Mucin Gene Expression in Immortalized Human Corneal–Limbal and Conjunctival Epithelial Cell Lines

Ilene K. Gipson, Sandra Spurr-Michaud, Pablo Argüeso, Ann Tisdale, Tat Fong Ng, and Cindy Leigh Russo

PURPOSE. The corneal and conjunctival epithelia, which cover the ocular surface, play an important role in preventing pathogen penetrance into the eye and maintaining a wet-surface phenotype by producing highly hydrophilic mucin molecules for their apical surfaces. Ocular surface infections, wounding, and pathologies resulting in dry eye threaten sight and can cause blindness. Understanding the ocular surface defense mechanisms that mucins provide has been hampered by the lack of immortalized human corneal and conjunctival epithelial cell lines that retain mucin gene expression patterns of the native tissue. The purpose of this work was to characterize newly developed immortalized corneal and conjunctival cell lines using mucin gene expression as markers of differentiation.

METHODS. The cell lines were derived as described by a previously published process. Primary cultures of corneal–limbal and conjunctival epithelia were sequentially transduced to express a dominant negative p53 protein and a $p16^{INK4A/Rb}$-resistant, mutant cdk4 protein, which enabled the cells to bypass a senescence mechanism recently identified for primary cultures of keratinocytes. These cells were then transduced to express the catalytic subunit of telomerase to permit them to retain their telomeres and divide indefinitely. Cellular morphology and expression of mucin genes in the two cell lines, designated HCLE for the human corneal–limbal line and HCjE for the human conjunctival cell line, were determined after culture on plastic, type I collagen, or Matrigel, in coculture with fibroblasts, and in severe combined immunodeficient (SCID) mice. Expression of the epithelial cell mucins was assayed by reverse transcription, real-time polymerase chain reaction, immunoblot analysis, or immunohistochemistry and compared with expression in native cornea and conjunctiva.

RESULTS. When grown in high-calcium medium on plastic and type I collagen, cells of both lines stratified, exhibiting multiple cell layers. In Matrigel, both cell lines formed cell aggregates that contained lumens. In the SCID mice, the conjunctival cell line formed stratified layers under the kidney capsule. The corneal cell line expressed keratins K3 and K12, the keratins that are corneal-epithelial–specific, and both cell lines expressed K19. As in native tissue, the HCLE and HCjE cell lines expressed the membrane-associated mucins, MUC1, -4, and -16, although their levels were generally lower. Levels of MUC4 and -16 mRNA were the most comparable to native tissue, particularly when cultured on plastic. Apical cells of the stratified cultures were the cells that expressed the membrane-associated mucins MUC1 and -16. Goblet-cell–specific MUC5AC mRNA and protein was detected in a small population of HCjE cells only when using type I collagen as a substrate or when cells were cocultured with fibroblasts. Both cell lines produced glycosylated mucins as indicated by binding of H185 antibody, an antibody that recognizes a carbohydrate epitope on mucins.

CONCLUSIONS. The immortalized corneal (HCLE) and conjunctival (HCjE) cell lines exhibit the mucin gene expression repertoire of their native epithelia. These cell lines will be useful in determining regulation of ocular surface mucin gene expression and, potentially, goblet cell differentiation. (*Invest Ophthalmol Vis Sci.* 2003;44:2496–2506) DOI:10.1167/iovs.02-0851

The corneal and conjunctival epithelia of the ocular surface protect the eye from pathogen invasion, desiccation, and injury. The corneal epithelium additionally provides an extraordinarily smooth, wet apical surface, which is the major refractive surface of the visual system.[1,2] Apical cells of both corneal and conjunctival epithelia express membrane-associated mucins for their tear film surface that, along with the secreted mucin of the conjunctival goblet cells, protects and maintains hydration of the ocular surface.[3,4]

Despite the well-developed defense mechanisms of the ocular surface epithelia, infections and diseases resulting in drying and keratinization of the epithelium often occur. Study of synthesis and production of defense and protective molecules by the ocular surface epithelia and their regulation would be facilitated by development of immortalized corneal and conjunctival epithelial cell lines that exhibit characteristics of their native epithelia.

Several methods for primary culture of corneal and conjunctival epithelia have been developed (for examples see Kahn et al.[5] and Risse Marsh et al.[6]). Primary cultures have been useful in determining aspects of stem cell location within the corneal and conjunctival epithelia of humans,[7] expression of corneal and conjunctival epithelial proteins,[8,9] goblet cell development,[10] cytotoxicity studies with the objective of replacing the Draize test,[11,12] and restoration of damaged ocular surface epithelium.[13,14] They have also been useful in constructing experimental corneal equivalents.[15,16] Although these studies have provided valuable information, immortalized epithelial cell lines that retain differentiation characteristics would facilitate studies of gene regulation specific to these epithelia. This is especially true in the case of conjunctival epithelial study, because there is not the tissue source that discarded donor corneal–limbal rims provide.

Several immortalized ocular surface epithelial cell lines have been reported. These include three corneal epithelial cell lines that were immortalized with a recombinant SV40 adenovirus vector.[5,17,18] The cell lines stratify and make the proteins that differentiated corneal epithelia make,[5,17,18] and they have been valuable resources for many studies.[19–22] In our hands, however, these cell lines did not synthesize the glycosylated mucin

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that we were trying to characterize. To our knowledge, immortalized epithelial cell lines from human conjunctiva have not been published, although several abstracts reporting experiments using a human conjunctival epithelial cell line, HCO597, have appeared (Hallberg CK, Hallberg SL, Trocme MC, Ward SD, Trocme SD, ARVO Abstract 3612, 1999; Trocme MC, Hallberg CK, Ward SL, Trocme SD, ARVO Abstract 3613, 1999; Ward SL, Walker TL, ARVO Abstract 4151, 1999). In addition, the so-called Chang conjunctival cell line American Type Culture Collection 20.2 (ATCC, Manassas, VA) is listed as conjunctival in origin; however, it is commonly acknowledged that it has a fibroblastic phenotype and an HeLa cell contaminant.

The development of techniques to immortalize epithelial cells by preventing telomere shortening by transduction with hTERT, the catalytic subunit of the telomerase holoenzyme, was originally purported to confer replicative immortality without loss of differentiation potential.[23,24] By comparison, immortalization with viral oncogenes, such as the SV40 large T-antigen, perturbs cell differentiation programs.[25] The goal of this study was therefore to develop and characterize hTERT-immortalized human ocular surface epithelial cell lines. During the course of the development of the cell lines, it became apparent that hTERT transduction was not sufficient to immortalize all cell types, including primary cultures of keratinocytes (Weinberg[25]). A two- or three-step process, including abrogation of either the p16$^{INK4A/Rb}$ pathway[26] or the p16$^{NK4A/Rb}$ and p53 pathways in the cell cycle, is required.[27] However, for those trying to obtain immortalized epithelial cells for study of their differentiation phenotypes (i.e., mucin expression), the inactivation of the cell cycle pathways may affect differentiation progress. We report here the differentiation characteristics and mucin gene expression profiles of corneal and conjunctival cell lines immortalized by stable transduction to express both a p16$^{INK4A/Rb}$-resistant mutant cdk4 protein and a dominant-negative p53 protein, followed by transduction with hTERT.[27] We report that the cells expressed the same mucin gene and keratin repertoire that their native epithelia produce, but they did not achieve normal morphologic differentiation seen in vivo.

