.

FIGURE 2. Confocal fluorescence micrograph of 5-bromo-deoxyuridine (BrdU)-labeled basal epithelial cell nuclei 6 hours after injection of BrdU. (A) The nuclei of basal epithelial cells demonstrate several staining patterns, including uniform light labeling, punctate labeling, and uniform intense labeling. (B) Confocal image of same field of cornea at a focal plane through the epithelium 16 μm anterior to the basal layer of epithelial cells. No labeled nuclei are evident. Bar = 25 μm.
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FIGURE 3. Confocal fluorescence micrograph of BrdU-bromo-deoxyuridine U (BrdU)-labeled basal cells in an epithelial whomount fixed 2 days after the injection of BrdU. In this single optical section, most of the basal cells with labeled nuclei are members of cell pairs. In a few cases, single labeled nuclei can be seen. When the focal plane was moved slightly, these single nuclei were nearly always found to be members of a cell pair. Background fluorescence enables the cell borders of unlabeled basal epithelial cells to be visualized. Bar = 50 μm.

We noted that labeled nuclei in the wing cell layer were nearly always members of a labeled pair, with both nuclei at a similar level in the epithelium (Figs. 4, 5, 6; Table 1). Figure 4A is a color-encoded projection of 18 serial optical sections through a cornea fixed 54 hours after injection of BrdU. The color bar in the upper left corner indicates the depth of labeled nuclei within the epithelium. Figure 4B is an anaglyph of the same image shown in Figure 4A. When this image is viewed with red–green glasses, the labeled nuclei appear to float in space, and the high percentage of paired nuclei can be appreciated. In a few cases, the pairing of nuclei is obscured because one member of a pair lies below another labeled nucleus. This problem is solved by viewing successive optical sections, such as those shown in Figure 6. Figure 5 is a three-dimensional reconstruction of a small region of a cornea fixed 54 hours after injection of BrdU. The three pairs of labeled nuclei appear suspended above Bowman’s membrane, which is identified by the higher level of background fluorescence. In some instances, one nucleus of a pair was tilted slightly below or above the other, as though the cytoplasm of the cells in which they resided overlapped (Figs. 4, 5). As did cells in the basal layer, pairs of nuclei in cells that had begun to differentiate shared the same BrdU labeling pattern.

The number of nuclei in cell pairs was striking, both because it was unexpected and because it was seen in more than 97% of labeled nuclei in the wing and superficial cell layers (Table 1). The simplest explanation for the presence of pairs of labeled nuclei in the more superficial layers of the epithelium was that daughter cell pairs differentiated (left the basal layer) at a similar time and were displaced together to more superficial layers of the cornea.

By 3 or 4 days after BrdU injection, labeled nuclei were present in all layers of the epithelium (Figs. 4B, 6). As they approached the corneal surface, the diameters of the labeled nuclei increased and they became flatter, as did the cells in which they resided. Labeled nuclei were still nearly always found in pairs (Fig. 6, Table 1). In a few cases, nuclei in the most superficial cells were not members of a pair. We suggest that this was because one member of the pair had recently desquamated from the corneal surface. Because labeled cells immediately below the corneal surface were found in pairs as frequently as in any other layer, we did not interpret the higher percentage of single labeled nuclei in the most superficial layer as a breakdown in the synchrony of cell differentiation. However, we still counted single labeled nuclei in the most superficial layer of the epithelium as single nuclei, not as one member of a pair.

At these later times after labeling, we sometimes saw clusters of three, four, or more labeled nuclei in the basal layer (Fig. 4B). It is likely that many of these groups resulted from the division of one or both members of a pair of labeled daughter cells. Some of these cell clusters also could have resulted from the proximity of two unrelated cell pairs, although the labeling patterns of the nuclei in these groups were usually similar, suggesting that they were derived from the same labeled precursor cell.

The remarkable synchrony with which daughter cells left the basal layer and differentiated appeared to be maintained in epithelia at 8 and 14 days after BrdU labeling. At these times, fewer labeled cells were detected in the superficial layers of the cornea than during previous periods (data not shown).

Until this time, the appearance of labeled cells in the central and peripheral regions of the cornea was similar. By 8 days after BrdU injections, however, labeled basal cells in peripheral regions were sometimes present in clusters of 8 to 12. Nuclei in these clusters were lightly labeled and always were motiled. Fewer of these large clusters of labeled nuclei were seen in the basal layer of the central region of the cornea. When labeled basal cells in the central region formed clusters, these groups consisted of fewer cells, and their nuclei were labeled more intensely than in the peripheral clusters (Fig. 7).

