The Immunomodulatory Role of Human Conjunctival Epithelial Cells

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PURPOSE. To characterize phenotypically the primary epithelial cells obtained from normal bulbar conjunctival biopsy specimens and a human conjunctival epithelial cell line (Wong-Kilbourne derivative of Chang conjunctiva; ChWK) with regard to their immunostimulatory function.

METHODS. The effects of expression of HLA-DR and costimulatory molecules (CD80, CD86, ICAM-1, and CD40) on normal conjunctival epithelial cells and ChWK treated with various cytokines (IFN-γ, TNF-α, IL-4, and IL-13) were determined using flow cytometry and confocal microscopy.

RESULTS. Epithelial cells were successfully grown from conjunctival explants and cultures passed three times, while retaining their cell surface markers. At least 97% of primary epithelial cells (n = 10) and more than 96% of ChWK cells (n = 10) were cytokeratin positive by flow cytometry and immunocytochemistry and demonstrated epithelial cell morphology. Both primary conjunctival epithelial cells and ChWK had a low basal expression of HLA-DR and ICAM-1, and both were upregulated by IFN-γ. For ChWK cells, CD80 and CD86 were constitutively expressed at low levels. CD80 was significantly upregulated after IFN-γ treatment (P = 0.043), whereas IL-4 induced a significant upregulation of CD86 (P = 0.039). Treatment with IL-13 and TNF-α did not induce significant effects.

CONCLUSIONS. The ability of conjunctival epithelial cells to express costimulatory molecules suggests a proinflammatory role for conjunctival epithelial cells. (Invest Ophthalmol Vis Sci. 2003;44:3906–3910) DOI:10.1167/iovs.02-04665

There are at least three forms of chronic allergic eye disease (CAED) in which T cells infiltrate the conjunctiva: vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), and giant papillary conjunctivitis (GPC). VKC and AKC are both severe forms of CAED and cause significant visual impairment due to corneal damage. Histologically, studies have demonstrated that activated CD4+ T cells infiltrate the subepithelial layer of the conjunctiva during all three forms of the disease.1 The localization of the inflammatory cells to the subepithelial layers suggests a role for conjunctival epithelial cells in the localized inflammatory response. In addition, there is no intercellular adhesion molecule (ICAM)-1 and HLA-DR expression in normal conjunctival tissue whereas in CAED, there is an upregulation of ICAM-1 and HLA-DR expression, which is localized to the subepithelial cells.2 The upregulation of these molecules raises the question of whether conjunctival epithelial cells can support T-cell responses. It has also been demonstrated by in situ hybridization that there is expression of interleukin (IL)-3, IL-4, and IL-5 in all three forms of disease, with an enhanced expression of IL-2 and human recombinant interferon (IFN)-γ by the CD3+ T cells in AKC.3 This pattern of cytokines is likely to affect the local tissue-resident cells at the site of inflammation, including the conjunctival epithelial cells. Other localized epithelial cell types, including intestinal epithelial cells4 and bronchial epithelial cells,5 express the necessary costimulatory molecules and have been suggested to function in a limited context as so-called “nonprofessional” antigen-presenting cells (APCs).

Studies have also demonstrated different patterns of conjunctival T-cell–derived cytokine profiles in each disease: in AKC, conjunctival T-cell lines produce increased levels of IFN-γ, IL-10, and IL-13, but little IL-4 and IL-5.6 In VKC, conjunctival T-cell lines produce increased levels of IL-5 and IL-13. Summarizing these different studies, it appears that there are Th2 cytokine-producing cells in the subepithelial layer of the conjunctiva in GPC, VKC, and AKC, as well as IFN-γ-producing T cells in AKC. The effects of these cytokines on resident conjunctival tissue cells, including epithelial cells, are as yet unclear.

T-cell cytokines are known to be potent inducers of immunologic molecules in several cell types,7–10 and these cytokines are thought to play a central role in allergic disease.11–13 The purpose of this study therefore was to investigate the expression of HLA-DR and costimulatory molecules in normal primary epithelial cells in comparison with a well-characterized immortalized conjunctival epithelial cell line (ChWK). The effects of T-cell cytokines on conjunctival epithelial cell surface expression were compared.

MATERIALS AND METHODS

Medium-199, heat-inactivated fetal calf serum (FCS), trypsin-EDTA, HEPES-buffered RPMI 1640, t-glutamine, 2-mercaptoethanol, human AB+ serum, trypsin-EDTA solution collagenase, cell dissociation solution, collagen type I, epidermal growth factor (EGF) and a serum substitute (Ultraser G; USG) were all purchased from Sigma-Aldrich (Poole, UK).

