Novel Enzymatic Isolation of an Entire Viable Human Limbal Epithelial Sheet

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OBJECTIVE. To develop a reproducible method of isolating an intact viable human limbal epithelial sheet.

METHODS. Human pigmented limbus was incubated at 4°C for 18 hours in supplemental hormonal epithelial medium (SHEM) containing 50 mg/mL dispase II and 100 mM sorbitol. A loose limbal epithelial sheet was separated by a spatula. The remaining stroma was digested and subcultured. The viability of isolated cells was assessed. Isolated epithelial sheets and remaining stroma were subjected to immunostaining. Sheets 1.5 mm in length were cultured in SHEM on plastic until confluence, and cell extracts were subjected to Western blot analysis.

RESULTS. Intact limbal epithelial sheets were consistently isolated. Pigmented palisades of Vogt revealed large superficial squamous cells and small basal cuboidal cells. No epithelial cells grew from the remaining stroma. Mean viability was 80.7% ± 9.1%. The basal epithelium was negative to keratin 5 and connexin 43, but was scatter positive for p63. The epithelial sheet showed negative staining for laminin 5 and collagen VII, but interrupted linear basal staining for collagen IV. The remaining stroma showed negative staining for laminin 5, positive linear staining for collagen IV in the basement membrane, and diffuse staining for collagen VII in the superior stroma subjacent to the basement membrane. Western blot analysis revealed that cells originating from the limbal sheets expressed keratin 3 and p63.

CONCLUSIONS. An intact limbal epithelial sheet can be consistently and reproducibly isolated and contains stem cell characteristics in the basal epithelium by degrading laminin 5 and part of collagen IV, and disassembling collagen VII. Presented in part at the annual meeting of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, Florida, May 2003. Submitted for publication January 28, 2003; revised April 11, 2003; accepted May 29, 2003.

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The maintenance of a healthy corneal epithelium under both normal and stressed conditions is achieved by a unique population of stem cells (SCs) located in the limbal basal epithelium.1,2 The existence of these limbal epithelial SCs is supported by the following facts: These cells do not express such corneal epithelial differentiation markers as keratin 3, keratin 12,3-5 and connexin 43,6-7 but have a superior proliferative capacity under both in vivo and in vitro conditions.2,8-10 Epithelial neoplasias, diseases affecting the SCs, frequently involve the limbal area.11 Destruction of the limbal region has catastrophic consequences for corneal wound healing and integrity.12,13 Transplantation of limbal tissue can replenish the SC population to support regeneration of the entire corneal surface epithelium.14,15

As a first step to investigate the aforementioned remarkable biological and clinical properties of limbal SCs, it is important to develop a consistent and reproducible method of isolating an intact and viable limbal epithelial sheet including the basal epithelium. Previously, conjunctival16 corneal,17-19 and limbal20 epithelium have been removed mechanically,17 chemically with EDTA20, or enzymatically with dispase II.18,19 However, no report has demonstrated the complete removal of an intact viable human limbal epithelial sheet.

Herein, we report our new technique of isolating an intact and viable human limbal epithelial sheet using dispase II under a special digestion protocol. We have further characterized the cleavage plane and reported their unique findings different from those of ethanol treatment used in laser assisted epithelial keratomileusis (LASEK). The significance of this new isolation technique is further discussed.

MATERIALS AND METHODS

Plastic cell culture dishes (60 mm) were from Falcon (Franklin Lakes, NJ). Amphotericin B, Dulbecco’s modified Eagle’s medium (DMEM), F-12 nutrient mixture, fetal bovine serum (FBS), gentamicin, Hanks’ balanced salt solutions (HBSS), HEPES-buffer, neomycin, penicillin, streptomycin, phosphate-buffered saline (PBS), RNA extraction reagent (Trizol), and 0.05% trypsin/0.53 mM EDTA were purchased from Invitrogen-Gibco (Grand Island, NY). A cell viability–cytotoxicity (Live/Dead) kit was from Molecular Probes (Eugene, OR). Disperse II powder was obtained from Roche (Indianapolis, IN). Tissue-Tek OCT compound and cryomolds were from Sakura Finetek (Torrance, CA).