Materials and Methods

Tissue Acquisition

All tissue was obtained in accordance with good clinical practice, Institutional Review Board and informed consent regulations of the Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, and the tenets of the Declaration of Helsinki. Human corneoscleral donor rims were provided by Roger Steinert and Ann Bajart of Ophthalmic Consultants of Boston, and conjunctival biopsy specimens from individuals with normal ocular surfaces were provided by C. Steven Foster of the Massachusetts Eye and Ear Infirmary. Corneoscleral donor rims consisted of peripheral and limbal corneas that were left after removal of central corneal buttons for transplantation. A small piece of bulbar conjunctiva was removed from the superior temporal region of patients undergoing routine cataract surgery.

Cell Culture

Primary cultures of human corneal–limbal and conjunctival epithelial cells derived from corneal donor rims and conjunctival biopsy specimens were immortalized by abrogation of p16 control and p53 function before immortalization by expression of hTERT, by the laboratory of James Rheinwald under contractual agreement on projects entitled, "Development of Human Conjunctival, Endocervical, and Tracheal Epithelial Cell Lines Expressing Mucins MUC4, 5AC, and 5B for Testing Agents that Affect Mucin Secretion" (funded by Inspire Pharmaceuticals, Inc., Durham, NC), and "Molecular Characterization of H185, a Membrane-Associated Mucin of the Corneal Epithelial Surface" (funded by Ciba Vision Corp., Duluth, GA).[27] Full details of the derivation of the two cell lines have been reported,[27] but their differentiation characteristics have not. Briefly, primary cultures of human corneal limbal epithelium were sequentially transduced with pBABE (cdk4R)hygro, which expresses a p16^{INK4A}-resistant point mutant (R24C) of cdk4,[28,29] and pL(p53DD)SN, which expresses a dominant-negative fragment of p53.[30,31] Primary cultures of human conjunctival epithelium were sequentially transduced with pL(p53DD)SN followed by pBABE (cdk4R)hygro. Both cell lines were finally transduced with pBABE(hTERT)puro, which expresses the catalytic subunit of human telomerase.[32,33]

The immortalized corneal and conjunctival epithelial cells, designated HuCl-22/cdk4R/p53DD/TERT (shortened here to HCLE) and ConjEp-1/p53DD/cdk4R/TERT (shortened here to HCjE), respectively, were plated at $2 \times 10^4/cm^2$ in a medium nutritionally optimized for growth of keratinocytes—keratinocyte serum-free medium (K-sfm)[34] (Gibco-Invitrogen Corp., Rockville, MD), supplemented with 25 μg/mL bovine pituitary extract (BPE), 0.2 ng/mL epidermal growth factor (EGF), and 0.4 mM $CaCl_2$,[35] and grown at 37°C in a 5% carbon dioxide atmosphere, as previously described.[27] To enhance nutrient composition, the cultures were switched at approximately half-confluence to a 1:1 mixture of Gibco K-sfm:low-calcium DMEM/F12 (Gibco) to achieve confluence (approximately 24 hours). After reaching confluence, cells were switched to DMEM/F12 medium with high calcium (1 mM $CaCl_2$) supplemented with 10% calf serum and 10 ng/mL EGF (stratification medium) for 3 to 7 days to promote stratification.

For all studies of the expression of mucins, cells were cultured in medium, as just described, on plastic, on inserts coated with type I collagen or Matrigel (Biocoat Cell Culture Inserts; BD Labware, Bedford, MA), or on inserts with corneal or conjunctival fibroblasts grown to confluence in the lower chamber. The corneal and conjunctival fibroblasts, obtained from James Zieske of the Schepens Eye Research Institute, were grown in DMEM/Ham F-12 plus antibiotic/antimycotic, 200 mM L-glutamine, and 10% fetal bovine serum (Sigma, St. Louis, MO).

To determine the effect of steroids on mucin gene expression, HCjE cells were grown to confluence, as described above, switched to serum-containing medium for 48 hours to achieve stratification, serum starved for 24 hours, and then cultured in DMEM/F12 plus 10^{-6} M dexamethasone for 24 hours.

To determine whether the immortalized conjunctival cells would differentiate into goblet cells, cells were grown to near confluence on clear cell-culture inserts (Transwell; Corning CoStar, Corning, NY), which were cut into 1×1-mm pieces and implanted, as previously described, into the renal subcapsular space of C.B-17-scid severe combined immunodeficient (SCID) mice homozygous for the Prkdc^{scid} mutation and lacking both T and B cells (Taconic, Germantown, NY).[36,37] Implanted HCjE cells were harvested for morphologic studies after 3, 5, or 21 days of growth in the SCID mice. The use of animals was approved by the Schepens Eye Research Institute Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Microscopy

Cell cultures were visualized and photographed during growth by phase-contrast microscopy. Cell cultures grown on type I collagen or Matrigel-coated inserts, and tissue excised from C.B-17-scid mice were fixed in 4% paraformaldehyde in phosphate buffer and processed for embedding in hydroxyethylmethacrylate embedding resin (Technovit 7100; Energy Beam Sciences, Agawam, MA). Cross sections were stained with hematoxylin and eosin.

Immunofluorescence Microscopy

Cultures grown on type I collagen inserts for 7 days in stratification medium were fixed in 4% paraformaldehyde for en face immunofluorescence detection of keratins and mucin proteins. Briefly, cultures were rinsed in phosphate-buffered saline (PBS), permeabilized with PBS plus 5% Triton X-100, and blocked with PBS with 1% bovine serum

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albumin (BSA). Cultures were then incubated for 1 hour at room temperature with primary antibody followed by fluorescein-conjugated secondary antibody and coverslipped with antifade mounting medium plus propidium iodide (Vectashield; Vector Laboratories; Burlingame, CA) as previously described.[38] The primary antibodies used were AE5, which recognizes keratin K3 (ICN Biomedical, Costa Mesa, CA); pAb55, which recognizes K12[39]; RCK 108, which recognizes K19 (ICN Biomedical); HMFG-2 (Biodesign, Saco, ME), which recognizes a peptide in MUC1; H185, which recognizes a carbohydrate epitope on MUC16[8,40]; Clone OC125, which recognizes a peptidic epitope on MUC16 (CA125; Dako Corp.; Carpinteria, CA); and 791, an antibody which recognizes a peptide in MUC5AC.[38]

Fluorescence In Situ Hybridization

Cultures grown on type I collagen inserts for 7 days in stratification medium were fixed in RNase-free 4% paraformaldehyde for en face fluorescence in situ hybridization (FISH) of mucin mRNA, essentially as previously described.[41] Briefly, FISH was performed using digoxigenin-labeled antisense and sense riboprobes (DIG RNA Labeling Kit; Roche Applied Sciences; Indianapolis, IN) generated from a previously described MUC5AC cDNA.[42] Probe labeling, prehybridization treatments (rinses, permeabilization, proteinase K treatment, and acetylation), hybridization with DIG-labeled riboprobes, and posthybridization washes were performed according to recommendations of the manufacturer. Hybridized riboprobes were detected with 20 μg/mL fluorescein-conjugated anti-digoxigenin antibody (Roche Applied Sciences).

