Hyperosmolar Stress Upregulates HLA-DR Expression in Human Conjunctival Epithelium in Dry Eye Patients and In Vitro Models

Piera Versura, Vincenzo Profazio, Costantino Schiavi, and Emilio C. Campos

PURPOSE. To investigate the immune response of human conjunctival epithelium to hyperosmolar stress.

METHODS. Tear osmolarity was measured in 15 normal subjects and 25 dry eye (DE) patients; conjunctival imprint cytology samples were obtained at the nasal bulbar area. Subconfluent primary human conjunctival epithelial cells (pHCECs) and human conjunctival organ cultures (hCOCs), both cultured in iso-osmolar medium (305 mOsm/L), were exposed for 24 hours to media with progressively higher osmolarity, with or without the ion channel inhibitor ruthenium red (RuR). Human leukocyte antigen (HLA)-DR expression was evaluated by immunocytochemistry, on imprints from subjects, on primary human conjunctival epithelial cells, on formalin fixed-paraffin embedded hCOCs, and by RT-PCR. Statistical evaluation was performed by applying the unpaired Student's t test, as well as Spearman's rho and Pearson's r correlation coefficients (significance $P < 0.05$).

RESULTS. HLA-DR expression increased in DE subjects with respect to control (% mean ± SD, respectively, 46.16 ± 7.2 vs. 7.48 ± 1.14, $P < 0.0001$) and exhibited significantly high correlations with tear osmolarity values ($r = 0.614; P < 0.0001$). In vitro experiments showed a progressive increase in HLA-DR expression as the osmolarity of the medium was increased from 6.75 ± 1.16 (% mean ± SD) in iso-osmolar-cultured cells to 9.96 ± 1.37 and 12.94 ± 4.04 in cells cultured in, respectively, 350 and 400 mOsm/L ($P < 0.05$). A stepwise progressive increase was also found in hCOCs. Results were confirmed by RT-PCR. Ruthenium red significantly reduced HLA-DR expression in hyperosmolar-cultured cells.

CONCLUSIONS. Data from complementary techniques demonstrate that extracellular hyperosmolarity induces HLA-DR overexpression in human conjunctival epithelial cells in both DE patients and in vitro cell culture models. (Invest Ophthalmol Vis Sci. 2011;52:5488–5496) DOI:10.1167/iovs.11-7215

The human leukocyte antigen (HLA)-DR is a transmembrane glycoprotein expressed primarily on antigen presenting cells (APCs). It binds to intracellularly processed peptides and presents them to helper T-cells. Like the other molecules in the human major histocompatibility complex (MHC) class II, HLA-DR plays a critical role in the initiation of the immune response. In fact, overexpression of HLA-DR has been demonstrated in the epithelial cells of both ocular and nonocular tissues affected by inflammatory disorders: in particular, it has also been seen in the ocular surface epithelium in both chronic conjunctivitis and in dry eye syndrome.

Dry eye (DE) is one of the most common ocular pathologies seen in ophthalmic practice; it is considered a disease of the ocular surface, a functional unit comprising the corneal and conjunctival epithelia, the lacrimal glands, the meibomian glands, the eyelids, and the connecting neural network. The latest International Dry Eye Workshop (DEWS), held in 2007, updated the definition of dry eye, having reached the consensus that, despite the different pathogenic mechanisms, the many forms of the disease are all characterized by variable amounts of ocular surface inflammation and tear film hyperosmolarity.

Indeed, tear hyperosmolarity is now thought to be the key pathogenic factor behind ocular irritation, acting via corneal epithelial cell inflammation, which is induced by activation of the epithelial signaling molecule cascade, leading to the release of proinflammatory mediators into the tears, such as local apoptotic cell death and a reduction in the number of conjunctival goblet cells, as demonstrated in both experimental models and humans. Furthermore, the tear instability resulting from these changes leads to increased tear evaporation and produces transient peaks in tear hyperosmolarity, adding to a detrimental cycle of events and thereby perpetuating the inflammatory process.

