Development of a Conjunctival Epithelial Equivalent with Improved Proliferative Properties Using a Multistep Serum-Free Culture System

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Purpose. To investigate the use of a multistep serum-free culture system in developing a conjunctival epithelial equivalent with improved in vitro and in vivo proliferative properties and to evaluate the effect of serum supplementation and culture conditions on the proliferative capacity of these cells.

Methods. Conjunctival epithelial cells were cultivated on human amniotic membrane (HAM) in a multistep serum-free culture system, under submerged and air-lifted conditions. The bromodeoxyuridine (BrdU) ELISA proliferation assay, colony-forming efficiency (CFE), and number of cell generations were compared with those in serum-containing medium. The in vivo proliferative capability of the tissue-constructs were evaluated by xenotransplantation to SCID mice. Cultured cells were evaluated for the expression of keratin-4, -19, and -3, as well as MUC5AC goblet cell mucin.

Results. The epithelial cells cultivated in serum-free medium (BrdU absorbance, 1.91 ± 0.08; cell generations, 25.6 ± 4.5) were more proliferative than those cultivated in serum-containing medium (BrdU absorbance, 1.06 ± 0.08; cell generations, 12.1 ± 3.0). The serum-free–derived epithelial equivalents demonstrated a significant increase in proliferation and stratification after transplantation. Cells that were air lifted for 6 and 12 days had a reduced proliferative capacity in vitro and in vivo compared with submerged cultures. Cultured cells expressed keratin-4 and -19, and MUC5AC mRNA was detected by RT-PCR. Electron microscopy demonstrated a basal lamina with numerous hemidesmosomes.

Conclusions. This is a multistep serum-free culture system for developing a conjunctival epithelial equivalent with improved proliferative and structural properties, which are crucial for enhancing graft survival and regeneration of the conjunctival surface after clinical transplantation. (*Invest Ophthalmol Vis Sci.* 2004;45:1789–1795) DOI:10.1167/iovs.03-1361

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The conjunctiva plays a critical role in supporting the normal milieu of the ocular surface and ensuring continued clarity and survival of the corneal epithelium and stroma. The conjunctival epithelium may be damaged by a variety of ocular disorders, such as chemical burns, Stevens-Johnson syndrome, and ocular cicatricial pemphigoid, or after wide-field excision of tumors. Bioengineered tissue equivalents have been used for corneal and limbal epithelial cell replacement in ocular surface diseases, but fail to address the problem of conjunctival damage that is often present.[1–4] The use of bioengineered conjunctival equivalents for the replacement of healthy conjunctiva may be important in a host of severe ocular surface disorders that involve the conjunctiva, or after surgical excision of conjunctival lesions, thereby removing the need to harvest conjunctival autografts and cause iatrogenic injury to the remaining ocular surface.

Most studies of tissue equivalents have focused on the ability to reconstitute a tissue equivalent that bears the structural and functional characteristics of the tissue of origin.[5–13] This has been achieved by differentiating cells in culture by modifying the culture conditions and air-lifting.[7–13] However, terminally differentiated cells have a limited long-term proliferative capacity, resulting in a lower regenerative potential after transplantation. In addition, the use of epithelial tissue constructs for ocular surface transplantation requires that cells be sufficiently attached to the underlying substrate that they are prevented from being sloughed off by direct mechanical or shearing forces, during or after surgical transplantation. A delicate balance is therefore necessary to preserve the proliferative potential of transplanted cells, while at the same time ensuring that transplanted cells have the necessary functional characteristics of the tissue organ. The ideal tissue-construct is one where transplanted cells possess a long-term regenerative capability for cellular renewal and replacement of the tissue.

Serum-containing medium, with or without a 3T3 feeder layer, remains the medium of choice for the ex vivo expansion of cells in corneal and conjunctival tissue equivalents.[1–4,10–15] Serum-free medium that was developed to overcome the problems associated with the use of serum-containing medium, has been used to study the effect of exogenous factors on cell proliferation and differentiation.[16–22] Various investigators have demonstrated the use of serum-free medium for the cultivation of human conjunctival epithelial cells.[23–25] However, the in vivo proliferative capacity of cells in bioengineered tissue equivalents is of paramount importance when used in clinical transplantation. As such, we studied the in vitro and in vivo proliferation of a serum-free ex vivo expanded conjunctival epithelial equivalent bearing the structural and functional characteristics of normal conjunctiva.

We used a multistep serum-free culture system in the development of a conjunctival epithelial equivalent with improved in vitro and in vivo proliferative properties and investigated the effect of serum supplementation and culture conditions on the long-term proliferative capacity of these cells. This is the first study that has focused on the proliferative and functional characteristics of the tissue equivalent, for the

purposes of improving graft survival and preserving the regenerative potential of transplanted cells. To our knowledge, a study of this nature has not been described. These findings have important clinical implications and provide valuable insights into the development of bioengineered tissue equivalents for clinical transplantation.

Materials and Methods

Human conjunctival biopsy specimens were obtained from prospective surgical patients after obtaining proper informed consent and approval, according to the statutes of the Institutional Review Board of the Singapore National Eye Center. All procedures conformed to the guidelines of the Declaration of Helsinki in Biomedical Research Involving Human Subjects.