RNA Isolation and Reverse Transcription

Total RNA was isolated from the cell cultures, human donor corneas, and human conjunctival biopsy specimens using TRIzol reagent (Invitrogen), according to the manufacturer's recommended protocol. Residual genomic DNA in the RNA samples was eliminated by digestion with DNase I (Amplification Grade; Gibco-Invitrogen). Digested total RNA was reverse transcribed with random hexamer primers and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol, as previously described.[38]

Conventional Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Conventional RT-PCR was performed on RNA from cell cultures grown on plastic to look for expression of MUC1, -2, -4, -5AC, -5B, -6, and -11, as previously described.[43–46] In addition, PCR primers were designed for the recently cloned membrane-associated mucins, MUC13,[47] -16,[48] and -17,[49] using Primer Express software (Applied Biosystems, Foster City, CA). The specificity of newly designed primers (MUC13, -16, and -17) and probe (MUC16) was confirmed by BLASTN (www.ncbi.nlm.nih.gov/blast/; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) searches against nucleotide databases. Furthermore, the identity of the amplified PCR products was verified by the DNA Sequencing Core of Massachusetts General Hospital (Boston, MA). The primer sets for MUCs 13, 16, and 17 are as follows: MUC13 Forward: TGCTTCTATCCCTCCAATGGA; MUC13 Reverse: TGGGTGAGGCTAGGTTGCA; MUC16 Forward: GCCTCTACCTTAACGGTTACAATGAA; MUC16 Reverse: GGTACCCCATGGCTGTTGTG; MUC16 *Taq*Man Probe: AGATGAGCCTCCTACAACTCCCAAGCCAG; MUC17 Forward: GGGCCAGCATAGCTTCGA; MUC17 Reverse: GCTACAGGAATTGTGGGAGTTGA.

Real-Time PCR

Real-time PCR amplification and relative quantitation of the mucin genes found to be expressed by the cell cultures was performed with double-labeled fluorogenic probes and primers (*Taq*Man; Applied Biosystems), as previously described.[38] PCR primers and probes for MUC16 are those listed herein, and those for MUC1, -4, -5AC and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been reported.[38] To validate the use of these primers and probes for relative quantitation of mRNA, real-time PCR assays were performed to confirm that the efficiency of the target gene amplification was equivalent to that of the endogenous control used for this study (GAPDH).

The important parameter for quantitation in real-time PCR is the C_T value, which is the fractional cycle number at which the amount of amplified target reaches a fixed threshold of detectable fluorescence. The threshold is set in the midlinear phase of the amplification plot. To standardize the amount of sample cDNA added to each reaction, the amount of target gene in each sample was normalized to the endogenous control by subtracting the C_T of the endogenous control, GAPDH, from that of the target gene (ΔC_T). For quantitation, the amount of mRNA for each target gene was expressed relative to the amount present in a calibrator sample using the ΔC_T method (Applied Biosystems). For this study, mucin expression in native corneal or conjunctival tissue (for HCLE or HCjE, respectively) were used as the calibrators. The level of mRNA for the calibrator sample was set at 1, and all other conditions were expressed relative to it. No template controls were included in all real-time PCR experiments to confirm the absence of DNA contamination in the reagents used for the amplification. Statistical comparisons of results from real-time PCR were done with the Fisher Protected Least-Significant Difference (Fisher's PLSD) test using StatView, version 5.0 (SAS Institute, Cary, NC).

Immunoblot

Protein was extracted from cell cultures by methods previously described,[8] except that a complete protease inhibitor cocktail was added (Roche Molecular Biochemicals, Indianapolis, IN). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose membrane and probed with antibodies recognizing MUC1 (HMFG-1; Biodesign), MUC16 (clone OC125), or the carbohydrate epitope on MUC16 (H185),[8] as previously described.[38]

Results

The purpose of the work reported herein was to develop immortalized corneal and conjunctival cell lines that would differentiate and express the mucins that are produced by native epithelia, to enable the study of regulation of expression of these differentiation products. The assays chosen to determine differentiation and mucin gene expression include (1) light microscopy of the epithelial architecture of the two cell lines after culture in high-calcium–containing medium on plastic and on culture inserts coated with type I collagen or Matrigel and after implant under the kidney capsule of C.B-17-scid mice; (2) immunolocalization of keratins specific to corneal and conjunctival epithelia; (3) real-time PCR to compare mucin gene mRNA expression from the same cultures listed above and from cocultures of the corneal and conjunctival epithelia with corneal or conjunctival fibroblasts, in situ hybridization for MUC5AC; and (4) mucin protein expression and mucin glycosylation assayed by immunohistochemistry or immunoblot analysis where possible.

Epithelial Architecture of Cultures of HCLE and HCjE Cells Grown on Various Substrates and in SCID Mice

Cells from both cell lines stratified when cultured with the serum-containing medium on plastic or culture inserts coated with type I collagen (Figs. 1, 2). The cells of all layers were elongated, with no apparent basal cell polarity. In Matrigel, both cell types formed cell aggregates (Figs. 1C, 1D; 2C, 2D). The conjunctival cell line formed a more regular, spherical, hollow aggregate, the walls of which had the three to four cell layers similar to that of a stratified conjunctival epithelium (Fig. 2D). By comparison, the HCLE cells formed aggregates, but the cells did not form a regular "epithelial-like" wall (Fig. 1D). If

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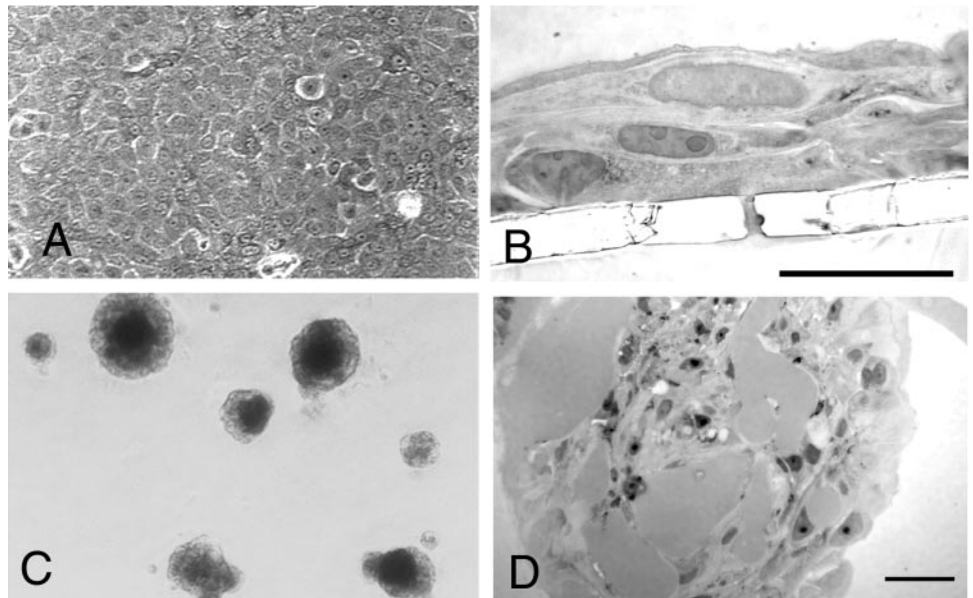

FIGURE 1. Microscopic appearance of cultures of HCLE cells. (**A**) Cells cultured in high-calcium media on plastic. (**B**) Cross-section of cells cultured in high-calcium medium on type I collagen-coated culture inserts. (**C**) Cell aggregates that formed when cells were cultured on Matrigel-coated inserts. (**D**) Cross-section of a cell aggregate similar to those shown in (**C**). Note the several lumens within the aggregate. Bars, 25 μm.

serum was not added to the media of HCjE cells, however, lumens did not form (data not shown).

To determine whether goblet cells differentiate from immortalized conjunctival epithelia, HCjE cells were grown on uncoated cell culture inserts to 95% confluence. The inserts with attached cells were cut into 1-mm^2 pieces and implanted into the renal subcapsular space of C.B-17-scid mice.[36,37] By 5 days, cells on the implants grew to a stratified epithelial-like architecture, with four to five cell layers, but completely differentiated goblet cells were not found. By day 21, implanted cells were no longer discernible. In pilot studies, we injected cells subcutaneously,[50] but we were not able to locate the injected cells after 11 days.

