Modulation of Keratin and Connexin Expression in Limbal Epithelium Expanded on Denuded Amniotic Membrane with and without a 3T3 Fibroblast Feeder Layer

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PURPOSE. Based on the knowledge that limbal epithelial stem cells (SCs) do not express keratin-3 (K3), connexin (Cx)43, and Cx50, a study was conducted to investigate amniotic membrane (AM) culturing conditions that promote limbal SC expansion.

METHODS. Human limbal epithelium was expanded on intact and epithelially denuded AM, with or without a 3T3 feeder layer, and subsequently transplanted to nude mice to induce epithelial stratification and differentiation. Immunostaining and Western blot analysis were used to determine protein expression of K3, Cx43, and Cx50. Expression of integrin-α3, -β1, -α6, and -β4 was investigated by immunostaining.

RESULTS. Protein levels of K3, Cx43, and Cx50 in limbal epithelium on intact AM was lower than those on denuded AM. Addition of 3T3 to denuded AM increased the level of Cx43 but decreased that of Cx50. After xenotransplantation, the basal layer of the resultant stratified epithelium on intact AM did not express K3, Cx43, and Cx50, whereas that on denuded AM expressed all three markers. The addition of 3T3 resulted in positive staining of Cx43 and K3 but negative staining of Cx50 in the basal epithelium. After stratification, integrin expression was detected at the basal epithelial-amnion basement membrane interface in all three culture conditions.

Conclusions. Limbal cultures on intact AM retain a limbal epithelial phenotype, whereas those on denuded AM differentiate into a corneal phenotype. The addition of 3T3 slows but does not prevent corneal differentiation on denuded AM. Such a difference may involve integrin-mediated extracellular matrix interactions. (Invest Ophthalmol Vis Sci. 2005;44:4230–4236)

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Ex vivo expansion of the limbal epithelium using amniotic membrane (AM) as a biological substrate is a new technique for corneal surface reconstruction in patients with partial or total limbal stem cell deficiency.1-5 Although studies have all shown favorable clinical outcomes, they adopted different culturing techniques for ex vivo expansion, especially regarding the preparation of AM and the inclusion of 3T3 fibroblast feeder layers. For example, Koizumi et al.6 seeded limbal explants on epithelially denuded AM with an additional feeder layer of mouse 3T3 fibroblasts on the plastic dish underneath the AM. Schwab et al.7 first expanded epithelial cells by a 3T3 fibroblast feeder layer and then seeded them on denuded AM. Tsai et al.,8 however, seeded limbal explants on intact AM without the use of a 3T3 fibroblast feeder layer.

It is obviously important to establish an effective culturing protocol for future clinical trials to ensure that ex vivo-expanded limbal epithelial progenitor cells indeed retain stem cell characteristics. As a first step toward resolving these culturing variables, we have recently reported that human limbal epithelial cells expanded ex vivo on intact AM without 3T3 fibroblast feeder layers remain slow cycling.7-9 When subsequently transplanted to athymic nude mice, these cells adopt a limbal epithelial phenotype.7-9 That is, the basal cell layer of the resultant stratified epithelium does not express such corneal differentiation markers as keratin-3 (K3) and connexin (Cx)43,7,9 and exhibits a label-retaining property that is resistant to the treatment of a tumor-promoting phorbol ester.9 In contrast, limbal epithelial cells ex vivo expanded on denuded AM become rapid cycling and after xenotransplantation in nude mice turn into a corneal phenotype expressing K3 and Cx43 in the basal layer and lose their label-retaining property.7 Based on these findings we propose that AM is a natural substrate ideal for restoring the stromal niche of the limbal epithelial SC pool, and speculate that the interaction between expanded limbal epithelial cells and AM matrix may play a crucial role in maintaining the said SC characteristics. In this study, we examined the beneficial role of 3T3 fibroblast feeder layers in human limbal explant cultures on denuded AM in comparison with those on intact AM alone.

MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium (DMEM), HEPES buffer, amphotericin B, and fetal bovine serum (FBS) were purchased from Invitrogen-Gibco (Grand Island, NY). The mouse monoclonal IgG antibodies against integrin-α3 and -α6 and laminin (Ln)-5 for immunohistochemistry were purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). The monoclonal anti-integrin-β4 antibody was purchased from Research Diagnostics, Inc. (Flanders, NJ). The rabbit polyclonal antibodies against integrin-β1 and the monoclonal antibodies against Cx43 and -50 were from Chemicon International (Temecula, CA). Dispase II was obtained from Roche Diagnostics, Inc. (Indianapolis, IN). The FITC-conjugated goat anti-mouse IgG antibody adsorbed with human serum proteins, gentamicin, hydrocortisone, dimethylsulfoxide, cholera-toxin subunit A, insulin-transferrin-sodium selenite medium supplement, mitomycin C, EDTA, and propidium iodide were all from Sigma-Aldrich (St. Louis, MO). The antifade (Vectashield) mounting medium and the biotinylated secondary antibody (goat anti-mouse IgG Elite ABC kit) were obtained from Vector Laboratories (Burlingame, CA). Tissue culture plastic plates (six-well) were from BD Biosciences (Lincoln Park, NJ). Culture plate inserts to which the AM was fastened were from Millipore (Bedford, MA). Protein extraction was performed using radioimmunoprecipitation assay (RIPA) buffer (150

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mM NaCl, 0.05 M Tris-HCl [pH7.5], 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS). The 7.5% Tris-HCl electrophoresis gel was from Bio-Rad Laboratories (Hercules, CA). A prestained broadband protein standard was purchased from Invitrogen (Carlsbad, CA). 3T3 Swiss/albino fibroblasts were purchased from American Type Culture Collection (Rockville, MD).

**Animals**

NIH-bg-nu-xidBR mice, which have no thymus-derived T-cells, T-independent B lymphocytes, and natural killer cells, aged 6 to 10 weeks, were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed under temperature-, humidity-, and light-dependent conditions in filter-covered cages in a laminar flow-equipped room and fed standard chow and water ad libitum. Before surgery, animals were anesthetized with an intramuscular injection of 0.1 mL ketamine (35 mg/kg). The mice were killed by craniocervical dislocation with an intramuscular injection of 0.3 mL ketamine (35 mg/kg) and xylazine (5 mg/kg). All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Human Tissue Preparation**

Human tissue was handled according to the Declaration of Helsinki. Corneoscleral tissue from human donor eyes was obtained from the Florida Lions Eye Bank (Miami, FL) directly after the central corneal button had been used for 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, iris, and corneal endothelium, the remaining tissue was placed in a culture dish and exposed to Dispase II (1.2 U/mL in Mg²⁺- and Ca²⁺-free Hanks’ balanced salt solution) at 37°C under humidified 5% CO₂ for 5 to 10 minutes. After one rinse with DMEM containing 5% FBS, the scleral rim was trimmed to obtain limbal tissue cubes approximately 1 × 1.5 × 2.5 mm in size.

**Human Limbal Explant Cultures on AM**

Preserved human AM was kindly provided by Bio-Tissue (Miami, FL). AM with the epithelial side facing up was fastened onto a culture insert, as previously reported. Three different culture conditions for limbal epithelium were analyzed: intact AM, denuded AM, and denuded AM with an additional 3T3 fibroblast feeder layer (Fig. 1). Twenty of 30 membranes used for limbal cultures were treated with 0.1% sterile EDTA solution for 30 minutes and then gently scrubbed with an epithelial scrubber (Amoils Epithelial Scrubber, Innova; Innovative Excimer Solutions, Inc., Toronto, Ontario, Canada) to remove the amniotic epithelium without breaking the underlying basement membrane. This technique has been described elsewhere. With this method, 90% to 100% of the epithelium was removed. The remaining 10 membranes were left untreated (as an intact AM). On the center of either intact or denuded AM, a limbal explant (1 × 1.5 × 2.5 mm) was placed after the membrane was briefly dried under the laminar flow bench. The stroma side of the explant was facing toward the AM. One drop of 100% FBS was placed on the explant and left in the incubator for 1 hour to ensure proper adhesion. After 1 hour, complete medium was carefully added to prevent detachment of the explant. Cultures were incubated at 37°C under 5% CO₂ and 95% air in a medium made of an equal volume of HEPES-buffered DMEM containing bicarbonate and Ham’s F12. The medium was supplemented with 0.5% dimethyl sulfoxide, 2 ng/mL mouse epidermal growth factor (BGF), 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 (supplemented hormonal epithelial medium [SHEM]). The medium was changed every 2 to 3 days. Ten of the 20 cultures with denuded AM were cocultured on a 3T3 fibroblast feeder layer that was prepared on the plastic dish with a method that has been reported. 3T3 fibroblasts were routinely