Isolation and Cultivation of Human Conjunctival Epithelial Cells

Conjunctival biopsy specimens were obtained from patients undergoing routine surgery for nasal pterygium or cataract. A small piece of normal conjunctiva (1 × 3 mm in size) was removed from the superior bulbar region, 10 to 15 mm from the limbus. Patients with extensive pterygia or with any other ocular surface disorders that might involve the area of biopsy were excluded from the study. Twenty-eight specimens were collected from donors aged 24 to 63 years (mean age, 57.3).

Preparation of HAM

Human placentas were obtained from mothers who had undergone cesarean sections. The membranes were washed with phosphate-buffered saline (PBS) to remove the blood clots. The HAM was peeled away from the chorion and flattened onto a sterilized nitrocellulose filter paper (Millipore, Bedford, MA). The HAM was then stored in 50% DMEM, 50% glycerol (Invitrogen-Gibco, Rockville, MD) at −80°C. In preparation for its use, the HAM was thawed, rinsed with PBS and incubated with 1.2 U/mL dispase II (Invitrogen-Gibco) for 2 hours, followed by gentle scraping to remove any remaining amniotic epithelial cells. The denuded HAM was trimmed to a 3 × 3-cm square and placed epithelial basement–membrane side up on a nitrocellulose support in a 35-mm culture dish.

Ex Vivo Expansion of Conjunctival Epithelial Cells on HAM

The conjunctival tissue was cut into 0.5- to 1-mm pieces, inoculated onto the epithelial basement-membrane side of the denuded HAM, and 500 μL of medium was added to submerge the explants. Ex vivo expansion of conjunctival epithelial cells was performed in serum-free medium, which consisted of keratinocyte growth medium (KGM; BioWhittaker, Walkersville, MD) supplemented with 10 ng/mL human epidermal growth factor (Invitrogen-Gibco), 5 μg/mL insulin (Sigma-Aldrich, St. Louis, MO), 0.5 μg/mL hydrocortisone (Sigma-Aldrich), 30 μg/mL bovine pituitary extract (Sigma-Aldrich), 50 μg/mL gentamicin (Invitrogen-Gibco), and 50 ng/mL amphotericin B (Invitrogen-Gibco). The calcium concentration of the medium was 0.15 mM. On the first day of culture, the calcium concentration was increased to 0.9 mM with calcium chloride solution (Sigma-Aldrich), which was equivalent to the calcium concentration present in a conventional 1:1 mixture of DMEM-Ham's F12.[11–13,23] From the second day onward, the calcium concentration was maintained at 0.15 mM.

The cells were incubated at 37°C, under 5%CO_2 and 95% air, with the medium changed every 2 to 3 days. Cultures were monitored under an inverted phase-contrast microscope (Axiovert; Carl Zeiss Meditec, Oberkochen, Germany). When the initial outgrowth of cells from the explants occurred, the volume of medium was increased to fully immerse the explants.

When a confluent layer of cells was obtained on the HAM after 8 to 10 days, the cultures were divided into three culture conditions.

Submerged Cultures. The calcium concentration of the serum-free medium was increased to 1.2 mM for 4 days to promote differentiation and stratification.

Air-Lifting: 6 and 12 Days. The calcium concentration of the serum-free medium was increased to 1.2 mM. These conjunctival equivalents were air lifted for either 6 or 12 days, to allow further stratification and differentiation of the epithelial cells. Air-lifting was performed by lowering the level of medium to the level of the membrane. Close monitoring of the fluid level was performed twice daily to ensure the desired medium level was maintained.

We compared the relative efficacy of this serum-free culture medium with that of the conventional serum-containing medium used for cultivating ocular surface epithelial cells.[2,3,5,10–12] The composition and growth factor supplementation of the serum-containing medium used for comparison was similar to previous reports,[2,3,5,10–12] and consisted of a 1:1 mixture of DMEM and Ham's F12 (Invitrogen-Gibco) supplemented with 10% FBS (Hyclone, Logan, UT), 10 ng/mL human epidermal growth factor (Invitrogen-Gibco), 5 μg/mL insulin (Sigma-Aldrich), 0.5 μg/mL hydrocortisone (Sigma-Aldrich), 0.1 nM cholera toxin (Sigma-Aldrich), 0.18 mM adenine (Sigma-Aldrich), 50 μg/mL gentamicin (Invitrogen-Gibco), and 50 ng/mL amphotericin B (Invitrogen-Gibco).

Subculturing was performed by enzymatic disaggregation with 0.125% trypsin and 0.02% EDTA (Invitrogen-Gibco), and cells were plated onto culture dishes at a seeding density of 4×10^3 cells/cm^2.

Quantitation of Growth and Proliferative Capacity of Cells

The following proliferation assays were used to assess the proliferative capacity of cells cultured on HAM under the various culture conditions.

