Development of a Three-Dimensional Organ Culture Model for Corneal Wound Healing and Corneal Transplantation

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PURPOSE. To develop and evaluate a three-dimensional organ culture system of the cornea anterior chamber that could replicate the in vivo processes occurring during corneal wound healing and corneal transplantation.

METHODS. Bovine corneoscleral buttons were clamped in a specially designed chamber through the sclera outside the limbus. The epithelium was exposed to air, and its anterior surface was automatically irrigated. The endothelial layer was perfused separately with media under normal intraocular pressure. Wound healing and corneal transplantation were observed using light, scanning, and transmission electron microscopy.

RESULTS. The organ culture system maintained the epithelium, the putative epithelial stem cells in the limbus, the stroma, and the endothelium in good condition for the 10-day period during which the system was evaluated. The authors observed that the processes of wound healing and corneal transplantation in the model appeared similar to those occurring in vivo.

CONCLUSIONS. In vitro model closely replicated the in vivo processes of wound healing and corneal transplantation. The authors believe this model will be useful for basic investigations into the cornea, such as study of the response of the cornea to surgery, wound healing, toxins, and therapeutic agents. (Invest Ophthal Vis Sci. 2006;47:2840–2846) DOI: 10.1167/iovs.05-1367

The role of in vitro models is becoming increasingly important in the field of ophthalmology. The use of primary and immortalized cell lines has meant that much work that previously could only be carried out on animals can now be performed more quickly and at greatly reduced cost in the cell culture laboratory. The use of corneal cell lines is particularly valuable in the early stages of investigation, such as when screening potential pharmacologic agents or novel biomaterials. In addition to these practical considerations, there are ethical issues to consider, and there is increasing pressure from the public and government, particularly in Europe, to find ways to minimize animal experimentation.

Although the use of cell lines is helpful, it has clear limitations, and cell lines cannot come close to replicating the complex interactions that occur between the different cell types in the cornea.

This has prompted investigators to attempt to develop an in vitro culture model for the whole cornea or anterior chamber. Several research groups have published various versions of this concept. One of the earliest models involved the use of human material and clamping of the corneoscleral button into a special holder where the posterior chamber could be perfused with medium. This system was successful for the endothelium, though no evaluation was made of the epithelium or stroma. Another group cultured human corneoscleral buttons in a simple system using Petri dishes and reported excellent results for the endothelium, epithelium, and stroma. Yet another group working with rabbit cornea showed that an in vitro model could be used to evaluate epithelial wound healing over a 72-hour period. Other workers developed a novel system with an agar-collagen gel to maintain the anterior chamber and demonstrated excellent epithelial wound healing in human and bovine cornea. Most recently, a sophisticated in vitro system was developed for pig corneas with separate epithelial irrigation and endothelial perfusion. This produced excellent results, but, because the epithelial irrigation was performed manually, it was only evaluated for 14 hours. In addition to these corneal models, an advanced and successful in vitro model exists of the trabecular meshwork.

All these models must be considered valuable and important contributions to the ultimate objective of producing an in vitro model for the cornea. However, it is clear that considerable scope remains for improvement. Ideally, an in vitro corneal model should include an epithelial exposure to the air, as occurs in vivo, and an automatic irrigation system that attempts to replicate the tear film and that assists in epithelial desquamation. The anterior chamber should be perfused separately to allow the anterior chamber to be maintained at normal intraocular pressure. It should be possible to maintain the cornea long enough for it to be useful for epithelial and endothelial wound healing studies, and the stroma and keratocytes should remain healthy and transparent during this period. Finally, the species of cornea used should be cheaply and readily available but should avoid the deliberate sacrifice of animals.

