Apoptosis in the Corneal Epithelium

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Purpose. To determine the viability of corneal surface cells and to determine whether apoptosis is present in the corneal epithelium of the rabbit.

Methods. The viability of epithelial surface cells and shed cells was examined using a calcein-ethidium assay. In this two-color fluorescence assay, viable cells fluoresce green, and the nuclei of nonviable cells fluoresce red. The number of nonviable cells on the normal corneal epithelial surface was quantified. Surface and shed cells also were examined from shear-stressed corneas. The TUNEL technique (TdT-mediated dUTP nick-end labeling) was used to detect DNA fragmentation characteristic of apoptotic cells.

Results. The calcein-ethidium viability assay revealed that the normal epithelial surface is composed mainly of viable cells, with nonviable cells making up a small percentage of the whole. There was an increase in the density of nonviable cells from the periphery of the epithelial surface to the center. In flatmounts of the cornea, TUNEL labeling demonstrated that a small percentage of surface cells exhibited DNA fragmentation, whereas cross-sections revealed that DNA-fragmented cells were found exclusively on the epithelial surface. Epithelial cells from shear-stressed corneas showed an increased number of apoptotic cells.

Conclusions. The normal epithelial surface consists mainly of viable cells, with only a small percentage of nonviable cells and apoptotic cells. The results suggest that nonviable epithelial cells are shed after terminal differentiation, whereas viable cells can be shed by classical apoptosis with the formation of blebs. Thus, there appears to be more than one mechanism for removal of cells from the corneal surface. Invest Ophthalmol Vis Sci. 1996; 37:1017-1025.

It has long been known that the corneal epithelium constantly renews its cell population, with cells added through mitosis in the basal cell layer and lost by shedding from the surface.1,2 More recently, hypotheses have been proposed for the regulation of cell proliferation and loss,3,4 and the contribution of limbal stem cells to the corneal epithelium has been elucidated.5-7

Several mechanisms have been suggested to explain loss of cells from the corneal epithelium. These include cellular necrosis and sloughing of cellular debris8 and the formation of exfoliation holes.9 Recent studies10 have suggested that apoptotic processes may affect both cell loss within the epithelium and shedding from the epithelial surface.11,12

Naturally occurring cell death (apoptosis) is considered distinct from pathologically induced cell death (necrosis).13 Necrosis is characterized by immediate cessation of cellular function, whereas apoptosis is gradual and orderly and functions as a critical control mechanism in cell population regulation and tissue development. In general, apoptosis is recognized morphologically by cell shrinkage, loss of cell-to-cell contact, aggregation of chromatin into dense masses attached to the nuclear membrane, formation of apoptotic bodies, and, finally, phagocytosis.14,15 Biochemically, apoptosis may be recognized by extensive cleavage of cellular DNA into oligonucleosome-sized fragments.14,16,17 Apoptosis has been identified in association with normal cell turnover in several types of mammalian epithelia.18-21

In this study, a viability assay using fluorescent probes allowed visualization of viable and nonviable cells on the epithelial surface and in shed cell collections. In addition, TdT-mediated DUTP nick-end la-
beling (TUNEL) labeling was used to identify apoptotic cells through the presence of DNA fragmentation. Some corneas were exposed to an increased shear force to see whether this altered the characteristics of shedding cells.

METHODS

Animals

Male New Zealand White rabbits, each weighing 1.5 kg, were fed a regular diet and kept in 12-hour cycles of light and dark. All experiments began between 9 and 10 o’clock in the morning to avoid possible circadian variation in cell death rates in the corneal epithelium. Rabbits were killed by intravenous injection of sodium pentobarbital solution (100 mg/kg). Care and maintenance of the rabbits conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Viability Assay

The combined calcein–ethidium assay (Molecular Probes, Eugene, OR) permitted the simultaneous determination of viable cells and nonviable cells. Calcein–acetoxymethyl ester (AM) indicates intracellular esterase activity, and the ethidium homodimer indicates membrane integrity. The plasma membrane is permeable to calcein–AM, which enters the cytoplasm of surface cells and shed cells. Intracellular esterase activity in viable cells is revealed by the intense green fluorescence of intracellular calcein generated by the enzymatic hydrolysis of calcein–AM. Nonviable cells are identified by the staining of nucleic acids by the ethidium homodimer that passes through compromised cell membranes. The emission spectra of calcein (530 ± 30 nm) and ethidium (645 ± 45 nm) are sufficiently different to permit clear separation of the labeling with appropriate filters.