BrdU ELISA Cell Proliferation Assay: Proliferation Index. The BrdU uptake by dividing cells was measured by an ELISA cell proliferation assay. We analyzed the proliferative capacity of primary cultures of conjunctival epithelial cells on HAM at the end of the culture periods for each condition. Conjunctival epithelial cells cultivated in these conditions were then subcultured onto culture dishes and their proliferation indices evaluated on the sixth day of passage. Cultured cells were incubated with 10 μM BrdU labeling solution (Amersham Biosciences, Freiburg, Germany) for 2 hours at 37°C, followed by washing with 250 μL of PBS containing 10% serum per well. They were fixed with 70% ethanol in hydrochloric acid for 30 minutes at −20°C and incubated with 100 μL monoclonal antibody against BrdU for 30 minutes, followed by 100 μL peroxidase substrate per well. The BrdU absorbance in each well was measured directly with a spectrophotometric microplate reader (Tecan, Grodig, Austria) at a test wavelength of 450 nm and a reference wavelength of 490 nm. This gave us a measure of the degree of cell proliferation, which we termed the proliferation index. Each sample was cultured in quadruplicate. The respective plain basal medium (KGM or DMEM-Ham's F12 medium) was used as a negative control.

Colony-Forming Efficiency. Subcultured cells were plated at a clonal density of 1000 cells onto 60-mm culture dishes. A colony was defined as a group of eight or more contiguous cells. The number of attached cells was determined on day 2 by counting the entire dish. The colonies were fixed on day 10, stained with rhodamine B, and counted under a dissecting microscope. The colony-forming efficiency was defined as follows:

$$\text{Colony-forming efficiency (\%)} = \frac{\text{Colonies formed at end of growth period}}{\text{Total number of viable cells seeded}} \times 100\%.$$

Number of Cell Generations. Primary cultures of cells in the various conditions were passaged until senescence in their respective media, to determine the number of cell that could be generated. Cells were subcultured when they achieved 70% confluence and plated onto

tissue culture dishes at a seeding density of 4×10^3 cells/cm^2. The number of cell generations (x) was calculated as follows: $x = \log_2(N/N_0)$, where N is the total number of cells harvested at subculture, and N_0 is the number of viable cells seeded.

Xenotransplantation of Conjunctival Epithelial Equivalents onto SCID Mice

Conjunctival epithelial equivalents that were prepared in submerged and air-lifted conditions were xenografted onto the subcutaneous tissue of severe combined immune deficiency (SCID) mice, aged 7 to 9 weeks. The SCID mice were anesthetized, and a three-sided rectangular incision was made through the dorsal skin. The skin flap was lifted from the exposed dorsal muscle fascia. The amniotic membrane with the overlying epithelial sheet was placed epithelial side up over the muscle fascia. The skin flap was returned to its original anatomic position and the wound edges sutured with 5-0 silk sutures. The mice were euthanized by asphyxiation with carbon dioxide at 8 to 10 days after grafting and the tissue excised for analysis.

Morphology of Conjunctival Epithelial Equivalents

Histologic analysis was performed by fixing the tissue equivalents in 4% paraformaldehyde and embedding in paraffin. Four-micrometer sections were cut and stained with hematoxylin and eosin. For transmission electron microscopic studies, the tissue equivalents were fixed in 2.0% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate buffer. They were postfixed in 1% osmium tetroxide and embedded in Epon 812. Ultrathin sections were prepared for viewing with uranyl acetate and lead citrate and examined under a transmission electron microscope (EM 10A; Carl Zeiss Meditec).

Assay of Differentiation-Related Markers

Tissues were embedded in optimal cutting temperature freezing compound (OCT, Tissue-Tek; Sakura Fintek, Torrance, CA), and 4-μm-thick sections were cut. The tissue was fixed with −20°C acetone for 10 minutes, followed by incubation for 1 hour with monoclonal antibodies to keratin-4 (Sigma-Aldrich), -19 (DakoCytomation, Carpinteria, CA), and -3 (AE-5 antibody was a kind gift from Tung-Tien Sun, New York University, New York, NY). Normal mouse immunoglobulin (Sigma-Aldrich) and pancytokeratin (AE-1 and AE-3; Sigma-Aldrich) were used as the negative and positive controls, respectively. The cells were subsequently incubated with secondary antibody (1:200 biotinylated horse anti-mouse immunoglobulin G) for 1 hour, detected with the mouse immunoperoxidase detection kit (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA), and stained with 3,3′-diaminobenzidine (DAB) substrate (Sigma-Aldrich).

The expression of conjunctival goblet cell mucin, MUC5AC, was determined by RT-PCR.[26–28] Total RNA was isolated from cultured conjunctival epithelial cells by using a guanidinium isothiocyanate protocol (RNeasy; Qiagen, Valencia, CA) and subjected to RNase-free DNase I digestion, extracted twice with phenol-chloroform-isoamyl alcohol (24:24:1), precipitated with ethanol, dissolved in RNase-free water, and quantified spectrophotometrically. One microgram of total RNA was used for cDNA synthesis (SuperScript II reverse transcriptase; Invitrogen, Carlsbad, CA). PCR was performed with primers for human MUC5AC.[26] The primer sequences were as follows: MUC5AC sense primer, 5′-TCCACCATATACCGCCACAGA-3′, and antisense primer, 5′-TGGACCGACAGTCACTGTCAAC-3′. The amplification reaction was performed in a thermal cycler (PCR Sprint; Thermo Hybaid, Ashton, UK). The conditions were: 3 minutes at 96°C, followed by 30 cycles of denaturation for 45 seconds at 96°C, amplification for 1 minute at 55°C, and extension for 1 minute at 72°C. The predicted length of the PCR product was 103 bp.[27] Amplified cDNA was analyzed by electrophoresis on a 1% agarose gel and viewed by ethidium bromide staining. Total RNA from forniceal conjunctival epithelium was used as a positive control. Actin PCR was conducted at the same time as a system control.