Human recombinant interferon (IFN)-γ, IL-4, IL-13, and tumor necrosis factor (TNF)-α were obtained from PeproTech (London, UK).

Mouse anti-human HLA-DRPE (clone Tu39) and mouse anti-human ICAM-1PE (clone HA58) mAbs were both from BD PharMingen (Oxford, UK). Mouse anti-human CD80PE (clone MEM-23) and mouse anti-human CD86PE (clone BU63) were from Serotec (Oxford, UK).

Mouse anti-human cytokerin mABFITC (clone C-11, recognizing cytokeratins 4, 5, 6, 8, 10, 13, 18) and mouse anti-human HLA-DR mABFITC (clone 5C3) were purchased from Immunotech (Marseille, France).

An irrelevant isotype-matched mAb mouse IgG1 negative controlFITC (clone F8–11–15) was used throughout as a negative control (Serotec). For confocal microscopy, the following reagents were used: mouse anti-human HLA-DR mAb (Dako, Glostrup, Denmark; clone CR3/43); mouse anti-human ICAM-1 mAb (R&D Systems Europe Ltd., Oxford, UK; clone BBIG-H1 [11C81]); mouse anti-human cytokeratin mAb (Novocastra, Newcastle-upon-Tyne, UK; clone 5D5) and goat anti-mouse IgG FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

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Cell Culture

**Primary Human Conjunctival Epithelial Cells.** Normal conjunctival tissues were obtained from noninflamed eyes undergoing vitrectomy. The local ethics committee approved all procedures, and all patients gave their informed consent at the time the biopsy was performed, in accordance with the Declaration of Helsinki. Tissue was transferred to medium-199 containing 10% FCS immediately and kept at 4°C before preparation for culture within 24 hours. Using a dissection microscope, the submucosal layers were removed and the epithelial layer was cut into 1- to 2-mm² pieces with a sterile scalpel. Individual explants were placed upside down on a collagen-coated 96-well plate. Explants were cultured in medium-199 containing EGF (5 ng/mL), USG (2%), 2 mM t-glutamine, and penicillin-streptomycin (100 U/mL and 100 µg/mL), and the epithelial cells were allowed to grow out from the explants. Culture medium was replaced every 5 to 4 days. The cells grew as monolayers and were cultured for at least 7 days before immunostaining. After 5 days, explants could be replated into new wells at least three times while maintaining cell growth. The size of the conjunctival sheet is 300 mm². A total of 20 primary human conjunctival biopsy specimens were used in these studies. Cells from each biopsy specimen were used in independent experiments and not pooled.

**Conjunctival Cell Line ChWK.** A human conjunctival cell line (Wong-Kilbourne derivative of Chang conjunctiva, ChWK; clone 1-5c-4; ECACC, Ildmiston, UK) was cultured under standard conditions (5% CO₂, 95% humidified air, 37°C) in medium-199 supplemented with 10% heat-inactivated FCS, 2 mM t-glutamine, and penicillin-streptomycin (100 U/mL and 100 µg/mL). Cells were replated at a density of 10,000 cells/well in 24-well plates (Falcon; BD Biosciences) for immunostaining or flow cytometry.

**Cytokine Treatment of Epithelial Cells.** Confluent monolayers of epithelial cells were cultured as previously described in the presence or absence of IFN-γ (50 or 500 U/mL), IL-1β (20 ng/mL), IL-13 (20 ng/mL), TNF-α (20 ng/mL), IL-4 with TNF-α or IL-13 with TNF-α for 24 hours or 48 hours in culture medium before nonenzymatic removal of cells.

**Flow Cytometry**

For two-color immunofluorescence staining, the cells were washed with Ca²⁺ and Mg²⁺-free Hanks’ balanced salt solution (HBSS) and detached with collagenase and cell dissociation solution (1:1 vol/vol). The cells were stained in suspension with 5 µL each of anti-ICAM-1FITC and anti-HLA-DRFITC, or anti-CD80FITC, anti-CD86PE, and anti-CD86H.TTC mAbs for 30 minutes on ice before washing twice and acquiring for flow cytometry (Facscan; BD Pharmingen). An isotype-matched control mAb was used to determine the level of background staining. For intracellular staining with anti-cytokeratin mAbs, 100 µL fixation/permeabilization solution (Cytofix/Cytoperm; BD Pharmingen) was added and washed with PermWash, an aqueous solution containing formaldehyde and saponin (BD Pharmingen), and adding anti-cytokeratinFITC, mAbs for 30 minutes on ice. Flow cytometric analyses were performed using the system software (CellQuest; BD Pharmingen). All the analyses shown were performed on a population of live cells gated by forward and side scatter to include the conjunctival epithelial cell population. At least 10,000 events were acquired, and the percentages of positive cells were calculated after subtracting the background staining.