The role of conjunctival epithelium in driving the immune response in dry eye pathogenesis has been suggested and demonstrated by many authors. Hence, conjunctival evaluation can be used to assess dry eye-induced surface damage: the earliest signs consist of mild staining in the exposed interpalpebral area of the nasal bulbar conjunctiva, and usually precede corneal involvement. This phenomenon has been attributed to prolonged contact between tears containing proinflammatory mediators—mainly cytokines and chemokines—or cytotoxic related oxidants — and the nasal bulbar conjunctiva during nasolacrimal drainage.

The aim of this study was therefore to determine whether there is a direct link between tear hyperosmolarity and the initiation of the immune response by the conjunctival epithelium in humans.

Thus, we evaluated HLA-DR antigen distribution and expression in patients suffering from mild DE, and correlated it with tear osmolarity values. To further verify the relationship, we also tested primary human conjunctival cells and human conjunctival organ cultures exposed to hyperosmolar stress in vitro experimental models.
METHODS

The study was based on two different but complementary experimental approaches, one clinical and the other in vitro, the latter exploiting primary cultures of human conjunctival cells and organ cultures.

Patients

This study complied with guidelines proposed by the Declaration of Helsinki for research involving human subjects, and was approved by the Ethics Committee of our Hospital. After thorough explanation of our research aims and procedure, informed consent was obtained from all participating subjects.

Fifteen normal adult volunteers (11 women and 4 men of mean age 40.7 ± 11.3 years; 15 eyes) and 25 DE patients (21 women and 4 men of mean age 51.6 ± 14.1 years; 32 eyes) were included in the study. The test subjects were diagnosed as having mild or moderate DE, according to their Dry Eye Workshop (DEWS) severity level.9 Exclusion criteria were: any form of systemic autoimmune disease, previous ocular surgery performed in the year leading up to the test, topical eye therapy, including immune modulators (but excluding tear substitutes), wearing contact lenses, punctual plug placement or cautery, ocular allergy. If topical therapy was in use, this had to have been suspended at least 8 days before the examination for the patient to be included in the study.

Subjective symptoms of dry eye were graded on the basis of the dry eye discomfort symptoms questionnaire (Ocular Surface Disease Index; OSDI23); the severe Ocular Surface Disease Index range was arbitrarily subdivided into two categories, 33 to 62 and 63 to 100, to coincide with DEWS severity levels 3 and 4.24

Tests and analyses applied in this study protocol are listed in Table 1, with their corresponding cut off values and methodology references.24–27

Human Conjunctival Impression Cytology (CIC) Samples for Immunohistochemistry

Impression cytology specimens were obtained from the lower nasal side of the bulbar conjunctiva of both patients and control subjects. CIC samples were obtained using a sterile, single-packed Biopore membrane (Millipore CM 0.4 μm PICM 0125; Millipore Corp., Bedford, MA). After topical anesthesia, the membrane was gently pressed for 3 to 5 seconds, and cells thereby collected were fixed by an isopropyl alcohol-based fixative (Kito-Fix, Kaltek, Padua, Italy), sprayed from a distance of 20 cm. Samples were then stored in the membrane package at 4°C until processing.

Human Conjunctiva Samples for In Vitro Studies

After informed consent, human conjunctival biopsy specimens (n = 41) were obtained in selected strabismus patients undergoing surgery to the horizontal rectus muscles for esotropia or exotropia. A 0.5- to 1-mm-wide strip of redundant conjunctiva was excised intraoperatively after surgical realignment and transferred to a sterile Falcon tube filled with PBS supplemented with 10% penicillin and streptomycin solution (10,000 U penicillin/mL, 10,000 μg streptomycin/mL, Cambrex).

Bulbar conjunctival specimens were collected from donor eyes (n = 5, mean age 67.2 ± 15.9 years) and treated as above.

