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Development of Immortalized Rat Conjunctival Epithelial Cell Lines: An In vitro Model to Examine Transepithelial Antigen Delivery.

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Abstract

The objective of these studies was to develop conjunctival epithelial cell lines for investigation of antigen translocation across a mucosal barrier. Conjunctival epithelial cells from Fischer 344 rats were immortalized with pSV3(neo) resulting in two cell lines - CJ4.1A and CJ4.3C. Each formed confluent cell layers with epithelial morphology when grown on permeable membrane filters. They expressed the SV40 T antigen, the conjunctiva-specific cytokeratin 4, the goblet cell-specific cytokeratin 7 and were negative for the corneal epithelial cell-specific cytokeratin 12. The cell lines have been in culture for over 60 passages, and the population doubling times were 22 ± 7 hours for CJ4.1A and 23 ± 9 hours for CJ4.3C. When grown on Transwell™ membranes, each cell line achieved a transepithelial electrical resistance of 600–800 Ωcm^2 by 3 to 4 days and maintained a high resistance for several days. Both cell lines expressed zona occludins-1 at confluence. At 24 h following addition of 250 μg of FITC-labeled ovalbumin to the apical chambers, $15 \pm 6 \mu\text{g}$ could be detected in the basal chamber of CJ4.1A and $6 \pm 1 \mu\text{g}$ in the basal medium of CJ4.3C. In contrast, $82 \pm 6 \mu\text{g}$ was detected in the lower chambers of cell-free Transwells. Similarly, Transwells containing confluent CJ4.1A or CJ4.3C cells impeded passage of 0.1 μm diameter polystyrene microspheres (5 ± 1 and 4 ± 1 %, respectively, of the apical input), compared to 26 ± 6 % of the input microspheres recovered from the basal chambers of cell-free Transwells™. Pretreatment with 4 mM EGTA for 10 min caused an increase in OVA-FITC translocation across CJ4.3C cells. Incubation in the presence of 4 mM EGTA significantly increased OVA-FITC translocation across both cell lines, relative to untreated cell layers. Morphological and functional characterization indicate that these cells provide a useful experimental tool to assess strategies for enhancing transepithelial antigen uptake.

Keywords

conjunctiva; rat; cell culture

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Introduction

The mucosal immune systems, including the ocular immune system, (O'Sullivan et al., 2005) is uniquely capable of mounting effective innate and adaptive immune responses to protect mucosal surfaces against potential microbial challenges from the environment (Holmgren and Czerkinsky, 2005). Administration of immunogenic substances through mucosal, but not parenteral routes, is a well-established approach to induce both systemic and mucosal immune responses. In the ocular-mucosal immune system the lacrimal gland (LG) is the principal effector site where secretory IgA antibodies are produced. These antibodies are transported, *via* the tears, to the ocular surface to protect against allergic, inflammatory or infectious disease and thus promote corneal and conjunctival health and visual acuity (Franklin and Montgomery, 1996; Montgomery and Whittum-Hudson, 1996; O'Sullivan et al., 2005). Earlier studies (Koornstra et al., 1991; Ridley Lathers et al., 1998) indicate that NALT (nasal-associated lymphoid tissue, the rodent equivalent of human Waldeyer's ring tissue (Koornstra et al., 1991)), and its draining posterior cervical lymph node (pCLN), acquire particulate antigen and appear to function as a major inductive site for eliciting tear IgA responses following ocular-topical immunization (Ridley Lathers et al., 1998). Non-penetrating antigen drains from the ocular surface *via* the nasolacrimal duct and is taken up by M cells overlaying the NALT. Alternately, antigen taken up by the conjunctiva is transported directly to the superficial (s) CLN, thus following an alternate inductive pathway which bypasses the NALT and pCLN (Egan et al., 2000; Ridley Lathers et al., 1998).