![Figure 1](image-url)
maintained by seeding a 1:1000 split of subconfluent cells in 75 cm² flasks containing DMEM and 10% FBS for 12 to 14 days with the medium changed every 2 to 3 days. Subconfluent monolayers were treated with 10⁻⁷ M mitomycin C for 2 hours, and seeded at a density of 2 x 10⁴ cells per 30-mm dish in DMEM containing 10% FBS. After the 3T3 cells attached, the medium was removed, and the culture inserts with AM and a limbal explant were placed on top of the 3T3 feeder layer. 3T3 fibroblasts and the limbal explant were separated from each other by the AM. The SHEM was changed every 2 to 3 days, as described earlier. When human limbal epithelial cells reached plateau growth in 3 to 4 weeks, they were embedded in optimal cutting temperature (OCT) compound and snap frozen for immunohistochemistry of monolayer cultures (three samples per condition), processed for protein extraction (four samples per condition), or used for xenotransplantation (three samples per condition).

**Xenotransplantation**

Nearly confluent limbal epithelium cultures (three on intact AM, three on denuded AM, and three on denuded AM with 3T3 fibroblast feeder layers) were transplanted to the subcutaneous plane of the abdomen of NIH-bg-nu-xidBR mice to induce stratification and differentiation, in manner identical with that used in our previous report. Brieﬂy, mice were narcotized by intramuscular injection of 0.1 mL ketamine (35 mg/kg) and xylazine (5 mg/kg). The skin was disinfected and an area of approximately 10 x 10 mm was dissected to the muscle fascia. Subconfluent cultures were rinsed with balanced salt solution and spread across the muscle fascia (expanded cells facing the skin). A second AM without expanded cells was placed on top of the transplant to protect the limbal epithelium. The skin flap was sutured back in place with 7-0 Vicryl sutures. After 7 days, the tissue including implanted AM and surrounding soft connective tissue were removed and embedded in OCT and snap frozen for cryosectioning.

**Immunostaining**

Frozen 5-μm sections from xenotransplantation samples were fixed in cold methanol for 20 minutes at -20°C. After rinsing with PBS three times for 10 minutes and preincubation with 5% BSA in PBS to block nonspecific staining, sections were incubated with the primary antibody (against integrin-α3, -α6, -β1, and -β4; Ln5; Cx43; Cx50; and K3) diluted 1:100 at 4°C overnight. After three washes with PBS for 15 minutes, they were incubated with a FITC-conjugated secondary antibody (goat anti-mouse or anti-rabbit IgG at 1:200) for 45 minutes at room temperature. In some sections, nuclei were additionally counterstained with propidium iodide for 5 minutes. After three additional PBS washes (15 minutes each), sections were mounted with an antifade solution (Vectorshield; Vector Laboratories) and analyzed with a fluorescence microscope (Axioskop; Carl Zeiss Meditec, Oberkochen, Germany).

**Western Blot Analysis**

Four donor corneas were used for this experiment. Two cultures from each donor rim were initiated and sampled to achieve sufficient cells for protein extraction. After limbal epithelium cultures on intact (n = 4), denuded AM with (n = 4) and without a 3T3 feeder layer (n = 4) reached plateau growth, 200 μL RIPA buffer (4°C) was added on top of the cultured limbal epithelium for 5 minutes. After that time, the limbal epithelium could be easily removed as a sheet from the remaining AM. The harvested limbal epithelial sheet was then further incubated in the same extraction buffer for 50 minutes. Using this technique, we obtained the total amount of extracted protein in all three groups, only from limbal epithelium without contamination from the amniotic epithelium, basement membrane, stroma, or adherent 3T3 fibroblasts.