Primary Conjunctival Epithelial Cell Culture

Primary human conjunctival epithelial cells (pHCECs) were established from bulbar specimens according to the methods of Berry et al.28 Specimens were divided into four parts, each placed directly, epithelium side down, in a fibronectin-coated (5 μg/cm², human fibronectin, BD Biosciences, Bedford, MA) chamber slide. Specimens were cultured in complete iso-osmolar medium (RPMI 300mOsm/L, as declared by the manufacturer and confirmed by freezing-point depression osmometry) supplemented with 5% FBS (lot: no: 806,006, Euroclone), 0.05% P-S solution, 0.05% amphotericin B solution (Sigma), 1% RPMI 1640 amino acid solutions (Sigma), and 5ng/L epidermal growth factors (E9644, Sigma); the medium was fed twice weekly until epithelial growth was established. After a further 3 to 5 days, explants were removed to avoid fibroblast contamination, and pHCECs were cultured for 7 days, checked for purity using Cytokeratin CK19 (clone RCK108, Dako, Germany) expression, and then used for hyper-osmolar stress experiments.

Human Conjunctival Organ Cultures (hCOCs)

Human conjunctival organ cultures (hCOCs) were established from donor eyes (n=4). Conjunctival fragments of 2 × 2 mm were placed in 12-well plates in 1.5 mL of iso-osmolar medium, as described above, for 24 h before their use in hyperosmolar stress experiments.

Hyperosmolar Stress Experiments

Sub-confluent pHCEC and hCOC fragments were exposed for 24 hours to media with progressively increasing osmolarity, obtained by adding NaCl 50 mM and 100 mM to RPMI, reaching peak osmolarity values of 350 and 400 mOsm/L, respectively, verified by freezing-point depression osmometry (Auto-Stat 6030, Kyoto, Daiichi, Kagaku). pHCECs were then exposed for 4 hours to hyperosmolar medium, as described above, in the presence or absence of ruthenium-red (RuR) (5μM), a non-selective Ca²⁺ channel blocker used to determine whether HLA-DR expression is dependent on the function of ion channels involved in osmoregulation.

Table 1. Methods and Tests Applied in the Study

<table>
<thead>
<tr>
<th>Method or Test*</th>
<th>Basis</th>
<th>Values to be Recorded</th>
<th>Pathologic Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tear osmolarity24 (TearLab Osmolarity System [OcuSense])</td>
<td>Tear osmolarity</td>
<td>mOsm/L</td>
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<td>Schirmer test25</td>
<td>Indirect measure of total tear secretion</td>
<td>mm wetting in 5 min</td>
<td>≈ 5 mm/5’</td>
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<td>Patient Allowed to Rest for 5 Min</td>
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<tr>
<td>Tear Film Break Up Time (TFBUT)25</td>
<td>An index of tear film stability</td>
<td>Seconds</td>
<td>≤ 10 s</td>
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<td>Ocular Surface Washing with Sterile NaCl 0.9% Water Solution</td>
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<tr>
<td>Lissamine green staining25</td>
<td>An index of conjunctival surface integrity</td>
<td>Score 0–3 for 6 areas</td>
<td>≥ 9 out of 18</td>
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<tr>
<td>Ocular Surface Washing as Above; Patient Is Allowed to Rest 15 Min</td>
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<td></td>
<td></td>
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<tr>
<td>Scraping cytology26</td>
<td>Degree of inflammation</td>
<td>Score 0–20</td>
<td>Score &gt; 4</td>
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<tr>
<td>Conjunctival impression cytology (CIC)27</td>
<td>Conjunctival epithelium squamous metaplasia</td>
<td>Score 0–5</td>
<td>Score &gt; 1</td>
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</table>

* Methods and tests are listed in their sequence of performance, and performed according to the methods quoted in the references.
Immunohistochemistry (IHC) for HLA-DR Expression on Impression Cytology in Patients

Biopore membranes were rehydrated in distilled water and then incubated in primary monoclonal human HLA-DR antibody diluted 1:40, following the manufacturer’s instructions, for 40 minutes at room temperature (r.t.). Secondary biotinylated bridge antibody and tertiary streptavidin-biotin-peroxidase complex were then applied for 30 minutes (all reagents purchased from Dako, Copenhagen, Denmark). Between steps, membranes were washed with a solution of 0.85% phosphate-buffered saline (PBS) and 0.05% Tween20. Before incubation with tertiary complex, membranes were immersed in phenyl hydrazine (Sigma-Aldrich, Italy) solution at 37°C for 1 hour, followed by prolonged buffer washings to inhibit endogenous peroxidases. Positive reaction sites were visualized by amino ethyl carbazole, applied for 2 minutes at r.t. Nuclear counterstaining was obtained using Harris’ hematoxylin. Intense red-brown membrane staining under light microscopy was considered a positive result.

