A Novel Method for Preservation of Human Corneal Limbal Tissue

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Cornea

PURPOSE. We investigated the efficacy of low-temperature airlift preservation of human corneal limbal tissue for ex vivo expansion and allograft keratolimbal transplantation.

METHODS. Human limbal tissue either was submerged or airlifted in Optisol-GS medium and preserved at 4°C for up to eight days. Hematoxylin and eosin, and E-cadherin staining was performed to investigate epithelial structure and cell–cell junction. Epithelial cell differentiation and proliferation were studied using the biomarkers, such as K10, K12, K14, Ki67, and p63. Cell apoptosis was detected with the TUNEL assay. The epithelial progenitor cell pool was evaluated by clonal culture of epithelial cells on 3T3 feeder layers. For clinical application, keratolimbal transplantation was performed in three patients with partial limbal stem cell deficiency, using limbal tissues preserved under the airlift manner. Pre- and postoperative evaluations were conducted by slit-lamp microscopy and fluorescein staining.

RESULTS. After eight days, intact epithelia with strong cell–cell junctions were retained only in airlifted tissues. Airlifting maintained a normal corneal differentiation pattern, along with low proliferation activity and increased proliferation potential, but little apoptosis. Epithelial cells harvested from the airlift preservation for up to eight days exhibited stable clonogenicity. Limbal tissues preserved under the airlift manner successfully reconstructed corneal and limbal surfaces in partial limbal stem cell–deficient patients.

CONCLUSIONS. Limbal tissues preserved under hypothermic airlift conditions maintain the intact structure, normal phenotype, high viability, and stem cell pool of limbal epithelium. Such a method may be used in eye bank tissue processing and corneal epithelial tissue engineering.

Keywords: cornea, limbus, stem cell, preservation

Corneal epithelial stem cells are located in the basal layer of the limbal epithelium,1 and they ultimately are responsible for the corneal epithelium under normal circumstances and during wound healing.2,3 Severe ocular surface diseases, such as chemical or thermal burns, ocular cicatricial pemphigoid, and Stevens-Johnson syndrome, can result in total limbal stem cell deficiency (LSCD).4 In such cases, keratolimbal transplantation can rebuild the limbus and corneal surface.5,6 Transplantation of ex vivo expanded limbal epithelial cells also is effective for corneal epithelial reconstruction.7,8

For both therapeutic approaches, limbal tissues from cadaveric donors are used commonly to provide limbal epithelial stem cells, and such tissues always come from eye banks. In European countries, it is common practice to preserve donor corneal tissue in organ culture medium at 31°C, whereby tissue can be stored for up to 4 weeks with high viability for corneal transplantation,9 and this preservation method also can maintain corneal epithelial proliferative potential effectively, which is pivotal for allo-limbal transplantation.10,11 However, eye banks in American and Asian countries normally use a hypothermic corneal storage method.12 Corneal tissues, including the limbus, are submerged in storage medium, such as Optisol-GS, Dexsol, or Chen’s medium, kept in a closed container, and preserved at 4°C. Although limbal tissues stored in Optisol-GS for as long as 16 days still can generate epithelial outgrowth in explant culture, the growth rate will decrease with preservation time.13 Previous studies also found that during hypothermic storage of corneal tissue, the epithelial cells gradually desquamate from the tissue,14 and the number of apoptotic cells increases with time in the epithelial layer, but not in the keratocytes or endothelium.15,16 Furthermore, prolonged donor storage under a hypothermic condition also can increase the risk of persistent postoperative epithelial defect.17 Therefore, currently widely used hypothermic preservation methods may not be feasible for midterm storage of limbal epithelial cells in keratolimbal transplantation and tissue engineering. A search for a better preservation method for limbal epithelia during corneal tissue processing is imperative.