It is now clear that the ocular surface and the LG function as a tightly integrated unit linked by interconnecting innervation (Stern et al., 1998a). Conjunctival or corneal insults are detected by afferent sensory neurons, the information is relayed to the central nervous system and then to the LG (via the efferent sympathetic and parasympathetic nerves) (Dannelly et al., 2001; Sinha et al., 2001), resulting in the release of neuropeptides in the LG (Stern et al., 1998b). This pathway has the potential of activating dendritic cells (DC) in the LG, since neuropeptides are known to be chemotactic for DC, and to affect the interactions between DC and T cells (Lambrecht, 2001). Further, components of the immune system localized in the corneal or conjunctival compartments also acquire feedback signals from the lacrimal gland delivered *via* tear-borne agents.

Prior to uptake and processing, antigen must overcome highly effective mucosal barriers. In the ocular compartment these include powerful mechanical and chemical cleansing components of the tear film, a tight epithelium, a more permeable stroma and, in the case of cornea, the endothelium. The conjunctival epithelium extends from the corneal-conjunctival limbus over the ocular bulb and lines the eyelid to the mucocutaneous junction and functions as a barrier that separates the ocular surface from the inside environment. The conjunctiva consists of non-keratinized, stratified squamous epithelial cells of which the superficial layers are sealed by peri-apical tight junctions which form the major barrier to the passive movement of fluid, electrolytes, macromolecules, and cells through the paracellular pathway.

The aims of this study were to develop immortalized conjunctival epithelial lines that retain differentiation characteristics and exhibit tight barrier properties as well as to investigate transepithelial soluble and particulate antigen delivery across these cell layers when grown on permeable supports. Several primary cultures of conjunctival epithelia (Kahn et al., 1993 and Risse Marsh et al., 2002) and several immortalized human conjunctival cell lines have been developed; the HCO597 line (Ward et al., 1998 and Lin et al., 2000), the IOBA-NHC spontaneously immortalized line (Diebold et al.) and the Chang conjunctival-derived cell line, which has a fibroblastic morphology and an acknowledged HeLa cell contamination. Since there were no reports of rat conjunctival epithelial primary cultures or immortalized cell lines, we developed SV40-transformed rat conjunctival cell lines suitable for *in vitro* investigation

of transepithelial antigen delivery. The cells formed functional, tight, epithelial barriers when grown on permeable supports using the approach employed by Saha et al.(1996). This in vitro model is expected to provide a more complete understanding the precise cellular and molecular mechanisms for antigen uptake from the ocular surface and has application to the rational design of effective ocular immunization regimes.

1. Materials and Methods

1.1 Isolation, Culture, Immortalization and Growth of Conjunctival Cell Lines

All treatment of animals and removal of tissues used in this study conformed to the guidelines established by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all animal procedures were approved by the Wayne State University Animal Investigation Committee based on compliance with the U.S. Public Health Service's *Guide for the Care and Use of Laboratory Animals*. Adult (15 weeks of age) male Fischer 344 rats were used in this study and obtained from Harlan (Indianapolis, IN).

The rats were killed by CO₂ narcosis followed by bilateral pneumothorax. Both eyes, including a rim of fornical conjunctiva, were surgically removed and rinsed in 4 changes of Dulbecco's modified Eagle medium (DMEM) with 10X concentrated antibiotics (1000 I.U./ml penicillin, 1 mg/ml streptomycin, 250 µg/ml gentamycin and 2.5 µg/ml fungizone; (all from Invitrogen-GIBCO, Grand Island, NY) then soaked in this mixture for 30 min. The eyes were incubated in 5 mM EDTA in phosphate buffered saline (10 mM) with a 1X antibiotic mixture for 2 h. at room temperature. The corneas were removed with fine forceps and discarded and the conjunctival epithelia peeled from the stroma. The conjunctival fragments were incubated with trypsin/EDTA solution (0.05%/0.53 mM, Invitrogen-GIBCO) for 15 min, triturated with a glass Pasteur pipette then washed 2X with growth medium. The dissociated cells were plated in the wells of a 24 well Primaria™ plate (Fisher, Pittsburgh, PA) which had been coated with a 50% Matrigel™ (BD Biosciences, San Jose, CA) in DMEM solution. In order to facilitate attachment, the wells contained just enough volume (0.1 ml) of cell suspension to cover the bottom. After 2.5 hours an additional 0.4 ml of culture medium was added. The serum-free culture medium (SFQMOM) was a modification of Oliver's medium (Oliver, 1980) and consisted of a 1:1 mix of DMEM (low glucose, MediaTech, Herndon, VA) and Ham's F12 (MediaTech) with 0.5% BSA, 10 mM HEPES, 1X trace elements B, 10 µg/ml insulin, 5.5 µg/ml transferrin, 50 ng/ml sodium selenate, 20 µg/ml ethanolamine, 0.3 µg/ml glucagon, 0.1 µM dexamethasone, 0.1 µM retinoic acid, 0.3 µM 3, 3', 5 triiodo-L thyronine, 80 ng/ml epidermal growth factor and 50 µg/ml gentamycin sulfate (Invitrogen-GIBCO).