Immunocytochemistry for HLA-DR Expression on Cultured pHCECs

After hyperosmolar stress experiments, pHCECs were fixed in 4% formaldehyde and immunostained for HLA-DR expression, using mouse monoclonal anti-human HLA-DR α-Chain (1:50, clone TAL.1B5; Dako). The antigen-antibody reaction was revealed (Novolink Polymer Detection System; Novocastra Laboratories, Newcastle, UK). Negative controls were performed by omitting the primary antibodies.

Immunohistochemistry for HLA-DR Expression on Cultured Human Conjunctival Organ Cultures (hCOCs)

hCOCs were formalin-fixed and paraffin-embedded after exposure to increasing hyperosmolar media or baseline iso-osmolar conditions. A total of five experiments were carried out. Four-μm sections were immunostained as described. Heat-induced antigen retrieval was performed in citrate buffer pH 6, as recommended.

Immunohistochemistry

Conjunctival Impression Cytology in Subjects and pHCECs. Adjacent microscopic high-power field (×40) micrographs were taken, the number of HLA-DR-positive cells and the total number of cells were counted, and the results were expressed as a percentage of HLA-DR positive cells (mean ± SD).

hCOCs. Micrographs were taken, as above, of immunostained histology sections, the number of HLA-DR positive cells were then counted and the results expressed as HLA-DR positive cells/mm² (mean ± SD).

Real Time PCR for HLA-DR Expression

Total RNA was extracted from pHCECs using reagent (TRI; Ambion Applied Biosystems), according to the manufacturer’s instructions. Total RNA (500 ng) was reverse-transcribed in 20 μL of reagent, using a high-capacity cDNA archive kit (Applied Biosystems). To assess HLA-DR and CK19, real-time PCR analysis was performed (Gene Amp 7000 Sequence Detection System; Applied Biosystems) (Power SYBER Green PCR Master Mix; Applied Biosystems). Assays purchased from Qiagen (10X Quantitect Primer; Hs_HLA-DR_A_1_SG, Cat. no: QT00089838; Hs_KRT19_1_SG, Cat. no: QT0081137) were used for real-time PCR. Product specificity was determined by melting-curve analysis. Samples were run in triplicate, and the average threshold cycle (Ct) value was used for calculations. The amount of mRNA was calculated relative to the housekeeping GAPDH (Quantitect Primer Assay, Qiagen, Hs_GAPDH_2_SG, Cat. no: QT01192646), according to Pfaffl.

Statistical Analysis

Statistical evaluation was performed (MedCalc, version 9.3; MedCalc Software, Mariakerke, Belgium, and SPSS 14 software; SPSS Inc., Chicago, IL), applying the Mann-Whitney and unpaired Student’s t tests. The correlation between HLA-DR expression and each of the other variables under investigation was determined using Pearson’s (r) or Spearman’s (ρ) correlation coefficients. Correlations were considered statistically significant at P < 0.05.

Descriptive statistics for tests and variables analyzed in subjects and in all in vitro experiments were reported as the mean ± SD.

RESULTS

Patients

Statistically significant differences were demonstrated between patients and controls in all parameters evaluated (Table 2). In particular, an increase in tear osmolarity values, conjunctival epithelium squamous metaplasia (CIC score), and degree of inflammation (scraping cytology score) was shown in patients with respect to controls.

HLA-DR expression analysis in conjunctival epithelial cells was performed in all CIC specimens collected from patients; both the number and the quality of sampled cells were considered adequate for the purpose. Only scattered dendritic cells were recognized in the impression samples, the majority of positive immunostained cells displayed an epithelial phenotype.

HLA-DR expression was increased in DE patients with respect to controls, with respective percentages of 46.16% ± 7.2% and 7.48% ± 1.14%, P < 0.0001. The percentage of HLA-DR⁺ conjunctival cells was positively correlated with increasing tear hyperosmolarity values in both patients and controls (Fig. 1a).