The total protein concentration was measured by spectrophotometry. Prestained broadband SDS-PAGE standard and protein samples (20 μg of total protein) from the different cultures were immediately electrophoresed in a 7.5% gradient polyacrylamide gel. After electrophoretic transfer to a nitrocellulose membrane, the membrane was immersed with 0.1% (vol/vol) Tween 20 in Tris-buffered saline (100 mM Tris, 0.9% NaCl [pH 7.5]; TTBS) for 30 minutes followed by 1 hour of blocking with 10% low-fat dry milk in PBS. The primary antibodies, 1:1000 against human Cx43 or 1:500 against Cx50 and K3, were placed on each membrane and incubated at room temperature for 60 minutes. After a wash with TTBS, each membrane was transferred to a 1:200 diluted solution of biotinylated second antibody goat anti-mouse or anti-rabbit IgG in TTBS containing 1% horse serum. After a 30-minute incubation, the membrane was incubated with 1:50 diluted avidin-biotin complex reagent (ABC; Vectastain Elite, Vector Laboratories) conjugated with peroxidase for 30 minutes and processed for color development in diaminobenzidine (DAB) for between 1 and 5 minutes. Blots were scanned using a flat-screen scanner (Hewlett-Packard, Palo Alto, CA). Densitometry analysis was performed on computer (Photoshop, ver. 5.5; Adobe Systems, Mountain View, CA) for each of the four sample triplets and the means and SDs were compared using the Wilcoxon matched two-tailed paired test. The result was normalized against 40-kDa actin visualized by Coomassie blue. As a positive control, we chose differentiated human central corneal epithelium, which was directly harvested from donor corneas and is known to express high levels of K3. This control allowed us to compare the expanded epithelium on the graft with the in vivo corneal epithelium.

**RESULTS**

**Expression of K3, Cx43, and Cx50 by Limbal Epithelium Monolayers on AM**

Densitometry measurements for each marker studied were normalized to the signals yielded by the respective Coomassie blue staining (Fig. 2, CB). Compared with that expressed by the human corneal epithelium in vivo (Fig. 2, CO), the amount of K3 expressed was overall much lower in limbal epithelium monolayers grown on AM under different conditions. The amount of K3 expressed by limbal epithelium on intact AM was lower than in that on denuded AM with or without a 3T3 feeder layer.
When grown on intact AM, the basal layer of the stratified limbal epithelium (LE) after expansion on intact AM (arrows). (B) LE on denuded AM with 3T3 showed K3 expression throughout all layers (arrows). (C) The same expression pattern was found in LE on denuded AM without 3T3. (D) Cx43 expression was absent in the basal layer of LE on intact AM. (E) LE on denuded AM with 3T3 showed strong Cx45 expression throughout all layers (arrow). (F) Less intense staining, but the same distribution was found in stratified LE on denuded AM alone. (G) Cx50 was not expressed in LE on intact AM. (H) Only minimal staining was discernible in the stratified LE on denuded AM with 3T3. (I) Strong immunoreactivity for Cx50 was found in all layers of LE on denuded AM alone (arrow). Representative samples of three samples for each condition are shown. Bar, 100 μm.

Expression of Integrins by Stratified Limbal Epithelium on AM

The basal layer of the stratified limbal epithelium on intact AM and on denuded AM with and without 3T3 fibroblast feeder layer (Fig. 2; each P < 0.05). The amount of K3 expressed by limbal epithelium on denuded AM with a 3T3 fibroblast feeder layer was less than that without a 3T3 fibroblast feeder layer (Fig. 2; P < 0.05).

Cx43 is normally expressed by the corneal epithelium on the basal layers in vivo and was detected by Western blot (Fig. 2, CO). The lowest expression of Cx43 was found in cultures grown on intact AM followed by denuded AM without a 3T3 fibroblast feeder layer (Fig. 2; P < 0.05). The strongest expression of Cx43 was found in cultures grown on denuded AM with a 3T3 feeder layer, being three times that on denuded AM without a 3T3 fibroblast feeder layer and four times that in limbal epithelium on intact AM (Fig. 2; P < 0.05).