Our previous study provided some indication that airlift culture of limbal tissue at 37°C could induce dramatic stratification and proliferation, meanwhile maintaining the intact structure of limbal epithelia.18 This finding prompted us to hypothesize that preservation of limbal tissue under the airlift manner might prolong the storage of limbal epithelia and help maintain their functional integrity. We compared here the effects of culturing limbal tissues in Optisol-GS medium at 4°C under airlift and submerged conditions on preserving epithelia integrity, viability, and phenotype. Our results indicated that in
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this medium airlifting at 4°C is more desirable than tissue submersion for preservation of these properties.

Materials and Methods

Materials

Dulbecco’s modified Eagle medium (DMEM), Ham’s/F-12 medium, HEPES buffer, fetal bovine serum (FBS), amphotericin B, and gentamicin were purchased from Invitrogen Corporation (Carlsbad, CA). Cholera toxin subunit A, dimethyl sulfoxide (DMSO), hydrocortisone, insulin-transferrin-sodium selenite (IT) media supplement, human recombinant epidermal growth factor (EGF), mitomycin C, acetone, Triton X-100, BSA, and FITC-conjugated antomouse, -goat, and -rabbit IgGs were obtained from Sigma-Aldrich Corporation (St. Louis, MO). Mounting medium with DAPI was obtained from Vector Laboratories, Inc. (Burlingame, CA). Mouse anti-cytokeratin 10 (K10) was obtained from Chemicon International (Temecula, CA). Goat anti-cytokeratin 12 (K12), mouse anti-cytokeratin 14 (K14), and E-cadherin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-p63 (clone 4A4) and Ki67 antibodies, and diamino benzidine (DAB) were from Dako North America, Inc. (Carpinteria, CA). ABC kits (VECTASTAIN Elite) for mouse, goat, and rabbit IgGs were obtained from Vector Laboratories, Inc. Cell culture inserts were obtained from Corning, Inc. (Corning, NY). A TUNEL apoptosis detection kit was purchased from Promega Corporation (Madison, WI). The NIH 3T3 mouse fibroblasts cell line was obtained from ATCC (Rockville, MD). Optisol-GS corneal storage medium was obtained from Bausch & Lomb, Inc. (Rochester, NY).

Preservation of Human Limbal Tissue

The study was approved by the ethics committee of the Xiamen University Affiliated Xiamen Eye Center. Human tissue was handled in accordance with the Declaration of Helsinki. Corneoscleral tissues from human donor eyes were obtained from the Xiamen Eye Center immediately after the central corneal button had been used for corneal transplantation. For this study, corneoscleral tissues from 10 human corneas were used; donor age ranged from 8 to 85 years (average 51.3 ± 7.9). All corneas were harvested within 24 hours postmortem and had been preserved in Optisol-GS corneal storage medium for one day before corneal transplantation. The tissue was rinsed three times with DMEM containing 50 μg/mL gentamicin and 1.25 μg/mL amphotericin B. After careful removal of excessive sclera, conjunctiva, and iris, the corneoscleral rim was trimmed to obtain limbal tissue cubes of 2 clock-hour width (i.e., a size of approximately 3 × 5 mm). The limbal tissue cubes were placed on the center of inserts in six-well plates containing Optisol-GS corneal storage medium and were preserved in airlift or submerged conditions (Fig. 1). In the airlift manner, 2 mL medium were added so that the limbal epithelium was exposed to the air, but the remaining limbal stroma was submerged in the medium. The preservation chambers then were sealed with Parafilm and preserved at 4°C under normal air, and the medium was changed every 2 days for 8 days before the preservation was terminated. In the submerged manner, 3 mL medium were added so that the entire limbal tissue was submerged in the medium.

Clonal Culture of Limbal Epithelial Cells

The human limbal cubes were removed from the preservation chambers at different time points and incubated in DMEM containing Dispase II (2 mg/mL) for 12 hours at 4°C. Limbal epithelial cells were harvested as described previously and rendered into single cells using TrypLE treatment for 5 minutes at 37°C. Single limbal epithelial cell suspensions then were seeded at 1 × 10³ cells per cm² into 6-well culture plates with mitomycin C-treated 3T3 fibroblast feeder layer, and cultured in supplemented hormonal epidermal medium (SHEM) for 9 days. SHEM medium contains equal amounts of DMEM and Ham’s/F12 medium supplemented with 5% FBS, 5 ng/mL EGF, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, 0.5 μg/mL hydrocortisone, 30 ng/mL cholera toxin A, 0.5% DMSO, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B. Colony-forming efficiency (CFE) was calculated as the percentage of the number of colonies generated divided by the number of epithelial cells plated in a well, and colony growth capacity was evaluated when cultured cells were stained with crystal violet on day 9.