In this article we describe how we have attempted to develop a model that meets these objectives. We describe how we have evaluated all the corneal cell types, including the putative epithelial stem cell population, in our model at the ultrastructural level and how we have assessed the usefulness of our model by monitoring epithelial wound healing and the model’s response to penetrating keratoplasty.
MATERIALS AND METHODS

The Culture System

Normal bovine eyes were obtained from a local abattoir within 2 hours of death, transported to the laboratory at 4°C, and used immediately. Corneoscleral buttons were dissected using standard eye bank techniques. Corneoscleral preparations were mounted on a perfusion chamber made of polycarbonate and secured with the clamping sleeve, which covered the sclera. The anterior chamber was perfused with Earle minimum essential medium (MEM; Gibco, Glasgow, UK) containing 4% fetal calf serum (FCS) at a flow rate of 2.5 µL/min. The perfusate from the outflow tube was collected in a sealed reservoir and recirculated. The culture medium was changed on a daily basis. The reservoir was elevated 25 cm above the level of the clamped cornea to create a positive pressure of 18 mm Hg inside the artificial anterior chamber, simulating physiological conditions. The apical surface was irrigated positive pressure of 18 mm Hg inside the artificial anterior chamber, simulating physiological conditions. The apical surface was irrigated

Corneal Epithelial Debridement and Re-epithelialization

After the corneoscleral button was mounted in the perfusion chamber, a 7-mm diameter circle with a disposable trephine, located in the center of the cornea, was produced. The encircled corneal epithelium was removed with a no. 15 Bard-Parker scalpel blade under a dissection microscope. For the whole cornea epithelial cell debridement experiment, epithelial cells were removed with a scalpel blade, and the corneal surface was rubbed with a cotton-tipped applicator so as to remove debris. Removal of the epithelial cells in the wounded area was confirmed by examining 10–0 nylon sutures. After saline wash, the exposed basement membrane retained the fluorescein stain. Images were taken digitally at different time points, and the rates of wound closure were calculated by examining 10–0 nylon sutures.

Light and Transmission Electron Microscopy

Specimens were fixed in 2.5% glutaraldehyde in PBS and postfixed in 2% aqueous osmium tetroxide. They were washed in PBS before passage through a graded ethanol series and then embedded in epoxy resin. Semithin (0.5–µm) sections were collected on glass slides and stained with toluidine blue before examination under a light microscope. Ultrathin (70-nm) sections were collected on copper grids and stained with uranyl acetate, 1% phosphotungstic acid, and Reynolds lead citrate before examination with a transmission electron microscope (JEM 1010; JEOL, Tokyo, Japan).

Scanning Electron Microscopy

Samples were fixed in 2.5% glutaraldehyde and postfixed in 2% osmium tetroxide. They were washed again in PBS before passage through an alcohol series. After two 20-minute changes of 100% ethanol, the samples were transferred to hexamethyldisilazane for 10 minutes and air dried. Samples were then mounted and sputter coated with gold before examination on a scanning electron microscope (JSM 5600; JEOL, Tokyo, Japan).

Statistical Analysis

All results are expressed as mean ± SEM. An unpaired Student t test was used for comparisons. Differences were considered statistically significant when P < 0.05.
RESULTS

Long-term Culture of Bovine Cornea

Fresh bovine corneas (n = 12) were mounted on the air-interface culture chambers and were cultured with surface irrigation solution containing 4% FCS for 10 days. Cultured corneas were then processed and examined using light and electron microscopy.

Light microscopy showed that the epithelial cell layers were not reduced in thickness or in number of layers and that the epithelial architecture was not changed. The stroma remained healthy and showed no evidence of edema (Fig. 3A). Scanning electron microscopy revealed that the epithelial surface consisted of flat, squamous, polygonal epithelial cells closely attached to each other with distinct cell boundaries, identical in appearance to the cornea in vivo (Fig. 3B). The morphology of the endothelial cells also resembled that seen in the cornea in vivo (Fig. 3C). Mean endothelial cell size (171 ± 15 μm) was not significantly different from that of the in vivo cornea (169 ± 11 μm) (P > 0.05). Percentages of hexagonal cells from cultured corneas and fresh corneas was 81% ± 9% and 82% ± 7% (P > 0.05), respectively. Data were obtained from three different random fields of each sample. Transmission electron microscopy demonstrated that the epithelial cell layer appeared to be healthy. The cell layer was stratified and differentiated into basal, wing, and superficial cell layers. Adjacent epithelial cells were connected with desmosomes (Fig. 3D). Examination of the putative epithelial stem cells in the limbal region revealed that their appearance and morphology were identical with those of in vivo bovine cornea (Fig. 3E). Examination of the stroma showed that the collagen fibrils had normal spacing and organization and no sign of edema. Both the density and the ultrastructure of stromal keratocytes appeared normal (Fig. 3F).

