Differential Cell Proliferation, Apoptosis, and Immune Response in Healthy and Evaporative-Type Dry Eye Conjunctival Epithelia

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Purpose. To assess cell viability and cell cycle kinetics of the conjunctival epithelium and to identify intraepithelial lymphocyte (IEL) subsets in patients with evaporative-type dry eye disease (ev-DED) caused by meibomian gland dysfunction and in healthy subjects. The effect of topical treatment and correlations between clinical symptoms and signs and epithelial and immune variables were also determined.

Methods. Inferior fornix and tarsal conjunctival cells were collected by brush cytology (BC) from patients with mild to moderate ev-DED (n = 23) before and after 2 months of treatment that included lid hygiene, artificial tears, and a marketed adolpid, Valladolid, Spain; 2Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Valladolid, Spain; and 3Allergan Inc., Irvine, California.

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Methods

Patients and Healthy Subjects

Twenty-three new patients ranging in age from 28 to 79 years (mean ± SD, 57 ± 12 years; 7 men, 16 women) and diagnosed with disturbance, tear film instability, increased tear osmolarity, and inflammation of the ocular surface. One of the major causes of this pathologic condition is posterior blepharitis caused by meibomian gland dysfunction (MGD). The oily secretion (meibum) of the meibomian glands forms the superficial layer of the preocular tear film and reduces evaporation of the aqueous phase. MGD is associated with a deficient lipid layer on the tear film and results in the excessive tear evaporation associated with evaporative-type DED (ev-DED). Patients with any type of DED report persistent dry eye-related symptoms such as burning, redness, dryness, grittiness, ocular pain, lid margin itching, swelling, light sensitivity, blurred vision, and foreign body sensation. However, clinical evidence indicates that the tests used for DED diagnosis do not correlate well with these symptoms, especially in mild to moderate forms of DED. Thus, there is a necessity for additional clinical tests or the finding of novel biomarkers that reflect the accurate symptomatology of these patients.

Increasing evidence indicates that inflammation and subsequent increased epithelial cell apoptosis are key mechanisms of tissue injury in many ocular surface disorders, including aqueous-deficient DED. The inflammatory component is thought to be mediated by an increase in CD4+ intraepithelial lymphocytes (IELs). These could interact with ocular surface epithelial cells and produce inflammatory mediators such as cytokines, chemokines, and matrix metalloproteinases that are capable of altering conjunctival epithelial homeostasis. The presence of conjunctival IELs and the increased expression of inflammatory and apoptosis-related markers in ocular surface cells have been extensively reported in patients with aqueous-deficient DED. Additionally, clinical studies of these DED patients have demonstrated the efficacy of anti-inflammatory agents to reduce cellular infiltrates and apoptotic pathways. However, many patients with dry eye symptoms do not have aqueous-deficient DED but, rather, have ev-DED, for which the knowledge of inflammatory markers is more limited.

The objective of this study was to assess the presence of IEL subtypes T, B, and NK cells, cell viability, apoptosis, and cell cycle stage in the conjunctival epithelia of ev-DED patients compared with healthy subjects. The results serve as potential indicators of conjunctival inflammation and may serve as prognosis markers in patients with DED. Additionally, we monitored the effect of therapy, including topical corticosteroids, on the immune variables that we measured.
ev-DED caused by posterior blepharitis resulting from MGD were enrolled in this prospective study. The study was approved by the Institutional Review Board of the University of Valladolid and followed the tenets of the Declaration of Helsinki. The nature of the study and the procedures involved were fully explained to all participants, and written informed consent for participation in this prospective research study was obtained from each subject. Detailed ophthalmologic examination was performed at baseline and after 8 weeks of treatment. There were six inclusion criteria for this study. The first was the presence of dry eye-related symptoms for more than 12 months, as assessed by two questionnaires. The symptoms of discomfort questionnaire (SODQ)\textsuperscript{30,33} consists of eight questions, scored from 0 to 4, with a maximum global score of 32. There is no cutoff defined for this test, so we assumed that anything but 0 meant that at least one mild symptom was present. The symptom assessment in the dry eye (SANDE version 1) questionnaire\textsuperscript{32} uses two 100-mm visual analog scales. The subjects are asked to put a mark on one line to depict the extent of symptom frequency and on another line to indicate symptom severity. The other five inclusion criteria included no previous treatment other than unpreserved artificial tears for the past 3 months, meibomian gland secretion alteration defined as plugging and pouting of the gland orifices and/or thick lipid secretions after digital expression,\textsuperscript{35} Schirmer test-I showing \( \leq 5 \) mm wetting in 5 minutes,\textsuperscript{34} tear break-up time (TBUT) \( \leq 10 \) seconds,\textsuperscript{35} and mild to negative (\( \leq 1 \)) corneal fluorescein and conjunctival rose bengal staining scored according to the Oxford scheme.\textsuperscript{36} These inclusion criteria ensured that patients with aqueous-deficient DED were excluded and that only ev-DED patients with posterior blepharitis were included.\textsuperscript{2,3,37} Conjunctival hyperemia was also evaluated using a slit-lamp and was graded on a scale from 0 to 4, according to Efron’s classification.\textsuperscript{38} Tear meniscus height,\textsuperscript{39} phenol red thread test,\textsuperscript{40} and tear lysozyme concentration\textsuperscript{41} were also determined. Patients with history of contact lens wear, ocular diseases other than ev-DED, rosacea, ocular surgeries in the past 12 months, systemic disease other than hypertension or hypercholesterolemia controlled with no drugs, ocular or extraocular allergies, and systemic or ocular therapies other than artificial tears for the past 5 months, as well as patients who were pregnant or nursing, were excluded from the study.

