Epithelial Desquamation, Regeneration and Renewal in Corneal Graft Prepared for Clinical Use

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ABSTRACT

Introduction: Corneal epithelium has been identified as an important element in human ocular surface homeostasis. Conservation cycle of corneal epithelium during warm storage has not been described. The aim of this study was to evaluate the complete regeneration and renewal of human corneal epithelium during the 35 days of organ culture.

Methods: Corneas maintained in organ culture were formalin-fixed and stained with H&E at day 0 (without organ culture), at day 1, 2, 3, 4, 6, 8, 9, 14, 25, 29, 31, 32, 35, 56 and 90. Proliferation, apoptosis and structure were evaluated by immunostaining with antibody against Ki67, Caspase 3 and E-cadherin respectively. Results: During the two first days, corneas were devoid of their epithelium, except at the limbal region. From day 2 to day 8, regeneration of the epithelium took place, starting from the limbal region towards the central cornea. From day 9 to day 35, corneal epithelium appeared thinner but healthy and formed. Proliferation happened in the whole cornea during the 35 days of organ culture, as shown by Ki67 positive cells. Apoptosis was rarely detected in the corneal epithelium at any time point.

Conclusions: Corneas conserved in organ culture showed a complete loss of the corneal epithelium followed by a full regeneration originating from the limbal epithelial cells. Corneal epithelium in organ culture for more than ten days exhibit a full regenerated epithelium, which could have, according to the literature, an important clinical impact by reducing post-graft complications.

Key Words: eye bank, corneal epithelium, conservation, regeneration

The techniques of corneal conservation have considerably evolved since the first successful human corneal transplant performed in 1905. In 1935, Filatov described the use of a tightly stoppered glass jars at 4°C for the storage of human whole globes, which essentially is the moist chamber preservation technique still in use today. Conservation of the whole globe in a moist chamber is limited to a short period of time (48 hours) because of the deterioration of the endothelium induced by the post-mortem modifications of the aqueous humour. For this reasons it is advantageous to collect only the scleral rim, which is then preserved at 4°C (cold storage) or in tissue culture at 30°C to 37°C (hot storage).

Surgery can be scheduled more easily with warm corneal storage because donor cornea can be stored for one month. Cold storage is the most common method in USA, in Asia and in countries where there is a relative sufficient number of corneal donor tissue compared to demand. In these countries Optisol GS is the most popular storage medium. In Europe and in the UK in particular, the corneoscleral rim is maintained primarily in tissue culture media, using either commercial (i.e. CorneaMax) or in-house produced media. These media are composed of Eagle’s minimal essential medium (MEM) or its variant Dulbecco’s MEM, supplemented with fetal calf serum (from 2 to 8%). In most of countries Optisol GS is the most popular storage medium.

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The quality assessment of corneal tissue is based on the end-of-storage endothelial health. The organ culture media used in eye banks have been developed to maintain the cellular integrity of the endothelium. As a consequence, less attention has been given to the integrity of the epithelium during conservation in the different media. Previous studies have demonstrated that in most cases the epithelium is lost during preservation, during the grafting procedure or in the early post-operative phase.

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clinical implications as the health of the epithelium is understood to impact surgical success in patients after ocular surface destruction. To date, it has been shown that the amount of epithelium loss is dependent on the time between death and graft harvesting, which was limited to 5-6 hours post-mortem. It is understood that a lack of the adenosine triphosphate and glycogen prompt this loss. In this study, a complete evaluation of the cell fate of the corneal epithelium during the full duration of the conservation process in organ culture medium, supplemented with FCS, has been reported. For this purpose, we evaluated the morphology of the corneal epithelium, the Ki67 proliferation index, E-cadherin staining and apoptosis during the 35 days of organ culture in CorneaMax.

MATERIALS AND METHODS

Human Corneal Tissue

All procedures conformed to the tenets of the Declaration of Helsinki for biomedical research involving human subjects. All corneas were received from Lausanne Eye Bank and were considered to be unsuitable for grafting because of either low endothelial cell density or donor medical contraindications. After retrieval, corneas were placed in CorneaMax (Eurobio, Les Ulys, France) at 32°C in a dry incubator, according to the standard organ culture in place in the Lausanne Eye Bank. In total, 72 organ cultured corneas (mean donor age: 70.9 +/- 9.6 years; mean time from death to procurement: 17 +/- 1 hours) were used (N>3 for each time point). Corneas were fixed after a period of organ culture of 1, 2, 3, 4, 6, 8, 9, 14, 25, 29, 31, 32, 35, 56 and 90 days. Five corneas were not organ cultured, but were excised and fixed directly in 4% formaline solution at day 0. Corneas enucleated for uveal melanoma were used as positive control.