Cx50 is normally expressed by the corneal epithelium on the suprabasal layers in vivo and was abundantly detected by Western blot (Fig. 2, CO). The highest expression of Cx50 was found in cultures on denuded AM without a 3T3 fibroblast feeder layer, being two times that on intact AM and 1.5 times that on denuded AM with a 3T3 fibroblast feeder layer (Fig. 2; P < 0.05).

K3, Cx43, and Cx50 Expression by Stratified Limbal Epithelium on AM after Xenotransplantation

Seven days after xenotransplantation of limbal epithelium monolayers obtained from each of the three AM conditions, the resultant epithelium was markedly stratified up to seven to eight layers. The dead amniotic epithelium in the intact AM group was no longer discernible after xenotransplantation, which was consistent with our previous findings. When grown on intact AM, the basal layer of the stratified epithelium was negative to K3 as shown by immunostaining, while the suprabasal cell layers were positive in all three samples (Fig. 3A) This finding was in sharp contrast to the full-thickness positive staining of the stratified epithelium grown on denuded AM with (n = 3) and without (n = 3) a 3T3 fibroblast feeder layer (Figs. 3B, 3C, respectively). Immunostaining for Cx45 was absent in the basal layer and weak in all other cell layers of limbal epithelium grown on intact AM (Fig. 3D). In contrast, positive staining throughout all cell layers was noted in cultures grown on denuded AM with a 3T3 fibroblast feeder layer (Fig. 3E), and predominantly in the suprabasal layers of limbal epithelium on denuded AM without a 3T3 fibroblast feeder layer (Fig. 3F). Immunostaining for Cx50 was not detected in cultures on intact AM (Fig. 3G) and in two of three on denuded AM with a 3T3 fibroblast feeder layer (Fig. 3H; in one sample, we found weak immunoreactivity for Cx50), but was strongly expressed in all layers of limbal epithelium on denuded AM without a 3T3 fibroblast feeder layer (Fig. 3I).

Distribution of integrin subunits in stratified limbal epithelium on AM after xenotransplantation. (A) Integrin α3 was detected pericellularly at the basal layer of the stratified epithelium expanded on intact AM. A faint staining was also visible in the suprabasal layers. (B) In the cultures in which the limbal epithelium was primarily expanded on denuded AM, an expression pattern similar to that in the intact AM culture system was noted, as was also the case when an additional 3T3 fibroblast feeder layer was used (data not shown). (C) The staining pattern obtained with an antibody against integrin-β1 was pericellular in the basal layer and less extensive in the suprabasal layers in the intact AM culture set up. (D) This pattern was also present in the denuded AM cultures. (E) Staining for integrin-α6 showed a linear pattern at the basal aspect of the basal cell layer, which faces the amniotic basement membrane. The suprabasal and superficial layers did not show any staining against this antibody. (F) No different staining pattern was noticed in the stratified epithelium obtained on denuded AM. (G) Immunofluorescent staining for integrin-β4 showed an even more distinct signal at the basal cell membrane of the basal cell layer. (H) A comparable signal was identified in cultures obtained on denuded AM. (I) Integrin-β5 was not discernible in cryopreserved intact AM alone without cultured limbal epithelium. Cell nuclei were stained with propidium iodide (red). (J) Ln-5 was found in cryopreserved AM. Bar, 100 μm.
layers had direct contact with the amniotic basement membrane after xenotransplantation (Fig. 1D). In contrast, during the expansion phase, the limbal epithelial monolayer on intact AM grew on top of amniotic epithelium and had no direct contact with the amniotic basement membrane (Fig. 1A). After induction of stratification in the xenotransplantation model, however, no amniotic epithelium was discernible, and therefore the basal epithelial layer had contact with the amniotic basement membrane in all three culture conditions. Immunostaining for integrin-α3, -α6, -β1, and -β4 showed an expression pattern similar to what has been described for the limbal and corneal epithelium in vivo (Fig. 4). Immunostaining for integrin-α3 and -β1 showed pericellular signals in the basal layer of the epithelium, which was in direct contact with the amniotic basement membrane. This finding was consistently noted in all three culture conditions. A slightly weaker staining was found between the more superficial layers of the stratified epithelium (Figs. 4A–D).