Although positive correlations between HLA-DR expression and scraping cytology, patients’ subjective symptom, and CIC scores were demonstrated, none of these results were found to be statistically significant (P > 0.05 in all cases; Figs. 1b–d).

Primary Human Conjunctival Epithelial Cell (pHCECs) Experiments In Vitro

Cell culture protocol ensured purity of pHCECs used for the experiments, as shown by full epithelial phenotyping and CK19 immunofluorescence staining (Fig. 2).

Being suitable for the purpose, a preliminary assessment of the maximum exposure time of cells to hyperosmolar medium without compromising cell viability was performed; 24 hours’ exposure was judged to be the correct balance between cell survival and death (data not shown). The hyperosmolar values

| Table 2. Summary of Results, in Control Subjects and DE Patients |
|-----------------|-----------------|------------------|-----------------|
|                 | Tear Osmolarity (mOsm/L) | Scraping Cytology Score | CIC Score | HLA-DR (%) |
| Control Subjects | 295.0 ± 10.8 | 2.06 ± 0.8 | 0.33 ± 0.48 | 7.48 ± 1.14 |
| DE Patients      | 310.8 ± 11.3 | 5.43 ± 1.8 | 1.36 ± 0.7 | 46.16 ± 7.2 |
| Significance (P) | 0.005          | 0.0001         | 0.0001         | 0.0001         |

Data are mean ± SD. A statistically significant difference (P always < 0.05) between the two groups was found for all the parameters analyzed.
of the media, obtained by adding NaCl in appropriate concentrations to reach 350 and 400 mOsm/L, respectively, were also checked with the same osmometer as above. Hyperosmolarities higher than 400 mOsm were not applied, due to extensive cell death above this level. Hyperosmolar media were prepared immediately before use.

HLA-DR positivity was judged on the basis of cell border staining, which slightly diffused into the cytoplasm. HLA-DR expression (% positive cells with respect to total) after 24 hours’ cell contact with hyperosmolar medium (Figs. 3b and 3c) was found to be increased with respect to cells grown in iso-osmolar medium (Fig. 3a); this increase was found to be dose-dependent and statistically significant (Fig. 3d).

Enhanced expression of HLA-DR in pHCECs as a consequence of increased osmolarity was also shown by RT-PCR estimation: HLA-DR expression showed a threefold increase from baseline to 400 mOsm (Fig. 3e).

**Human Conjunctival Organ Culture (hCOC) Experiments**

Cell culture can be viewed as a simplification of the in vivo situation, because cells are maintained in isolation from other cell types, thereby minimizing biological complexity.

The effects of hyperosmolar media (350 and 400 mOsm/L) were tested on human conjunctival organs established from conjunctival explants. Hyperosmolar media were prepared and administered as described above for pHCECs. Exposure time was also 24 hours in this case.

The results were comparable to those found in pHCEC experiments. HLA-DR positivity was mainly found in the basal cells of the conjunctival epithelium.

HLA-DR expression (number of positive cells per mm² tissue area) increased in organ cultures grown in iso-osmolar medium (Fig. 4a) after 24-hour contact with hyperosmolar medium (Figs. 4b and 4c); this increase was also found to be dose-dependent and statistically significant (Fig. 4d).

**Transient Receptor Potential Vanilloid (TRPV) Ion-Channel Blocker Experiments In Vitro**

The effects of hyperosmolar media (350 and 400 mOsm/L) were tested on human conjunctival epithelial cells analyzed by blocking ion channels, using ruthenium red (RuR), a general noncompetitive antagonist channel blocker, before exposure to hyperosmolarity (350 and 400 mOsm/L). RuR concentration was estimated on the basis of preliminary experiments evaluating the blocking effect versus cell survival; an RuR concentration of 5 µm was chosen as
the best compromise. Similarly, an RuR contact time of not more than 4 hours was selected as a reliable time permitting cell survival (data not shown).

Cells grown in iso-osmolar medium with RuR showed a slight, non-significant reduction in HLA-DR expression with respect to cells grown in iso-osmolar medium without RuR.

Cells grown in hyperosmolar medium with RuR, on the other hand, showed a statistically significant reduction in HLA-DR expression, compared with cells grown in either 350 or 400 mOsm/L medium without RuR (Fig. 5).