Histology and Immunostaining

After the different preservation time, the human limbal explants were embedded in ornithine carbamoyltransferase (OCT). Cryostat sections (4 μm) of the limbal explants were fixed in acetone for 10 minutes at −20°C and prepared for immunostaining by rehydration in PBS, followed by incubation in 0.2% Triton X-100 for 10 minutes. After three rinses with PBS for 5 minutes each and preincubation with 2% BSA to block nonspecific staining, the sections were incubated with primary antibodies for 1 hour at different dilutions (K12 and K10 at 1:200, K14 and Ki67 at 1:100, E-cadherin at 1:100). After three washes with PBS for 15 minutes, they were incubated with an FITC-conjugated secondary antibody (goat antirabbit or antigoat IgG at 1:100) for 45 minutes. After three additional PBS washes for 15 minutes, they were mounted with an antifade solution with DAPI and photographed with a fluorescence microscope. For immunohistochemical staining of p63, endogenous peroxidase activity was blocked by 0.6% hydrogen peroxide for 10 minutes after fixation, and nonspecific staining.
was blocked by 1% normal goat serum for 30 minutes. The sections then were incubated with anti-p63 antibody (1:50) for 1 hour at room temperature. After three washes with PBS for 15 minutes, the sections were incubated with biotinylated anti-mouse IgG (1:100) for 1 hour, followed by incubation with ABC reagent for 30 minutes. The reaction product then was developed with DAB for 2 minutes.

In Situ TUNEL Labeling

To measure end-stage apoptosis, in situ TUNEL labeling was performed on the limbal tissues after airlift preservation at different durations, using the DeadEnd Fluorometric TUNEL System according to the manufacturer’s instructions. The cryosections were fixed in acetone at 4°C, rinsed with PBS, and permeabilized by 0.2% Triton X100, followed by incubation in equilibration buffer for 10 minutes. The sections were incubated further with TdT reaction mix for 60 minutes, and then immersed in standard saline citrate to stop the reaction. After a rinse, the sections were counterstained with DAPI and mounted, and photo images were taken with a confocal laser scanning microscope (Fluoview FV1000; Olympus, Tokyo, Japan). For a positive control, sections were incubated in DNase I before addition of equilibration buffer, while distilled water was used instead of TdT reaction mix in the negative control.

Participants and Clinical Study

The clinical study was approved by the ethics committee of Xiamen University Affiliated Xiamen Eye Center and was performed in accordance with the principles of the Declaration of Helsinki. All participants were informed of the purpose of this study, and their written consent was obtained before limbal transplantation. Three consecutive patients with ocular surface disease, including one patient with corneal ulcer (case 1: male, age 36) and two patients with partial limbal stem cell deficiency resulting from thermal burn (case 2: male, age 43; case 3: male, age 50) were enrolled in this study. Donor limbal tissues were preserved under the airlift manner for 4, 6, and 8 days, as mentioned previously. The graft size varied among the eyes from 3 to 5 clock hours, including the limbal area, and 1 to 2 mm peripheral cornea. During the surgery, a conjunctival peritomy adjacent to the deficient area in the recipient cornea was performed, and the limbal conjunctiva was undermined and allowed to retract posteriorly. The donor graft was secured in the recipient bed using a 10-0 nylon suture after the pannus was dissected and catarized. The limbal portion of the graft was sutured to the underlying episcleral tissue, and the corneal edge of the graft was sutured to the peripheral cornea. At the end of the procedure, the eyes were patched to maintain pressure of the graft on the recipient bed. Patients were given oral prednisolone (30 mg/d) for one week before surgery, and intravenous dexamethasone sodium phosphate (10 mg/d) combined with cefotaxime sodium (1.5 mg/d) for three days after surgery. When the cornea was re-epithelialized, the recipient eye was treated with topical dexamethasone sodium phosphate (0.1%) four times daily, and topical tear substitutes four times daily. Topical corticosteroids were tapered three to four months after surgery. None of the patients received systemic cyclosporin A or other systemic immunosuppressants. The patients’ ocular surfaces were examined by slit-lamp microscopy and fluorescein staining.