Immunohistochemistry

Immunohistochemistry was performed on serial 5 µm sections. Briefly, tissue sections were dewaxed in xylene, rehydrated through graded alcohols, and washed with phosphate-buffered saline. Antigen retrieval was achieved by heat treatment by autoclave (11 min at 121°C) using a pH6 citrate retrieval solution. Sections were incubated for 60 min with primary antibodies at room temperature. The reference and dilution factor of primary antibodies were as follows Ki67 (1:200, DAKO, MIB-1), E-cadherin (1:200, DAKO, NCH-38) and cleaved-Caspase 3 (1:300, BD Biosciences, C92-605). Sections were further incubated using a sensitive polymer-based detection system (EnvisionPlus, DAKO) for 30 minutes and Dako’s AEC+ High sensitivity substrate Chromogen or DAB+ were used as chromogens according to the manufacturer’s instructions. Negative controls were obtained by omitting the primary antibody. For positive controls, we immunostained 3 eyes enucleated for uveal melanoma to control the pattern of staining in normal cornea and limbus.

Proliferation Index Evaluation

Ki-67 antibody (clone MIB-1) was used to determine the proliferation index for all specimens (the number of stained cells for Ki-67 against the counted epithelial cells expressed as a percentage).

Statistical Analysis

All data are presented as the mean +/- SEM.

RESULTS

In the corneas conserved in organ culture (CorneaMax at 32°C), during the early stages of organ culture extensive detachment of epithelial cells was observed. The epithelium lost its adherence to the basal lamina of the cornea creating a large epithelial sheet (Fig. 1). In the histological sections of the corneas without conservation which were immediately sectioned, three types of conditions were observed: in the first the epithelium was intact and attached; in the second, it was intact but detached centrally and finally it was detached peripherally and absent centrally (Fig. 2A). Healthy cell junctions (Fig. 2B, E-cadherin staining) and an absence of cell apoptosis (Fig. 2C, cleaved-Caspase 3 antibody) were also observed in these sections. The series of histological sections of organ-cultured corneas are shown in figure 3. During the first three days of organ culture, most corneas had complete loss of the
Epithelium with erosion starting from the center. By day 2-3 only a residual peripheral cluster of cells could be detected, cellular edema and an increase in intercellular space were also evident, at this time point none of the corneas had central epithelium remaining. At day 4 some regeneration of the epithelium was observed, starting from the limbus towards the center of the cornea. At day 6, epithelial cells are present centrally and at day 8 the whole cornea was covered. From day 9, corneal epithelium retained a minimal but formed structure, consisting of only few cell layers (Fig. 3). At the end of the epithelium regeneration, several morphologies could be identified, varying from two to five cell layers (Fig. 4A). Long-term evaluation of the cornea (56 and 90 days of organ culture) demonstrated that epithelial

**Figure 2.** Histology of human cornea at day 0 (without organ culture). Corneal epithelium is stained with hematoxilin-eosin (A), for the calcium-dependent adhesion protein E-cadherin (B) and for the cleaved-Caspase 3 protein, a marker of apoptosis (C) ((A), (B) and (C), original magnification: 40x).

**Figure 3.** Hematoxilin-eosin staining of section of human corneal epithelium corresponding to a representative histology of a control cornea and human corneas maintained in organ culture during 35 days. Following the loss of the corneal epithelial cells, regeneration of the epithelium took place, starting from the limbus towards the center of the cornea (Original magnification: 40x).

**Figure 4.** Hematoxilin-eosin staining of section of human corneal epithelium. Representative histology of the variation of the regeneration of the epithelium after 29 to 32 day of organ culture (A). Evaluation of the corneal epithelium after long term conservation. Regenerated epithelium is still present after 56 and 90 days of organ culture (B) ((A) and (B), original magnification: 40x).
cells were still present in the limbal region, but also in the central cornea with no evidence of separation of epithelium from the basal layer (Fig. 4B).

At the same time points a series of histological section were examined with Ki67, Caspase 3 staining and E-Cadherin staining. At day 1, no regeneration of cells, no cell apoptosis and good cell junction preservation were observed. At day 6, clear evidence of regeneration was observed; Ki67 positive cells could be identified in the limbal area or the central epithelium during the regeneration of the cornea and until day 35 coinciding with a near complete lack of cell apoptosis. The Ki67 proliferation index, which is normally around 4% in normal cornea increased to 13.3% +/- 1.3 at day 6, to 12.6% +/- 1 at day 9 and to 8.6% +/- 1.3 at day 14. At day 25, the proliferation index had returned close to normal (4.6% +/- 0.5). Cell junction integrity was not optimal by day 6 centrally but was completely formed from day 9, after this time point all the epithelial layers were positive for E-cadherin immunostaining. This indicates that the regenerated tissues developed the intercellular junctions required to form a functional barrier (Fig. 5).

**DISCUSSION**

In this study, the cycle of regeneration of the corneal epithelium in organ culture was described; here we reported the loss of epithelial cells as a sheet during the first three days of organ culture storage, which is followed by an intense regeneration of the corneal epithelium starting from the limbus toward the center of the cornea (day 3 to day 9). From day 9 to day 35 a self-renewal process took place resulting to an epithelium limited to two to five cell layers. In this article we expanded on previous observations, demonstrating the survival and proliferation of limbal epithelial cells in organ culture. This regeneration cycle allows the restoration of a complete corneal epithelium (Fig. 6).