DISCUSSION

This study has succeeded in demonstrating that class II MHC antigen HLA-DR expression in human conjunctival epithelial cells is positively correlated with tear hyperosmolarity in DE patients. The finding was confirmed by in vitro experimental cell and organ cultures grown in hyperosmolar media, obtained using incremental concentrations of sodium to mimic the in vivo conditions.

DE is now considered a multifactorial progressive disease, where evolutionary changes to its final stage, proposed by Bron and coworkers,30 comprise the loss of compensatory responses over time, with a stepwise increase in tear osmolarity and reduction in sensory drive. Therefore, rather than a diagnostic “gold standard,” tear osmolarity should be interpreted as an index of progressive shift to compensation failure and severity increase31 in DE.

Hence, the key role played by tear osmolarity imbalance in ocular irritation has piqued scientific interest in its long-term effects on ocular surface tissues, as many daily activities and typical indoor environments can cause such an imbalance. The main body of knowledge regarding the issue is derived from typical indoor environments can cause such an imbalance. The main body of knowledge regarding the issue is derived from typical indoor environments can cause such an imbalance.

Corneal epithelium impairment is a major concern in ophthalmic practice; although it manifests at a later stage of the disease, changes in conjunctival epithelium have been noted in the initial stages, according to the natural history of the disease.35 In fact, moderate vital staining in the nasal side of the exposed conjunctiva is a common observation in early conjunctival epithelium damage, presumably occurring as a consequence of contact with “inflammatory” tears during nasolachrimal drainage.5,21,22 In advanced stages, the damage can also involve the temporal side of the conjunctiva and the lower corneal area.

Interestingly, the presence of inflammatory markers in conjunctival cells has been demonstrated by many authors.3–7 In particular, HLA-DR expression in the conjunctival epithelium of dry eye patients has shown to be directly correlated to the severity of the disease.6

The HLA test is routinely applied to blood cells in tissue compatibility typing to match donor and recipient in transplantation. However, HLA-DR has also been detected in other types of human cell, such as sinus epithelial cells, where its overexpression has been shown in chronic rhinosinusitis2 and allergic rhinitis.31 In fact, there is increasing evidence for the role of HLA-DR as a marker in systemic inflammatory disorders. As an example, in postoperative infectious complications, HLA-DR expression appears to be downregulated in immunocompetent cells, albeit without any predictive correlation.32,33

Thus, the detection of HLA-DR is not yet the final demonstration of antigen presentation functions in these types of cells. Nevertheless, HLA-DR overexpression does appear to be involved in the immune response in human conjunctival epithelium from dry eye patients, as previously demonstrated, a fact which could one day prompt innovative tests for the condition and perhaps even novel therapeutic strategies.7,34,35

In our study, HLA-DR was detected in conjunctival epithelial cells sampled in the bulbar area of DE patients, who were observed after an 8-day washout period to minimize the role of any pharmaceuticals in the surface antigen assay. The method of detection we used was impression cytology, a technique that provides a suitable number of pure populations of conjunctival cells. With this technique, however, recognition of the “true” antigen presenting cells (APCs) can only performed by analysis of cell morphology. A recent demonstration of HLA-DR overexpression in CK19+ conjunctival cells, but not in CD45+ leukocytes, from DE patients was obtained by flow cytometry7 although further research is needed to investigate the role of these cells as functional APCs.

We used immunocytochemistry to demonstrate HLA-DR positivity on cell plasma membranes in both the control and patient groups, in agreement with previous authors exploiting other techniques.5–7 However, slight cytoplasmic staining was
also shown, as indicated by the manufacturer for the specificity of the clone in use.

The area selected for sampling of the bulbar conjunctiva was the lower nasal side, where a longer resident time of concentrated tears occurs, making the impact of hyperosmolality stress theoretically more detectable than in other conjunctival regions.36

In agreement with previous authors, HLA-DR appeared to be overexpressed in DE patients with respect to normal subjects, although no relationship with clinical severity was demonstrated; this is conceivably due to the population under investigation, which comprised mild/moderate cases, but not severe or Sjogren’s dry eye patients.