Immunoreactivity to integrin-α6 and -β4 was concentrated at the basal aspect of the cell membrane facing the amniotic basement membrane. A faint staining was found on the lateral aspect between basal cells. This pattern was found in the basal layer of the stratified epithelium in all three culture conditions (Figs. 4E–H). We did not detect any specific immunoreactivity to the antibodies analyzed in the control sample of intact AM alone that had been cryopreserved for sometime, after repeated freezing and thawing cycles and after 3 weeks of culturing (Fig. 4I shows the staining for integrin-α3 as an example). Ln-5, a major basement membrane component, was recognized in the AM sample by a monoclonal antibody, as shown in Figure 4J.

**DISCUSSION**

In this study, we reaffirmed our recent finding that intact AM is superior to denuded AM in preserving stem cell characteristics of ex vivo–expanded human limbal epithelium. Using intact AM as a positive control, we further demonstrated the beneficial role of 3T3 fibroblast feeder layers in restoring stem cell characteristics that were lost on denuded AM. K3 is not expressed in the in vivo limbal basal epithelium, which contains the limbal epithelial stem cell population, but is present when the limbal stem cell differentiates into the corneal epithelium.11 Similarly, the gap junction protein Cx43 is also absent in the basal limbal epithelium, but is expressed in the corneal basal epithelium. In contrast, Cx50 is expressed only in the suprabasal and superficial layers of limbal and corneal epithelia.12 Our study provides experimental evidence to support the notion that the expression of K3 and Cx43, two corneal epithelial differentiation markers, by ex vivo–expanded limbal epithelium is substantially modulated by direct contact with the underlying extracellular matrix. On intact AM, the expanded limbal epithelium had direct contact with the amniotic epithelium, which had lost its viability and proliferative activity after cryopreservation.13 This devitalized amniotic epithelium therefore precluded limbal epithelium from making direct contact with the amniotic basement membrane. At this time, we do not know whether such deprivation of epithelium–basement membrane interaction or release of modulating cytokines by the devitalized amniotic epithelial cells influences epithelial differentiation. Compared with the in vivo level of K3 expression, the overall expression by limbal epithelium on intact and denuded AM was low, suggesting that epithelial differentiation was prevented by AM, whether intact or denuded. This notion was also supported by the low expression of Cx43 (Fig. 2). The lower protein level of Cx43 was consistent with our previous immunohistochemical findings and with reduced gap junction–mediated intercellular communication in flattened samples.7,9 These data collectively suggest that limbal epithelium on intact AM is more undifferentiated than that on denuded AM, as judged by the level of K3, Cx43, and Cx50 expression.

To further confirm that the status of epithelial differentiation by limbal epithelial monolayers on AM cultures was still maintained in the stratified epithelium after xenotransplantation, we performed immunofluorescence studies that clearly showed that epithelium cultures on intact AM did not express K3 and Cx43 in the basal layer and Cx50 in the suprabasal layers (Figs. 3A, 3D, 3G). The absence of K3, Cx43, and Cx50 expression in the basal layer of the stratified limbal epithelium on intact AM was not due to negative staining of the amniotic epithelium, because we have previously reported that the amniotic epithelium is no longer discernible after xenotransplantation. In contrast, limbal epithelium on denuded AM showed positive expression of K3 and Cx43 in the basal layer and Cx50 in the suprabasal layers (Figs. 3C, 3F, 3I). The data regarding K3 and Cx43 were consistent with findings reported in our recent study.7 Collectively, these data show why it is efficacious to use ex vivo–expanded limbal epithelial cells on intact AM to treat patients with partial or total limbal deficiency.15