Other reagents and chemicals, including bovine serum albumin (BSA), cholora-toxin (subunit A), collagenase A, dimethyl sulfoxide, hydrocortisone, insulin-transferrin-sodium selenite (ITS) media supplement, mouse-derived epidermal growth factor (EGF), prestained broad-band SDS-PAGE standard, and sorbitol, were purchased from Sigma-Aldrich (St. Louis, MO). An immunoperoxidase staining kit (Vestatin) and antifade mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vectashield) were obtained from Vector Laboratories (Burlingame, CA). We obtained the following monoclonal antibodies: keratin 5 (AE5; ICN, Aurora, OH), integrin β4 (Chemicon, Temecula, CA); laminin 5 (Accurate Chemicals, Westbury, NY); mouse anticycligon VII antibody, rhodamine-conjugated rabbit anti-goat antibody, and fluorescein-conjugated goat anti-mouse antibody (Sigma-Aldrich, St. Louis, MO); and a goat polyclonal antibody against collagen IV (Southern Biotech, Birmingham, AL).
Enzymatic Isolation of Limbal Epithelial Sheets

Twelve pigmented human corneoscleral rims from donors younger than 50 years and less than 4 days after harvesting were obtained from the Florida Lions Eye bank within 8 hours after penetrating keratoplasty. Figures 1A and 1C show the amount of pigmentation in the selected rims and Figures 1B, 1D and 1E show a view of Vogt’s palisades with a high magnification. After corneal transplantation, they were immediately transferred to supplemental hormonal epithelial medium (SHEM), which was made of an equal volume of HEPES-buffered DMEM and Ham’s F12 containing bicarbonate, 0.5% dimethyl sulfoxide, 2 ng/mL mouse-derived 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 subunit, 5% FBS, 50 µg/mL gentamicin, and 1.25 µg/mL amphotericin B. They were then transported at 4°C within 2 hours to the laboratory, where the rims were rubbed off the endothelium and the uveal tissue with a cotton tip and cut by a razor blade into four symmetrical segments, each spanning three clock hours starting from 12 o’clock. Each segment was incubated at 4°C in SHEM containing 50 mg/mL dispase II and 100 mM sorbitol for 18 hours. Under a dissecting microscope, an already loose limbal epithelial sheet was separated by inserting and sliding a noncutting stainless-steel spatula into a plane between the limbal epithelium and the stroma. This maneuver was videophotographed. The video is available online at http://www.iovs.org/cgi/content/full/44/10/4275/DC1.

Cell Culture of Remaining Stroma

To determine whether there was any epithelial cell left, 16 remaining stromal segments from eight different donor rims were incubated at 37°C for 5 minutes in Hanks’ balanced salt solution (HBSS) containing 0.05% trypsin and 0.53 mM EDTA. After a brief pipetting to achieve a single cell suspension, cells were centrifuged at 800g for 5 minutes and resuspended in PBS containing 2 µM calcein AM and 4 µM ethidium homodimer for 45 minutes at room temperature before cells were counted under a fluorescence microscope at 100X. A mean percentage of live cells was calculated by counting both dead (red fluorescence) and live (green fluorescence) cells at 10 different locations in a plastic dish. Cultured human corneal epithelial cells expanded from limbal explants23 that were exposed to methanol for 1 hour were used as a positive control as dead cells.

Viability Evaluation

To determine the cell viability, six isolated limbal epithelial sheets from six different donor rims were incubated at 37°C for 5 minutes in Hanks’ balanced salt solution (HBSS) containing 0.05% trypsin and 0.53 mM EDTA. After a brief pipetting to achieve a single cell suspension, cells were centrifuged at 800g for 5 minutes and resuspended in PBS containing 2 µM calcein AM and 4 µM ethidium homodimer for 45 minutes at room temperature before cells were counted under a fluorescence microscope at 100X. A mean percentage of live cells was calculated by counting both dead (red fluorescence) and live (green fluorescence) cells at 10 different locations in a plastic dish. Cultured human corneal epithelial cells expanded from limbal explants23 that were exposed to methanol for 1 hour were used as a positive control as dead cells.