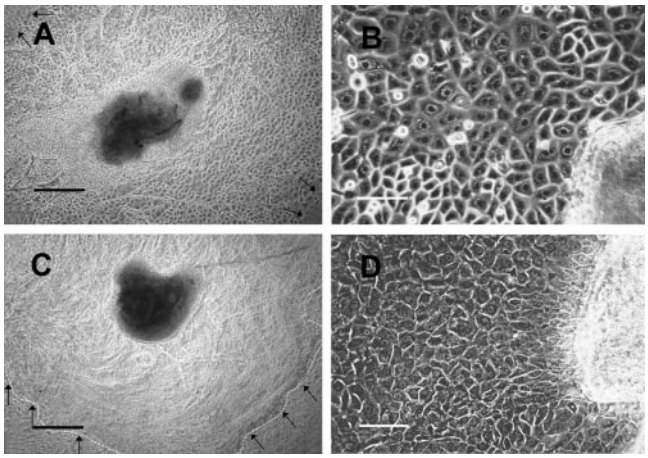

FIGURE 1. Phase-contrast appearance of human conjunctival epithelial cells cultivated on HAM (**A**, **B**) Serum-free medium on day 4. (**C**, **D**) Serum-containing medium on day 4. (**A**) Low-power view demonstrating epithelial cell migration from an explant. *Arrows*: The leading edge. (**B**) High-power view of the epithelial sheet demonstrating a cobblestone morphology with fairly uniform small, ovoid cells. (**C**) Epithelial cell migration from the explant cultivated in serum-containing medium. (**D**) Migrating cells had a more elongated and squamous appearance, and were less uniform in size. Bar: (**A**, **C**) 500 μm; (**B**, **D**) 125 μm.

RESULTS

Epithelial Morphology

Human conjunctival epithelial cells cultivated in serum-free medium began to migrate from the explants on the first day. The initial migratory cells were small and ovoid, with a prominent nucleus and scanty cytoplasm (Figs. 1A, 1B). Continued migration and proliferation of the cells resulted in an epithelial sheet that became more densely populated and had a cobblestone morphology. This confluent sheet of epithelial cells covered the 3×3-cm^2 area of HAM after 8 days in culture. Increasing the calcium concentration resulted in the epithelial sheet's becoming more densely populated and more stratified, with cells becoming more elongated in appearance. Air-lifting for 6 and 12 days resulted in a greater number of elongated cells.

Epithelial cells cultivated in serum-containing medium migrated from the explants by day 1, forming an epithelial sheet. The cells were more elongated and squamous in appearance than those in serum-free cultures at corresponding time intervals (Figs. 1C, 1D). Air-lifting for 6 and 12 days resulted in further stratification and an increase in the number of large, elongated, and squamous cells.

Morphology of Conjunctival Epithelial Equivalents

The conjunctival epithelial sheets in serum-free cultures were more stratified than those in serum-containing cultures at corresponding time intervals.

Submerged Cultures. In serum-free medium, the conjunctival epithelial sheet was one to two layers in thickness by day 6. Four days of exposure to a calcium concentration of 1.2 mM resulted in an increase in stratification to two to four layers and the appearance of columnar basal cells (Fig. 2A). Serum-containing cultures consisted mainly of flattened, elongated cells and were two to three layers thick (Fig. 2D).

Air-Lifted Cultures: 6 Days. Serum-free cultures that were air-lifted demonstrated greater stratification, with columnar basal cells and progressive flattening toward the surface (Fig. 2B). The serum-free–derived epithelial sheets were five to nine