Plasmid DNA bearing the pSV3-neo gene was prepared from *E. coli* HB101 (ATCC, Manassas, VA) using the Wizard®PureFectin, Plasmid DNA Purification System (Promega, Madison, WI) according to the manufacturer's protocol. After one subculture on Primaria™ (CJ4.1A) or two subcultures on Matrigel™-coated wells followed by subculture on Primaria™ (CJ4.3C), using a trypsin/EDTA solution to dissociate the cells, the cultures were transfected with SV40 large T antigen using LipofectaminePlus™ reagent (Life Technologies, Carlsbad, California) in SFQMOM according to the supplier's protocol. Transfected cells were selected by culture in geneticin- (G418, Invitrogen-GIBCO) containing SFQMOM either before subculture (CJ4.3C) or after two subcultures (CJ4.1A).

The cells were grown to 70–100% confluency in 75 cm² tissue culture flasks (Corning, Inc., Corning NY) then removed using a trypsin/EDTA solution. Viable cell counts were obtained using trypan blue exclusion then the cells were plated into tissue culture flasks at a seeding density of 6.67×10^3 cells/cm². Population doublings were calculated as follows: $x = \log_2(N/N_0)$, where N is the total number of viable cells harvested at subculture, and N_0 is the number

of viable cells seeded (Ang et al., 2004). The doubling time was calculated by dividing by the days in culture.

1.2 Transwell Cultures and Solute or Particulate Translocation

Cells were grown to confluency (3–5 days) on polyester Transwell-Clear™ inserts (0.4 µm pore size, Corning, Inc.) and used when the transepithelial electrical resistance (TER) was greater than 300 Ωcm², as measured using a volt-ohm meter (EVOM; World Precision Instruments, Sarasota, FL). The cultures were given a change of fresh medium; then 0.5 ml of a 1:1000 dilution of 0.1 µm fluorescent microspheres (MS – FluoSpheres, Molecular Probes/Invitrogen Detection Technologies, Eugene, OR) or fluorescein isothiocyanate-conjugated ovalbumin (OVA-FITC - 250 mg/ml) was added to the apical chambers (input) followed by incubation at 37°C. At 24 hours the culture plates were shaken for 20 seconds at 100 rpm using an Orbis shaker (Mikura, Ltd., UK) to evenly suspend the contents of the basal chambers, then 100 µl samples were transferred to a black flat-bottomed microtiter plate (Nalge Nunc International, Rochester, NY). Fluorescence was measured using a SPECTRAFluor Plus microplate reader (Tecan US, Durham, NC).

The percentage of input MS or the concentration of OVA-FITC translocated to the basal chambers was calculated using standard curves prepared from the input MS or OVA-FITC (in culture medium and also incubated at 37°C for 24 hours). The total percentage of the input MS present in the basal chamber was calculated by multiplying by 15 (the inverse of the proportion sampled: 0.1 ml = 1/15 of 1.5 ml). Statistical significances were determined using a two-tailed Student's *t*-test.

1.3 Preparation of Conjunctival and Corneal Sections

The entire globe was removed from male rats and then frozen in Histo Prep (FisherDiagnostics, Fair Lawn, NJ). Sagittal sections (6 µm), which included both corneal and conjunctival tissue, were cut using a cryostat microtome (Lipshaw, Detroit, MI) and placed on gelatin-coated slides for use in immunofluorescence staining.