To verify that the corneal and conjunctival cell lines retain the keratin expression patterns of their native epithelia, the keratins K3, K12, and K19 were immunolocalized in the HCLE and HCjE cells, grown on type I collagen–coated inserts for 7 days in stratification medium. As demonstrated in Figure 3, the cornea-specific keratins K3 and K12 were present within the corneal cells, with only occasional cell binding in the conjunctiva-derived cells (Figs. 3A–D), and K19, a widely expressed keratin, was present in both cor-

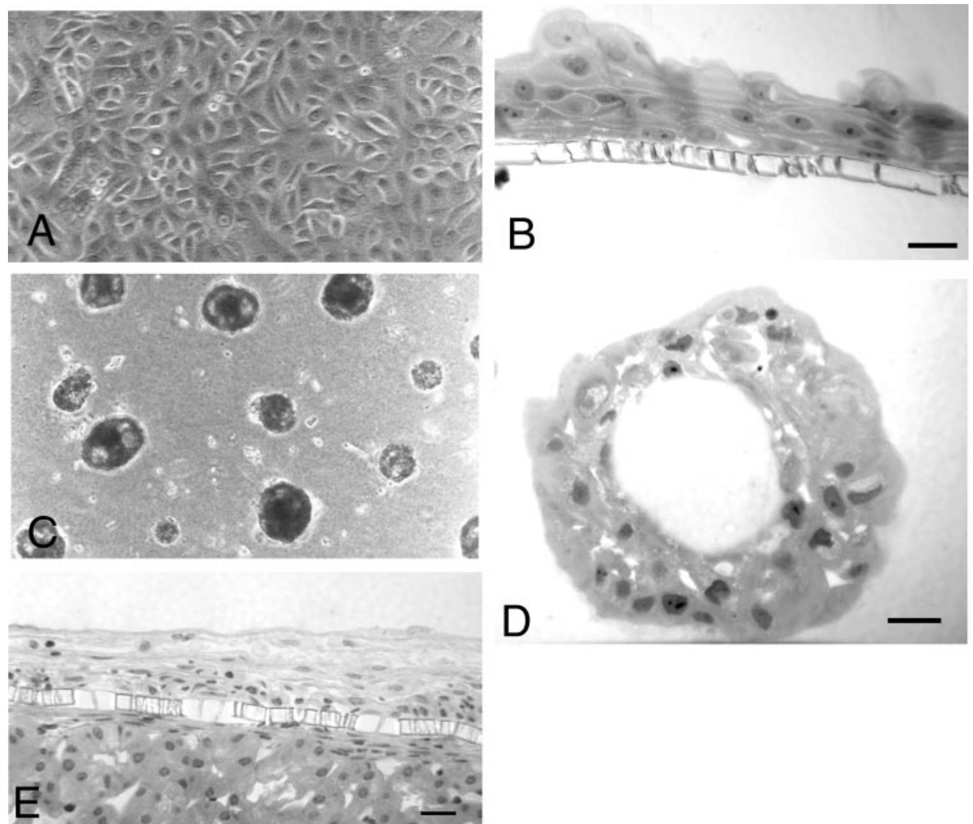

FIGURE 2. Microscopic appearance of cultures of HCjE cells. (**A**) Cells cultured in high-calcium medium on plastic. (**B**) Cross-section of cells cultured in high-calcium medium on culture inserts coated with type I collagen. (**C**) Spherical cell aggregates that formed when cells were grown on Matrigel-coated culture inserts. (**D**) Cross section of a cell aggregate similar to those in (**C**). Note the central lumen and the three to four cell layers of the wall of the spherical cell aggregate. (**E**) Cells grown on culture inserts and then implanted under kidney capsule for 5 days. Stratified layers of cells were present along the insert, but no cells with goblet cell morphology were observed. Bars, 25 μm.

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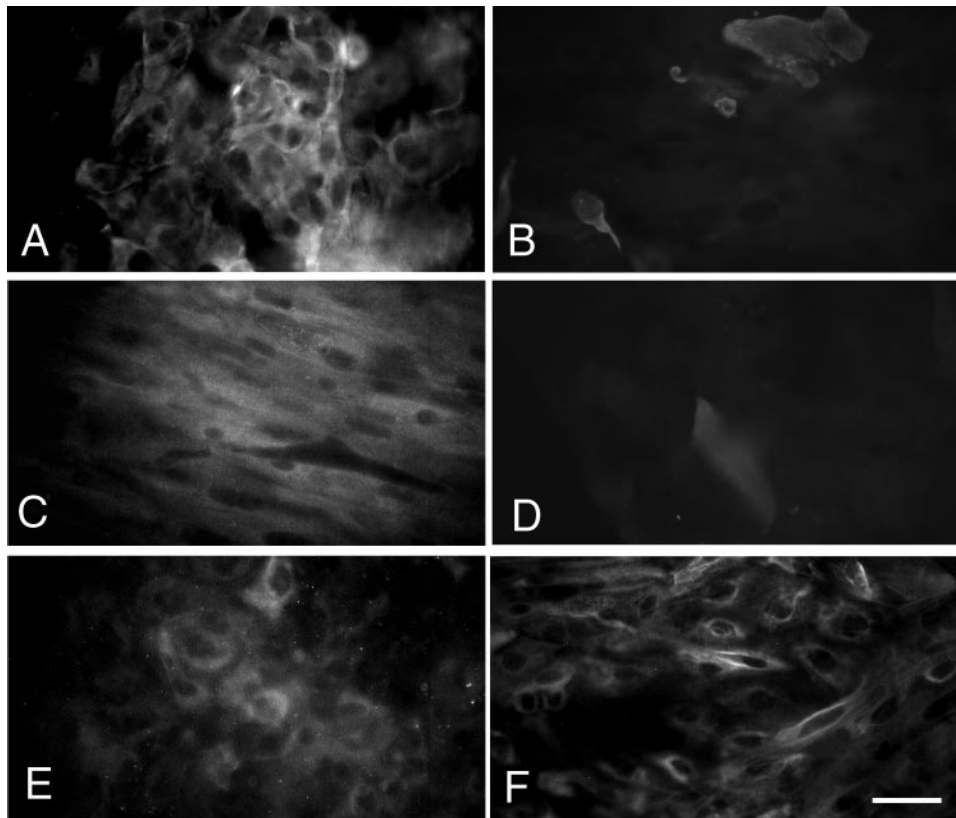

Figure 3. Immunofluorescence microscopy demonstrating keratin expression in HCLE and HCjE cells cultured on type I collagen–coated culture inserts. Keratin K3 antibodies, considered to be corneal epithelium specific, bind to the HCLE cells (**A**); only occasional HCjE cells (**B**) bind the antibody. Similarly, antibodies to keratin K12, also considered to be corneal epithelium specific, bind to HCLE (**C**), but not to HCjE (**D**). Antibodies to keratin K19 bind to both HCLE (**E**) and HCjE (**F**) cells. All micrographs are of the same magnification. Bar, 25 μm.

nea- and conjunctiva-derived cells (Figs. 3E, 3F). These data indicate that the immortalized cell lines expressed the keratins of their native epithelia, as summarized by Risse Marsh et al.[6] and Kurpakus et al.[39]

Mucin mRNA Expression in Immortalized Human Keratinocytes

In initial studies to determine which mucin genes were expressed by the HCLE and HCjE cells, conventional RT-PCR assays were performed on cells grown on plastic for 7 days in stratification medium. mRNAs for MUC1, -4, and -16 were found, whereas MUC-2, -5B, -6, -13, and -17 were not found. In addition, mRNA for MUC11 was detected by RT-PCR in the HCLE and HCjE cells, as well as in native cornea and conjunctiva (data not shown), but because only tandem repeat sequence is available for MUC11, further studies of this poorly described mucin were not conducted.

