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Multi-layered culture of primary human conjunctival epithelial cells producing MUC5AC

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Abstract

The purpose of our study was to establish a system for culturing normal human conjunctival epithelial (NHCE) cells under serum-free culture conditions without compromising their ability to differentiate into a mucous epithelium. To this end, small pieces of normal conjunctiva were biopsied from patients undergoing cataract surgery. Obtained NHCE cells were cultured in bronchial epithelial growth medium (BEGM) under serum free culture conditions and passage 3 cells were air-lifted. Cultured NHCE cells displayed typical epithelial morphology. Expression of cytokeratin 19 and conjunctival epithelial specific carbohydrate residue were detected. Air-lifted NHCE cells demonstrated an increase in stratification and differentiation into goblet cells up to 3 weeks under air-liquid interface (ALI) culture condition. NHCE cells expressed *MUC1*, *MUC4*, *MUC16*, and *MUC5AC* mRNA, and MUC5AC production and secretion increased in a time dependent manner after culture under ALI conditions. Exposure of cells to proinflammatory cytokines (TNF-α or IFN-γ) resulted in upregulation of *MUC1*, *MUC4*, *MUC16*, and *MUC5AC* gene expression. In conclusion, differentiated NHCE cells showed features of a multi-layered conjunctival epithelium, including goblet cells, and retained functional characteristics similar to those seen *in vivo*. This cell culture system can better facilitate investigation of conjunctival epithelial cell biology and goblet cell differentiation.
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Keywords: bronchial epithelial growth medium; conjunctiva; epithelium; goblet cell; MUC5AC

1. Introduction

The conjunctival mucous epithelium, a stratified squamous non-keratinizing epithelium of 2–10 cell layers, is critical in protecting the eye from external stimuli and maintaining a healthy ocular surface (Gipson et al., 2005). Unlike other stratified squamous epithelia, the conjunctival epithelium has intercalated secretory goblet cells (Gipson et al., 2005). Apical cells of the conjunctival epithelium express membrane-associated mucin. Along with mucin secreted by conjunctival goblet cells, the membrane-associated mucin of apical corneal and conjunctival epithelium protects and hydrates the ocular surface (Jumblatt et al., 1999; Argueso and Gipson, 2001; Gipson and Argueso, 2003). The inflammatory cytokines secreted by the conjunctival epithelium are involved in the pathogenesis of ocular surface diseases such as keratoconjunctivitis sicca (Pflugfelder et al., 1999).

Several conjunctival epithelial cell lines have been described. The Chang conjunctival cell line has a fibroblastic phenotype and HeLa cell contaminants (Chang, 1954; Lavappa et al., 1976). A second conjunctival cell line, immortalized with the catalytic subunit of telomerase, expresses the mucin repertoire of native conjuctival epithelium (Gipson

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et al., 2003). Finally, the IOBA-NHC cell line has typical epithelial morphology but does not show the property of fully differentiated conjunctival epithelium (Diebold et al., 2003).

Several in vitro systems for the primary culture of normal human conjunctival epithelial (NHCE) cells have been developed. To retain the physiologic functions of *in vivo* conjunctiva, these cell lines should be able to produce and secrete mucus. Primary culture methods using a fibroblast feeder layer have resulted in a stratified epithelium with goblet cell differentiation (Tsai et al., 1994). Primary monolayer cultures have shown ultrastructural evidence of mucus (Diebold et al., 1999) and the production of MUC5AC (Ang et al., 2005). These culture methods for conjunctival epithelial cells require the use of serum with or without feeder layer. Serum-free media, which have been used to study the effect of exogenous factors on cell proliferation and differentiation, have also been used in the cultivation of human conjunctival epithelial cells (Risse Marsh et al., 2002; Ang et al., 2004), but none of these cultures has been shown to produce or secrete mucin-type glycoproteins. In contrast, primary cultures of simple epithelia of tracheal and nasal origin with serum-free media express and synthesize the secretory gel-forming mucins MUC5AC and MUC5B (Gray et al., 1996; Thorton et al., 2000; Yoon et al., 2002).

Conjunctival epithelium has several characteristics in common with respiratory epithelia. We therefore sought to establish a NHCE cell culture system under serum-free conditions without compromising their ability to differentiate into a mucous epithelium. We modified a culture model to study the proliferation and differentiation of normal human airway epithelial cells. Here we show that this system resulted in goblet cell differentiation, allowing us to investigate mucin gene expression and mucin secretion in NHCE cells.

