The organ-cultured cornea: An in vitro study

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A presentation of techniques for organ culture and an evaluation of the effects of organ culture on human and animal corneas is presented. The gross and microscopic appearance of cultured corneas, phase contrast microscopic study, and histopathology of organ-cultured corneas are discussed. These observations show that corneas can be stored in organ culture and that after prolonged in vitro preservation by this method remain grossly clear and appear to have extraordinary viability. Further application of the organ culture technique to study the cornea is discussed.

Key words: organ culture, cornea, viability, storage, preservation, morphology, phase-contrast microscopy, epithelium, endothelium, histopathology, transplantation, allografts, isografts, xenografts, histocompatibility, antigens, skin, rabbit, chicken, guinea pig, human.

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It has been proven that organ-cultured skin remains functionally viable for extended periods. Furthermore, during organ culture, skin loses immunogenicity. This paper represents an attempt to extend the original research to include an additional epithelial covered organ, the cornea. The viability of organ-cultured skin through a series of autologous human skin transplants has been established. Immunogenic modification through an analogous series of human allografts from mis-matched female donors to male recipients has been shown. It has been possible to adapt this phenomenon to an animal model using isografts and allografts of cultured skin in mice. Major mouse histocompatibility barriers have been crossed. The model even permits use of cultured skin across species barriers as well (xenografts). Paradoxically, the modification of immunogenicity during the culture process is not attributa-
able to loss of antigens during the culture process, and in the allografts of both mice and men successful transplantation is achieved while major histocompatibility antigens can be demonstrated to be present in the grafts in apparently normal amounts. In light of the above, a series of studies was begun at the University of Minnesota to evaluate this system for use with corneal transplants. Our initial study involves an evaluation of materials, methods, techniques, gross morphology, microscopic morphology, phase contrast microscopy, and histopathology of corneas of humans, rabbits, guinea pigs, and chickens adapted to prolonged organ culture.

Methods

Animals. For the purpose of this study, organ culturing techniques were applied to corneas from chickens, rabbits, and guinea pigs. The animals were killed as follows: rabbits, by air embolus via marginal ear vein or heart puncture; guinea pigs, air embolus via heart puncture; and chickens by decapitation.

Human donor eyes. The human eyes used in the experiments were those that were donated to the Minnesota Lions Eye Bank and deemed unacceptable for human transplantation for various reasons.

Interim storage. Both human and animal eyes were placed in a moist saline chamber at 4°C. The human eyes used in most cases had been stored in a moist chamber at 4°C for at least 48 hours. The animal eyes were used within two to three hours after enucleation.

Preparation of corneal button with scleral rim. Human and animal eyes were prepared for organ culture by sectioning the cornea from the eye with a 2 to 3 mm. rim of sclera. Vitreous, lens, and iris were then gently removed, avoiding damage to the corneal endothelium. Sterile techniques were used throughout.

Organ culture media. The organ culture media consisted of the following ingredients: (1) minimum essential medium (Eagles) 1× with Earle's salt without L-glutamine, (2) calf serum, in a 10 per cent concentration, (3) L-glutamine in a one per cent final concentration, and (4) an antibiotic-antimycotic mixture consisting of penicillin 10,000 units per milliliter, amphotericin 25 μg per milliliter, and streptomycin 10,000 μg per milliliter added at a concentration of one per cent in the final mixture. The above ingredients are standard readily available products. The minimum essential medium was stored according to label directions at 4°C. prior to use. The calf serum and L-glutamine were stored in amounts appropriate to quickly mix with 100 to 500 ml. of media. The antibiotic-antimycotic mixture was the last ingredient to be added. The media was prepared 500 ml. at a time, and allowed to come to room temperature before use.

Placement and maintenance in media. The corneal buttons obtained were placed in sterile Falcon tissue culture dishes containing sterile organ culture media. A gravity feed technique was used to pipette the organ culture media into the tissue culture dishes. The culture media was completely changed on the second and fifth days of each week. All manipulations of the culture were performed under sterile conditions, aspirating the media with the sterile pipette from the culture dishes and replacing it with fresh media by the sterile gravity feed method, as above.

The corneal buttons were placed epithelial side up on a glass cover-slip in the Falcon dishes. One drop of fibrinogen and one drop of thrombin were placed on the cover slip to hold the cornea in place. The fibrinogen and thrombin clot technique was used only on initial placement in culture.

Incubation. The dishes containing corneal buttons were then placed in a water-jacketed tissue culture incubator at 37°C. The atmosphere consisted of five per cent CO₂ and 95 per cent air.

Results

Gross morphology. Twenty corneas each from human, chicken, rabbit, and guinea pig were placed in organ culture and all noncontaminated* corneas remained clear over an observation period arbitrarily terminated at one month. The phenol red in the medium imparted a slight reddish tinge to the corneal tissue. The human corneas all became slightly swollen in appearance, but remained quite transparent over the entire period of culture (Fig. 1). It was also noted that several human corneas which were cloudy at the time of placement into the organ culture media subsequently cleared to gross observation within one week in organ culture.

Phase-contrast microscopy. Under the phase-contrast microscope, good growth of the epithelium, endothelium, and stromal cells was observed in human and animal corneas. Epithelial and endothelial cells were seen alone and in sheets by the second
day. A three to four day period elapsed before the spindle-shaped stromal cells could be identified. Sheets of cells were readily observed growing from the edge of the specimens, particularly where a wedge biopsy had been taken.

**Microscopic morphology.** In vitro specimens of organ-cultured corneas from human, rabbit, guinea pig, and chicken were examined daily for the first ten days and then at weekly intervals for the remainder of the first month.

