Integrity of Epithelium and Endothelium in Organ-Cultured Human Corneas

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PURPOSE. To examine components of the junctional complex and the actin cytoskeleton and the incidence of apoptosis in epithelium and endothelium of organ-cultured human corneas.

METHODS. Human corneas, either organ-cultured for 1 to >28 days or excised directly from eyes stored in moist chambers, were stained with antibodies to ZO-1, vinculin, and caspase 3 coupled to FITC-conjugated secondary antibody. These markers were combined with rhodamine-phalloidin staining for F-actin and DAPI labeling for DNA. The corneas were examined by confocal microscopy.

RESULTS. The depth of the epithelium was reduced during organ culture, but no changes were observed in the distribution of ZO-1 or vinculin, or in the F-actin cytoskeleton. The appearance of apoptotic epithelial cells positive for caspase 3 or with condensed DNA increased with time after 14 days in organ culture, but there was no correlation with donor age. ZO-1 and F-actin staining patterns in endothelium were similarly undisturbed by organ culture, but apoptotic endothelial cells were only rarely seen and then only after >28 days in organ culture.

CONCLUSIONS. Organ culture maintained the integrity of tight junctions and the actin cytoskeleton in epithelial and endothelial cell layers. Apoptosis was evident in epithelium but was observed rarely in the endothelium and then only after extended periods in organ culture. (Invest Ophthalmol Vis Sci. 2001;42:1757–1761)

Both organ culture and hypothermia have their limitations as methods of corneal storage for transplantation. Hypothermia has the general beneficial effect of slowing metabolism and reducing cellular demand for energy, but there are also deleterious cellular changes that effectively limit the hypothermic storage time to less than 2 weeks. These include depletion of high-energy phosphates, altered ionic balance owing to suppression of the Na⁺/K⁺-pump, cellular edema, loss of Ca²⁺ homeostasis, and cellular acidosis. To these can now be added cell loss through apoptosis and breakdown of intercellular junctions. Conversely, organ culture attempts to supply the nutrients needed to maintain cellular metabolism. Even though the culture conditions have not been optimized, storage for 1 month is routinely accepted, and successful grafts have been achieved with corneas organ-cultured for even longer periods. As with hypothermic storage, there is some loss of both epithelial and endothelial cells. Moreover, the integrity of these cell layers could be further compromised by dissociation of intercellular junctions, which would have implications for corneal homeostasis and the recovery of postoperative graft function. Disruption of the actin cytoskeleton and apical junction complex is evident after 4°C storage, but the state of endothelial junctions in organ-cultured corneas is not known. It is also uncertain whether the superficial cells of the remaining layers of epithelium retain the tight junctions that are responsible for the low permeability and high electrical resistance of the normal corneal epithelium.

Here we report on the distribution of components of the junctional complex and the actin cytoskeleton and on the incidence of apoptosis, in the epithelium and endothelium of organ-cultured human corneas compared with corneas from eyes stored in moist chambers.

METHODS

Human Corneas

Twenty-seven corneas were obtained from 25 donors (aged 50–84 years, 10 men and 15 women) from the Corneal Transplant Service Eye Bank Bristol. All corneas were obtained with consent for research consistent with the Human Tissue Act 1961. The corneas were deemed unsuitable for transplantation because of either low endothelial cell density (<2200 cells/mm²) or donor medical contraindications to transplantation. Seven corneas were not organ-cultured but were excised and fixed (details below) within 24 hours of death from eyes that had been stored in moist chambers at 4°C. Twenty corneas were fixed after periods in organ culture at 54°C, ranging from 1 to 35 days. These corneas were from eyes enucleated within 24 hours of death. The eyes were transported to the Bristol eye bank in moist chambers on ice, cleaned, and processed according to our standard protocols for organ culture. The culture medium was Eagle’s MEM buffered with Hepes and containing 26 mM sodium bicarbonate, 2% fetal bovine serum, 2 mM L-glutamine, penicillin, streptomycin, and amphotericin B. The corneas were fully immersed, being suspended by a suture in 80 ml medium in a glass DIN bottle closed with a silicone rubber stopper. Some corneas were fixed after a further 2 to 11 days in culture medium containing 5% dextran T500, which is used before transplantation to reverse the stromal edema that occurs during organ culture. In the Bristol eye bank, the normal time limits for corneas that are to be transplanted are 28 days in organ culture and a further 4 days in dextran medium.