The calcein–ethidium working solution was prepared in basic tear solution (BTS), which has been shown to be benign to the epithelial surface. The BTS contained 116.3 mM NaCl, 18.8 mM KCl, 0.4 mM CaCl2·2H2O, 0.6 mM MgCl2·6H2O, 0.08 mM NaH2PO4, 26 mM NaHCO3. Osmalities were checked using a vapor pressure osmometer (Wescor, Logan, UT) and adjusted to 304 ± 2 mOsm/kg before beginning the experiment. Basic tear solution was gassed with 5% CO2–20% O2–75% N2 to a pH of 7.4 ± 0.1. Stock solutions of fluorophores were diluted with BTS to yield concentrations of 2 μM calcein–AM and 4 mM ethidium homodimer in the working solution.

Viability of Surface Cells. Eight rabbits were used in this viability assay. For epithelial loading, the calcein–ethidium working solution (40 μl) was dropped carefully onto the corneal surface and remained there for 10 minutes immediately before the rabbits were killed. The cornea was then excised, and a whole flatmount cornea (Fig. 1) was prepared. Corneal surface cells were observed using epifluorescence microscopy.

Viability of Shed Cells. Corneal epithelial cells were collected from the precorneal tear film using a corneal irrigation chamber. After collection, the cells in suspension were centrifuged onto a microscope slide at 2000 RPM for 10 minutes. Calcein–ethidium working solution (20 μl) was then placed directly onto the slide. After 10 minutes at room temperature, the slide was coverslipped and observed under epi-illumination.

Density of Nonviable Cells. The number of nonviable, ethidium-positive cells on the corneal surface was counted on flatmount preparations using a 0.82-mm2 frame in the eyepiece of the microscope. The frame was moved across two orthogonal meridians on each cornea (Fig. 1). For the purpose of analyzing regional variations in cell density, the cornea was divided into center, midperiphery, and periphery. Ethidium-positive cells on 16 corneas of eight rabbits were counted.

DNA Fragmentation Assay

In situ TUNEL labeling uses certain reagents for non-isotopic DNA end-extension. Residues of digoxi-
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genin–nucleotide are added catalytically to the DNA by terminal deoxynucleotidyl transferase (TdT), an enzyme that catalyzes a template-independent addition of deoxyribonucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA. The antidigoxigenin antibody fragment carries a fluorescein molecule to the reaction site. When excited by light of 494 nm wavelength, the fluorescein generates an intense signal at 523 nm. This allows for specific staining of the very high concentration of 3'-OH ends that are localized in apoptotic cells using a commercially available kit (ApopTag In Situ Apoptosis Detection Kit, Oncor, Gaithersburg, MD).

Flatmount Corneas. Immediately after each rabbit was killed, the lids were held open and 10% formalin in phosphate-buffered saline (PBS), pH 7.4, was dropped on the corneal surface for immediate fixation of corneal surface cells. After 10 minutes, the corneas were excised from each eye 2 mm posterior to the limbus. Excised corneas were transferred to a beaker containing fixative for a further 10 minutes. For microscopy, corneas were flatmounted on a slide, as shown in Figure 1, and labeled as described below.

Corneal Sections. Corneas were bisected in the vertical meridian, embedded in OCT compound (Tissue-Tek, Mishawaka, IN), sectioned at 6-μm intervals in a vertical meridian, and mounted on slides coated with poly-L-lysine.

Labeling. For DNA nick-end labeling, flatmount corneas and corneal sections were incubated with TdT for 1 hour at 37°C. After two 5-minute rinses in PBS, the tissue was incubated with fluorescein-tagged antidigoxigenin antibody for 30 minutes at room temperature. After two more 5-minute rinses, specimens were coverslipped with anti-fade (Molecular Probes, Eugene, OR).

As a positive control, randomly selected serial sections were incubated with DNase 1 (Sigma, St. Louis, MO) 1 μg/ml in PBS, pH 7.4, for 10 minutes at 37°C. After a 5-minute rinse in PBS, the slides were processed according to standard TUNEL procedure. As a negative control, serial sections adjacent to positive controls were processed following standard procedures; in this case, incubation with TdT during DNA nick-end labeling was replaced by incubation with distilled water. Flatmount corneas were prepared in a similar way.