The quantitation of mucin transcripts in the immortalized human corneal and conjunctival keratinocytes cultured under different conditions was performed with real-time PCR, selecting the mucin mRNA values of corneal and conjunctival biopsy specimens as the calibrator (relative expression = 1). All membrane-associated mucins expressed by the native corneal and conjunctival epithelia, MUC-1, -4, and -16,[4,40,50] were detected in the immortalized corneal (HCLE) and conjunctival (HCjE) epithelial cell lines (Figs. 4A, 4B).

The expression of MUC1 was similar in cells grown on plastic, type I collagen and Matrigel, although, of the membrane-associated mucins, the number of MUC1 mucin transcripts was the lowest compared with primary cultures of corneal and conjunctival epithelia and native tissue. HCLE and HCjE cells grown on plastic expressed MUC1 mRNA at levels 21- and 7-fold lower than native tissue, respectively. By comparison to MUC1, the expression of MUC-4 and -16 varied between culture substrates. The HCLE and HCjE cells grown on plastic maintained a greater capacity to express the MUC4

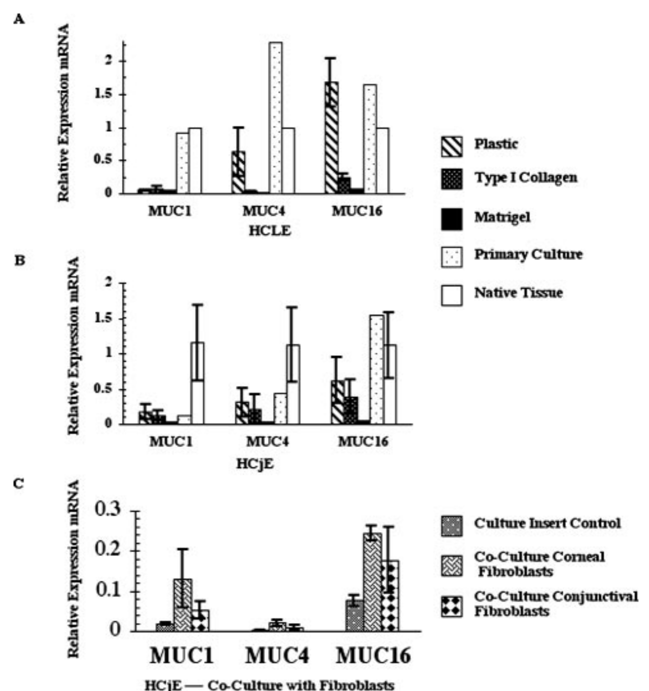

Figure 4. Real-time PCR assay of relative mRNA levels of the membrane-associated mucins MUC1, -4, and -16 in HCLE (**A**) and HCjE (**B**, **C**) cells after culture for 7 days in stratification medium on plastic-, type I collagen–, or Matrigel-coated culture inserts (**A**, **B**), or after coculture with fibroblasts for 4 days in stratification medium (**C**). mRNA levels were compared with that in native tissue, which is set at 1. For experiments in (**A**) and (**B**), the minimum number of replicates was three, except for primary cultures and native cornea, where $n = 1$. For (**C**), $n = 2$ for control and conjunctival fibroblast coculture, and $n = 5$ for coculture with corneal fibroblasts. Data are shown as mean ± SEM (error bars) where possible.

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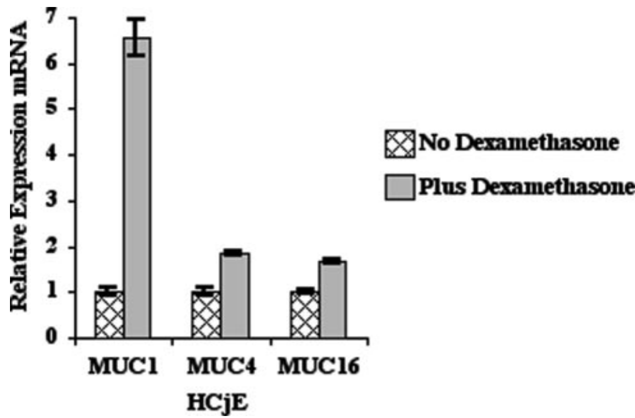

FIGURE 5. Real-time PCR assay of relative mRNA levels of the membrane-associated mucins, MUC1, -4, and -16 in stratified cultures of HCjE cells treated for 24 hours with 10^{-6} M dexamethasone after 48 hours of serum starvation. mRNA levels of the dexamethasone-treated cultures are compared with the mean of the no dexamethasone control cultures, which is set at 1. Data are shown as the mean ± SEM (error bars); $n = 2$ for each group.

and -16 mRNA mucins, HCLE being nearly equivalent to primary cultures of corneal epithelium and native tissue. However, when cell lines were grown on type I collagen and, especially, Matrigel, the expression of MUC4 and -16 mucin mRNA was reduced (Figs. 4A, 4B).

To test the effects of coculture with either corneal or conjunctival fibroblasts on expression of membrane-associated mucins, HCjE cells were grown on culture inserts, with or without fibroblasts in the lower chamber (Fig. 4C). Although there was a slight increase in expression of MUC-1, -4, and -16, when HCjE cells were grown with corneal or conjunctival fibroblasts, the increase did not reach significance. The source of fibroblasts, either corneal or conjunctival, did not differentially affect mucin mRNA expression.

To determine whether the mucin genes were inducible in the conjunctival cell line, cells were cultured in the presence of 10^{-6} M dexamethasone. MUC1 mRNA was 6.5-fold higher in the dexamethasone-treated cells compared with the control (Fig. 5). By comparison, MUC4 and -16 showed minimal increases (1- and 0.75-fold, respectively) in response to dexamethasone.

The most dramatic difference between HCjE and native tissue was the low expression of the conjunctival goblet cell mucin MUC5AC (Fig. 6). Note that data in Figure 6 are graphed on a logarithmic scale. The level of MUC5AC expression in HCjE cells was approximately 4.3×10^4-fold lower than that of native tissue when cells were cultured on plastic, indicating that only a very small population of cells in culture expressed MUC5AC transcripts (as verified by immunohistochemistry and in situ hybridization, discussed later). When the different substrates were compared, the HCjE cells grown on type I collagen had the highest levels of MUC5AC transcripts, approximately 80-fold higher as compared to plastic substrate ($P < 0.05$), but still 500-fold less than native tissue. Cocultures with corneal or conjunctival fibroblasts enhanced MUC5AC expression slightly (11- or 6-fold higher than on plastic, respectively). The goblet-cell–specific MUC5AC mucin was not detected in any of the immortalized human corneal epithelial cell cultures nor in native corneal tissue, as determined by real-time PCR (data not shown).

Mucin Protein Synthesis in Immortalized Keratinocytes as Determined by Immunohistochemistry and Immunoblot Analysis

Immunohistochemical localization of MUC1 and -16 in both the HCLE and HCjE cell lines after culture on type I collagen is shown in Figure 7. Scattered apical cells of the stratified cultured epithelia bound the antibody to MUC1 (HMFG-2; Figs. 7A, 7B) and to MUC16 (OC125; Figs. 7C, 7D). Confocal imaging of the cells allowed reconstruction of the cross-section of the cultures, demonstrating that the binding of the antibodies was on the apical surfaces of the cultured cells (Fig. 7E). Despite attempts to make antibodies to MUC4 peptides and trials with commercially available MUC4 antibodies, to date we have found no antibodies that work well in immunohistochemistry. Therefore, we did not immunolocalize that membrane-associated mucin.

