Regulation of Human Leukocyte Antigen Expression in Human Conjunctival Epithelium

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PURPOSE. To demonstrate that interferon-γ (IFN-γ) is the key cytokine responsible for the upregulation of HLA-DR antigen in conjunctival epithelial cells of Sjögren syndrome (SS) patients.

METHODS. Flow cytometry of conjunctival epithelial cells from SS and non-SS dry eye patients was performed for the quantification of HLA-DR surface expression. With a conjunctival epithelial cell line (ChWK), HLA-DR regulation by various cytokines was evaluated, and confocal immunocytochemical and western blot analyses were performed to evaluate the activation of nuclear factor-kappa B (NF-κB) and signal transducers and activators of transcription 1 and 3 (STAT1 and STAT3, respectively).

RESULTS. HLA-DR expression was upregulated in conjunctival epithelial cells of SS patients but not in non-SS dry eye patient or healthy control subject. IFN-γ was the only cytokine that effectively upregulated HLA-DR expression in ChWK, which was synergistically enhanced by tumor necrosis factor-α (TNF-α). IFN-γ induced the nuclear translocation of NF-κB, but did not activate STAT1 or STAT3 in ChWK.

CONCLUSIONS. Upregulation of HLA-DR antigen in the conjunctival epithelium of SS patients may be regulated by IFN-γ through the activation of NF-κB. (Invest Ophthalmol Vis Sci. 1999;40:28–34)

The human conjunctival epithelium is a nonkeratinized squamous tissue three to four layers thick that covers 80% of the ocular surface. It is exposed directly to various environmental insults such as desiccation and exposure to foreign bodies, UV light, and air pollution. In ocular surface disorders such as dry eye or allergic conjunctivitis, inflammatory monocytes and neutrophils infiltrate the conjunctival epithelium1,2 and are believed to play a major role in the pathogenesis of inflammatory reactions. The expression of HLA-DR antigen and intracellular adhesion molecule-1 (ICAM-1) in the epithelium of asthmatic patients is thought to accelerate antigen presentation and the recruitment of inflammatory cells.3–5

The mounting evidence now suggests that expression of HLA-DR antigen, ICAM-1, or both in human conjunctival epithelium is upregulated in trachoma,6 allergic conjunctivitis,7 and dry eye associated with Sjögren’s syndrome (SS).7 HLA-DR antigen in epidermal Langerhans cells and monocytes is upregulated in response to inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-6 (IL-6), and IL-1,8,12,15 which are upregulated in the lacrimal gland of SS patients as previously reported by our laboratory.1,3 The cytokines present in tears may thus be important for the upregulation of HLA-DR in SS conjunctival epithelium. The activation of the cells by IFN-γ and tumor necrosis factor-α (TNF-α) is considered to involve the activation of signal transducers and activators of transcription 1 (STAT1) or nuclear factor-kappa B (NF-κB),14–15 although, it is not known whether the same mechanism exists in the conjunctival epithelium. In contrast, IL-10, epidermal growth factor (EGF), TNF-α, and transforming growth factor-β (TGF-β) are known to downregulate the expression of HLA-DR and ICAM-1 in the epithelium.16–19

This study was designed to shed light on the regulation of HLA-DR expression in conjunctival epithelial cells in vitro and in conjunctival brush cytology samples from SS dry eye patients. The cytokine profile responsible for HLA-DR upregulation and the involvement of STAT1, STAT3, and NF-κB was also investigated.

MATERIALS AND METHODS

Reagents and Monoclonal Antibodies

The following reagents and monoclonal antibodies were obtained from the indicated sources: recombinant human IFN-γ (specific activity, 4.75 × 107 U/mg), IL-1α (specific activity, 2.36 × 106 U/mg) and TNF-α (specific activity, 1.43 × 108 U/mg; Genzyme, Cambridge, MA); human recombinant IL-4 (specific activity, 5 × 105 U/mg), and human recombinant IL-6 (specific activity, 1 × 109 U/mg; Boehringer, Mannheim, Germany), human recombinant IL-10 (Becton-Dickinson Labware, Bedford, MA), and human recombinant EGF (GIBCO-BRL, Grand Island, NY); anti–HLA-DR and anti–ICAM-1 monoclonal antibodies and fluorescein isothiocyanate (FITC) conjugate (Becton-Dickinson, San Jose, CA); biotin-conjugated anti-phosphotyrosine monoclonal antibody (Transduction Laboratories,}