Statistical Analysis

All experiments were conducted at least three times, each in triplicate. Summary data were reported as means ± SD. Group means were analyzed using Student’s t-test, where *P < 0.05 is considered statistically significant. The statistical analysis was conducted with software (GraphPad Prism for Windows, version 5.00; GraphPad Software, Inc., La Jolla, CA).

RESULTS

Morphologic Change of Limbal Epithelium During Preservation

Human limbal explants were preserved under an airlift or submerge manner for different durations. Hematoxylin and cosin (H&E) staining demonstrated that the limbal epithelium was nearly intact, and only a few superficial cells had detached from the epithelium on day 0. Under the submerge manner, the epithelium showed progressive fragmentation and desquamation from days 2 to 8. However, the epithelial integrity was well maintained by airlift preservation from days 2 to 6, and there was a slight increase in epithelial stratification on day 6. Although there was desquamation of superficial cells on day 8, the basal and suprabasal cells maintained close contact throughout the preservation (Fig. 2). We then performed E-cadherin immunostaining to investigate epithelial cell–cell adhesion during preservation. As expected, E-cadherin expression dramatically diminished from days 4 to 8 in tissues preserved in the submerge manner. However, E-cadherin was well maintained in tissues preserved under the airlift manner from days 0 to 8 (Fig. 3). These data indicated preserving limbal tissues under the airlift manner helps maintain the integrity of limbal epithelium.

Epithelial Differentiation During Preservation in Airlift Condition

Our previous study demonstrated that airlift culture of limbal epithelia could induce abnormal differentiation of epithelial cells, whereby epithelial cells switched their phenotype from corneal to epidermal.18 As such, we wondered whether hypothermic preservation of limbal tissue under the airlift condition could cause abnormal differentiation of limbal epithelial cells. We then performed keratin K12 and K10 immunostaining to elucidate epithelial differentiation status. The results showed that K12 was expressed in the limbal epithelial cells, except for some basal cells, during storage under the airlift manner, while there were no keratin K10-positive cells present throughout the airlift storage duration (Fig. 4). As reported previously, the epithelial cells also showed K12-positive and K10-negative in the submerge manner (Fig. 4). These results suggested that hypothermic preservation of limbal tissue under the airlift condition for 8 days did not induce epidermal differentiation of limbal epithelial cells.
To characterize the proliferation status of the limbal epithelial cells during airlift preservation, we performed immunostaining for K14 keratin, Ki67, and p63. Expression of K14, a marker thought to be expressed in basal epithelial progenitor cells of all stratified epithelia, was noted in basal and suprabasal epithelial cells in the normal limbus before preservation. There was dramatic decrease of K14 expression in tissues preserved under submerge manner from days 2 to 8, and the positive cells were confined to basal epithelium at day 8. However, there was a mild increase in K14 expression under the airlift condition from days 4 to 6, and there were much more positive cells in the airlift than in the submerge condition at day 8 (Fig. 5). There were only a few Ki67-positive cells present in the basal layer of the limbal epithelium on day 0, while limbi preserved under the airlift manner showed an increase in Ki67-positive epithelial cells from days 2 to 8, and there were no Ki67-positive cells present in submerge tissues from days 2 to 8 (Fig. 5). The p63-positive cells were noted mostly in basal and suprabasal layers before preservation, and they increased dramatically on day 2, and spread to full thickness of the limbal epithelium in airlift tissues from days 4 to 8. However, there was a decrease of p63 expression in submerge tissues from days 4 to 8 (Fig. 5). Collectively, these data indicated that the proliferation potential of limbal epithelial cells increased during airlift preservation, while it decreased during submerge preservation.