1.4 Immunofluorescence

Conjunctival cells grown on Transwell membranes or tissue sections were fixed with cooled 100% methanol for 10 min then incubated for 30 min in blocking buffer which contained 0.3% BSA in PBS. The cells or sections were then incubated with the following dilutions of primary antibodies for one hour at room temperature: Antibody to SV40 large T/small t (1:10, clone PAb 108, BD Biosciences, San Diego, CA); anti-cytokeratin 4 (CK-4), specific for conjunctival stratified, squamous, non-goblet epithelial cells (Krenzer and Freddo, 1997; Shatos et al., 2001) (1:10, clone 6B10, MP Biomedicals, Inc/Cappel, Aurora, OH); anti-cytokeratin 7 (CK-7) which recognizes a goblet cell cytokeratin (1:5, clone RCK 105, MP Biomedicals); anti-cytokeratin 12 (CK-12) recognizing keratinized corneal epithelial cells (Krenzer and Freddo, 1997; Shatos et al., 2001) (1:5, rabbit polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA); a pan-cytokeratin (pCK) cocktail of monoclonal antibodies (1:20, clones C-11, PCK-26, CY-90, KS-1A3, M20, A53-B/A2, Sigma-Aldrich, Saint Louis, MO), and anti-zona occludens-1 (ZO-1) which recognizes a tight junction component (1:50, rabbit anti-human, Santa Cruz Biotechnology, Inc.). The FITC-conjugated secondary antibody (Sigma-Aldrich) was diluted 1:100 and incubated with the cells or sections for one hour at room temperature. Negative controls consisted of substituting PBS or isotype control antibodies for the primary antibody. Positive controls included frozen sections of the rat orbit containing both conjunctival and corneal tissues. Slides or membranes were washed 3 times in PBS then coverslips were mounted using ImmunoMount (Thermo-Shandon, Pittsburgh, PA). Confocal imaging was performed using a Leica TSC SP2 Laser Confocal Microscope (Leica Microsystems, Heidelberg, GmbH).

2. Results

2.1 Establishment of immortalized conjunctival epithelial cell lines

Primary epithelial cultures were initiated in MatrigelTM-coated tissue culture wells using cells dispersed from sheets of bulbar and fornical conjunctiva. Upon reaching confluency, the cells were passaged by trypsinization and replated into uncoated culture wells either directly or after two passages into MatrigelTM-coated wells using a serum-free modification of Oliver's medium (Oliver, 1980). Transfection with SV40 large T antigen resulted in two cell lines, CJ4.1A and CJ4.3C. Figure 1A–D are phase-contrast photomicrographs which show the epithelial “cobblestone” morphology of confluent three day cultures of CJ4.1A (Fig 1A) and CJ4.3C (Fig 1B). After confluence CJ4.1A cells retained monolayer growth but began to develop phase-bright intracellular vesicles (Fig 1C) while the CJ4.3C cells began to stratify (Fig 1D) as seen in these four day cultures. After longer culture CJ4.3C also developed phase-bright vesicles (data not shown). Both cell lines expressed SV40 large T antigen as demonstrated by the positive immunofluorescence staining shown in Figure 1 (E and F). Expression was nuclear, as expected due to the transforming activity of the SV40 large T antigen. SV40 T antigen expression was not detected in Caco-2 cells, a human colonic adenocarcinoma cell line (data not shown).

To date, the cell lines have been in culture for more than 300 days. The growth kinetics of CJ4.1A and CJ4.3C are not significantly different, as shown in Figure 2. Over the interval of passages 15–60, encompassing a total of 72 days (CJ4.1A) or 65 days (CJ4.3C), the CJ4.1A cell line had a doubling time of 22 ± 7 hours while CJ4.3C doubled in 23 ± 9 hours. There was no evidence of senescence, which would be indicated by a downward deflection of the growth curve at later time points. Cryopreservation for one month followed by thawing then by continued culture (arrows in Fig. 2) had no effect on the slope of the growth curve, indicating that both cell lines can be readily recovered from liquid nitrogen storage. Further, each cell line has been recovered after over two years of storage in liquid nitrogen (data not shown).