Corneal Re-epithelialization after Epithelial Cell Debridement

A brief latency period was observed after epithelial debridement, though our measurement intervals were not short enough to accurately evaluate this phase. In the 7-mm diameter corneal epithelial debridement experiment, wound healing occurred in a circularly symmetrical manner, and the corneas remained transparent throughout the healing process. Results revealed that the process of re-epithelialization occurred more rapidly in the corneas with surface irrigation solution containing FCS than in corneas irrigated without FCS (Figs. 4A, 4B).

Complete wound closure occurred in 49 ± 2.5 hours (n = 12) for the surface irrigation solution containing FCS and in 132 ± 5.2 hours (n = 10) for that without FCS. Wound healing rates were 0.78 ± 0.08 mm²/h and 0.29 ± 0.05 mm²/h (P < 0.001), respectively (Fig. 5). In the total epithelium debridement corneas (n = 2), complete re-epithelialization was achieved 7 days after surface irrigation with a solution containing FCS (Fig. 4C).

In the 7-mm diameter epithelial cell scraping experiment, light micrographs showed that in both experimental groups, the epithelium covering the wound area had regained full thickness after 7 days of culture. The epithelial sheet resembled normal corneal epithelium with superficial squamous layers, wing layers, and a single basal layer of columnar cells (Figs. 6A, 6B). However, vacuoles were present in some of the regenerated epithelial cells in the samples that did not have FCS in the irrigation solution (Fig. 6B). Examination of the apical surface by scanning electron microscopy showed that a continuous layer of flat polygonal epithelial cells was regenerated in both experimental groups. In the samples with corneal surface irrigation solution containing FCS, the junctions between the superficial cells appeared more prominent and more tightly opposed (Fig. 6C) than in the group without FCS (Fig. 6D). Apical surfaces of both groups’ cells were covered with numerous small microvilli. Results from transmission electron microscopy demonstrated that the regenerated basal epithelial cell formed hemidesmosomes with the basal lamina in both experimental groups. However, the gaps between the basal cells and the neighboring basal lamina were significantly larger in samples irrigated using solution without FCS (Fig. 6D) than in those whose solution contained FCS (Fig. 6E). The average space was 0.15 μm² in the group for surface irrigation solution with FCS and 0.28 μm² without FCS (P < 0.001). Data were obtained from three random fields from each sample consisting of 3-μm-long interfaces between epithelial basal cells and adjacent basal lamina.

Wound Healing after Corneal Transplantation

Lamellar and penetrating transplantation were performed. In the lamellar transplantation group (n = 10), corneas maintained transparency 1 week after surgery (Fig. 7A). Two days after surgery in the penetrating transplantation group (n = 12), corneal edema became apparent along the junction between
the donor explant and the recipient. Edema subsided substantially 1 week after surgery (Fig. 7B). Cultures were terminated 1 week after surgery, and samples were processed and examined with the use of light and electron microscopy.

In the lamellar transplantation group, though sutures were placed snugly to achieve adequate tissue apposition, light microscopy showed some space between donor and recipient tissues in the vertical direction. Epithelial cells migrated along this region of exposed stroma, and, 1 week after transplantation, this corneal anterior stromal defect had been covered. Epithelial cells from donor and recipient became incorporated (Fig. 8A). Scanning electron microscopic examination showed that apical polygonal epithelial cells from the explant appeared normal 1 week after lamellar transplantation. These epithelial cells were still undergoing the normal process of desquamation (Fig. 8B). Transmission electron microscopy showed that the arrangement of fibrils was disorganized along the wound margins at each side of the stroma. Within a range of 200 μm from the cutting edge, keratocytes showed signs of apoptosis, including chromatin condensation, bleb formation inside the cell, and shrinkage (Fig. 8C). In undamaged areas of the corneas, the lamellae were well organized, and keratocytes appeared healthy (Fig. 8D). In the wound margins, the new migrating epithelial basal cells attached to the underlying stromal substrate with few hemidesmosomes (Fig. 8E). In contrast, hemidesmosomal connections were common between the basal epithelial cells and the basal lamina in the unwounded areas (Fig. 8F).