An association between elevated pathologic conjunctival inflammatory score, as detected by scraping cytology, and elevated HLA-DR expression in conjunctival epithelial cells in DE patients has been shown. In the present study, we applied an inflammatory score based on inflammatory cell semiquantitative analysis26 of conjunctival secretions containing cells scraped from the outer layers of inflamed conjunctiva and those carried in tears. The HLA-DR overexpression noted could, therefore, be explained by the role played by HLA-DR in mononuclear cell recruitment. However, as our data cannot discriminate between events preceding inflammatory cell recruitment and those resulting as a consequence thereof, further study would be required to clarify this point.

An association has also been found between HLA-DR overexpression and a high degree of ocular surface metaplasia as evaluated by Tseng’s impression cytology score.27 The functional role of proinflammatory cytokines (IL-1) as inducers of squamous metaplasia in the pathogenesis of dry eye has been investigated in animal models to test the hypothesis of autoimmune-mediated keratinization of the ocular surface in this condition.37 In humans, however, HLA-DR has only been investigated in metaplastic diseases of other tissues.38 Nonetheless, our data may indirectly support the postulated relationship between HLA-DR and MUC5AC expression, as suggested by conjunctival impression samples in glaucoma patients subjected to preservative or preservative-free topical medications.39

**FIGURE 3.** Immunocytochemistry (ICC) (24-hour) and real-time PCR (4- and 24-hour) assessment of HLA-DR expression in pHCECs after hyperosmolar stress experiment. Representative images of HLA-DR+ cells revealed by ICC in (A) RPMI 300 mOsm/L (control), (B) RPMI 350 mOsm/L and (C) 400 mOsm/L. Magnification, ×20. Micrograph inserts (magnification, ×32) showed that HLA-DR was expressed at the membrane. (D) Adjacent microscopic high-power field (×40) micrographs were taken, and total cells were counted. Results were expressed as percentage HLA-DR positive cells (mean ± SD). *Significantly different versus untreated (control) cells (P < 0.05). (E) HLA-DR mRNA expression was measured by real-time PCR. Cells were treated for 4 and 24 hours in hyperosmolar media (350 mOsm/L, gray column, and 400 mOsm/L, black column). Data were normalized using GAPDH as a housekeeping gene. The abundance of HLA-DR mRNA in untreated cells (white column, RPMI 300 mOsm/L) was defined as 1, and the amounts of HLA-DR mRNA from hyperosmolar-treated cells were plotted relative to that value (mean ± SE; n = 4). *Significantly different from untreated cells (controls).
In our experiment, high HLA-DR expression was found to be associated with an elevated subjective symptoms score, in agreement with findings in rheumatoid arthritis, in which a relationship between serum HLA-DR concentration and pain score has been demonstrated, and the role of proinflammatory cytokines in pain development hypothesized.40

However, no statistically significant correlation coefficients between each of these three parameters (conjunctival inflammatory score, ocular surface metaplasia, and subjective symptoms score) and HLA-DR expression have been found, perhaps due to a certain degree of biological variability in expression of the antigen itself.

The main purpose of this study was to investigate the correlation between tear osmolarity values and HLA-DR expression, which was demonstrated in both patients and controls. Previously, besides those published on ocular surface cells and already cited above, few reports in the literature indicate that hyperosmolarity may cause inflammation.31,42 To our knowledge, this is the first report on extracellular (tear) hyperosmolarity as a possible inducer of class II MHC antigen HLA-DR expression in humans.

To shed light on this finding and refine the hypothesis suggested by the in vivo data, the in vitro behavior of cultured human conjunctival cells was analyzed. Matrix metalloproteinase 9 (MMP9), IL-1beta and TNF-alpha activation has already been demonstrated in human corneal cells after hyperosmolar medium culture.43 In this study we used primary cultured human conjunctival epithelial cells due to constitutive differences between both spontaneously immortalized IOBA-NHC cell lines44 and transfected ChWK Wang cell lines34 as regards increased expression of HLA-DR (in addition to other co-inducer molecules).