In the literature, the corneal epithelium damage during harvesting process or organ culture was already described. In short term storage, the loss of epithelium has been reported during the first 4 days of storage at 4°C in both Optisol GS and Dexsol storage media. These results were corroborated by the results of other studies conducted by Means et al, which described a general loss of epithelial integrity after 6 days of storage in Optisol GS or by Wagoner et al, who did not find any cornea without epithelial defect after a storage time of 7 days in Optisol GS at 4°C. Combined these results indicate that the loss of corneal epithelium is a general event that happens independently of the type of eye banking conservation. This loss of epithelium has been correlated with post-mortem time, which should be inferior to 6 hours. For example Van Meter reported the complete sloughing of the epithelium in cornea that was harvested more than 12 hours post mortem. In the Lausanne eye bank and probably in most eye banks, the interval between death and graft harvesting exceeds this limit (for example, 17 +/- 1 hour in Lausanne eye bank). This indicates that most eye banks, including those using organ culture media, will observe a complete loss of corneal epithelium during the first days of conservation. However, only the organ culture system, containing fetal calf serum, has the potential for the regeneration of the corneal epithelium.

In this study CorneaMax was used, which consist of a commercially manufactured MEM-based medium supplemented by fetal calf serum. According to the European eye bank association directory, about 75% of the European eye banks...
use a similar organ culture system, where the percentage of fetal calf serum is varied between centers (2-8%). This suggests that the majority of European centers, even those whom do not use the CorneaMax conservation medium, could expect to observe a similar epithelium cell regeneration cycle (Fig. 7).

Our study demonstrated the expression profile of E-cadherin, a calcium-dependent cell-cell adhesion molecule, was localized in the cell periphery of all epithelial cells, including the basal cell layer during organ culture process. In the literature, it was shown that 14 days of organ culture maintained the appearance of the circumferential band of F-actin, the distribution of the actin-binding protein, vinculin and the tight junction protein ZO-1 in the different epithelial cell layers. Combined these results indicate the presence of an effective epithelial barrier throughout organ culture storage after 9-10 days of conservation. This barrier is maintained during the following day of conservation until day 35, which is the legal limit for corneal use. Therefore the use of cornea preserved between 10 and 35 days should ensure the presence of an active barrier against penetration of bacteria and fungi into the stroma, thus reducing the risk of post-operative complications.

The presence of an intact epithelium immediately following graft may also have anti-inflammatory effect on the graft. Epithelial cells can express antimicrobial proteins in response to invading pathogens, for example the epithelial cytokeratins have innate defense properties because they constitutively produce cytoprotective antimicrobial peptides. Furthermore there are several molecules expressed by the corneal epithelium which are involved in the modulation of the corneal inflammation. Two examples of these molecules are heme oxygenase 1 and 2, which contribute to the regulation of inflammation, by modulating leukocyte migration and inhibiting expression of the inflammatory cytokine.

The clinical data supports the supposition that presence of an intact viable epithelium is advantageous for the survival of the graft. One clinical study demonstrated that after penetrating keratoplasty (PK) the postoperative healing time of re-epithelialisation was directly related to the presence of an epithelium on the corneal graft at the end of surgery. In a consecutive series of 1819 PK procedures, Price et al estimated that 25% of secondary graft failure could be attributed to problems with the external surface of the graft, just after endothelial failure as a result of immunologic allograft reactions (27%). More recently, in a series of 901 consecutive penetrating keratoplasty procedures, Anshu et al., identified postoperative epithelial problems as an important risk factors affecting corneal graft survival, representing 15.6% of the failures. Therefore, increasing the survival of donor corneal epithelial cells could even better expand graft survival and we know from the literature that these cells have a high potential of survival as they can survive more than 18 months after implantation. Irregularity of the superficial layers of the corneal epithelium could decrease the quantity and the quality of the post-operative best corrected visual acuity.

As a final point, human cornea conserved in organ culture are used for ex vivo investigation of the corneal epithelium, for example as a model of wound healing or as a model of corneal epithelium homeostasis. The regeneration cycle described here, will be a large confounder in the interpretation of any such experiments. Caution should be exerted when extrapolating experimental data concerning normal human corneal epithelium renewal or wound healing which have been maintained in organ culture.

In this report, three distinct phases have been identified: firstly the desquamation of the corneal epithelium, secondly the regeneration of the corneal epithelium from the limbal epithelium and lastly the continued self-renewal. These results, allow us to better understand the state of the epithelium during cold storage (through the literature) and long term organ culture (Fig. 7). This study could have an important clinical impact in Europe, were warm storage is common, as it indicates how to optimize conservation of an healthy epithelial which has the potential to reduce post-graft complications and improve graft survival.
REFERENCES