Immunocytochemistry of Intact Cornea

Whole corneoscleral discs were fixed for 2 minutes in 1% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) containing calcium chloride (0.1%) and magnesium chloride (0.1%). The corneas were then rinsed in PBS and trimmed to remove all but a 1-mm rim of sclera. Corneas were cut into five equally sized sectors. The pieces were polished and cleaned, and processed according to our standard protocols for organ culture and immunocytochemistry. These procedures are described in detail elsewhere (2). The corneal discs were cut into five equally sized sectors. The pieces were polished and cleaned, and processed according to our standard protocols for organ culture and immunocytochemistry. These procedures are described in detail elsewhere (2).
Epithelium cells seen in each matic cell loss or damage were scored for the frequency of apoptotic densed DNA were counted as apoptotic. Areas of epithelium and Cells that labeled positively with anti-caspase 3 or those with con-

Apoptosis was adjusted to give an optimum optical section of approximately to a Leica DMIRBE inverted epifluorescence microscope. The pinhole detection of fluorescence/reflectance. The confocal unit was attached with an argon-krypton laser (488-, 568-, 647-nm lines) and argon-UV UK) TCS NT confocal laser scanning microscope. This was equipped Full-thickness corneas were examined using a Leica (Milton Keynes, Laser Confocal Imaging

Full-thickness corneas were examined using a Leica (Milton Keynes, UK) TCS NT confocal laser scanning microscope. This was equipped with an argon-krypton laser (488, 568, 647-nm lines) and argon-UV laser (351and 364 nm), providing three channels for simultaneous detection of fluorescence/reflectance. The confocal unit was attached to a Leica DMIRBE inverted epifluorescence microscope. The pinhole was adjusted to give an optimum optical section of approximately 1-μm thickness. Images, obtained using a ×40 apochromatic oil immersion objective lens, were collected in 1- to 5-μm steps from the apical surface of the most superficial epithelial or endothelial cell surface, through the basement membranes, to the stromal keratocytes. For direct comparison of immunocytochemical staining in triple-labeled specimens, simultaneous imaging was used to capture the FITC and TRITC images. UV images were collected separately without changing the X, Y, and Z settings and merged with the FITC/TRITC images.

Apoptosis
Cells that labeled positively with anti-caspase 3 or those with condensed DNA were counted as apoptotic. Areas of epithelium and endothelium with complete cell coverage and without obvious traumatic cell loss or damage were scored for the frequency of apoptotic cells seen in each ×40 objective field (Table 1).

RESULTS
Epithelium

Moist-Chamber Corneas. Confocal imaging in the XZ plane of full thickness corneas stored for <24 hours revealed a typical 5- to 7-layered, stratified, squamous epithelium (Fig. 1A). Filamentous actin (F-actin) was labeled in all cells throughout the depth of the epithelium and in keratocytes (not shown). In moist-chamber corneas, the depth of the epithelium recorded between the apical and basal cell borders was containing 1% bovine serum albumin (BSA), were directed against ZO-1, a component of tight junctions (61-7300; Zymed Laboratories, South San Francisco, CA); vinculin, an intracellular attachment protein linking the actin cytoskeleton and the plasma membrane at points of contact or adhesion (V-4505; Sigma, Poole, Dorset, United Kingdom); and caspase 3, a protease activated during apoptosis (AF835; R&D Systems, Abingdon, United Kingdom). Negative controls were incubated without primary antibody. Corneal pieces were then rinsed in PBS, and a fluorescein (FITC)-conjugated secondary antibody added simultaneously with rhodamine (TRITC)-phalloidin (P1951; Sigma) for 2.5 hours at room temperature in the dark. Unbound excess labels were removed by rinsing in PBS, and the tissue was postfixed for 1 hour in 1% paraformaldehyde in PBS. The full-thickness corneal pieces were mounted in cavity slides in Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI), a counterstain for DNA (H-1200; Vector, Peterborough, United Kingdom), with either the epithelial or endothelial surface uppermost toward the coverslip. Laser Confocal Imaging

Five-staining procedures were evaluated for the presence of epithelial cells with characteristic apoptotic features (Fig. 3). There was no correlation between the apoptosis score and donor age (Pearson’s r = -0.18, P = 0.45), but there was a positive correlation between the appearance of apoptotic epithelial cells and the length of time in organ culture (Pearson’s r = 0.67, P = 0.001).