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Lexington, KY). anti-cytokeratin monoclonal antibodies AE-3 and AE-5 (clone: KC20; Transformation Research, Framingham, MA); and anti-4-Von Willebrand factor VIII (vWF VIII) monoclonal antibodies (Cedarlane Laboratory Limited, Hornby, Ontario, Canada) and Hoechst 33258 (Sigma, St Louis, MO).

Analysis of HLA-DR and ICAM-1 Expression in Dry Eye Patients

Ten SS dry eye patients (mean ± SD age, 49.8 ± 13.1 years; all women) and 17 non-SS dry eye patients (mean ± SD age, 49.7 ± 11.0 years; 2 men and 15 women) were recruited. Patients gave consent for their participation after the study procedures were explained to them, which is in accordance with the tenets of the Declaration of Helsinki. Dry eye was diagnosed as described previously by the presence of symptoms, abnormal tear dynamics, and ocular surface abnormalities.20,21 and SS was diagnosed according to the criteria of Fox et al.22 Healthy volunteers (mean ± SD age, 37.0 ± 15.2 years; 2 men, 5 women; n = 7) with only refractive errors served as controls.

For FACScan (flow cytometric machine; Becton-Dickinson, San Jose, CA) samples, a miniature brush (Cytobrush S; Medscand AM, Malmo, Sweden) was used to scrape the epithelial side up on a Labtek tissue chamber slide (Nunc). The tissue was stored in DMEM/Hams F-12 (Dulbeccos modified Eagle's medium/Nutrient Mixture F-12) containing antibiotics (TC-199 containing 10% FBS. The medium was changed every 2 to 3 days.

Effects of Cytokines on the Expression of HLA-DR in ChWK Cells

The effects of IFN-γ, IL-1α, IL-4, IL-6, IL-10, and TNF-α on HLA-DR expression in ChWK cells were examined by flow cytometry. Subconfluent ChWK cells in 12-well plates (approximately 10^5 cells/well) in assay medium (TC199) with 0.5% FBS was coincubated with various concentrations of each cytokine for 48 hours at 37°C. At the end of the incubation period, cells were washed three times with TC199 and cultured with trypsin to obtain a single cell suspension. Cells were labeled with antibodies to HLA-DR and ICAM-1 (IFN-γ only) and were subjected to flow cytometry. Each experiment was repeated in triplicate. To see the effects of TNF-α, IL-10, EGF, Cyclosporine A, and betamethasone phosphate on the upregulation of HLA-DR expression in addition to IFN-γ, each drug was given with IFN-γ together to the cell culture medium and cultured for 24 hours before flow cytometry.

Immunocytochemistry

HLA-DR expression in primary human conjunctival epithelial cells was determined by immunocytochemistry using a laser image analyzer equipped with a confocal optical system (ACAS 470; Meridian Instruments, Okemos, MI). Slides were preincubated with 10% goat serum for 30 minutes and with the anti-HLA-DR antibodies for 1 hour. Cells were washed and incubated with 1:100 diluted FITC-labeled goat anti-mouse IgG-1 (Southern Biotechnology, Birmingham, AL) with 1:100 diluted FITC-labeled goat anti-mouse Ig F(ab)'Then, the cell nuclei were stained with Hoechst 33258.