Epifluorescent Microscopy and Photography

Flatmount corneas, corneal sections, and shed cells were examined using a Leitz Aristoplan microscope (Wild Leitz, Wetzlar, Germany) equipped with epifluorescence. Two different filter combinations were used to optimize the fluorescence appearance: The first provided a band-pass filter for excitation (470 to 490 nm) and a long-pass filter for viewing (>520 nm), and the second provided longer wavelengths for both excitation (515 to 560 nm) and viewing (>590 nm). The second filter combination was used to quantify ethidium-positive cells on flatmount corneas. Some corneas also were viewed by differential interference contrast and epifluorescence successively.

Whole Eye Culture

After rabbits were killed, the eyes were excised intact and suspended in a constant temperature chamber. Each eye was positioned looking downward, and the corneal surface was immersed in BTS while the anterior chamber of the eye was perfused with an aqueous humor substitute solution at 20 mm Hg pressure. For the control eyes, cells were harvested from unstimred BTS; for the test eyes, a shear stress was generated at the epithelial surface by stirring the BTS with a magnetic stir bar. Cells were harvested from the BTS at hourly intervals for 8 hours and were centrifuged onto a microscope slide as described for the viability assay.

RESULTS

Viability of Surface Cells

The great majority of surface cells were viable cells that fluoresced uniformly green (Fig. 2A), an indication of widespread intracellular distribution of calcein after the cleaving of calcein–AM by intracellular esterase. These viable cells were polygonal and similar in size, and they overlapped each other. A small number of these viable cells were washed easily from the corneal surface (Fig. 2B). Some nonviable ethidium-positive cells appeared morphologically normal and usually were observed as isolated single cells. Ethisdium-positive cells were washed easily from the corneal surface with a stream of BTS from a syringe, which indicated that they were not well connected to neighboring cells. Occasionally, ethidium-positive cells were seen on the surface in contiguous groups of three to five cells.

Viability of Shed Cells

Shed cells showed the fluorescent red nuclear ethidium labeling characteristic of nonviable cells on the epithelial surface (Fig. 2C). Punctate green staining of the cytoplasm of some cells revealed that esterase activity had not ceased completely in the subcellular structures. Morphologically, shed cells had intact nuclei, but a small number showed various stages of nuclear degeneration (not shown). The polygonal morphology, characteristic of viable surface cells, was maintained in many shed cells (Figs. 2C, 2D). Shed cells usually were found singly, although connected groups of three to five cells occasionally were ob-
FIGURE 2. Fluorescence and differential interference contrast (DIC) micrographs of the flatmount corneal surface and shed epithelial cells. (A) Epithelial surface at low magnification. Most surface cells fluoresced green (calcein-positive cells) with more intense fluorescence in the nuclear regions. Bar = 100 μm. (B) Epithelial surface at higher magnification. The two ethidium-positive cells (arrows) are seen against a background of calcein-positive cells. One ethidium-positive cell (open arrow) is squamous. This cell shows no esterase activity. The polygonal shape of the cell is preserved through the shedding process and centrifugation onto the slide. The relative position of cells on the slide does not reveal their position on the epithelial surface. Bars = 20 μm. (C,D) Fluorescence and DIC micrographs of the same shed cells. Shed cells showed red nuclei, some with punctate green fluorescence in the cytoplasm indicating minimal esterase activity. The polygonal shape of the cell is preserved through the shedding process and centrifugation onto the slide. The relative position of cells on the slide does not reveal their position on the epithelial surface. Bars = 20 μm. (E) Epithelial surface from a control cornea after whole eye perfusion. As in A and B, most cells fluoresce uniformly green, and there is more intense fluorescence in the nuclear region. The large arrow points to a terminally differentiated cell (ethidium positive–calcein negative), and the small arrow points to an apoptotic cell (ethidium negative–calcein positive). Bar = 20 μm. (F) DIC appearance of the surface after 6 hours of shear stress. (large arrow) Main bodies of apoptotic cells with attached blebs. (small arrows) Blebs that have become detached from the parent cell body. In the center of the photomicrograph are two blebs that lie on the surface of a polygonal squamous cell. Bar = 20 μm. (G,H) Fluorescence and DIC micrographs of shed cells after 6 hours of shear stress. The two classes of shed cells are represented: an apoptotic cell (larger arrow) and four terminally differentiated cells (smaller arrows). The blebs attached to the apoptotic cell vary up to 12 μm in diameter. There are no blebs associated with the terminally differentiated cells. (insets) Two views of the same apoptotic cell collected from the control cornea after 6 hours without a shear force. The largest bleb on this cell is 10 μm in diameter. There is no obvious difference in size or appearance of apoptotic cells collected with or without shear stress. Bars = 20 μm.