Endothelium

Moist-Chamber Corneas. Confocal imaging of the endothelium of moist-chamber corneas revealed a staining pattern for F-actin (Fig. 4A), that was consistent with that seen in cat, rabbit, and keratoconic human corneas.2,7,8 F-actin labeling consisted of distinct cytoplasmic bands forming continuous circumferential bundles of fibers around the perimeter of each cell. Double labeling for F-actin and vinculin showed that vinculin was restricted to a single discontinuous line of labeling between the double F-actin bands. At the junctions between three adjacent cells, there

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<th>Apoptotic Cells per Field</th>
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* One apoptotic cell per five fields.

36 ± 10.5 μm (mean ± SD, range, 20–56 μm, n = 7). In sections through the epithelium F-actin was seen as a single band between adjacent cells in all layers (Figs. 1E, 1G).

Separation of the three pseudocolor channels showed that ZO-1 labeling was restricted to the most superficial cells, where it was localized at the cell borders, completely encircling the cells (Fig 1C). There were no gaps between adjacent superficial cells, rather there was an increased density of ZO-1 labeling at points where three cells abutted at the Y junction. (Fig. 1C). The negative control, with only secondary antibody, showed no background FITC labeling. No ZO-1 labeling was seen in the wing or basal epithelial cells (Figs. 1E, 1G).

Apoptotic cells, positive for caspase 3 or with condensed DNA, were observed only very occasionally in moist-chamber corneas. Obvious epithelial defects, however, were found to have high numbers of cells positive for caspase 3 or containing condensed DNA adjacent to the wound edge and up to 20 cells (ca. 200 μm) distant from the damaged area (data not shown). Organ-Cultured Corneas. Corneas stored in organ culture showed a reduction in the depth of the epithelium (Fig. 1B); for example, compared with the 54 μm epithelial thickness in the moist-chamber cornea in Figure 1A, the epithelium of the contralateral cornea from the same donor was only 42 μm thick after 14 days in organ culture. The number of cell layers was correspondingly reduced from 5-7 in the moist-chamber corneas to <5 after organ culture. Variations in epithelial thickness were observed between corneas, and in some instances only 2 layers of epithelium were present. Whether this loss was due entirely to organ culture was uncertain because there was no initial measure of epithelial depth before storage of these corneas. All superficial cells expressed continuous ZO-1 labeling, regardless of the number of underlying cell layers (Fig. 1D). This did not vary even with extended storage up to 35 days with or without added 5% dextran. ZO-1 labeling was not observed in any cells other than the most superficial (Figs. 1F, 1H). The F-actin labeling appeared to be continuous around all cells in all layers, independent of storage time (Figs. 1F, 1H). The staining pattern was consistent with that in moist-chamber corneas (cf. Figs. 1E, 1G).

After 14 days in organ culture some epithelial cells could be seen to be positively labeled with anti-caspase 3 or to have condensed DNA (Fig. 2). When this particular image, which is a compilation image of the three selected wavelengths, was separated into its component channels, it could be seen that the cells labeled by anti-caspase 3 also had condensed DNA. Cells were also seen, however, that were labeled with anti-caspase 3 but that did not have condensed DNA. Many cells positive for caspase 3 appeared to be encapsulated by extra layers of F-actin (see inset Fig. 2).

Corneas, both moist-chamber and organ cultured, were evaluated for the presence of epithelial cells with characteristic apoptotic features (Fig. 3). There was no correlation between the apoptosis score and donor age (Pearson’s r = -0.18, P = 0.45), but there was a positive correlation between the appearance of apoptotic epithelial cells and the length of time in organ culture (Pearson’s r = 0.67, P = 0.001).

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appeared a distinct Y-shaped area of high-density labeling of vinculin (Fig. 4B).