Confocal Microscopy of NF-κB, STAT1, and STAT3

ChWK were seeded in glass-bottomed culture dishes (MatTek, Ashland, MA) at a concentration of 1 X 10^5 cells/ml overnight and then were stimulated by 10 ng/ml or in combination with IFN-γ (100 ng/ml). Selected cells were preexposed to dexamethasone (10^{-6} M) or Cyclosporine A (10^{-5} M) for 3 hours. The reactions were terminated by washing with ice-cooled PBS at 10, 30, and 60 minutes after stimulation. Cells were then immediately fixed with 4% paraformaldehyde (pH 7.2) for 60 minutes at room temperature, followed by permeabilization with 0.1% Triton-X (Sigma) for 30 minutes at room temperature. Cells were then treated with 1:200 diluted rabbit anti-p65/RelA antibody (Rockland, Gilbertsville, PA), with 1:200 diluted murine anti-human STAT-1 monoclonal antibody (Pharmingen, San Diego, CA), or with 1:200 diluted murine anti-human STAT-3 monoclonal antibody (Pharmingen) for 1 hour at room temperature. After washes with PBS, cells were incubated with FITC-labeled goat anti-rabbit IgG antibodies.
Electrophoresis for 80 minutes at 40 mA. Gels were blotted once with cold PBS.

Cold lysis buffer and tates were washed three times with 100 

Conjunctival Epithelial Cells from Dry Eye

overnight and reacted with anti-human STAT1 or STAT3 mono-

antibody conjugated with biotin (clone: RC20: Transduction Laboratories, Lexington, KY), and 12.5 ml Streptoavidine-

horseradish (Amersham, Buckinghamshire, UK) for 1 hour at room temperature. Blots were reacted with enhanced chemiluminescence detection reagents for 1 minute and were developed onto film for 1 minute.

IL-la, IL-4, IL-6, IL-10, and TNF-α did not change HLA-DR expression (Fig. 3). IFN-γ also upregulated ICAM-1 expression in this cell line in a dose-dependent manner (Fig. 4). When IFN-γ-treated cells were coincubated with either TNF-α, IL-10, EGF, Cyclosporin A (10 ng/ml), or dexamethasone (10 ng/ml), only TNF-α showed a synergistic effect with IFN-γ on HLA-DR expression (Fig. 5). This effect was observed clearly when TNF-α was given after IFN-γ but not when they were given in the reverse order (Fig. 6).

Unstimulated cells showed diffuse fluorescein staining for NF-κB in the cytosol (Fig. 7). Stimulation with TNF-α (10 ng/ml) and IFN-γ (100 ng/ml) for 30 minutes produced dark fluorescein staining within the nuclei of the cells (Fig. 7D).

Activation of STAT1 and STAT3

Immunocytochemistry of STAT1 and STAT3 did not reveal a difference between unstimulated cells and cells treated with IFN-γ alone or in conjunction with TNF-α (data not shown). The hybridization with RC20 failed to detect clear phosphorylation of STAT1 (Fig. 8A), although rerehybridization with anti-STAT1 antibody revealed the existence of STAT1 molecules in

RESULTS

Expression of HLA-DR Antigen on Human Conjunctival Epithelial Cells from Dry Eye Patients

The human conjunctival cells collected by brush cytology were predominantly (>99%) epithelial, determined on the basis of positive keratin markers (AE-3 and AE-5), and negative vien-
FIGURE 1. (A) Primary culture of human conjunctival epithelial cells cultured as described in the Methods section. (B) These cells stained positively with the anti-cytokeratin monoclonal antibody AE-3, which is a keratin marker for epithelial cells. (C) Interferon-γ (100 pg/ml)-induced HLA-DR antigen expression was detected by immunofluorescence. (D) Nuclei as seen in (C), except stained with Hoechst 33258.

the cell lysates (Fig. 8B). In the same way, the phosphorylation of STAT3 was not observed (data not shown).

DISCUSSION
We have shown that HLA-DR expression in conjunctival epithelium in SS dry eye patients is upregulated compared with that in non-SS dry eye patients and healthy control subjects. IFN-γ proved to be the candidate cytokine for upregulating HLA-DR expression, which was enhanced by TNF-α. The class II major histocompatibility complex (MHC) molecules are cell-surface receptors mediating specific cell-cell recognition needed for precise immunoregulatory control. A previous study of corneal epithelial HLA-DR expression showed the possibility of antigen presentation by epithelial cells. The upregulation of HLA-DR in the conjunctival epithelium in SS patients may contribute to that antigen presentation, although it may be nonspecific as reported in labial salivary glands of SS patients. Although the conjunctival epithelium in SS patients has not proved to present antigen for HLA-DR expression, it can be targeted by CD4+ T cells. Thus, the upregulation of HLA-DR can be the second mechanism of ocular surface cell destruction by immunologic reaction in addition to the desiccation of the ocular surface in SS.