In the penetrating transplantation group, in addition to the features of wound healing already described for lamellar transplantation in the epithelium and stroma (data not shown), endothelial cells migrated onto the exposed posterior stroma in the wounded area and covered this area completely 1 week after operation (Fig. 9A). Transmission electron microscopy showed that the migrated endothelial cells covering the exposed posterior stroma had normal morphology (Fig. 9B). Scanning electron microscopy revealed that a continuous layer of flat polygonal endothelial cells completely covered the wound area (Fig. 9C). However, the shape of these cells was slightly irregular, and the interdigitations at the cell boundaries were not as distinct as those in the nonwounded area (Figs. 9C, 9D).

**DISCUSSION**

As we attempted to develop an in vitro model of the cornea, we had several key objectives. We thought it was critical to try to replicate the air interface of the in vivo corneal epithelium because this has been shown to improve epithelial integrity and tight junction formation. In our model, the epithelium was exposed to the normal atmospheric rather than to the 5% CO₂ environment inside an incubator. A similar air-interface model existed but had only been monitored for 14 hours. Some form of irrigation was also necessary to try to replicate (however crudely) the natural tear film and wash away desquamated epithelial cells. We believe the automated irrigation system in our model to be unique. The culture system also had to be able to support the epithelial stem cells, which are essential for the epithelium's ability to regenerate. We therefore included the basal limbal cells in our evaluation of the model, which we describe as putative (presumed) stem cells. This classification is based on previous work on bovine cornea by us and others. Sun and coworkers describe undifferentiated basal (stem) cells in the bovine limbus. Our detailed ultrastructural examination showed that the appearance of these cells was consistent with that previously described for stem cells (Fig. 3E). We also show that after total debride-
ment of the epithelium, new epithelial cells migrated from the limbus (Fig. 4C). To simulate the physiology of the anterior chamber, our model had to be under normal intraocular pressure. We used 18 mm Hg, which is physiological intraocular pressure for cattle and within the range of normal human intraocular pressure. In addition, the endothelium should be perfused independently at physiological flow rates. Our posterior chamber perfusion rate of 2.5 L/min was based on the mean of reported physiological aqueous humor flow rates. Finally, we wanted to be able to determine whether the cornea could be maintained long enough for it to be useful for epithelial and endothelial wound healing studies. Our results show that all these objectives can be achieved with the model we have described.

We chose bovine cornea because it is readily available and because the scientific literature includes numerous studies on bovine cornea that show it has a structure and a biochemical makeup similar to that of human cornea. Our long-term culture study showed that the transparency and normal shape were maintained over a period of 10 days. Epithelial cells appeared healthy, and epithelial layers were not reduced. Putative stem cells in the limbal region of our organ-cultured corneas appeared identical with those in vivo. The keratocyte population in the corneal stroma also appeared normal. Two parameters for evaluating the quality of endothelial cells, mean cell size and percentage of hexagonal cells, did not change, and the stroma showed no sign of edema. The inclusion of 4% FCS in our endothelial perfusate resulted in excellent endothelial preservation and wound healing; we did not use higher levels because they could potentially result in endothelial proliferation.

With regard to the usefulness of this model for wound healing studies, our study demonstrates that epithelial migration over a debridement area occurred in a manner similar to that of in vivo studies. When the epithelial surface was irrigated with a solution containing FCS, the epithelial closure rate was comparable to that of clinical observations of healing in human cornea. However, when the irrigation solution did not contain FCS, the closure rate was much slower, and the basal junctions of the cells were abnormal. These results reflect the importance of tear substances, particularly growth factors and other cytokines, in the process of corneal wound healing.