The expression of the integrin subunits α3, β1, α6, and β4 in the stratified epithelium was comparable to the pattern of limbal and corneal epithelium in vivo, as described by others14–16 (Fig. 4A–H). Integrin-αβ1 and -αβ4 are major ligands to the basement membrane component Ln-5,17 which is also a component of the amniotic basement membrane18 (Fig. 4J). Furthermore, integrin-αβ4 participates in hemidesmosome formation.19 One may speculate that the lack of integrin–mediated attachment to the underlying amniotic basement membrane component laminin-5 due to the presence of devitalized amniotic epithelial cells during the expansion phase (monolayer stage) causes reduced hemidesmosome formation for limbal epithelium grown on intact AM. Consistent with this notion is the electron microscopy finding, which showed that limbal epithelium on intact AM forms desmosomal contacts between the expanded epithelium and amniotic epithelial cells, but not between expanded epithelium and amniotic basement membrane.20 We did not detect any of the integrin subunits analyzed in cryopreserved intact AM alone (i.e., without expanded limbal epithelium) using immunohistochemistry (Fig. 4I), a finding consistent with others (Kuo CL, personal communication, 2002; Kuo CL, et al. IOVS 2002;43:ARVO E-Abstract 4195).

Because the limbal epithelium expanded on intact AM is separated from the amniotic basement membrane through the amniotic epithelium during the expansion phase but not after stratification, as shown by the same expression pattern of integrin-α3, -β1, -α6, and -β4 for all three analyzed conditions at the latter stage (Fig. 4), one may speculate that expanded limbal epithelium interaction with its underlying extracellular matrix plays an important role in maintaining stem cell characteristics and preventing epithelial differentiation, a view that has been proposed for keratinocytes.21–25 Further studies are needed to verify this hypothesis and to elucidate the involved mechanisms.

Although recent data from our7–9 and other1 laboratories indicate that intact AM as a biological substrate is a new culture system to help expand limbal epithelial stem cells, Rheinwald and Green in 197526 actually first demonstrated that long-term survival and serial propagation of epidermal keratinocytes and other types of epithelial cells can be accomplished only by seeding them directly onto a 3T3 fibroblast feeder layer.
This so-called 3T3 fibroblast culture system has also been successfully applied to growing rabbit\textsuperscript{29,30} and human\textsuperscript{1,32} limbal epithelial stem cells.

It remains unclear whether AM and 3T3 fibroblast culture systems share a common mechanism. In this study, we provided experimental evidence supporting the idea that a more differentiated epithelial phenotype of limbal explant cultures on denuded AM is downregulated by 3T3 fibroblast feeder layers. Provision of 3T3 fibroblast feeder layers on the plastic surface reduced the level of K3, increased that of Cx43, but decreased that of Cx50 expressed by limbal epithelium on denuded AM (Fig. 2), suggesting that a less differentiated phenotype was maintained at the monolayer stage. After xenotransplantation to induce stratification and differentiation, the resultant epithelial phenotype continued to be corneal, as shown by the full-thickness expression of K3 and Cx43. When compared with that of limbal cultures on denuded AM, addition of 3T3 fibroblast feeder layers reduced the expression of Cx50 (Fig. 3). These results suggest that although addition of 3T3 fibroblast feeder layers inhibits epithelial differentiation, it does not prevent its differentiation into a corneal phenotype when grown on denuded AM. If our experimental data could be extrapolated to humans, we would propose that inclusion of 3T3 fibroblast feeder layers is beneficial in preventing epithelial differentiation when denuded AM is used for ex vivo expansion of limbal epithelial progenitor cells.\textsuperscript{3,4} Because air-lifting was also used by the latter investigators,\textsuperscript{3,4} but was not tested in our experiments, we do not know whether limbal epithelial stem cell characteristics are preserved.

Because the expanded epithelium was not in direct contact with 3T3 fibroblast feeder layers, as seen in the conventional 3T3 fibroblast culture system, such modulation may be mediated by diffusible factors or cytokines. Our laboratory previously reported the presence of an antiapoptotic survival factor in 3T3 fibroblast-conditioned medium.\textsuperscript{53}

In conclusion, our data confirm that intact AM is a suitable substrate for limbal epithelium expansion, in that it preserved limbal stem cell characteristics, whereas removal of amniotic epithelium in our culture system promoted differentiation toward corneal epithelium. Limbal epithelium–extracellular matrix interaction appeared to be the cue for this observation. However, further studies are needed to confirm this hypothesis and to elucidate the specific signaling pathways. The additional use of a 3T3 fibroblast feeder layer partially prevented the limbal epithelial cell differentiation promoted by the denuded AM culture system. This modulation is at least in part mediated by soluble factors that should be identified in future studies.

References