After culture on type I collagen, HCjE cells expressed some message for MUC5AC (Fig. 6). In those cultures, MUC5AC was detected by antibody binding to scattered cells (Figs. 8A, 8B), whereas no binding was found to cell cultures grown on cross-linked type I collagen, which did not express MUC5AC mRNA (Figs. 8C). The binding of the antibody appeared to be in endoplasmic reticulum and Golgi apparatus (Figs. 8A, 8B). In situ hybridization using riboprobes to MUC5AC verified the expression of goblet cell mucin by the small subpopulation of cells (Figs. 8E, 8F). Many attempts were made to induce the expression of MUC5AC and thereby induce differentiation of goblet cells in the HCjE cultures. Neither culture on Matrigel or in SCID mice, nor coculture with fibroblasts (Fig. 6), nor feeding cells nutrient-rich medium from the basal aspect of the cell culture (data not shown) induced MUC5AC mRNA. Nevertheless, there was a small population of cells within the cultures, particularly those on type I collagen, that expressed MUC5AC.

Immunohistochemistry using an antibody to a carbohydrate epitope on MUC16, (designated H185; see Ref. 40, in the current issue) demonstrate that both HCLE and HCjE cell lines glycosylate MUC16 in culture (Fig. 9). As with immunolocalization of the membrane-spanning mucins MUC1 and -16, scattered apical cells of the stratified cultures bound the H185 antibody. Immunoblot analysis (Fig. 10) verified the immunohistochemical analysis. Both MUC1 and -16 proteins were detected by immunoblot of cell lysates of HCLE grown on type I collagen and HCjE grown on plastic, and the presence of glycosylated MUC16 in both cell lines is confirmed by the binding of the H185 antibody (Fig. 10).

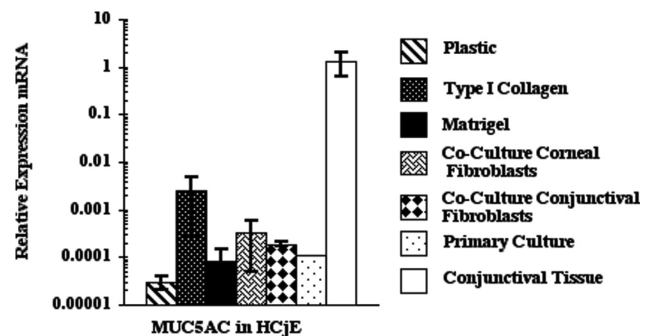

FIGURE 6. Real-time PCR assay of mRNA levels of the goblet cell mucin MUC5AC in HCjE cells cultured for 7 days in stratification medium on plastic, type I collagen, or Matrigel. Message on plastic and Matrigel was extremely low, whereas culture on type I collagen was detected at C_T's of 30 to 37. MUC5AC mRNA levels on type I collagen were significantly higher than on the other substrates examined ($P < 0.05$). As demonstrated by the logarithmic scale, native conjunctival tissues express MUC5AC mRNA at levels significantly greater than those of the primary culture and HCjE cells ($P < 0.0001$). For cells grown on plastic, collagen, or Matrigel, $n = 7$ to 10. For coculture experiments, $n = 2$ to 5. For primary culture, $n = 1$. For conjunctival tissue, $n = 8$. Data are shown as the mean ± SEM (error bars).

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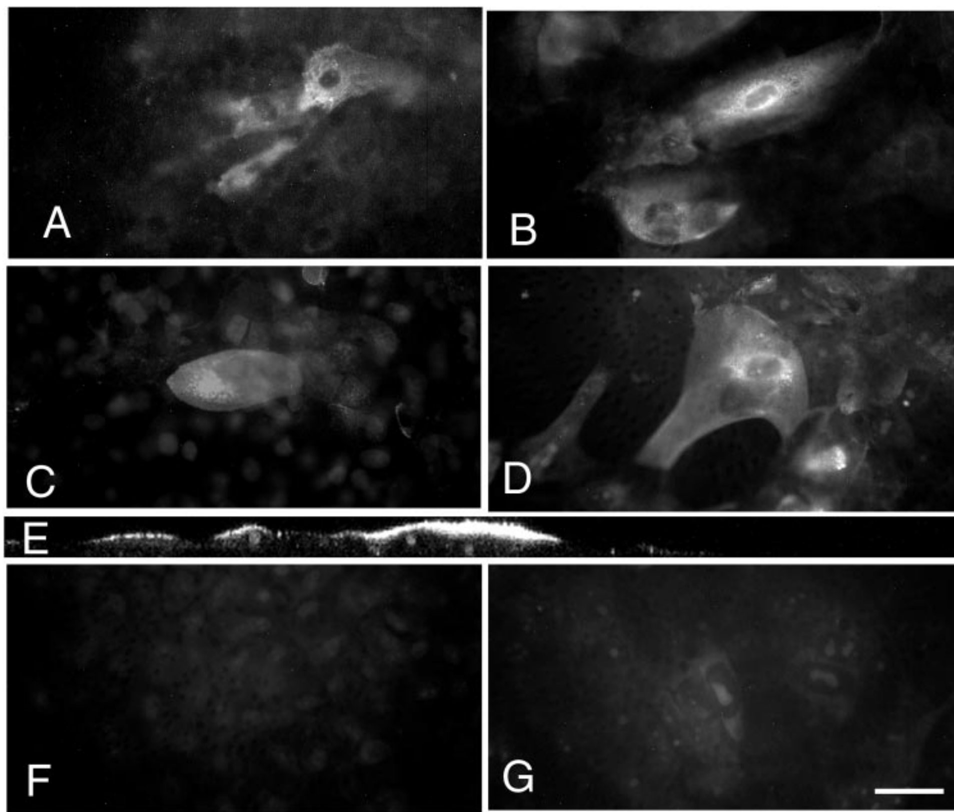

Figure 7. Immunolocalization of membrane-associated mucins on HCLE and HCjE cells after culture on type I collagen for 7 days in stratification medium. MUC1, localized by using HMFG-2 antibody, was present on apical cells in stratified cultures of HCLE (**A**) and HCjE (**B**). MUC16, localized by using a CA125 antibody, also was present on apical cells of both HCLE (**C**) and HCjE (**D**) cells. Proof that the membrane-associated mucins are on apical cells was demonstrated by reconstruction of vertical sections from stacked confocal images of the labeled cultures (**E**). The micrographs in (**E**) are of cultures immunolabeled to localize MUC16. (**F**) and (**G**) show lack of nonspecific binding of secondary antibody to corneal and conjunctival cells, respectively. All images are of the same magnification. Bar, 25 μm.