We also evaluated the usefulness of our in vitro model by performing penetrating keratoplasty. To our knowledge, this is

**Figure 6.** Light micrographs (A, B) and scanning (C, D) and transmission (E, F) electron images of corneal re-epithelialization. Corneas after surface irrigation with solution containing FCS (A, C, E). Corneas with surface irrigation with solution not containing FCS (B, D, F). Full-thickness epithelium was regenerated in both experimental groups after 7 days of culture (A, B). However, vacuoles formed inside the regenerated epithelium irrigated with a solution without FCS (B). In samples irrigated with a solution containing FCS, the junctions between regenerated superficial cells were more tightly opposed (C) than those irrigated with a solution not containing FCS (D). Migrated basal epithelial cells formed hemidesmosomes with the basal lamina (E, F). Average space between the base of the cell and the basal lamina was 0.15 μm² in the group for surface irrigated with solution containing FCS (E) and 0.28 μm² for the group irrigated with solution not containing FCS (P < 0.001) (F). Values are given for a 3-μm-long interface between epithelial basal cells and adjacent basal lamina. Arrows: how the spaces were defined (E, F).

**Figure 7.** Representative images after corneal lamellar (A) and penetrating (B) transplantation. One week after corneal lamellar transplantation, the donor explant apposed well with the recipient, and the whole cornea maintained its transparency (A). At time 0 after corneal penetrating transplantation, there is slight edema (B1). Two days after the operation, the recipient cornea along the adjacent area with the donor explant is highly edematous (B2). One week after the operation, the corneal edema has greatly subsided (B3).
the first time this procedure has been carried out on an in vitro corneal model.

A series of changes occurs during and after corneal transplantation in vivo. Immediately after incision, the wound edges separate and Descemet membrane curls inward toward the stroma at the margins of the wound. The corneal stroma becomes relatively dehydrated, which induces apoptosis of the keratocytes. After the donor explant is sutured on the stroma in the wound region, the kerocytes become thin and flat as they migrate over the exposed tissue. Some keratocytes along the wound edges undergo the process of desquamation only 1 week after lamellar transplantation. In our model, for the first time this procedure has been carried out on an in vitro corneal model. Results show that, at least over the limited time period of our study, the in vitro corneal culture system behaves similarly to the in vivo corneal system. Clearly, any results obtained from this model must be interpreted with the understanding that our in vitro model has no immune system. However, one could argue that because of the immune-privileged status of the cornea, this may be less important in an in vitro corneal model than in any other tissue. In addition, though this model does have limitations, we suggest it is clearly superior to in vitro systems based on isolated corneal cell lines.

Although we developed this system for use in fundamental scientific research, it has potential applications in applied research, particularly within the European Union (EU). A recent EU amendment places new restrictions on animal testing beginning in 2009. Hence, a real need exists for in vitro test systems to be developed and validated before these restrictions are put into effect.

In conclusion, it is clear that this model replicates the in vivo cornea well over the period of this study. It can be used for studies on corneal wound healing or stem cell biology and could also be used to evaluate the effect of therapeutic agents or toxins. The model could be easily modified for human tissue.

FIGURE 8. Seven days after lamellar transplantation. Light micrograph of the junction area between recipient and donor tissues (A). Scanning electron micrograph of the apical epithelial cells of donor explant in Figure 8A. Transmission electron micrographs (C–F). Stroma along the margin of the wound area includes an apoptotic keratocyte with chromatin condensation, bleb formation, and cell shrinkage and fibril disorganization (C). In the nonwounded area, the keratocytes appeared healthy and the lamellae well organized (D). In the wounded area, the epithelial cells showed few hemidesmosomes are evident between the epithelial cells and the basal lamina (E).

FIGURE 9. Seven-day culture after penetrating transplantation showing the posterior surface. Light micrograph of endothelial cells on the posterior stromal wounded area (arrow), between the donor Descemet (DD) and recipient Descemet (RD) membranes. Transmission electron micrograph of migrated endothelial cells on the stromal wounded area (B) appear normal. Scanning electron micrographs of corneal endothelial cells on the stromal wounded (C) and nonwounded (D) areas. Endothelial cells have completely covered the stromal wounded area (C). However, interdigitations between cells on the wounded area (C) were not as evident as on the nonwounded area (D). Endothelial cells on the nonwounded area appear normal (D).
and used for preliminary investigations into innovative surgical procedures, such as in the use of new endothelial or epithelial stem cell transplantation techniques or for the evaluation of novel keratoprosthesis.

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References