Immunofluorescent Staining

After the corneoscleral rims were incubated in dispase II, as described earlier, one piece of corneoscleral rim was embedded in optimal cutting temperature compound (OCT), without removing the epithelium, and snap frozen in liquid nitrogen for 5-µm frozen sectioning. As a comparison, epithelial sheets and remaining stroma were separately subjected to frozen sectioning. After fixation in cold acetone for 10 minutes at −20°C, immunofluorescence staining was performed as previously described,22 using antibodies against the following antigens: keratin 3 (1:100), connexin 43 (1:100), p65 (1:40), integrin β4 (1:100), collagen IV (1:50), collagen VII (1:100), and laminin 5 (1:100). The primary antibody was detected with a fluorescein-conjugated secondary antibody, except for collagen IV, in which a rhodamine-conjugated antibody was used. Sections were mounted in a nonfading solution containing DAPI (Vectashield; Vector Laboratories), and analyzed with an epifluorescence microscope (Te-2000u Eclipse; Nikon, Tokyo, Japan).

Characterization of Isolated Epithelial Sheet Outgrowth on Plastic

Segments of isolated limbal epithelial sheets (n = 7) of 1.5 mm of arc length were cultured until confluence in 60-mm dishes containing
SHEM. To determine the expression of keratin 3, which is regarded as a corneal differentiation marker, and p63 nuclear protein, which is a presumed corneal SC marker, proteins of confluent cultures were extracted (TRIzol; Invitrogen-Gibco) and precipitated by centrifuging at 12,000g in 100% isopropyl alcohol. After washing and centrifugation three times, the protein pellet was precipitated with a solution of 95% ethanol containing 0.3 M guanidine hydrochloride. A final wash was performed with 100% ethanol and the protein pellet was air dried for 10 minutes. Prestained broad-band SDS-PAGE standard and protein samples were dissolved into 1× SDS loading buffer: 50 mM Tris Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 1% bromophenol blue, and 10% glycerol. Ten micrograms of total proteins were electrophoresed in a 7.5% gradient polyacrylamide gel. After proteins were electrophoretically transferred to a nitrocellulose membrane, the membrane was immersed for 50 minutes in Tween-Tris-buffered saline (TTBS), which contained 0.1% (vol/vol) Tween 20 in 100 mM Tris and 0.9% NaCl (pH 7.5), followed by 1 hour of blocking with 5% low-fat dry milk in TTBS. Membranes were incubated for 1 hour at room temperature with primary antibody against p63 (1:250 dilution) and keratin 3 (1:1000 dilution). After being washed with TTBS, each membrane was transferred to a 1:200 diluted solution of biotinylated goat anti-mouse antibody in TTBS containing 1% horse serum. After incubation for 30 minutes, the membrane was incubated with 1:50 diluted avidin biotin complex (ABC) reagent (Vectastain Elite; Vector Laboratories) conjugated with peroxidase for 30 minutes and developed in diaminobenzidine (DAB; Dako, Carpinteria, CA) for between 1 and 3 minutes.

RESULTS

Isolation of Epithelial Sheet

Intact limbal epithelial sheets were consistently removed from 48 limbal segments, demonstrating the procedure’s simplicity and reproducibility. As shown in Figure 2, the entire isolated limbal sheet with pigmented palisades of Vogt can be obtained (Fig. 2A as an example). Microscopic evaluation of the remaining limbal stromal surface revealed the lack of pigmented tissue (not shown). A phase-contrast microscopic view of the isolated
limbal sheets showed large superficial cells on the surface (Fig. 2B) and small basal epithelial cells on the basal surface of the sheet (Fig. 2C). The isolated limbal epithelial sheet was easy to handle and could be transferred to a culture dish in a medium using a transfer pipette to maintain the sheet’s integrity in all cases.