Discussion

The immortalized human corneal and conjunctival epithelial cell lines described in the current study exhibited some but not all the characteristics of their native epithelia. Since the corneal cell line expressed the same membrane-associated mucins, as well as keratins K3 and K12, that are expressed in native corneal epithelium, and since the conjunctival cell

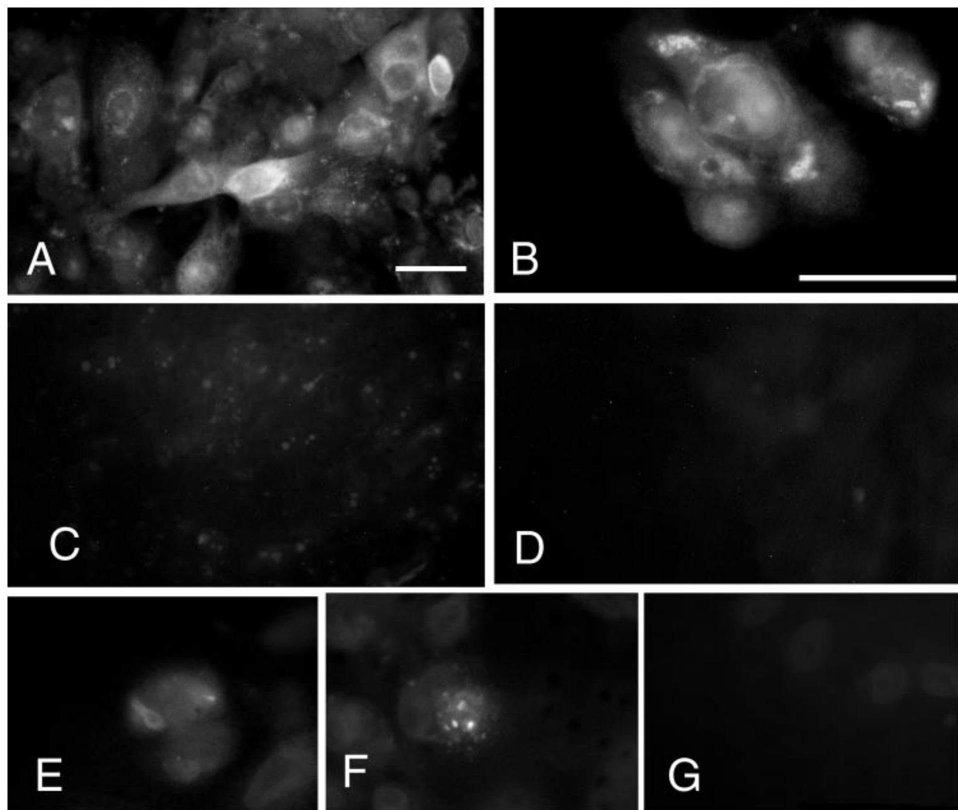

Figure 8. Immunolocalization of MUC5AC protein and in situ hybridization of MUC5AC mRNA in HCjE cells cultured on type I collagen for 7 days in stratification medium. (**A**, **B**) Two examples of binding of MUC5AC antibody to subpopulations of cells within the cultures. (**C**) MUC5AC antibody did not bind to cells that did not express MUC5AC mRNA, when cultured on cross-linked type I collagen. (**D**) No nonspecific binding of secondary antibody (primary antibody omitted) was observed. Fluorescence in situ hybridization of the MUC5AC riboprobe shows message for the mucin in individual cells of the HCjE culture (**E**, **F**). Sense control incubations showed no binding to cells (**G**). Magnification is the same in (**A**), (**C**), and (**D**) and in (**B**), (**E**), (**F**), and (**G**). Bars, 25 μm.

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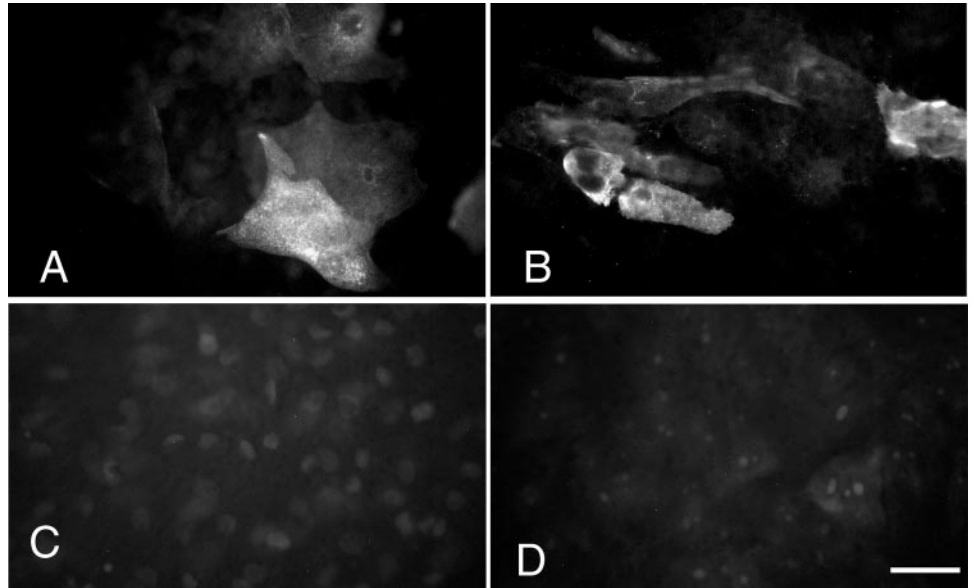

FIGURE 9. Immunolocalization of H185 antibody, which recognizes a carbohydrate epitope on MUC16 on HCLE (**A**) and HCjE (**B**) cells. These data indicate that both cell lines glycosylate MUC16. No nonspecific binding of secondary antibody (primary antibody omitted) was present in each culture (**C**, HCLE; **D**, HCjE). Bar, 25 μm

line expressed MUC5AC (at low levels) and keratin K19, but not K3 or K12, it can be said that the cell lines retained several of the unique characteristics of their native epithelia. As in the native state, the cultures stratified, and as in the native tissue, it was the flattened apical cells that expressed the membrane-associated mucins. In addition, there was evidence of glycosylation of membrane-associated mucins. Under the culture conditions used to date, neither cell line fully differentiated to form well-polarized epithelia exhibiting cuboidal basal cells, nor, in the case of the conjunctival cell line, did fully differentiated goblet cells form, despite some MUC5AC expression. We conclude from the studies performed with these cell lines that they will be useful tools, particularly for studies of the regulation of expression, glycosylation, and function of membrane-associated mucins. The data showing that MUC1 can be upregulated by dexamethasone in the HCjE cell line demonstrate the potential of the cell line. Moreover, the HCLE cell line has been used to demonstrate that the H185 antibody recognizes a carbohydrate epitope on MUC16.[40]