2.2 Cytokeratin expression

To verify that the cells in the conjunctival cell cultures were indeed epithelial cells, immunofluorescence staining was performed using a pan-cytokeratin (pCK) antibody mixture that recognizes all major keratins. Sections of rat cornea and bulbar conjunctiva tissue were also stained for control and comparative purposes. Specific cytokeratin staining was detected in both CJ4.1A and CJ4.3C cells, as well as in corneal and conjunctival tissue, confirming the epithelial lineage of the cell lines (Fig. 3A).

Expression of CK -4, -7 and -12 was assayed to further characterize the cell lines. CK-4, specific for intermediate filaments in stratified non-keratinized epithelial cells such as found in the conjunctiva, (Krenzer and Freddo, 1997) was expressed by the majority of CJ4.1A cells and by a portion of the CJ4.3C cells. This was consistent with the positive staining seen in conjunctival sections and lack of staining in cornea sections from normal rat. (Fig. 3B). The CK-7 intermediate filament is a marker of glandular epithelia but is expressed by goblet cells in the human conjunctiva (Krenzer and Freddo, 1997). Conjunctival goblet cell specificity was demonstrated in the rat by the single strongly fluorescent goblet cell with surrounding unstained epithelial cells seen in the conjunctival section in Fig. 3C. Immunostaining demonstrated that both CJ4.1A and CJ4.3C contained CK-7 intermediate filaments (Fig. 3C). The specificity of this antibody was further demonstrated by lack of staining of two intestinal epithelial cell lines, Caco-2 and IEC-6 (data not shown). CJ4.1A and CJ4.3C were both negative for CK-12 (Fig. 3D) which, with its CK-3 partner, is widely used to identify differentiated corneal epithelial cells (Schermer et al., 1986; Sun et al., 1985). Corneal expression along with lack of expression in conjunctiva showed the expected specificity in CK-12 stained tissue sections (Fig. 3D).

Taken together, the data indicate that CJ4.1A and CJ4.3C have conjunctival, rather than corneal characteristics.

2.3 CJ4.1A and CJ4.3C cells grown on permeable filters express tight junction proteins and exhibit barrier function

In keeping with their function of providing a boundary between the outer environment and underlying compartments, epithelial cells exhibit polarity (Handler, 1989). CJ4.1A and CJ4.3C cells grown on permeable Transwell™ membranes formed confluent monolayers in three to four days. When examined in the horizontal (xy) plane using an antibody to detect zona occludens-1 (ZO-1), one of the tight-junction-associated proteins, immunofluorescence staining and enface (xy plane) confocal microscopy revealed that ZO-1 was localized to the cell-cell contacts in CJ4.3C cells (Figure 4B, large panel). Although ZO-1 staining also marked the cell-cell contacts of CJ4.1A cells, diffuse staining was evident throughout the cytoplasm (Figure 4A, large panel). Vertical optical sectioning (xz and yz planes) through the thickness of the cell layer revealed that ZO-1 was localized to sites of cell-cell contact in the lateral but not the basal or apical regions of the cells (Fig. 4A and B, lower and right panels). Note that the diffuse cytoplasmic staining in CJ4.1A is evident in the reconstructed side view of the cells (Figure 4A, lower and right panels). Claudin-1, a membrane-expressed tight junction protein, was also expressed by both cell lines in a similar perijunctional pattern (data not shown).