After the first visit, all ev-DED patients began treatment with the same eye as the worst eye was chosen based on a randomization scheme. In all cases, the worst eye was defined as the most symptomatic; global SODQ scores were 0, and the SANDE version 1 was symptomatic; complete improvement was considered to have occurred when symptoms had improved and no further therapy was required. Partial improvement was considered to have occurred when symptoms decreased but some additional therapy was recommended, and no improvement was considered to have occurred when the symptoms persisted.

To provide normal reference values, 17 healthy volunteers (9 women, 8 men; age, 75 \( \pm \) 11 years) scheduled to undergo cataract surgery were evaluated. These patients were examined with similar procedures, and brush cytology (BC) samples were taken before their surgery. They were otherwise healthy, had no systemic or previous ocular disease other than cataract, and were under no systemic or ocular therapies.\textsuperscript{2} Additionally, these volunteers were totally asymptomatic; global SODQ scores were 0, and the SANDE version 1 was negative. Additionally, the TBUT, Schirmer’s test-I, and lysozyme tear levels were within normal limits, and corneal and conjunctival staining scores were always 0.

**Conjunctival Brush Cytology**

BC was the sampling technique of choice based on previous reports.\textsuperscript{42-45} Conjunctival cells were always collected by the same clinician (EC), as previously described.\textsuperscript{44} Briefly, eyes were anesthetized with topical 0.4% oxybuprocaine drops, and one rotation of the cytobrush (Cytobrush-Plus GT; Medscand Medical AB, Molnö, Sweden) was performed on the inferior fornix and tarsal conjunctiva. Cells were then detached from the brush by gentle rotation for 30 seconds in an Eppendorf tube containing a 1:1 mixture of Dulbecco’s modified essential medium and Ham F12 medium (DMEM/F-12) supplemented with 10% fetal bovine serum (Gibco-Invitrogen, Inchinnan, UK). BC was then performed three more times at the same location, and the samples were pooled before transport to the laboratory for flow cytometry (FC) analysis, which was conducted less than 30 minutes after sampling. Only one eye was examined to avoid any bias related to immune intereye dependency.\textsuperscript{25} In all cases, the worst eye was chosen for BC collection. The worst eye was defined as the most symptomatic one chosen by the patient. If both eyes were equally symptomatic, then the study eye was chosen based on a randomization scheme. The same eye was sampled at the first and second visit.

**Flow Cytometry Analysis**

Immunophenotype, viability, and proliferative capacity of the BC-recovered cells were analyzed by FC. First, each cell suspension was divided equally in four different tubes of 300 \( \mu \)L each. Each tube was then washed with 2 mL cell wash solution (BD Biosciences, San Jose, CA), centrifuged at 500g for 5 minutes, and analyzed by a different panel of specific immune cell markers (Table 1). After removal of the supernatant, the remaining cells were processed for three different analyses: cell linage (tubes 1 and 2), viability (tube 3), and cell cycle (tube 4). For each assay, at least 5000 conjunctival cells were analyzed. FC analysis