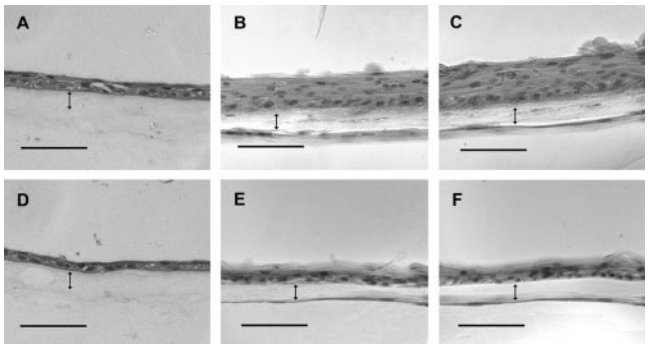

FIGURE 2. Light microscopic appearance of conjunctival epithelial equivalents cultivated in (**A–C**) serum-free medium and (**D–F**) serum-containing medium. (**A**) Submerged culture on day 12 (after 4 days of exposure to a 1.2-mM calcium concentration). The epithelial sheet consisted of two to four layers of cells, with the appearance of columnar basal cells. (**B**) Air-lifted culture (6 days). Note the increase in stratification of up to six to seven layers of cells, with columnar basal cells and progressive flattening toward the surface. (**C**) Air-lifted culture (12 days). A slight increase in stratification was noted compared with the 6-day air-lifted culture. The epithelial sheet was seven to eight layers in thickness. (**D**) Submerged culture in serum-containing medium on day 12. The epithelial sheet consisted of one to two layers of cells. (**E**) Air-lifted culture (6 days). An increase in stratification was noted, with the epithelial sheet being three to four layers in thickness. (**F**) Air-lifted culture (12 days). There was no significant increase in stratification compared with the 6-day air-lifted culture. Stratification of the epithelial sheet was more pronounced in serum-free cultures than in serum-containing cultures at corresponding time intervals. The HAM is indicated by the area within the *double-headed arrow*. Bar, 100 μm.

layers in thickness. Corresponding cultures in serum-containing medium were less stratified, and the epithelial sheets were three to five layers in thickness (Fig. 2E).

Air-Lifted Cultures: 12 Days. A slight increase in stratification was noted in 12-day air-lifted cultures (Figs. 2D, 2F), compared with 6-day air-lifted cultures. Serum-free derived epithelial sheets were 5 to 10 layers in thickness, compared with those derived from serum-containing medium, which were 3 to 6 layers in thickness. The nonkeratinized conjunctival epithelial cells were found to be particularly prone to desiccation, and close monitoring of the cultures and the level of the medium was necessary to prevent excessive desiccation of the cells.

Proliferative Capacity

In primary cultures, submerged conjunctival cells demonstrated a higher proliferation index than did air-lifted cultures. Cells cultivated in serum-free medium had a higher proliferation index than did cells cultivated in serum-containing medium (Fig. 3A). This difference was most marked in air-lifted cultures, where the serum-containing cultures demonstrated a greater drop in proliferation index than did the corresponding serum-free cultures.

After subculturing, submerged serum-free cultured cells (BrdU absorbance, 1.91 ± 0.08) had a significantly higher proliferation index compared with submerged serum-containing cultures (BrdU absorbance, 1.06 ± 0.08; Fig. 3B). Air-lifting resulted in a drop in proliferation index for serum-free as well as serum-containing cultures. In both submerged and air-lifted conditions, serum-free cultures had a higher proliferation index than serum-containing cultures.

Cells cultured in serum-free medium that remained submerged had a higher colony-forming efficiency (23.1% ± 3.1%) than 6-day air-lifted cells (11.0% ± 1.7%) and 12-day air-lifted cells (7.6% ± 1.6%; Fig. 3C). The colony-forming efficiencies of serum-containing cultures were lower than serum-free cultures for corresponding submerged and air-lifted culture conditions. The number of cell generations achieved by cells cultured in