When grown on 1 cm² permeable membranes having 0.4 μm pores the CJ4.3C cells obtained a transepithelial electrical resistance of over 800 Ωcm² by day 3 and maintained this resistance for at least three days (Fig. 5). The CJ4.1A cells required an additional day to reach peak TER which was not significantly lower than the TER achieved by CJ4.3C (Fig. 5). Both cell lines maintained a TER of over 300 Ωcm² for an additional 3–4 days (7–9 days of culture) after which the resistance dropped to the level of cell-free Transwell™ membranes (90–120 Ωcm²) as the cultures became overgrown and deteriorated (data not shown). Both cell lines exhibited barrier function against soluble as well as particulate substances. The cells were grown on permeable (0.4 μm pore diameter) membranes and used when the TER was over 300 Ωcm², indicating confluence. When 250 μg of OVA-FITC in SFQMOM was placed in the apical chambers of CJ4.1A cultures, 15 μg ± 6 μg was recovered from the basal chamber after 24 hours of incubation at 37°C. This was significantly lower than the 82 μg ± 6 μg recovered from the “basal” chambers of inserts containing no cells (Fig. 6A). Similarly, CJ4.3C cultures allowed only 6 μg ± 1 μg of OVA-FITC to translocate to the basal chambers, compared to the significantly greater 62 μg ± 2 μg that passed to the “basal” chambers of cell-free inserts (Fig. 6A). The conjunctival cell lines also impeded the passage of 0.1 μm carboxylated polystyrene microspheres. 5% ± 1% of the beads placed in the apical chamber were recovered from the basal chambers of inserts containing CJ4.1A cells after 24 hours of incubation, compared to 26% ± 6% recovered from cell-free inserts (Fig. 6B). A comparable 4% ± 1% and 21% ± 4% of the input microspheres were recovered from the basal chambers of CJ4.3C cell cultures and cell-free inserts, respectively (Fig. 6B). The data indicate that both CJ4.1A and CJ4.3C cell lines modeled the ocular conjunctival barrier as far as having significant barrier function in impeding the passage of both solutes and particulates.

An essential step to initiate an immune response against topically applied antigen involves delivery of the antigen across the epithelium to components of the immune system (i.e. dendritic cells or macrophages) located in the underlying stroma. Tight junctions create a barrier to the diffusion of solutes by forming a seal between the apical and basal-lateral membrane domains. (Cereijido et al., 1998; Madara, 1998). We utilized EGTA to open the calcium-dependent tight junctions and allow paracellular passage of a soluble macromolecular antigen, OVA-FITC, across confluent CJ4.1A and CJ4.3C epithelial cultures grown on permeable membranes. Transient removal of Ca²⁺ by pretreating the cultures for ten minutes

with 4 mM EGTA, then restoring the Ca^{2+} by replacing the culture medium did not increase (compared to untreated cultures) the amount of OVA-FITC translocated from the apical chamber to the basal chamber of CJ4.1A cultures after 24 hours of further incubation (Figure 7). EGTA pretreatment did result in a small but significant increase in protein that crossed CJ4.3C epithelial layers (Fig. 7). EGTA pretreatment for 20 or 30 minutes did not result in a further increase in OVA-FITC recovered from the basal chambers after 24 hours (data not shown). EGTA pretreatment of the cells for 10 minutes reduced the TER to near that of cell-free Transwells. By 3–4 hours following replacement of the culture medium the TER had recovered to pretreatment levels (data not shown). As a positive control for total disruption of the tight junctions, the cultures were subjected to prolonged Ca^{2+} removal by the continued presence of EGTA for 24 hours during the OVA-FITC incubation, a large proportion of the input protein was recovered from the basal chambers of both cell lines (Fig. 7). The viability of the EGTA-treated cells was not significantly different from untreated cells at 24 hours, as determined by trypan blue exclusion (data not shown). The data indicate that these cell lines can be utilized to model the uptake of antigens across a conjunctival barrier and to study agents that enhance their translocation.