**Immunocytochemistry**

Epithelial cells were grown on chamber slides (Laboratory-Tek; Nalge Nunc International Corp., Hereford, UK) and, after reaching confluence, cells were cultured with or without IFN-γ, as described earlier. The cell monolayers were fixed with cold methanol at −20°C for 5 minutes and cultured in PBS with 10% goat serum for 30 minutes at room temperature to block nonspecific binding of the mAb. Cells were then incubated with the following dilutions of primary mAbs for 1 hour at room temperature: mouse anti-human HLA-DR and mouse anti-human cytokeratin, diluted 1:50 in PBS, and mouse anti-human ICAM-1 diluted 1:20. After cells were washed three times with PBS, goat anti-mouse IgG(H+L) (1:200) was added and cells were incubated for 1 hour at room temperature. Cells were washed a further three times in PBS and mounted. Negative controls consisted of substituting the primary mAb with PBS. Immunostaining was examined by confocal microscopy (Carl Zeiss Meditec, Ltd., Welwyn Garden City, UK), and serial 0.7-µm thick Z-sections were collected through the thickness of the ChWK monolayer and combined to give a single projection.

**Statistical Analysis**

Results were calculated as the mean ± SEM (or SD), and Student’s t-test was used to determine the levels of significance, with P < 0.05 regarded as significant. All experiments in this study were performed at least three times.

**RESULTS**

**Flow Cytometry and Immunocytochemical Characterization**

As early as 2 hours after establishment of the culture, adherent cells were visible at the outer edges of the tissue. Within 4 to 5 days of culture, cells were visibly growing outward from the conjunctival tissue. On reaching confluence, the cultures were trypsinized and replated. Not every explant grew successfully, but frequently cells could be passaged three times without losing their morphologic integrity. Primary cultures of human conjunctival epithelial cells grew as contact-inhibited monolayers and exhibited a typical cobblestone epithelial morphology (Fig. 1A).

All cells of epithelial origin express cytokeratin, a characteristic marker of the epithelial phenotype. Cytokeratin expression was detected by flow cytometry which showed more than 97% of gated primary conjunctival epithelial cells (n = 10) and more than 96% of ChWK cells (n = 10) to be cytokeratin positive (Figs. 1B, 1C).

**Expression of Immune Markers on ChWK and Primary Epithelial Cells after Cytokine Treatment**

The expression of HLA-DR and costimulatory molecules on both epithelial cell types was compared. Both primary conjunctival epithelial cells and ChWK had a low basal expression of HLA-DR but expressed significant levels of HLA-DR after 48 hours’ stimulation with IFN-γ (500 U/mL; primary epithelial cells 22% ± 3.6%, P = 0.005, ChWK 39.6% ± 2.5%, P = 0.0002, Fig. 2). Both cell types expressed ICAM-1 constitutively, which was upregulated after 24 hours’ culture in the presence of IFN-γ (50 U/mL; primary epithelial cells 75.6% ± 5.9%, P = 0.00005, ChWK 84.1% ± 4.3%, P = 0.00002, Fig. 3). These data were also confirmed by immunocytochemistry, as illustrated for ChWK (Fig. 4).

To compare the effect of different cytokines on expression of these immune molecules, we performed flow cytometric analysis of ChWK cells treated with and without IFN-γ, IL-4, IL-13, TNF-α, IL-4+TNF-α, and IL-13+TNF-α (Fig. 5). CD80 was significantly upregulated after IFN-γ treatment (P = 0.043), whereas IL-4 induced a significant upregulation of CD86 (P = 0.039). Treatment with IL-13 and TNF-α did not induce statistically significant effects. CD40 expression was detected constitutively at very low levels, and after 48 hours’ IFN-γ treatment, there was a slight increase that did not reach significance (data not shown).
DISCUSSION

In previous studies, investigators have used impression cytology for exploring superficial conjunctival epithelial cells by flow cytometry and immunocytochemistry and in conjunctival biopsy specimens for culturing for immunocytochemical studies. The purpose of this study was to examine the deeper layers of conjunctival epithelial cells from biopsy tissues. We have developed a modified explant culture system and have shown that the cells can be grown and propagated on collagen-coated plastic and maintained in culture with a basic culture medium supplemented only with serum substitutes and EGF. Similar levels of expression of cytokeratin, ICAM-1, and HLA-DR were detected by both confocal microscopy and flow cytometry on primary conjunctival epithelial cells and ChWK, and therefore flow cytometry was used throughout to quantitate the differential effects on the ChWK in response to various cytokines.