To verify the growth capacity of human limbal epithelia preserved in different conditions, we harvested the limbal epithelial cells at the end of different storage durations, seeded the cells on 3T3 feeder layers for 9 days, and calculated CFE. The results showed that there was a gradual decrease of CFE with storage time in tissues preserved under the submerge manner. After preservation in the submerge manner for 6 days, no epithelial colony had formed. In contrast, there was only a minor decrease of CFE in the epithelial cells of tissues preserved under the airlift manner for 8 days, compared to those from day 0. The CFEs were significantly lower in the submerge manner at all time points from days 2 to 8, compared to those preserved under the airlift manner (Fig. 7). These results suggested that airlift preservation at 4°C for 8 days did not affect the proliferative property of limbal stem cells.

**Figure 3.** E-cadherin expression in limbal tissue during hypothermic storage. Immunostaining showed that E-cadherin expression decreased gradually in the limbal tissue preserved under the submerge manner. However, E-cadherin was maintained throughout the storage duration under the airlift condition.

**Figure 4.** Phenotype of the limbal epithelial cells during hypothermic storage. Immunostaining revealed that keratin K12 was expressed in the limbal epithelial cells, except for some basal cells, during storage under the submerge and airlift manners. No keratin K10-positive cells were present throughout the storage duration under the submerge and airlift manners.

**Figure 5.** Proliferation status of the limbal epithelial cells during hypothermic storage. K14 expression decreased in tissues preserved under the submerge manner from days 2 to 8, while there was a mild increase in K14 expression under the airlift condition. Ki67-positive cells were located sporadically in the basal and suprabasal epithelium in the airlift, but not in the submerge preservation from days 0 to 8. p63 was expressed in the basal and suprabasal epithelium on day 0, and decreased gradually in the submerge condition, but expanded to full thickness of the limbal epithelium in the airlift condition from days 2 to 8.

**Apoptosis of Limbal Epithelium During Preservation**

A previous study showed that corneal epithelial cells will go into apoptosis during hypothermic storage, and the apoptotic cells will increase dramatically with time. We performed a TUNEL assay to detect apoptosis of limbal epithelial cells, and the results showed that no apoptotic cells were present in the limbal tissues on day 0. There was a gradual increase of apoptotic cells in the limbal epithelium and stroma in tissues preserved under the submerge manner from days 2 to 8. However, only a few apoptotic cells were present on superficial epithelia in tissues preserved under the airlift manner on day 8 (Fig. 6), indicating that the epithelial cells maintained high viability under airlift preservation.
cells; however, submerged preservation could cause severe limbal stem cell loss.

Clinical Outcome of Keratolimbal Allograft Transplantation

To confirm further the feasibility of limbal tissue preserved under the airlift manner, we performed keratolimbal allograft transplantations in three cases using limbal tissues preserved for durations of 4, 6, and 8 days, respectively. One week after surgery, complete corneal re-epithelialization was seen in all the eyes. One month after surgery, the patients’ corneal surfaces maintained integrity, and fluorescein staining revealed no epithelial defects. Fibrovascular tissue did not recur in any of the eyes (Fig. 8). These results suggested that limbal tissues preserved under the airlift manner for 4 to 8 days can be used for keratolimbal allograft transplantation.