3. Discussion

Although the ocular surface is endowed with a variety of non-specific defense mechanisms against environmental challenges, it is highly desirable to develop vaccination strategies to protect it, along with other mucosal portals of entry, from pathological agents. Ocular topical immunization with soluble or microparticle encapsulated protein antigens in the presence of microbially-derived adjuvants or of certain cytokines induces tear IgA antibody responses (O'Sullivan et al., 2005). These responses tend to be of modest magnitude and not long lasting. This is likely due to the formidable barriers that topical antigen must pass to reach local components of the immune system. The tear film (with its lipid, aqueous and mucus compartments, antimicrobial effects and cleansing flow) prevents much contact with the ocular epithelia. After breaching the tear film barrier, antigen encounters the stratified epithelium which is sealed by tight junctions. The epithelium possesses transporters which facilitate the influx and egress of specific solutes and ions. The underlying stroma provides support for the epithelium and contains embedded cells of the innate and adaptive immune systems. These cells, particularly the dendritic cells, represent an attractive target for ocular topical immunization. Recently, cell culture models of ocular barriers have been developed to study small molecule drug transport into ocular tissues (Hornof et al., 2005).

As a first step in engineering a functional conjunctival equivalent to study antigen delivery, we have developed two immortalized cell lines that retained several characteristics of rat conjunctival tissue. These cell lines possessed epithelial morphologies and expressed a conjunctival rather than a corneal cytokeratin profile. They each formed continuous polarized cell layers when grown on permeable Transwell™ membranes, as evidenced by high TERs and expression of tight junction proteins. Both cell lines exhibited Ca^{2+} -dependent barrier function against soluble macromolecular and particulate substances.

The cell cultures expressed cytokeratin-4, an intermediate filament associated with nonkeratinized, stratified squamous epithelial cells (Krenzer and Freddo, 1997) and localized to the conjunctiva and not the cornea of the adult eye in the mouse (Kurpakus et al., 1994). This keratin was expressed by all of the CJ4.1A cells, similar to the staining pattern seen in normal rat conjunctival tissue. In contrast, only a few of the CJ4.3C cells expressed CK-4. This may be due to the culture conditions since the lack of extracellular laminin in the culture substrate has been reported to correlate with the loss of conjunctival epithelial keratin-4 localization (Kurpakus and Lin, 1999).

It was somewhat unexpected to find cytokeratin 7 (a “glandular epithelium” type of keratin, (Kasper, 1991)) expressed by the CJ4.1A and CJ4.3C cells since this is considered to be a goblet cell-specific marker in the conjunctiva (Krenzer, 1997). Our positive CK-7 staining and the accumulation of translucent vesicles seen in Figure 1 may be due to differentiation during culture. Such differentiation from epithelial cells has been tied to cell division and specific time points by clonal analysis of human conjunctival stem cells (Pellegrini et al., 1999). Alternatively, while CK-7 is expressed by goblet cells, it is also detected on other epithelial cell types. Diebold et al. (2003) reported that a spontaneously immortalized epithelial cell line from normal human conjunctiva was positive for CK-7. This was not considered a goblet cell line since the epithelial mucin genes MUC-1, -2 and -4 were expressed and the goblet-cell gene products MUC5AC and MUC-7 were not detected.

While vimentin is not expressed by conjunctival epithelial cells in vivo (Kasper, 1991) it was expressed by both cell lines (data not shown). This intermediate filament is associated with mesenchymal cells in vivo but is found in most cells in culture. Vimentin-keratin co-expression in cultured epithelial cells is thought to indicate proliferative activity (Kasper et al., 1989).

Our tissue culture methods resulted in the development of functional conjunctival epithelial sheets on permeable supports. The CJ4.1A and CJ4.3C cell lines each exhibited high TERs and impeded the passage of protein (ovalbumin) and particulates (0.1 μ m polystyrene microspheres). Each cell line expressed laterally localized tight junction proteins (ZO-1 and claudin-1). The cultures maintained barrier properties for more than four days after reaching confluence (7–9 total days in culture), a practical time-span for antigen transport studies. Barrier function was affected by removal of calcium from the culture medium using EGTA chelation as indicated by a rapid drop in TER (data not shown) and increased recovery of OVA-FITC from the basal chamber of the Transwells™, indicating that the paracellular route can be manipulated to translocate antigens across the epithelium. These findings show that the CJ4.1A cell line and especially the CJ4.3C cell line will be useful in modeling the conjunctival epithelial barrier and in developing strategies for delivering macromolecular antigens across this barrier to underlying components of the immune system such as dendritic cells. Whether these cells can be utilized to construct more complex bioengineered conjunctival equivalents by growing them on an extracellular matrix substrate in which dendritic cells are incorporated and/or at an air-interface to facilitate a more differentiated, stratified epithelium are the focus of future studies (Hornof et al., 2005).