Culturing the Stromal Remnants after Epithelial Sheet Removal

No epithelial outgrowth was seen in any of 16 limbal stromal remnants that were digested by collagenase A and cultured for 2 weeks. Instead, abundant fibroblasts grew out of these stromal remnants in every remnant. In contrast, all five control samples with an intact limbal epithelium showed a characteristic epithelial outgrowth. These findings confirmed that there was no epithelial cell remaining on the stroma after the isolation.

Cell Viability

The isolated epithelial sheet was then subjected to a brief trypsin-EDTA treatment to render single-cell suspensions. The mean viability rate of six different samples was 80.7% ± 9.1% (ranging from 66.3% to 90.7%) when 31.23 ± 5.9 cells were counted per 100× field. The positive control of methanol-treated cultured human corneal epithelial cells showed a viability of 0% (i.e., 100% of dead cells).

Characterization of Epithelial Phenotype of Isolated Limbal Sheets

Hematoxylin staining of the isolated limbal epithelial sheet showed a stratified and organized epithelium identical with that noted in vivo human limbus. This stratified epithelium consisted of superficial large squamous cells, intermediate wing cells, and small basal epithelium, which was associated with pigmentation (Fig. 3A, 3B). The superficial surface was smooth, whereas the basal surface was undulating. Immunostaining of the isolated limbal epithelial sheet showed strong intracytoplasmic staining for the AE-5 antibody, which recognizes keratin 3, in the full-thickness stratified epithelium, corresponding to the peripheral corneal epithelium (Fig. 3C, to the right of the arrow), and suprabasal cell layers of the limbal epithelium (Figs. 3C, 3D, to the left of the arrow). This AE-5 staining pattern showing the basal negativity of keratin 3 has been reported as a proof of limbal epithelial SCs.1 The intercellular punctuate staining of connexin 43 was found in the

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suprabasal cells, but was absent in the basal cells (Fig. 3 E), a pattern noted previously as well.6,7 The staining for the transcription factor p63, a reportedly limbal SC marker,23 was located exclusively in the basal cell layer (Fig. 3F).

Characterization of the Basement Membrane Adhesion Complex after Complete Digestion

After complete dispase digestion, we analyzed the basement membrane-adhesion complex before the limbal epithelium was separated. Hematoxylin staining showed that the limbal epithelium was loosely adherent to the underlying stroma, as evidenced by the spaces created between (Fig. 4A, asterisk). The staining for collagen IV was positive in the blood vessels and the superficial stroma of the limbus, with discontinuous staining in the basement membrane area of the basal surface of the loose limbal epithelial sheet (Fig. 4B). The staining for collagen VII was linearly positive in the superficial stroma of the corneal portion, but was weak in the superficial stroma of the limbus after digestion (Fig. 4C). Under higher magnification, a strong linear pattern of staining was located in the basement membrane zone of the peripheral cornea (Fig. 4D). Nevertheless, the staining was diffuse in the superficial stroma of the limbus (Fig. 4E). Staining for laminin 5 was negative in the basement membrane zone of the entire region (Fig. 4F), suggesting total digestion of this protein during the 18-hour incubation.

Characterization of the Basement Membrane Adhesion Complex after Epithelial Sheet Isolation

After digestion, we isolated limbal epithelial sheets and then analyzed the sheet and the remaining stroma separately by immunostaining. The staining for integrin β4 was linearly positive on the basal epithelial cell surface (Fig. 5A), but was absent on the remaining stroma (not shown). The staining for laminin 5 was negative in the total epithelial sheet (Fig. 5B) and negative on the remaining stroma (not shown). The staining for collagen IV was sporadically positive on the basal surface of the isolated sheet (Fig. 5B), but was strongly positive in a linear pattern on the superficial surface of the remaining stroma (Fig. 5F). Staining for collagen VII was negative on the isolated limbal sheet (Fig. 5D), but was diffusely positive in the superficial stroma of the stromal remnant (Fig. 5G). These findings were consistent with those described in Figure 4.