Double-labeling for F-actin and ZO-1 showed that ZO-1 was also localized pericellularly between the F-actin bundles. ZO-1 labeling was interrupted at the Y-junctions between neighboring endothelial cells (Figs. 4C, 4D), which has been reported previously.7,8 This was not, however, a consistent finding in all the moist-chamber corneas examined in this study.

There was no evidence of apoptotic cell death in the endothelium of moist-chamber corneas. No cells labeled with anti-caspase 3, and none had condensed DNA.

Organ-Cultured Corneas. Corneas that were stored for up to 28 days in organ culture retained similar staining patterns for F-actin and ZO-1 (Figs. 4E, 4F) compared with moist-chamber corneas (Figs. 4C, 4D). There was no loss of F-actin bundles nor were any changes observed to the Y-junctions between adjacent cells. Endothelial cells retained the same dimensions in the XY (Figs. 4D, 4F) and XZ (not shown) planes (ca. 20 and 5 μm, respectively) throughout organ culture storage. The addition of 5% dextran to the organ culture medium, which is used routinely to reduce stromal edema before transplantation, induced no alterations in either F-actin structures or in the distribution of ZO-1 labeling in endothelial cells. Endothelial cells that stained positively for caspase 3, indicative of apoptosis, were observed rarely and then only in corneas that had been in organ culture for longer than 28 days or had been in dextran medium for >5 days.

**DISCUSSION**

We found that the epithelial and endothelial cell layers of human corneas stored in organ culture for up to 28 days did...
not exhibit any fundamental changes in the appearance of the circumferential band of F-actin, in the distribution of the actin-binding protein, vinculin, or in the distribution of the tight junction protein ZO-1 when compared with corneas excised within 24 hours of death from eyes that had been stored in moist chambers. This is in contrast to the findings of Hsu et al.,2 who recently reported that the endothelial cells of corneas stored in Optisol-GS (Bausch and Lomb, Claremont, CA) for 8 days at 4°C lost normal F-actin staining and appeared to have enlarged gaps at the Y-junctions when stained for ZO-1. They reported that this was reversed after 24 hours storage at 37°C in either serum-free medium or Optisol-GS, and they suggested that the changes observed were a consequence of low-temperature disruption to regulation of the junctional complex. This seems a likely explanation because depletion of ATP occurs during hypothermic storage,1 and loss of ATP causes dissociation of intercellular junctions.9

Discontinuity in the distribution of ZO-1 has been reported as a normal feature in corneal endothelium, in particular leaving gaps at the Y-junctions between three neighboring cells.7,8 This supports the concept of the endothelium being a leaky barrier sealed by focal macula occludens at the apical border between adjacent cells rather than by a continuous zonula occludens completely encircling the cells. Although we also observed a similar discontinuous distribution of ZO-1, this was not a consistent finding in all the corneas we examined. Regulation of the permeability of the endothelial barrier has been proposed as part of the mechanism of stromal hydration control.10 This could be achieved by alterations in the structure of the apical junctional complex, which would explain the variation we observed in ZO-1 distribution between different corneas.

In agreement with previous observations,11 we found a loss of epithelial cell layers during organ culture, but this was highly variable and may have reflected events before corneal storage and preexisting epithelial damage. Our concern was that this loss of cell layers may have had implications for the effectiveness of the epithelial barrier because it is only the superficial cells that are encircled by complete tight junctions.12 The distribution of ZO-1 in the epithelium of those corneas that had not been organ cultured agreed with this. Loss of this feature would greatly increase the permeability of the epithelium, which would have consequences for corneal homeostasis. It was, however, remarkable how consistent the presence of ZO-1 labeling was in the most superficial epithelial cells of the organ-cultured corneas regardless of the loss of underlying cell layers. It would appear from this structural evidence that organ culture does indeed maintain an effective epithelial barrier, but functional measurements, such as trans-epithelial resistance or epithelial permeability, would provide corroboration.

We also sought to determine whether epithelial and endothelial cells were undergoing apoptosis during organ culture. Classically, apoptotic cells display nuclear shrinkage, chromatin condensation, and DNA fragmentation into high-molecular-

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933589/ on 12/01/2018)

**Figure 2.** Compilation image in XY plane of epithelium of a cornea after 14 days in organ culture showing cells positively labeled for caspase 3 (green). Higher magnification of the central caspase 3-positive cell (inset) labeled only for F-actin suggests cell engulfment.