<table>
<thead>
<tr>
<th>Tube/Panel</th>
<th>Antibody/Marker</th>
<th>Clone</th>
<th>Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD3-PC5</td>
<td>UCHT1</td>
<td>Pan T-cell marker</td>
<td>BK</td>
</tr>
<tr>
<td></td>
<td>CD4-RD1</td>
<td>SFC12T4D1I</td>
<td>T-helper cell</td>
<td>BK</td>
</tr>
<tr>
<td></td>
<td>CD8-EC8</td>
<td>SFC121Thy2D3</td>
<td>T-cytotoxic cell</td>
<td>BK</td>
</tr>
<tr>
<td></td>
<td>CD45-FITC</td>
<td>B382I1FA</td>
<td>Pan leukocyte marker</td>
<td>BK</td>
</tr>
<tr>
<td>2</td>
<td>CD3-FITC</td>
<td>33-2A5</td>
<td>Pan T-cell marker</td>
<td>IS</td>
</tr>
<tr>
<td></td>
<td>CD16-PE</td>
<td>B75.1</td>
<td>NK cells</td>
<td>CL</td>
</tr>
<tr>
<td></td>
<td>CD19-PC5</td>
<td>J4.119</td>
<td>Pan B-cell marker</td>
<td>BK</td>
</tr>
<tr>
<td></td>
<td>CD45-PC7</td>
<td>J53</td>
<td>Pan leukocyte marker</td>
<td>BK</td>
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<td>CD56-PE</td>
<td>NCAM 16.2</td>
<td>NK cells</td>
<td>CL</td>
</tr>
<tr>
<td>3</td>
<td>Annexin V-FITC</td>
<td></td>
<td>Viability/apoptosis</td>
<td>BK</td>
</tr>
<tr>
<td></td>
<td>Propidium iodide</td>
<td></td>
<td>Viability/apoptosis</td>
<td>BK</td>
</tr>
<tr>
<td>4</td>
<td>Propidium iodide</td>
<td></td>
<td>Cell cycle</td>
<td>BK</td>
</tr>
</tbody>
</table>

BK, Beckman Coulter (Fullerton, CA); IS, Immunostep (Salamanca, Spain); CL, Caltag Laboratories (Burlingame, CA).
was performed with a cytometer (Cytomics FC 500; Beckman-Coulter, Fullerton, CA) using 488 nm excitation with an argon-ion laser for fluorescein isothiocyanate (FITC), phycoerythrin (PE or RD1), phycoerythrin-Texas Red-X (ECD), propidium iodide (PI), phycoerythrin-cyanine 5 (PC5), and phycoerythrin-cyanine 7 (PC7). The data collected were analyzed with a software program (Cytomics RXP; Beckman-Coulter, Fullerton, CA). Controls included cross-reactivity of the fluorescence signals of each channel as well as isotype-matched unspecific monoclonal antibodies used as negative controls. FC gates were applied as appropriate to analyze the cell phenotype, apoptotic stage, and cell cycle.

**Cell Lineage Analysis**

The phenotype of the BC-recovered conjunctival cells was determined by staining with two panels of fluorochrome-conjugated mouse-anti-human monoclonal antibodies (Table 1). Cell suspensions were incubated in the dark with these antibodies at room temperature (RT) for 15 minutes. They were then incubated in the dark with 1 ml solution (FACS Lysing; BD Biosciences) at RT for 15 minutes to fix the cells. Afterward, the cells were gently agitated, and FC analysis was performed. At least 100 IELs were necessary for further analysis of the subtypes.

**Cell Viability Analysis**

The percentages of live, early and late apoptotic, and dead BC-recovered conjunctival cells were determined using an annexin V-FITC and PI commercial kit (Beckman-Coulter) as previously described. The mean proliferation index (MPI) was calculated as the ratio of \( S \times G_2/M \), where \( S \) is the percentage of cells in S-phase, \( G_2/M \) is the percentage of 4n cells in G2 and M phases, and \( G_0/G_1 \) is the percentage of 2n cells in G0 and G1 phases.

**Statistical Analysis**

Statistical analysis was performed by a biostatistician (IF) using the SPSS software package (SPSS 15.0 for Windows; SPSS, Chicago, IL). Percentages of events obtained by FC analysis were expressed as means ± standard deviations. Nonparametric Mann-Whitney U and Wilcoxon signed-rank tests were used for comparisons of two independent sample groups (DED vs. control) and treatment-dependent changes, respectively. When the data were normally distributed, a parametric Student’s t test was used. Correlations between FC data and clinical parameters for DED patients were determined by the Spearman’s rho correlation coefficient. \( P < 0.05 \) was the criterion of significance for all statistical tests.