Based on our preliminary experiments, a time of contact with hyperosmolar medium of only 24 hours was chosen, because more prolonged exposure affects cell survival, as already shown in other cell models. To preserve cell survival in a steady state model culture, 400 mOsm/L was selected as the highest hyperosmolar value although, albeit unproven, it is theoretically recognized that tear osmolarity could reach 500 mOsm/L over the cornea in extreme conditions.10

HLA-DR overexpression was demonstrated in both pHCECs and hCOCs, and in both models a statistically significant, dose-dependent relationship was found after 24 hours of contact with hyperosmolar medium. Moreover, a doubling of HLA-DR expression under hyperosmolar conditions with respect to baseline was demonstrated by IHC cell staining.

**FIGURE 4.** HLA-DR expression in hCOCs exposed to hyperosmolar media for 24 hours. Representative images of hCOCs cultured in (A) iso-osmolar medium, (B) 350 mOsm/L, and (C) 400 mOsm/L are shown. (D) Samples were observed, digital photos were taken and analyzed (Image-Pro Plus software; Media Cybernetics, MD). Results were expressed as HLA-DR positive cells/mm² tissue area analyzed. *Significantly different versus untreated (control) organ culture. White column, iso-osmolar medium; gray column, 350 mOsm/L, and black column, 400 mOsm/L.

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**FIGURE 5.** HLA-DR mRNA expression measured by real-time PCR after RuR (5 μM) blocking. Cells were treated for 4 hours in hyperosmolar media (350 mOsm/L and 400 mOsm/L respectively) in the absence (black columns) or presence (gray columns) of ruthenium red (RuR, 5 μM). Data were normalized using GAPDH as a housekeeping gene. The abundance of HLA-DR mRNA in untreated cells (white columns) was defined as 1, and the amounts of HLA-DR mRNA from treated cells were plotted relative to that value (n = 5). *P < 0.05.
RT-PCR analyses also confirmed HLA-DR overexpression in hyperosmolar-cultured cells: an almost threefold increase was shown in cells subjected to 4-hour contact with 400 mOsm/L media, compared with baseline cells cultured in iso-osmolar medium.

The specificity of the relationship between osmolarity and HLA-DR expression was revealed by performing experiments with ruthenium red (RuR), a nonspecific ion channel blocker. RuR is considered a general noncompetitive antagonist channel blocker that interferes with the pore complex, and it is widely applied in studies of transient receptor potential vanilloid (TRPV) ion channel family members, critical contributors to the perception of various environmental stimuli and nociceptive pain that have already been demonstrated to exist in human corneal epithelial and endothelial cells, and very recently in human conjunctival epithelium. RuR also acts on intracellular concentrations of Ca by blocking both Ca uptake and release from mitochondria and ryanodine-sensitive intracellular stores. As well as blocking TRPV channels, in this context, RuR also blocks the voltage-sensitive surface membrane Ca channels.

In fact, in iso-osmolar cultured cells, the addition of RuR ion channel blocking reduced HLA-DR expression, albeit not significantly. Furthermore, adding RuR ion channel blocker to hyperosmolar cultured cells significantly reduced HLA-DR expression compared with baseline. These findings suggest that TRPV channels in human conjunctival epithelial cells might be involved in the hypertonic stress-induced effect, in agreement with that suggested for human corneal epithelial cells.

In addition, our findings indicate a role for TRPV channels in HLA-DR expression, as already demonstrated in several blood cell types. Further studies are therefore warranted to define the physiological and molecular role of TRPV channels in HLA-DR expression. In this context, it is interesting to note that TRPV1-mediated signaling has been shown to raise the pro-inflammatory cytokine profile of human corneal cells subjected to hyperosmolar stress.

In conclusion, a positive correlation between expression of the HLA-DR antigen in human conjunctival epithelial cells and elevated tear osmolarity in mild/moderate DE patients was shown.

Furthermore, this study also showed that hyperosmolarity, achieved by addition of sodium chloride to cultured primary human conjunctival epithelial cells or human conjunctival organ cultures, results in a stepwise increase in HLA-DR antigen expression over time, dependent on the degree of osmolarity of the medium.

These findings further confirm the role played by tear osmolarity in initiating and perpetuating the cycle of inflammation in DE diseases. More studies are needed to elucidate the involvement of selected ion channels in the process, and to define a suitable means of pharmacological control.

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