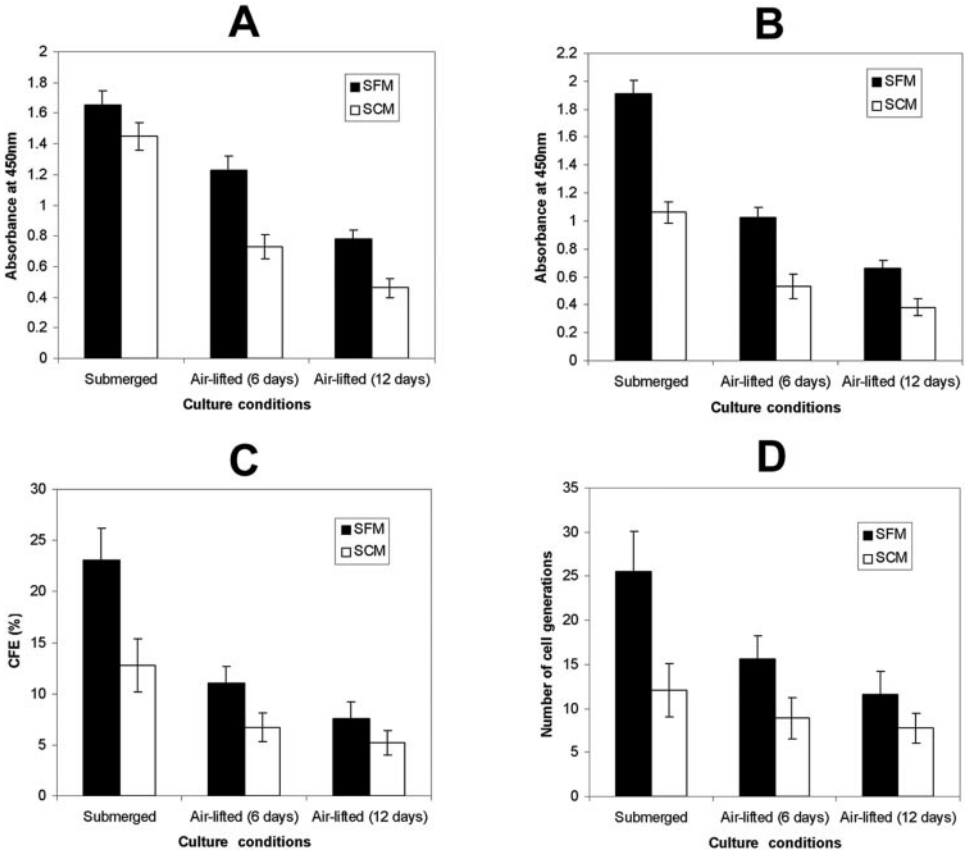

FIGURE 3. Proliferation assays of conjunctival epithelial cells cultivated in serum-free and serum-containing culture conditions. (**A**) BrdU ELISA cell proliferation assay of primary conjunctival cultures in the various culture conditions on HAM. The data describe the BrdU absorbance in each culture condition. (**B**) BrdU proliferation assay of subcultured passage 1 conjunctival cells on day 6. There was greater incorporation of BrdU in cells cultivated in serum-free medium than in serum-containing medium. Submerged cells had a higher proliferation index than air-lifted cells. (**C**) Colony-forming efficiency (CFE) of subcultured passage 1 conjunctival cells. Cells cultured in serum-free medium had a higher CFE than did cells cultured in serum-containing medium. Air-lifted cells had a lower CFE than did submerged cells. (**D**) Number of cell generations achieved. Cells cultured in serum-free medium were able to achieve a greater number of cell generations than cells cultured in serum-containing medium. Air-lifting resulted in a decrease in the number of cell generations achieved.

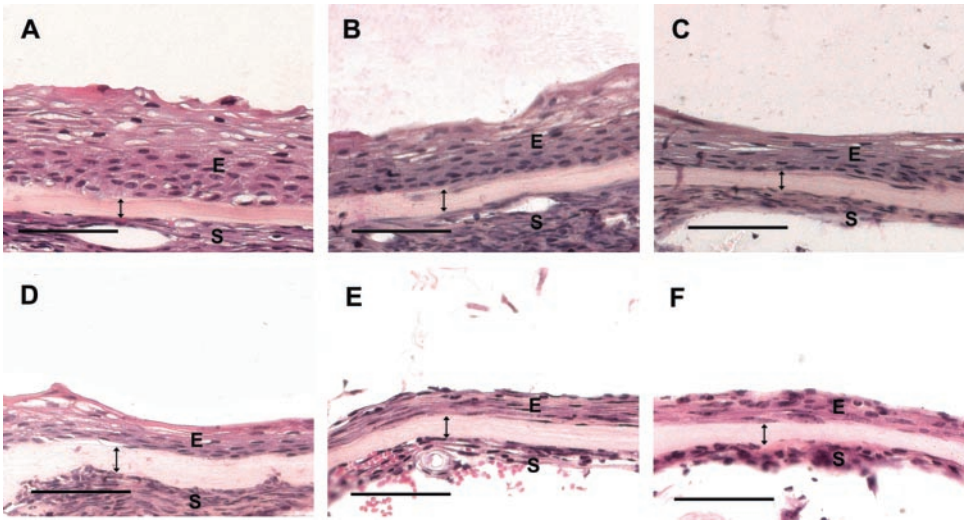

FIGURE 4. Light microscopic appearance of conjunctival epithelial equivalents xenotransplanted in SCID mice. (**A–C**) grafts cultured in serum-free medium; (**D–F**) grafts cultured in serum-containing medium. (**A**) Transplanted graft developed in serum-free medium under submerged conditions. Note the highly stratified epithelial (**E**) sheet, with numerous basal columnar cells and progressive flattening of the cells toward to surface. (**B**) Transplanted 6-day air-lifted conjunctival equivalent. (**C**) Transplanted 12-day air-lifted conjunctival equivalent. Note the less stratified epithelium with more flattened cells in 6- and 12-day air-lifted conjunctival equivalents. (**D**) Transplanted graft developed in serum-containing medium under submerged conditions. The degree of stratification are less than in the corresponding serum-free derived graft. (**E**) Transplanted 6-day air-lifted conjunctival equivalent. (**F**) Transplanted 12-day air-lifted conjunctival equivalent. Note that there was little increase in the degree of stratification in transplanted 6- and 12-day air-lifted grafts. The epithelium was also more disorganized than the transplanted submerged graft. E, epithelial sheet; S, stroma of SCID mice. The HAM is indicated by the area within the *double-headed arrow*. Bar, 100 μm.

serum-free medium (submerged, 25.6 ± 4.5) was 2.1 times greater than that achieved in serum-containing medium (submerged, 12.1 ± 3.0; Fig. 3D). Air-lifted cultures produced fewer cell generations than submerged cells. There were no significant differences in the cell proliferation between cells harvested from patients of different ages.

Xenotransplantation of Conjunctival Epithelial Equivalents in SCID Mice

In the serum-free–derived grafts, the greatest degree of stratification was noted in those cultured under submerged conditions (Fig. 4A). Proliferation of transplanted cells resulted in a significant increase in stratification of up to 8 to 12 cell layers in thickness. These serum-free derived epithelial sheets were also the most organized among the various culture conditions, and consisted of columnar basal cells, progressive flattening of the cells toward the surface, and squamous cells in the uppermost layers. Transplanted epithelial cells that were air-lifted for 6 and 12 days were less stratified and more flattened compared with submerged cultures (Figs. 4B, 4C).