**Histopathology of human corneas.** The following observations were made on human corneas maintained for four weeks in organ culture. Grossly, the specimens were uniformly clear. For the following routine and special staining techniques, we stained a human cornea which was six hours post-mortem as a parallel control.

Hematoxylin and eosin staining revealed the following: the epithelium of the cultured cornea showed some variability in thickness but this was not marked. The thinnest areas still contained at least two cell layers of epithelium and were found at the periphery, while the central area contained the usual five to six cell thickness. Growth of a single layer of epithelial cells extending over the cut edge of the specimen was noted. Bowman's membrane was intact. The corneal stroma showed a moderate decrease in the number of stromal cells, Descemet's membrane was intact and the endothelium remarkably well preserved. Usually no edema was seen and no shrinkage could be detected.

The PAS (periodic acid–Schiff) stain showed good preservation of Descemet's membrane. The basement membrane of the epithelium stained well and was intact throughout the anterior surface of the specimens. Stroma, epithelium, and endothelium stained appropriately. Van Gieson stain for collagen fibers revealed no remarkable pathology.

Alcian Blue stains with and without hyaluronidase for acid mucopolysaccharide also showed no abnormalities when compared with fresh tissue.

**Flat preparation of corneal endothelium.** The endothelial layer from organ-cultured human corneas was removed with a dissecting needle under a dissecting microscope, mounted on a glass slide, and stained by the silver-hematoxylin-celestine blue staining method. Cell membranes were intact and were found to stain as well as fresh normal corneas. Normal to increased evidence of cell division was present morphologically in that sheets of mononucleated and multinucleated endothelial cells were observed (Figs. 2 and 3). These findings were consistently noted in rabbit endothelium as well as human. We did not study endothelium from guinea pig or chicken corneas.

**Serial staining of animal corneas.** The corneal tissue appeared viable and was difficult to distinguish from fresh normal corneal specimens. Cultured tissues were slightly increased in thickness as compared to fresh cornea; this finding was especially noted in guinea pig and rabbit corneas in side-by-side cross-section comparison to normal control corneas. Both epithelial and endothelial cell populations grew over the scleral rim of the corneal buttons, and even obscured the limbal edge of the cornea in some instances. Serial sections of corneas stained with hematoxylin and eosin showed good growth of the epithelium, preservation and growth of endothelium, and changes in the stroma consistent with a slight decrease in the number of stromal
Fig. 2. Human corneal endothelium organ cultured 2 to 3 weeks showing intact cell membranes and one nuclei per cell.

cells. Collagen stains revealed a normal stroma. Minimal edema and shrinkage changes in the stroma were seen with a cross-section preparations. In general, the specimens maintained good epithelium, stroma, and endothelium up to the end of the first month of observation in organ culture.

Discussion

Human and animal corneas maintained their clarity in organ culture for the period of this study which was arbitrarily concluded at one month. Not only was clarity maintained, but several cloudy human corneas initially preserved in a moist chamber at 4° C. for 48 hours or more cleared during organ culture.

Phase-contrast microscopy revealed viability of the corneas in culture through observable cell growth in organ-culture media. During the second 24 hour period in culture we began to see epithelial and endothelial cell outgrowth, and after three to four days stromal cells appeared as has been previously observed by Stocker in his tissue culture of the various cell layers.

Under the dissection microscope, the animal corneas appeared somewhat swollen. Growth of epithelium and endothelium was evidenced by proliferation about the edges of the specimens. Specimens cultured for one month looked well preserved in all regards with the exception of a slight decrease in the number of stromal cells.

Histopathologic analysis of human corneas cultured for one month revealed a consistently viable organ as demonstrated by various histochemical staining techniques. The five layers of the corneas were well preserved including Bowman's and Descemet's membranes. No remarkable pathology was noted with hematoxylin and eosin, PAS, Van Gieson, or Alcian Blue stains.

Human corneal endothelium showed remarkable evidence of viability in organ culture. Endothelial preparations evidenced normal cellular integrity as well as growth on flat mount preparations. To us this was a unique experience with stored corneas.

The fact that nuclear division exceeded cytoplasmic division (revealed as multinucleated cells) was interpreted as evidence for stimulated growth kinetics. This growth of the endothelium was induced through organ culture techniques in corneal endothelial cells which are usually thought to be capable of little growth at best.

The possible significance of the in vitro phenomenon herein described is many fold. Skin stored for up to eight months has been successfully transplanted autologously and no indication of loss has been encountered in these successful transplants (Summerlin, personal communication). Organ-culture techniques may be used for intermediate or long-term preservation of human and
animal corneas. Currently short, intermediate, or long-term preservation by storage in a moist chamber, with host serum, and by special freezing techniques, respectively, have been developed and used clinically. These methods are not ideal and by comparison our cornea culture technique seems to have some advantages. Organ culture may provide a means to use clinical material that might otherwise go unused for transplantation purposes. The method may also permit viability studies during in vitro maintenance. Further, organ culture could provide a laboratory model of a functioning cornea for studies useful in elucidating the physiologic contributions of the various cell layers that make up the organ. Finally, fundamental disease processes may be addressed by use of this laboratory model.

These investigations show that cornea, like skin, can be surprisingly well maintained in organ culture. Indeed, initial morphologic observations suggest that the cornea may be as well suited to maintenance in organ culture as skin, which has already yielded such extraordinary findings when transplanted after a period in culture. Further investigations are underway to evaluate the practical application of this simple organ-culture technique using allogenic and xenogenic transplantation.

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