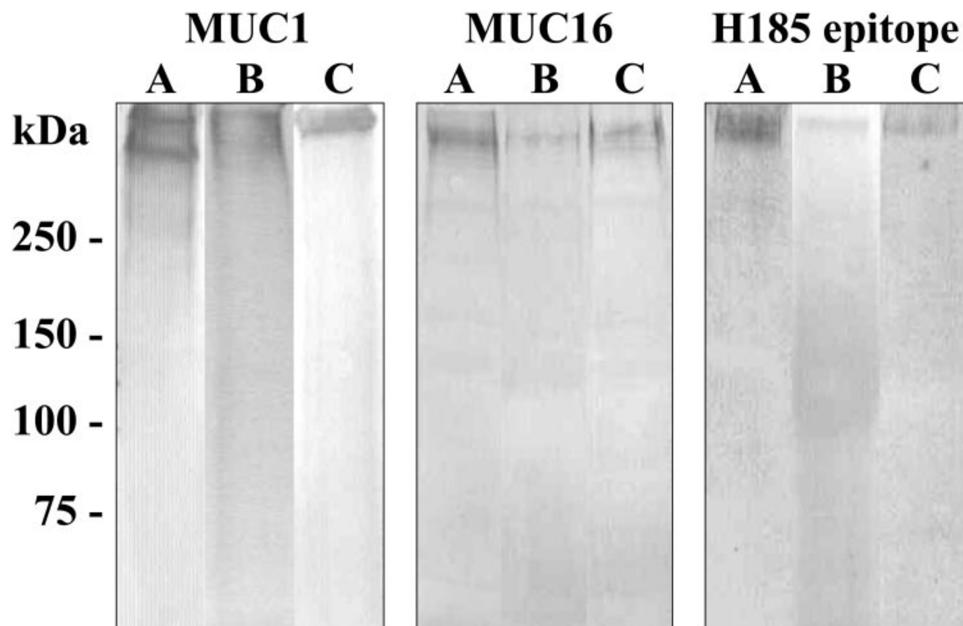

FIGURE 10. Western blot experiments demonstrating the presence of the MUC1 and -16 mucins and the H185 epitope in protein extracts of cultures of HCLE and HCjE cells. *Lane A*: primary cultures of nontransformed human corneal keratinocytes grown on plastic, with 100 μg protein loaded per lane. *Lane B*: cultures of immortalized HCLE grown on type I collagen, with 50 μg protein loaded per lane. *Lane C*: cultures of immortalized human conjunctival keratinocytes (HCjE) grown on plastic, with 50 μg protein loaded per lane. Western blot analysis was performed with the HFMG-1 monoclonal antibody against the MUC1 mucin, the CA125 against the MUC16 mucin, and the H185 hybridoma cell culture supernatant against the H185 epitope. Experiments were performed on two to three preparations with reproducible results. Negative controls included blots probed with primary antibody omitted and blots of cell culture medium supernatants of equivalent protein load (data not shown).

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There is considerable literature in which cell cultures have been used to study regulation of mucin gene expression. To date, most of the studies of mucin gene expression have been performed in primary cultures of epithelia or cancer cell lines. Because of the role of mucins in cystic fibrosis, studies of primary cultured tracheal epithelia have been undertaken to determine regulators of tracheal mucin expression.[52,53] Similarly, because mucin genes are upregulated and aberrantly expressed in breast, colonic, and endometrial cancers, there have been studies of these cell types in culture.[54–56] To our knowledge, three cell lines immortalized from normal tissue have used expression of MUC1 and -4 as an indicator of differentiation. Breast epithelial and endometrial cell lines immortalized from normal epithelia by E6, E7, and SV40, respectively, express MUC1,[57,58] and tracheal gland cells immortalized by SV40 express MUC1 and -4.[59] With the exception of the cancer cell lines, the results of studies to determine mucin expression patterns on the primary cell cultures or cell lines derived from normal epithelia indicate that each epithelial cell culture retains the repertoire of mucin genes expressed by their native tissue. Data from our corneal and conjunctival epithelial cell lines are thus comparable to those in previous studies in this regard. As in the native corneal epithelium, the corneal cell line expresses MUC1, -4, -11, and -16 but not MUC13 or -17. Similarly, the conjunctival cell line expresses MUC1, -4, -11, -16, and -5AC but not MUC2, -5B, or -6. Because specific epithelia express their unique repertoire of mucins, and because primary cell cultures and cell lines immortalized from normal epithelia retain that repertoire, the value of having ocular surface epithelial cell lines for study of specifics of tear film mucin expression, glycosylation, and function are apparent.

Other corneal epithelial cell lines that have been developed have been immortalized by the SV40 large T antigen.[5,17] In our hands, as evidenced by the absence of binding of the H185 antibody that recognizes a carbohydrate epitope on MUC16, these cell lines did not glycosylate mucins (data not shown). It was for this reason that the hTERT method of immortalization, with its potential to preserve differentiation characteristics, was undertaken for the corneal and conjunctival epithelia. Both HCLE and HCjE glycosylate MUC16, but glycosylation of the other mucins has yet to be studied.

Primary cultures of the simple epithelia of tracheal and colonic origin express and synthesize the large gel-forming mucins MUC5AC and -5B.[60] Cells similar to goblet cells differentiate in these cultures and secrete mucin that has a gel-like character at the culture surfaces.[61] Although a few cells in the HCjE cell line described herein expressed MUC5AC, particularly with culture on type I collagen, we did not see goblet cell morphology or evidence of secreted mucins in the cultures. A variety of culture conditions were used to induce MUC5AC expression and goblet cell differentiation. The variations included, in addition to the culture on type I collagen, Matrigel, and coculture with fibroblasts reported herein, culture media (RPMI-1640, stratification medium from day 1), time in culture (up to 17 days), plating density, and addition of galactose, a precursor for O-linked mucin-type sugars.[62] None of these variations dramatically enhanced MUC5AC expression, nor did we see goblet cell morphology (data not shown).

The question remains, is there a small subpopulation of cells of a goblet cell lineage in the immortalized conjunctival cell line, and are there culture deficiencies by way of the effector molecules, substrates, or coculture conditions that are required for goblet cell differentiation that have not been met by our experiments to date? The fact that we found no evidence of mature goblet cells when the epithelial cells were cultured under the kidney capsule in SCID mice, where conditions are purportedly ideal for differentiation, suggests that the differentiation pathway for goblet cells within the cell line may have been altered by the immortalization procedures. Indeed, Weinberg et al.[25] caution that inactivation of cell cycle pathways may affect the differentiation process. The possibility also exists that most of the cells transduced in the primary cultures were cells that had left the pluripotent cell compartment. Perhaps only a few stem cells were transduced, and it was these pluripotent stem cells that gave rise to the small subpopulation of cells that expressed MUC5AC. Isolation and culture of this subpopulation may be informative.

The second conclusion of this study is that culture substrate can influence mucin gene expression. Matrigel negatively affected MUC4 expression in both cell lines, and there was upregulation of MUC5AC mRNA when the conjunctival cell line was cultured on type I collagen. This observation has been made with culture of other types of epithelial cells. Airway epithelial cells, grown on collagen gels or a type I collagen gel matrix in the presence of retinoids, produce mucin-like glycoproteins.[63,64] Similarly, Caco-2 cells, a colon cancer cell line, produces a greater amount of mucin if grown on type I and IV collagens.[65] The later studies measured the effect of collagens on mucin glycoprotein product, not on mucin mRNA. In contrast, studies of the effect of Matrigel on expression and SMC protein levels (SMC is the rat homologue of MUC4) have demonstrated no effect on mRNA transcripts, but protein levels were significantly reduced.[66,67] Perhaps this difference is a species variation. Perhaps collagen upregulates mucin production because this substrate fosters epithelial differentiation and cell polarization. Polarization supports synthesis of mucins that are either in apical membranes or secreted apically.

Several studies have demonstrated that mucin gene expression and mucin glycosylation are altered in ocular surface disease. MUC5AC levels are decreased in conjunctival epithelia of patients with Sjögren dry eye.[38] There is alteration in expression and/or glycosylation of the cell-associated MUC16, as evidenced by altered H185 binding in non-Sjögren dry eye.[68] In a rat model of vitamin A deficiency,[69] both rMuc4 and rMuc5AC were downregulated. Finally, there is a loss of GalNAc-transferases, the isoenzymes that initiate O-glycosylation of mucins, in the keratinized dry ocular surface of conjunctival epithelia of patients with ocular cicatricial pemphigoid. This suggests alteration of glycosylation of ocular surface mucins in keratinized, human ocular surface disease.[70] Study of mucin gene expression in the HCLE and HCjE cell lines will yield information regarding regulation, glycosylation, and functions of mucins specific to the surface of the eye. Such information may provide a better understanding of and potential therapeutic treatments for ocular surface diseases.

Acknowledgments

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