For grafts that were cultured in serum-containing medium, 6- and 12-day air-lifted grafts were less stratified and less organized than submerged grafts (Figs. 4D–F). For both submerged and air-lifted conditions, serum-free derived grafts underwent greater proliferation to form epithelial sheets that were more stratified than grafts cultured in serum-containing medium.

Differentiation of Conjunctival Epithelial Cells on HAM

Pancytokeratin, keratin-4, and keratin-19 were expressed in all layers of the normal conjunctival epithelium. In the conjunctival equivalents, pancytokeratin, keratin-4, and keratin-19 were similarly expressed throughout the epithelium in submerged cultures exposed to a normal calcium concentration for 4 days. A similar staining pattern was noted in air-lifted cultures. Keratin-3, which is expressed in corneal cells, was not expressed by the conjunctival epithelial cells in all conditions. These findings were similar to the staining pattern of normal conjunctiva in vivo.

The message for MUC5AC goblet cell mucin was detected as a 103-bp band[27] by RT-PCR in conjunctival epithelial cells cultured in serum-free medium (Fig. 5). The integrity of RNA was confirmed by the amplification of actin mRNA. These results confirmed the existence of MUC5AC gene expression in serum-free cultured cells. A slightly reduced message was noted in cells cultured in serum-containing medium.

Ultrastructural Appearance of the Conjunctival Epithelial Equivalent

Transmission electron microscopy showed that on day 6 of culture, the conjunctival epithelial sheet was one to two cell layers in thickness (Fig. 6A). On day 12 of culture, after exposure to a 1.2-mM calcium concentration for 4 days, a multilayered epithelial sheet was formed. Numerous microvillus extensions were present on the apical surfaces of the superficial cells (Fig. 6B), and the basal cells were columnar in appearance (Fig. 6C). Numerous intercellular interdigitations and desmosomes were demonstrated (Fig. 6D), and a fairly continuous basal lamina with hemidesmosomes was observed at the basal cell–HAM junction (Fig. 6E). These cell–substrate adhesion structures are important for maintaining the integrity of the epithelial sheet during and after transplantation.

DISCUSSION

Previous studies of ocular surface equivalents have relied primarily on serum-supplemented medium for the cultivation of epithelial cells.[8–13] These studies focused mainly on the formation of a differentiated epithelial equivalent bearing the morphologic characteristics of the original tissue.[8–13] These terminally differentiated cells contribute to the structural and

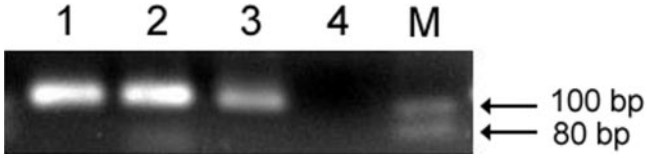

FIGURE 5. Identification of MUC5AC transcripts in normal conjunctiva and cultured conjunctival epithelial cells by RT-PCR. MUC5AC transcripts were detected in cDNA from human forniceal conjunctiva (*lane 1*), conjunctival epithelial cells cultured in serum-free medium (*lane 2*), and conjunctival epithelial cells cultured in serum-containing medium (*lane 3*). PCR was performed with water replacing cDNA as the negative control (*lane 4*). *Lane M*: molecular weight standard.

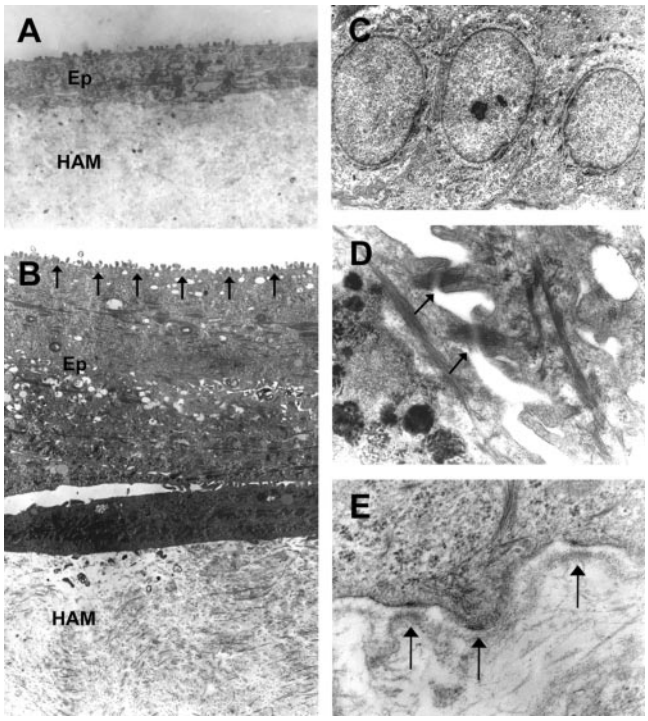

FIGURE 6. Transmission electron microscopy of conjunctival tissue equivalents cultured in serum-free medium. (**A**) Day 6 of culture, demonstrating an epithelium consisting of one to two layers of cells on HAM. (**B**) Day 12 of culture, demonstrating a multilayered epithelial sheet with numerous microvilli over the apical surface of superficial cells (*arrows*). (**C**) Prominent columnar basal cells were noted. (**D**) Intercellular interdigitations and numerous desmosomes (*arrows*) were present. (**E**) A fairly well-defined basal lamina with numerous hemidesmosomal attachments (*arrows*) was present at the basal cell-HAM interface. Ep, epithelial sheet; HAM, human amniotic membrane. Magnification: (**A**, **B**) ×10,000; (**C**) ×140,000; (**D**) ×40,000; (**E**) ×36,000.