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Figure 1. Microscopic appearance and nuclear SV40 T antigen expression. Phase-contrast micrographs of CJ4.1A cells at 3 (A) or 4 days (C) after plating and CJ4.C at 3 (B) or 4 days (D) after plating. Magnification bars = 20 μ m. Confocal micrographs of CJ4.1A (E) and CJ4.3C (F) immunostained for SV40 large T and small t antigen. Bar = 8 μ m.

Figure 2.

Replicative characteristics. CJ4.1A (■) and CJ4.3C (□) were serially passaged following transfection with pSV3-neo. The graph depicts the cumulative population doublings from passage 15 or 17 for CJ4.1A or CJ4.3C, respectively, to passage 60. The arrows indicate times at which the cell lines were cryopreserved then later thawed for further culture. The insert shows the mean doubling times of CJ4.1A and CJ4.3C

Figure 3.

Immunofluorescence confocal microscopy demonstrating cytochrome expression. CJ4.1A and CJ4.3C cells lines grown on Transwell membranes or corneal and conjunctival sections were stained with (A) an anti-pan-cytokeratin antibody cocktail to identify epithelial cells, (B) anti-cytokeratin 4, considered to be conjunctival epithelial cell specific, (C) anti-cytokeratin 7, a goblet cell marker or (D) anti-cytokeratin 12, corneal epithelial cell specific. The corneal and conjunctival surfaces are oriented to the left and the stroma to the right of the sections. All micrographs are of the same magnification. Bar = 20 μ m.

Figure 4.

Immunolocalization of ZO-1 in CJ4.1A and CJ4.3C cells. The cells were grown on Transwell™ membranes for 4 days, fixed and stained using an antibody against zona occludens 1 (ZO-1) and imaged by confocal microscopy. The larger panels depict horizontal (xy) full thickness projections of CJ4.1A (A) and CJ4.3C (B). The bottom and right side panels are vertical optical sections (xz and yz, respectively) acquired at the horizontal and vertical lines seen on the xy projections. Arrows indicate representative areas of bright staining in the vertical sections and the corresponding point in the enface image (Solid arrows indicate lower, xz, panel and open arrows indicate the right, yz, panel) Bar = 20 μm.

Figure 5. Transepithelial electrical resistance (TER). CJ4.1A ($\square$) and CJ4.3C ($\blacksquare$) cells were grown on TranswellTM membranes and the TERs were determined daily using EVOM. Each data point represents the mean $\pm$ SEM of 3–10 cultures, each having triplicate wells.

Figure 6.

Protein and microsphere translocation. CJ4.1A and CJ4.3C cells were grown to confluency on Transwell™ membranes. OVA-FITC (A) or 0.1 µm FluoSpheres™ were added to the apical chambers of Transwell™ inserts with or without cells. At 24 hours the fluorescence intensity of aliquots of the basal chamber fluid was determined and expressed as total µg of OVA-FITC recovered or percentage of the input microspheres recovered. The data represent four separate experiments having triplicate determinations. Significant differences (determined by a two tailed Student's t-test) in transport across Transwell membranes with and without epithelial cell layers is indicated by asterisks.

Figure 7.

Effect of EGTA on OVA-FITC translocation. CJ4.1A and CJ4.3C cells were grown to confluency on Transwell™ membranes. The cells were incubated with 4 mM EGTA for 10 minutes (gray bars) or for 24 hours (open bars). Control wells contained untreated cells (hatched bars) or were cell-free (solid bars). 250 µg OVA-FITC was added to the apical chambers and the OVA-FITC concentration in the basal chamber was determined at 24 hours. Data represent the mean $\pm$ SEM µg OVA-FITC present in the basal chamber using four experiments performed in triplicate. Significant differences, compared to untreated cells (determined by a two tailed Student's t-test), are indicated by asterisks.