DISCUSSION

Corneal epithelium of donor tissue eventually will be replaced by the recipient epithelium in penetrating keratoplasty. Therefore, much attention has been paid to corneal endothelia instead of epithelia during corneal storage, and endothelial cell viability long has been the major criterion for quality control of donor tissue. However, in keratolimbal transplantation, where limbal epithelial cells are the main therapeutic target, and in corneal epithelial tissue engineering, which specifically uses...
In our previous study, airlift culture of limbal tissues was employed, and the integrity of limbal epithelia, as well as the cell–cell junction, was well preserved at 4°C under the airlift manner for different durations. One month after surgery, the donor tissues were well integrated into the host tissues, and the limbal epithelium remained intact in all cases. Therefore, we found that hypothermic preservation of limbal tissue under the airlift manner could help maintain the integrity of limbal epithelia, as well as the cell–cell junction, for at least 8 days. The airlift method originally was developed to culture skin cell sheets for transplantation. Minami et al. first adopted this technique for corneal epithelial culture to generate a well-stratified corneal epithelial sheet. Subsequently, airlift culture was shown further to make the cell–cell attachment tighter, reduce intercellular space in the superficial epithelial cells, and promote formation of the barrier function. Because the previous studies were conducted under cell culture conditions, which is at 37°C with complete cell culture medium, in our study we showed that the effect of the airlift condition on epithelial integrity was well preserved at 4°C with corneal storage medium. This result also suggests that certain cell physiology, such as the cell–cell junction, could be well maintained under relatively low temperature.

The unique phenotype of corneal epithelial cells is characterized by the expression of keratins K3 and K12 in many species. In our previous study, airlift culture of human limbal tissue induced hyperproliferation of limbal epithelial cells; however, some epithelial cells transdifferentiated into epidermal phenotype, which showed K10 expression. Another study from our group showed that airlift culture of human limbal tissue in hypoxia conditions can induce hyperproliferation, yet prevent transdifferentiation of limbal epithelial cells. In the current study, hypothermic preservation of limbal tissues under the airlift condition does not cause abnormal differentiation of corneal epithelial cells. We presumed that the cell differentiation machinery under low temperature might be different from that under higher temperature. Another explanation might be the media used in different studies. Previous studies have used SHEM medium, which contains serum and growth factors, such as EGF and ITS, to culture limbal tissues. In our study, we used Optisol-GS medium, which is serum-free. Moreover, the preservation chambers were sealed with Parafilm during storage; we suspect the oxygen concentration in the chambers may decrease constantly during preservation, which may affect epithelial differentiation. Further study is needed to explore the underlying mechanism, which may help us understand the mechanism of corneal epithelium transdifferentiation.

As we know, cell metabolism decreases significantly under hypothermic conditions. Interestingly, we found that limbal epithelial cells could proliferate even under low temperatures. This was proved by the Ki67 staining of the limbal epithelia. Although the proliferation rate was maintained at a relatively low level, consistent proliferation also might contribute to structural maintenance of the limbal epithelium during preservation. The fact that there was a mild increase in epithelial stratification on day 6 of airlift preservation also supported the notion that limbal epithelial cells still can proliferate at 4°C. Moreover, apoptosis of epithelial and stromal cells was eliminated almost completely in the airlift preservation, but was prominent in the submerged manner. This high cell viability could affect greatly the outcome of clinical transplantation and cell yield in ex vivo expansion.

The major purpose of our study was to preserve limbal epithelial stem cells during storage. The expression of K14 and p63 did not decline, but rather increased, at the end of airlift storage of the limbal tissues. In addition, the CFE of the limbal epithelia did not decline noticeably with preservation time. All these results suggested that airlift storage at 4°C preserved well the stem cell pool of limbal epithelia. Based on these results, we conducted keratolimbal transplantation in three cases of partial limbal stem cell deficiency, and successfully reconstructed the limbus in all cases. Because this is a small-sized, uncontrolled serial case study, further research is needed to compare the clinical outcomes of keratolimbal transplantation using limbal tissues preserved under different conditions.

In summary, our study set up a novel method for ex vivo preservation of human limbal tissues. Using this method, limbal epithelial integrity, epithelial cell phenotype, epithelial proliferative potential, and the stemness of limbal stem cells can be well maintained for at least 8 days. This method could be used by eye banks to preserve limbal tissues specific for allograft limbal transplantation, to increase the success rate, or to increase the yield of limbal progenitor cells for tissue engineering of corneal epithelia. In basic research and clinical applications, this method also might be helpful for extending the storage time of limbal tissues for more flexible surgery or research planning. Furthermore, this method also might be used in the preservation and geographic transportation of tissue engineered ocular surface epithelia.

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