Density of Nonviable Cells

There was no difference in the number of nonviable (ethidium-positive) cells in the two orthogonal corneal meridians studied, so all the data were analyzed together. Data were plotted (Fig. 3) as nonviable cells/mm² in three regions of the cornea. Nonviable cells had the highest density in the central cornea and were less abundant in the midperipheral and peripheral cornea. For the eight rabbits studied, the mean densities of nonviable cells in the central, midperipheral, and peripheral corneas were 20.2 ± 3.9, 15.2 ± 3.0, and 8.2 ± 1.3 cells/mm² (mean ± SEM, n = 8), respectively. Differences among these three regions are statistically significant (P < 0.05, two-tailed paired t-test). The mean density of nonviable cells for all the regions was 14.5 ± 2.7 cells/mm² (mean ± SEM, n = 8).

DNA Fragmentation

Cells with DNA fragmentation were found on the epithelial surface, but not in deeper layers of the epithelium. Figure 4B shows a flatmount of the normal corneal surface labeled using the TUNEL technique. The technique identified the nuclei of surface cells with fragmented DNA by an intense green fluorescence. In the positive control (Fig. 4A), in which DNA fragmentation had been artificially induced with DNase 1, all the nuclei of surface cells fluoresced green. In the negative control, in which TdT was replaced with distilled water, there were no fluorescent nuclei (not shown). TUNEL-labeled nuclei of epithelial cells were identified in frozen corneal sections (Fig. 5). In the positive control, all nuclei in the epithelium and stroma were labeled. In normal corneas (inset), nuclei were labeled only in surface cells; no TUNEL-positive cells were found in the wing or basal layers. No labeled nuclei were found in the negative control (not shown).

Whole Eye Culture

The great majority of surface cells were viable cells that fluoresced uniformly green in both the control corneas (Fig. 2E) and the shear-stressed corneas when labeled with calcein and ethidium. These viable cells were polygonal, overlapped each other, and had a longest dimension greater than 30 μm. A much smaller number of cells showed nuclei that fluoresced red, demonstrating failure of the plasma membrane to exclude ethidium. These nonviable, ethidium-positive cells appeared morphologically normal, were the same size as calcein-positive cells, and usually were observed as isolated, single cells (Fig. 2E, large arrow). After

Served. The pattern of grouped shed cells corresponded to grouping patterns observed in nonviable cells on the epithelial surface.
several hours perfusion, the surface of both the control and the shear-stressed corneas showed more cells that fluoresced intensely green (Fig. 2E, small arrow) compared to the normal, viable, green-fluorescent surface cells. These cells were small and rounded, with maximum diameters of 20 to 25 μm, and were more numerous on the shear-stressed corneal surface. Around the plasma membrane of the cell, spherical membrane-bound cell fragments (blebs) often were observed (Fig. 2F). Blebs also were found that had become detached from cells, but many remained attached. Small cells with blebs attached also were retrieved from the superfusate (Figs. 2G, 2H).

DISCUSSION

The normal corneal epithelial surface consisted mainly of viable cells with intact membranes (Fig. 2A). However, there were a small number of nonviable cells on the normal epithelial surface (Fig. 2B) identified by their diminished intracellular esterase activity and increased membrane permeability to ethidium. These cells could be easily removed from the corneal surface by irrigation, as has been reported previously. The density of nonviable cells was higher in the central corneal epithelium than in the peripheral epithelium (Fig. 3). Increased shedding of cells from the central cornea is compatible with the theory that eyelid movement sets up a gradient of shear forces, with the result that shedding is greatest at the corneal center. An alternative explanation arises from the limbal origin of epithelial cells. It will take cells migrating from the limbus longer to reach the central cornea than the peripheral cornea. Consequently, central cells have undergone more cycles of mitosis than peripheral cells and are, therefore, more likely to proceed to terminal differentiation.

It is not known how long ethidium-positive cells remain on the surface before they are shed, although it has been reported that they are the first to shed, even before the micropunctate cells observed clinically when the cornea is stained with fluorescein. The density of exposed cells on the surface of the rabbit epithelium has been reported to be approximately 2000 cells/mm². This suggests that the density of 14.5 cells/mm² for nonviable cells is approximately 1% of the exposed cells on the corneal surface. Thus, almost all the normal epithelial surface is made up of viable cells with intact membranes.