HLA-DR expression can be induced by a variety of inflammatory mediators including IL-1, IL-6, IFN-γ, and TNF-α. The markedly reduced tear production in SS patients makes it impossible to collect sufficient tears to determine which inflammatory cytokines contribute to the upregulation of HLA-DR in conjunctival epithelium. However, the increased IFN-γ production in the lacrimal glands of SS patients suggests an interaction between tears and ocular surface HLA expression. Cytokines often have synergistic effects on the expression of various proteins. We added IL-4, TNF-α, IL-10, and anti-inflammatory drugs to cultured conjunctival epithelium previously exposed to IFN-γ and observed a synergistic effect only for the combination of IFN-γ and TNF-α. The effect of TNF-α varies between cell types: It has been reported to downregulate HLA-DR expression in fibroblasts. Because both cytokines are upregulated in the lacrimal gland of SS patients, the increased HLA-DR expression in the conjunctiva of SS patients may be due to an additive effect.

In contrast, we were not able to detect the involvement of STAT1 or STAT3 phosphorylation in the conjunctival cell line.
Because the STAT1 and STAT3 proteins were detected, the assay system was considered to be working. Although it is not possible to conclude that the conjunctival epithelium does not use the STAT1 or STAT3 system for the upregulation of HLA-DR expression, other intracellular promoters such as NF-kB may be responsible. Determination of the precise mechanism of HLA-DR expression and its physiological role would shed light on the involvement of conjunctival epithelium in ocular surface disorders such as dry eye or SS.

Recently, the intracellular mechanism of activation by IFN-γ or TNF-α was revealed in other cell types. The phosphorylation of STAT or translocation of NF-κB is the major pathway for HLA-DR expression, other intracellular promoters such as NF-κB may be responsible. Determination of the precise mechanism of HLA-DR expression and its physiological role would shed light on the involvement of conjunctival epithelium in ocular surface disorders such as dry eye or SS.
FIGURE 6. Amplification of interferon-γ (IFN-γ)-induced HLA-DR antigen expression by tumor necrosis factor-α (TNF-α) in Wong-Kilbourne Derivative of Chang Conjunctival Cells, clone CCL20.2. HLA-DR expression was analyzed by flow cytometry as described in the Methods section. Open circles show the group simultaneously treated with IFN-γ (100 pg/ml) and TNF-α for 24 hours. IFN-γ and TNF-α were given together. Closed circles show the group pretreated with TNF-α only.

of cell activation. In this study, we have shown that the combination of IFN-γ and TNF-α caused the translocation of NF-κB in a human conjunctival epithelial cell line. Similar synergistic interactions between TNF-α and IFN-γ have been reported such as the expression of the chemokine interferon inducible protein (IP-10) in human fetal fibroblasts (NIH/3T3) cells and the expression of ICAM-1 in epithelial and endothelial cells.

This may be one of the mechanisms responsible for inflammatory reactions in the conjunctival epithelium in response to cytokines such as IFN-γ.

The MHC antigen class II transactivator (CIITA) has been found to act as a master regulator of MHC class II expression, and CIITA expression can only be detected in MHC class II-positive cell types and tissues. CIITA and MHC class II expression are not only qualitatively but also quantitatively correlated. CIITA is also the obligatory mediator of IFN-γ-inducible MHC class II expression. Because IFN-γ induces CIITA expression, which in turn activates MHC class II transcription, further studies to elucidate its involvement in human conjunctival cells are in progress.

References


15. Jahnke A, Johnson JP. Synergistic activation of intercellular adhesion molecule 1 (ICAM-1) by TNF-alpha and IFN-gamma is mediated by p65/p50 and p65/c-Rel and interferon-responsive factor STAT 1 alpha (p91) that can be activated by both IFN-gamma and IFN-alpha. FEBS Lett. 1994; 354:220.