HLA-DR expression has been observed on various types of epithelial cells involved in chronic allergic diseases, including chronic allergic conjunctivitis. In agreement with our data, De Saint-Jean et al. detected similar levels of IFN-γ induced HLA-DR expression on ChWK using flow cytometry. ICAM-1 was maximally upregulated with IFN-γ, in agreement with others. It has been observed in vitro that there is a significant increase in ICAM-1 expression within the conjunctiva during CAED, which correlates with the degree of granulocyte and lymphocytic infiltration detected by immunohistochemical analysis. In addition, it has been found that allergic subjects have a marked ICAM-1 expression on conjunctival epithelial cells after allergen challenge.

IL-4 is a key Th2 cytokine that is involved in the development of many allergic responses. In this study, the modulatory effects of IL-4 on the expression of costimulatory molecules by

FIGURE 1. (A) Phase-contrast micrograph showing a representative explant monolayer culture of primary conjunctival cells at day 7 of confluence. Magnification, ×400. (B) Flow cytometry of primary conjunctival epithelial cells. The live cells were gated based on forward and side scatter. (C) Flow cytometric histogram demonstrating intracellular cytokeratin expression. Dotted trace: background staining (negative control); bold line: cytokeratin expression, with 97% of these cells being cytokeratin positive.

FIGURE 2. HLA-DR expression by normal human primary conjunctival epithelial cells and ChWK cells. Cells were stained with anti-HLA-DR-DP, -DQ, and -DR mAb. HLA-DR was expressed at a low level in untreated cells. Forty-eight hours of treatment with 500 U/ml IFN-γ induced a small but significant increase in HLA-DR expression in both primary and ChWK cells. Results are calculated as percentage expression (mean ± SEM). **P < 0.01 and ***P < 0.001 comparing untreated and IFN-γ-treated cells.

FIGURE 3. ICAM-1 expression by normal human primary conjunctival epithelial cells and ChWK cells. Both cell types were stained with anti-ICAM-1 mAb. ICAM-1 was constitutively expressed in untreated cells by both primary epithelial and ChWK cells. There was a significant increase after 24 hours of treatment with 50 U/ml IFN-γ. Results are calculated as percent expression (mean ± SEM). *P < 0.01 comparing untreated and IFN-γ-treated cells.
ChWK have been investigated. IL-4 did not induce expression of HLA-DR and ICAM-1. The expression of CD86, but not of CD80, was upregulated by IL-4 to a greater level than with the other cytokines. To our knowledge this has not been demonstrated for conjunctival epithelial cells, although a similar effect has been reported for B cells in allergic bronchopulmonary aspergillosis. Abu-El-Asrar et al. used immunohistochemical techniques to study the expression of CD80 and CD86 in conjunctival biopsy specimens from patients with active VKC and normal control subjects and found that CD86 is more widely and prominently expressed by Langerhans’ cells than is CD80. Tesavibul et al. used immunohistochemistry as well and also found CD86 expression to be significantly higher in ocular cicatricial pemphigoid (OCP) conjunctival substantia propria than in normal conjunctiva.

Preliminary data in this study suggest that the expression of CD40 on ChWK cells is slightly upregulated by IFN-γ at 48 hours but not by any of the other cytokines investigated in this study, including TNF-α. However, Bourcier et al. found that ChWK cells constitutively expresses CD40 which was significantly increased after 24 hours of IFN-γ exposure and after 48 hours’ exposure to TNF-α. The reason for these conflicting data could be the different cytokines and culture conditions used by the two groups. The expression of CD40 and CD40L have been detected by immunohistochemistry in conjunctiva of patients with active VKC, and CD40 immunoreactivity by epithelial cells is stronger in VKC specimens than in noninflammatory conjunctival tissues.

The epithelial layer of the conjunctiva has two distinct surfaces, the apical and the basolateral. The apical surface is exposed to the environment directly, whereas the basolateral surface appears to be the site of recruitment of CD4+ T cells, although the mechanisms involved in T-cell migration to this site are poorly understood. Therefore, future work should be focused on investigating the ability of the epithelial cells to support T-cell migration and identifying the cytokines and chemokines that are involved.
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References


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