During a 24-hour period, epithelial cells in the peripheral cornea of the rat divide at least one and one-half times as often as those nearer the center. The large groups of lightly labeled basal cells in the peripheral cornea were probably the progeny of a single progenitor cell. If this is correct, their BrdU label would have been diluted by several rounds of DNA.
synthesis, leaving only the heterochromatic regions of the nucleus with label of sufficient intensity to be detected. This could account for the mottled appearance of the fluorescence in these nuclei. By 14 days, few labeled basal cells could be detected in the peripheral cornea. Presumably, continued rounds of DNA synthesis had diluted the BrdU-labeled DNA in most of the remaining peripheral basal cells below the limit of detection. However, labeled cells were still detectable in the basal layer of the central cornea at this time (Fig. 7). Cell pairs could be found throughout the thickness of the central epithelium, though at a lower frequency than at shorter time periods after labeling (data not shown).

To determine whether the data obtained using BrdU labeling could be replicated by another method, we labeled rat eyes with $^3$H-thymidine. Animals were killed 3 days later, and corneas prepared for autoradiography. Sections were taken parallel to the surface of the cornea to approximate the plane of optical section
FIGURE 4. (A) A two-dimensional projection of 5-bromo-deoxyuridine (BrdU)-labeled cells in the full thickness of the corneal epithelium 54 hours after labeling. Depth within the epithelium is color encoded. Nuclei deeper in the epithelium (more basal) and background staining in Bowman's membrane are blue or green. Nuclei nearer the epithelial surface (more superficial) and some superficial staining appear orange or red. This projection is taken from eighteen 2 μm confocal optical sections and includes all labeled nuclei from the basal layer to the superficial layer in this volume of the corneal epithelium. The scale in the upper left shows the relationship between color and depth within the epithelium. Members of cell pairs are at similar levels within the epithelium. Color values were not identical for all cell pairs because cell nuclei sometimes extended just above or below the other member of the pair. However, color values for a given pair of nuclei nearly always overlapped. (B) A three-dimensional reconstruction, presented as ananaglyph, of the full thickness of corneal epithelium from the same sample shown in A. To see the image in three dimensions, look at it through red-green glasses with the green lens in front of the right eye. When viewed in this manner, the basal surface of the epithelium appears farthest from the viewer. An impression of depth within the epithelium can be obtained without red-green glasses by examining the offset between the red and the green images of each nucleus. Deeper cells (more basal) have a greater offset than more superficial cells. The figure shows pairs of BrdU-labeled nuclei at different levels throughout the epithelium. Labeled nuclei in the more superficial epithelial cells are flattened, compared to those in basal epithelial cells. The horizontal width of the fields in A and B is 148 μm.

FIGURE 5. Three dimensional representation of corneal epithelial cells labeled with 5-bromo-deoxyuridine 54 hours previously. Two hundred nineteen serial 0.18 μm optical sections were taken through the full thickness of the epithelium and reconstructed using Voxel View. Three pairs of cells are shown. One pair is in the basal layer, the second is just above the basal layer, and the third pair of more flattened cells is in the wing cell layer.

DISCUSSION

The stratified epithelia of the body, especially the skin and cornea, have been studied intensively. However, it seems our studies are the first in which the progeny of dividing cells in an epithelium have been followed up throughout a significant period of their lifetimes. In previous studies, BrdU-labeled cells were detected in histologic sections. The extension of this method to wholemounts, as in the current study, provides an informative view of the life history of the mitotically active cells in a population and their descendants. Relationships between the daughter cells of a mitosis that were not evident using two-dimensional sectioning became obvious when viewed in three dimensions.

With this approach, we found that the daughter cells of a mitotic division frequently remained near each other in the basal layer of the corneal epithelium for periods of 2 to 14 days. When daughter cells left the basal layer and began to differentiate, they moved into the wing cell layer together and began to differentiate synchronously. These observations were supported by studies of corneal epithelial cells labeled with 3H-thymidine. Rafferty and Rafferty, using wholemounts of mouse lens epithelia, also showed that nearly all thymidine-labeled differentiating lens fibers were present as cell pairs.

For the cornea to function properly, a balance must be maintained between the loss of cells, resulting from continued differentiation and desquamation, and the formation of new cells by mitosis. There must
be some mechanism to balance the rate of division of basal cells with the continued differentiation of this same cell population. There is ample evidence for such a link. In the normal cornea, the thickness of the epithelium is maintained within narrow limits in spite of continued mitosis in the basal cells. Removal of a portion of the epithelium triggers increased mitosis in remaining basal cells (reviewed in ref. 1). In addition, mitosis normally occurs more frequently and cells leave the basal layer more rapidly in the peripheral epithelium of the rat cornea than in the center, yet the thickness of the epithelium remains constant in the two regions.