Characterization of Epithelial Outgrowth Derived from Isolated Limbal Epithelial Sheets in Culture

One small segment of isolated limbal epithelial sheet having a size of 1.5 mm of arch length was seeded on the center of each 60-mm plastic dish and cultured in SHEM. Cells rapidly grew out of the sheet and reached the border of the dish in 17.7 ± 3 days (Fig. 6A). Epithelial cells continued to grow onto the side wall of the dish to the level of the medium (Fig. 6B). Phase contrast microscopy showed that cells appeared to be small and formed a compact monolayer (Figs. 6C, 6D). Western blot analysis of proteins extracted from these cells after confluence showed a positive band of p63 at 60 kDa and another at 64 kDa (keratin 3).

DISCUSSION

Our technique of isolating the entire limbal epithelial sheet is based on digestion by dispase II, a neutral protease from Bacillus polymyxa that was originally used to separate the epidermis from the dermis.24 In the skin, the proteolytic action of dispase II is thought to target fibronectin and collagen IV of the basement membrane.25 Spurr and Gipson19 demonstrated disappearance of immunoreactivity to laminin in the rabbit cornea after 6 hours of incubation with 2.4 U dispase II at 37°C. We noted that an 18-hour incubation of 50 mg/mL dispase II at 4°C degraded laminin 5 completely and nearly all the collagen IV in the corneal and limbal basement membranes. In addition, such a digestion regimen did not degrade...
collagen VII, which forms anchoring fibrils in the corneal basement membrane, but caused their complete disassembly in the limbal basement membrane. Dispase II did not alter integrin $\beta 4$ of the basal epithelium. Taken together, these findings support that the cleavage plane created by dispase II is at the lamina densa of the basement membrane. The differences in the composition and anatomy of the limbal and central corneal basement membranes may explain why a different digestion regimen is needed to separate an intact human limbal epithelium from the stroma.

The digestion by dispase II would have to be extended to 18 hours to remove the limbal epithelial sheet completely. This notion was verified by the absence of epithelial outgrowth from the remaining stroma after subculturing for 2 weeks. In contrast, we noted that some basal epithelial cells remained in the stroma when the same dispase dose was incubated at 37°C for 1 hour or at 4°C for 14 hours (not shown). Because it was necessary to incubate in dispase II for such a long period, it is important to keep it at a low temperature (4°C) to reduce metabolic activity and maintain the viability and in the presence of 100 mM sorbitol to prevent cell swelling by increasing the osmolarity. By doing so, we confirmed that isolated limbal epithelial sheets indeed retained a high viability of 80–90%. Because human limbal rings used in this study were not fresh and were studied at variable times after death, stored in Chen medium, and transported to the laboratory, there may have been cell death before digestion. Therefore, we applied the same digestion protocol to several samples of fresh, pigmented rabbit limbus, and obtained a mean maximum cell viability of 93% (not shown).

Because the cleavage plane is within the basement membrane zone, the cell membrane and intercellular junctions remain intact. As a result, cells retain high viability and preserve such intercellular structures as cadherins, integrins, and connexins. In this study, we noted that the basal epithelium of the isolated limbal epithelium retained pigmentation, did not express keratin 3 and connexin 43, but actively expressed p63. These characteristics are identical with SC features reported in human limbal epithelium in vivo.

The cleavage plane created by this dispase digestion is different from the brief treatment of 20% ethanol used to prepare an epithelial flap before excimer laser ablation in the LASEK procedure, in which the cleavage plane is characterized as located in the lamina lucida and the hemidesmosomes of the basement membrane, and there is ethanol-induced cell membrane damage. To further verify that isolated limbal epithelial sheets indeed were viable and functioned properly, we seeded the fragment rapidly grew into a confluent epithelial outgrowth markers of the limbal epithelial SCs, respectively.

With such an intact and viable epithelial sheet isolated from the human limbus, one can begin to study limbal SCs with respect to their properties, proliferation, and differentiation into the corneal epithelium and their interaction with the underlying stromal niche. This technique may also facilitate the purification of limbal SCs once the surface marker has been identified. Furthermore, it may also be useful to use isolated limbal epithelial sheets to expand limbal epithelial SC ex vivo for therapeutic epithelial transplantation. Such a new method of separating the epithelial sheet may also improve the clinical efficacy of LASEK when viable epithelial flaps are better preserved.

References