2. Materials and methods

2.1. Tissue specimens

Six 3×3-mm conjunctival biopsy specimens were obtained from the superior temporal bulbar conjunctiva of six patients (2 male, 4 females) undergoing routine cataract surgery who ranged in age from 43 to 65 years. Specimens used in these studies were from normal areas by slit lamp microscopic examination preoperatively. Each patient provided written informed consent, and the study protocol was approved by the Institutional Review Board of the Ethics Committee of the Yonsei University College of Medicine in Seoul, Korea, in accordance with the Declaration of Helsinki.

2.2. Conjunctival epithelial cell isolation and culture

Conjunctival specimens were incubated for 16–20 h at 4 °C with 0.1% Protease (Sigma-Aldrich, St Louis, MO, USA) in a 1:1 mixture of a Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (DMEM/F12, Gibco, NY, USA) supplemented with 1% penicillin-streptomycin (Gibco). The loosened cells were scraped with a pipette, washed three times and suspended in DMEM/F12 supplemented with 1% penicillin-streptomycin (Gibco) and 10% fetal bovine serum (FBS; Gibco). Cells were preplated on 60 mm plastic culture dishes for 1 h at 37 °C in a humidified, 5% CO_2 atmosphere in order to eliminate fibroblasts by differential attachment. Suspended epithelial cells were seeded at 3×10^4 cells per dish in 60 mm plastic culture dishes. Two cultures were seeded from each biopsy specimen. The culture medium used was bronchial epithelial growth medium (BEGM; Clonetics Corp, Walkerville, MD, USA) supplemented with insulin (Clonetics, 5 μg/mL), hydrocortisone (Clonetics, 0.5 μg/mL), epinephrine (Clonetics, 0.5 μg/mL), triiodothyronine (Clonetics, 6.5 ng/mL), transferrin (Clonetics, 10 ng/mL), retinoic acid (Clonetics, 10 ng/mL), bovine pituitary extract (Clonetics, 0.13 mg/mL), gentamicin:amphotericin (Clonetics, 50 μg/mL:50 ng/mL), human epidermal growth factor (Sigma-Aldrich, 10 ng/mL), and bovine serum albumin (BSA) (Sigma-Aldrich, 0.15 mg/mL). The culture medium was changed 1 day after seeding and every other day thereafter until the cultures reached 60%–70% confluence for 5–6 days, at which time they were dissociated with 0.25% trypsin-EDTA (Clonetics). The cell number was determined using hemocytometer, and >2000 cells were seeded per square centimeter for subsequent passage. Three subcultures were seeded from each culture on 10 cm plastic culture dishes. Cells not used for reestablishing cultures were suspended at 1–2×10^6 cells/mL in culture medium containing 10% dimethyl sulfoxide and stored frozen in liquid nitrogen for future use. Passage 2 NHCE cells (10^5 cells/0.5 mL culture medium) were seeded onto 24 mm polyester membranes, 0.4 μm pore size, with Costar® Transwell-clear 3450 culture inserts (Corning, NY, USA). They were cultured in a 1:1 mixture of BEGM:DMEM containing the same concentrations of all supplements as above, except for epidermal growth factor, which was used at 0.5 ng/mL. The cultures were grown submerged for the first 4 days, during which time the culture medium was changed on day 1 and every other day thereafter. On day 5, when the cells reached 80–90% confluence, ALI cultures were developed by removing the apical medium and feeding the cultures daily only from the basal compartment. Day zero marked the first day of ALI culture conditions.

2.3. MTT cell viability assay

Cell viability was detected by the MTT cell proliferation kit (Roche, Penzberg, Germany), in which the MTT is converted into formazan granules in the presence of molecular oxygen. The intracellular formazan can be solubilized and quantified by spectrophotometric means. Triplicate samples in Transwell-clear 3450 culture inserts were incubated in a medium containing 25 μl of 5 mg/ml MTT for 4 h at 37 °C in a humidified, 5% CO_2 atmosphere. Formazan was incubated with 250 μl of solubilization solution overnight at 37 °C. Solubilized formazan were put in a 96 well plate and the optical densities were measured using an ELISA plate reader (SPECTRA max; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 550 nm with background subtraction at 690 nm. Each experiment included a blank control (culture medium).

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