functional attributes of the tissue, but have a limited proliferative capacity in the long term. The ability of the cultivated cells to remain proliferative in vivo is important for the long-term regeneration of the tissue after transplantation. We describe the use of a multistep serum-free culture system in developing a conjunctival epithelial equivalent with improved proliferative and structural characteristics, which are crucial for the regeneration of the conjunctival surface after clinical transplantation.

The multistep approach that we adopted takes into account the various modulating effects by exogenous factors during the culture process, so as to improve the functional and structural characteristics of the transplantable tissue.[16–18] The calcium concentration of the culture system was modified to promote cell attachment and migration from the explant in the initial period and to increase cell stratification and enhance the formation of cell-to-substrate adhesion structures before transplantation. We demonstrated that under both submerged and air-lifted conditions, conjunctival epithelial cells cultured in serum-free medium had a greater proliferative capacity than cells cultivated in serum-containing medium, without the attendant disadvantages associated with the use of serum and a 3T3 feeder layer.

Air-lifting has been used to promote differentiation, stratification, and the formation of cell–substrate adhesion structures in tissue equivalents.[7–13] Tsai et al.[12] showed that epithelial cultures on collagen matrices that were air-lifted were three to four cell layers thick, compared with submerged cultures that were one to two cell layers thick. Similar results were noted by Meller and Tseng[13] who kept cultures air-lifted for up to 4 weeks. However, the promotion of differentiation and stratification by air-lifting may be disadvantageous for transplantation, because the proliferative capacity of these differentiated cells would be reduced. In our study, we showed that air-lifted conjunctival epithelial cells achieved a greater degree of stratification than submerged cultures in vitro, but the long-term proliferative potential of these cells was reduced, as evidenced by the lower BrdU cell proliferation assay, colony-forming efficiency, and number of cell generations achieved. There was no significant difference in the appearance or degree of stratification between cultures that were air lifted for 6 or 12 days. The duration of air-lifting was also a factor in influencing the proliferative capacity of cells, as demonstrated by the fact that cells exposed to a longer period of air-lifting (12 days) had a lower proliferative capacity.

Several authors have used long-term labeling with BrdU as an indirect method for determining the presence of putative progenitor cells in epithelial equivalents, by their label-retaining nature.[29–31] However, the in vivo proliferative capacity of ex vivo expanded cells in submerged and air-lifted tissue equivalents has not been assessed. The ability of serum-free derived epithelial equivalents to remain proliferative in vivo was confirmed in our study. There was a dramatic increase in the degree of stratification and proliferation of cells that were originally cultured under submerged conditions. These cells proliferated more rapidly than air-lifted cultures and formed a stratified epithelium that was better organized into a basal columnar population of cells with progressive flattening of cells toward the surface. Air-lifted cultures that were transplanted did not undergo a significant increase in stratification, suggesting that prolonged incubation in vitro may be counterproductive, resulting in cells that were more differentiated and less proliferative. Xenografted tissue equivalents demonstrated a greater degree of stratification compared with in vitro tissue equivalents, which suggested that the in vivo environment provided a more conducive environment for cell growth and differentiation. As such, when considering the ex vivo expansion of conjunctival cells for transplantation, an earlier return to the in vivo environment, rather than prolonged in vitro incubation, may be beneficial in preserving a greater proportion of proliferative cells at the time of transplantation.

Studies have shown that air-lifting is necessary to promote the formation of a better-developed, continuous basal lamella, with a higher density of hemidesmosomes, whereas submerged cultures exhibited only a rudimentary, discontinuous basement membrane structure.[9,11–13] By altering the calcium concentration of the culture medium in our multistep approach, thereby making use of the growth modulating effect of this exogenous factor, submerged cultures elaborated a continuous basement membrane with numerous hemidesmosomes, as well as numerous intercellular desmosomes. In addition, the conjunctival epithelial equivalent expressed the differentiation-related markers that are consistent with that of normal conjunctiva.

In summary, we describe the development of a conjunctival epithelial equivalent, using a multistep serum-free culture system to enhance cell proliferation, differentiation, and attachment for clinical transplantation and tissue regeneration. The elimination of serum and feeder cells is a significant and important improvement over existing serum-containing methods of cultivating cells for clinical transplantation. These findings have important clinical implications and are important for the development of a safe and effective bioengineered tissue-equivalent for clinical use, such as in the regeneration of the ocular surface in conditions in which the normal conjunctiva is damaged or deficient.

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