We expected to find that, when a labeled basal cell divided, one of the daughter cells would soon leave the basal layer and begin to differentiate, leaving the other daughter in the proliferating pool of basal cells (Fig. 9A). If such a mechanism were operating, it would maintain the number of cells in the basal layer while contributing a constant supply of cells to replace those shed from the epithelial surface. In terms of the X, Y, Z hypothesis, both X, the rate of proliferation, and Z, the rate of cell loss from the cornea, would be functionally linked, because each cell division would produce one cell that would soon be shed from the cornea and one that would remain behind to proliferate. Regulation of epithelial renewal would depend only on maintaining sufficient cell division to produce an adequate supply of superficial epithelial cells. A small number of “new” basal cells, supplied from the limbal stem cells, would replace any basal cells that die or lose the capacity to divide. This view of epithelial differentiation is shared by many authors (see ref. 31) and is consistent with kinetic studies of the rate of loss of labeled DNA from the cornea.

However, our data showed that, rather than hav-
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FIGURES. An autoradiograph of 3H-thymidine-labeled nuclei in a section of the corneal epithelium from a rat labeled with precursor 72 hours earlier. The cornea was sectioned nearly parallel to its surface, causing the plane of section to approximate the plane of the corneal epithelium. The section passes through basal cells at the bottom of the figure and through superficial cells at the top. Therefore, cells later in the process of differentiation are located nearer the top of the figure. The surface of the epithelium (nearest the tear film) is indicated by the solid circles. Several pairs of labeled nuclei can be seen (double arrows), along with scattered labeled nuclei. Not all nuclei were expected to be visible as members of a pair in these sections because the nucleus of one cell of a pair is often slightly above or below the other, as shown in the three-dimensional representations in Figures 4, 5, and 6. For this reason, in some cases only one nucleus of a pair will be present in a section.

...ing different fates, both daughter cells of a mitotic division behaved in a similar manner. They often remained in the basal layer of the epithelium, in some cases for as long as 2 weeks (Fig. 9B). Some of these daughters divided several more times, forming clusters of labeled basal cells (Fig. 7). Alternatively, labeled daughter cells often left the basal layer and began to differentiate. Remarkably, in a high percentage of cases, both labeled daughter cells differentiated at the same time (Fig. 9C).

The results described above question the existence of a direct causal or temporal relationship between cell division and the terminal differentiation of corneal epithelial cells. In our studies, some cells began to differentiate (left the basal layer) within 2 days of cell division, others remained in the basal layer for many days before differentiating, and some basal cells divided several additional times before some of their progeny left the basal layer and began to differentiate. To maintain corneal renewal, there must be coordination between cell division and cell differentiation. However, our results suggest that the link between these processes is more indirect than previously suspected.

Recently, Masters and Thaer found that human corneal epithelial cells may differentiate in pairs. They used a new scanning slit confocal microscope to follow the behavior of basal epithelial cells and wing cells in vivo. In these studies, the same field was relocated at successive time intervals, using the pattern of subepithelial nerve plexuses as a landmark. The authors observed two adjacent basal epithelial cells, both apparently in the process of differentiating into wing cells. This observation is consistent with our findings in the rat cornea.

Our observations showed that epithelial cell differentiation was not linked temporally to the division of precursor cells. The loss of contact between a basal cell and its underlying basal lamina appeared to be an independent event that frequently occurred many days after cell division. Furthermore, the finding that daughter cells differentiated in a coordinated manner raised the possibility that these cells inherited a "differentiation timetable" from their progenitor cell. Finally, the synchronous separation of daughter cells from the substratum suggested that changes in the expression of cell-substrate adhesion molecules, perhaps members of the integrin family, could play an important role in regulating the rate of epithelial cell differentiation.

Thoft and Friend identified three points at which...
corneal epithelial differentiation might be regulated: the entry of cells into the epithelium from the limbus, the division of basal cells, and the desquamation of cells from the corneal surface. Our data suggest a fourth control point, the loss of contact between a basal cell and its basal lamina, which signals the beginning of terminal differentiation. This event has received little attention as a potential control point regulating corneal renewal, probably because most investigators assumed that one of the daughter cells of each mitosis was fated to differentiate. It should be possible to define experimentally the relationship between cell division and differentiation in the corneal epithelium using methods similar to those used in the current study.

Key Words
cell adhesion, corneal epithelium, cell lineage, cell proliferation, three-dimensional confocal microscopy

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References
1. Lemp MA, Mathers WD. Renewal of the corneal epithelium. CLAO J. 1991;17:258–266.
24. Haskjold E, Refsum SB, Bjerknes R. Two cell kinetic
methods studied on the rat corneal epithelium. 