![Figure 3](https://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933589/ on 12/01/2018)

**Figure 3.** Epithelial apoptotic score as a function of donor age (A) and time in organ culture (B). The lines were fitted by least squares to indicate the linear association between the variables and Pearson product-moment correlation coefficients were calculated (see text).

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933589/ on 12/01/2018)

**Figure 4.** Dual-labeled XY images of endothelium. After moist chamber storage: F-actin (A) and vinculin (B); F-actin (C) and ZO-1 (D). After 14 days in organ culture: F-actin (E) and ZO-1 (F).
weight pieces. These cells are ultimately phagocytosed without leakage of their cellular contents. It has been suggested that this process occurs rapidly within 30 to 60 minutes and, as a result, few apoptotic cells are seen in steady-state tissues. In humans, >10 caspases have been identified that become activated during apoptosis. Caspase 3 is considered to be the key to apoptotic activation. It is responsible for the promotion of chromatin condensation and disruption of actin regulation dynamics, thereby promoting cellular packaging and subsequent engulfment. In this study, we considered positive staining for caspase 3 and/or nuclear shrinkage to be indicative of apoptosis.

Apoptotic endothelial cells were seen occasionally but only in those corneas that had either been in organ culture for >28 days or in dextran medium for >5 days, which are beyond the storage time limits applied in the Bristol eye bank, where organ culture is the storage method of choice. Apoptosis, based on our observations of caspase 3 staining and nuclear shrinkage, would not therefore appear to be a significant mechanism of endothelial cell loss during organ culture until the culture medium perhaps becomes significantly depleted (the medium is not changed during organ culture in our eye bank). This apparently conflicts with recent findings by Albon et al. of a high incidence of apoptosis in the endothelium of organ-cultured corneas. One explanation for these seemingly disparate observations is likely to be found in the different techniques used for identification of apoptosis. TUNEL staining, which identifies cells with fragmented DNA, has been extensively as a marker for apoptosis, but whether this method exclusively identifies apoptotic cells has been questioned because DNA fragmentation is also a feature of necrosis. Caspase 3 activation, by contrast, is an early event in apoptosis and cells positive for caspase 3 are committed to the apoptotic pathway of cell death. Although some caspase 3 staining was observed by Albon et al., its incidence was far lower than that of TUNEL-positive cells, all of which they assumed to be apoptotic. Whether caspase 3 or TUNEL gave a truer indication of the incidence of endothelial apoptosis clearly needs to be resolved, but, we suggest that in the absence of traumatic endothelial damage (including heavy folding of Descemet’s membrane), which would in any case render a cornea unsuitable for penetrating keratoplasty, apoptosis is not a significant mechanism of endothelial cell loss during organ culture.

Conversely, a rise in numbers of apoptotic cells was evident in the epithelium with increasing time in organ culture, especially after 14 to 21 days, which was independent of donor age. Kumoro et al. also observed that moist-chamber corneas had almost no apoptotic cells in any section of the cornea but that such cells became detectable in the epithelium after as little as 5 days of storage at 4°C. Many of the epithelial cells that were either positive for caspase 3 or had condensed DNA were encapsulated in double layers of F-actin (Fig. 2), which suggests that these cells were being engulfed by neighboring epithelial cells. Phagocytosis of apoptotic cells has been described in rat corneal epithelium and in rat lens epithelium. Our observations therefore led us to speculate that apoptosis, as a mechanism for the orderly elimination of unwanted cells, would allow epithelial cell turnover in vivo without compromising the epithelial barrier.

Organ culture storage of human corneas offers a longer storage time and increased microbiologic safety compared with hypothermic storage. Our morphologic studies demonstrate another advantage in that the changes to the tight junctions and actin cytoskeleton of the epithelial and endothelial layers that occur during hypothermic storage are not observed with organ culture. Apoptotic epithelial cells were apparent only after 14 days in organ culture, but there was little evidence of apoptosis in the endothelium. The presence of an active apoptotic cell turn over system in normal epithelium is suggested and this warrants further investigation.

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References