In addition to their presence on the epithelial surface, ethidium-positive cells were found in collec-
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The term apoptosis refers to the process of cell death in which a genetic mechanism programs orderly steps by which a cell can be dismantled. Single cells are deleted in apoptosis, whereas in necrosis large groups of cells die. Apoptosis can be identified morphologically by the presence of apoptotic bodies (blebs) in the cell membrane and biochemically by the characteristic manner in which the nucleus breaks into nucleosomes. The finding of TUNEL-positive cells in the corneal epithelium (Fig. 4B) supports the presence of apoptosis, but such labeling was found only on the epithelial surface and not in deeper layers (Fig. 5 inset). In the positive control, TUNEL-positive cells were identified at all levels of the epithelium and stroma (Fig. 5). Evidence of apoptotic blebbing was found on the surface of control corneas (Fig. 2E) but was more abundant on shear-stressed corneas (Fig. 2F). Blebs also were found in collections irrigated from the surface of the eye (Figs. 2G, 2H). Blebs and ethidium-positive cells could be identified in the same collection of shed cells.

The evidence presented here suggests that there are two pathways by which cells can be deleted from the corneal surface: They can leave by classical apoptosis (bleb formation) or by terminal differentiation. The essential identifying feature of a terminally differentiated cell is the presence of ethidium labeling of the nucleus. Punctate esterase activity is sometimes visible in the cytoplasm (Figs. 2C, 2E, 2G) of these cells, but this is not uniform (as shown in Fig. 2B), and the cell is described as calcein negative. Thus, terminally differentiated cells are calcein positive, ethidium positive, polygonal, and without blebs, and they shed as whole cells. Classically apoptotic cells are calcein positive, ethidium negative, rounded, and smaller as a result of blebbing. Although we have identified TUNEL labeling on the epithelial surface, we cannot be sure whether these cells (Figs. 4B, 5 inset) are classically apoptotic cells or are terminally differentiated cells. Nor do we know whether terminally differentiated cells can form blebs and become classically apoptotic.

The distinctions that have been made in the literature between apoptosis and necrosis include widespread involvement of contiguous cells as a characteristic of necrosis. Cell death by terminal differentiation does not require widespread loss of cells because ethidium-positive cells were observed frequently to be isolated from other ethidium-positive cells (Figs. 2B, 2F). In the normal epithelium, terminal differentiation appears to be the response of an isolated cell to internal programming, whereas classical apoptosis requires an external stimulus to trigger the death pathway. We have stopped short of referring to terminal differentiation as apoptosis to distinguish it from classical apoptosis with bleb formation. However, terminal differentiation may be a specialized form of apoptosis that is unique to epidermal tissue.

Apoptosis within the corneal epithelium has been inferred from the discovery of dark cells during the recovery stage from debridement of the rat corneal epithelium. In the epidermis, it has been suggested that similar dark cells represent deletion within the tissue because of hyperproliferation and intraepithelial lysosomes observed after ultraviolet irradiation.
tion may be apoptotic. Thus, there is evidence that apoptotic cell deletion occurs within the epithelium. However, the finding reported here, that TUNEL labeling occurs only in the surface epithelium, provides no evidence of subsurface apoptosis in the normal epithelium. It might be that apoptosis in subsurface epithelial layers only occurs during trauma or recovery from trauma. Apoptotic bodies have not, as yet, been found on the surface of the normal human corneal epithelium or in human shed cells, but structures described as blebs have been reported in keratoconic corneas examined by scanning electron microscopy.

In general, the mechanism for disposing of cells will depend on tissue morphology. In closed tissues, phagocytosis of apoptotic cells by neighboring cells or macrophages is necessary, whereas in epithelia with exposed surfaces, shedding provides complete and instantaneous disposal of unneeded cells. Widespread removal of sheets of contiguous cells from the corneal surface would be disruptive to the precorneal film and does not normally occur in the rabbit or human epithelium. The orderly deletion of single cells or small groups of cells could be essential for the maintenance of the stable film.

Shedding appears to be the normal route for the removal of cells from the cornea, and this is accomplished by at least two mechanisms—classical apoptosis and terminal differentiation. Which mechanism is used might depend on the amount of stress placed on the surface and other factors as yet unidentified.

Key Words
apoptosis, cell shedding, cornea, corneal epithelium, programmed cell death, epithelial kinetics

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References