**Results**

**Diagnostic Symptoms and Clinical Tests**

Based on the SODQ questionnaires and clinical examinations, all the ev-DED patients had mild to moderate symptoms. The SODQ values at first visit were 12.19 ± 5.43 out of a maximum of 32. At the pretreatment visit, all tests except BUT and quality of meibomian gland secretion were within normal limits, thus ensuring the exclusion of aqueous-deficient DED patients.2,5,37 After treatment for 2 months, all symptoms decreased, and the SODQ significantly improved to 9.14 ± 4.89 (\( P = 0.032 \)). The frequency and intensity of symptoms evaluated with SANDE decreased at the second visit (\( P < 0.001 \)). For the clinical tests in the pretreatment and posttreatment eyes, conjunctival hyperemia improved from 1.57 ± 0.59 to 0.71 ± 1.01, and tear meniscus height increased from 1.91 ± 0.73 mm to 2.55 ± 0.69 mm. There were slight but not significant improvements in the quality of meibomian gland secretion, from 1.43 ± 0.79 to 1.14 ± 0.73, and in TIBUT, which increased from 4.17 ± 3.14 seconds to 4.48 ± 2.46 seconds. Values for these two variables remained abnormal. In summary, most ev-DED symptoms improved after 2 months of treatment. At the end of the study period, four patients experienced complete improvement, 16 experienced partial improvement, and three experienced no improvement. Thus, 87.0% of patients experienced improvement.

**Flow Cytometry**

No patients or healthy controls undergoing BC sampling reported any side effects from this technique. Based on immunotyping by forward scatter (FS) and side scatter (SS) dot plots of the FC data, the vast majority (>97%) of the BC-recovered conjunctival cells for both ev-DED and healthy eyes were of epithelial origin (Fig. 1A, Table 2). However, based on the positive CD45 staining and low SS signal (cellular complexity or cytoplasmic granularity), a small population of IELs was also detected (Fig. 1B, Table 2). There were significantly fewer CD45+ IELs in DED patients (1.8% ± 0.9%) compared with healthy subjects (2.9% ± 1.5%), and these values did not change between patient visits (Table 2). Most of these lymphocytes (>60%) were CD3+ T cells (Figs. 1C, 2A). Within the CD3+ population, the pretreatment patients had more CD4+ than CD8+ T cells (Figs. 1D, 1E, 2A). The CD4/CD8 ratio was 1.6 ± 0.8, indicating the predominance of T-helper cells over T-cytotoxic cells (Fig. 2B). This same pattern was present in healthy subjects, in whom the CD4/CD8 ratio was 2.4 ± 1.5. After 2 months of treatment, the number of CD4 cells decreased whereas the number of CD8 cells increased (Fig. 2A). Consequently, the CD4/CD8 ratio decreased significantly to 0.8 ± 0.4 (\( P < 0.05 \); Fig. 2B). Other lymphocytic populations found in BC-recovered conjunctival cells were CD19+ B lymphocytes (Fig. 1F) and CD3-CD16-/CD56+ natural killer (NK) lymphocytes (Fig. 1G). There were no significant differences within these populations between the ev-DED patients and control subjects or between the ev-DED patients at pretreatment and posttreatment visits (Fig. 2A). In all cases, the percentage of CD19+ B cells was above 56%, whereas the percentage of NK cells remained very low (<1.2%; Fig. 2A).

For both ev-DED patients and healthy subjects, two different cell populations differentiated by cell size (FS scale) and complexity (SS scale) were present in the apoptosis assay (Fig. 3A). In normal eyes, 54.4% ± 17.0% of the recovered cells were larger and more complex, with 29.2% ± 22.4% viability. In contrast, in pretreatment ev-DED patients, 85.4% ± 3.2% (\( P < 0.001 \)) of the recovered cells were larger, more complex, and had only 15.9% ± 7.6% viability (\( P < 0.05 \); Table 3, Fig. 3B). In normal eyes, 38.7% ± 22.1% of the recovered cells were smaller, less complex, and had 95.8% ± 1.0% viability. In contrast, in pretreatment ev-DED, 10.9% ± 2.9% (\( P < 0.001 \)) of the recovered cells were smaller, less complex, and had 64.5% ± 17.6% viability (\( P < 0.001 \); Table 3, Fig. 3C).

After treatment, the percentage of live cells in the small, less complex population of cells decreased in the ev-DED patients to 54.6% ± 18.7% (Table 3). In the larger, more complex population of cells, it decreased to 11.4% ± 5.4%. Simultaneously, the percentage of apoptotic and dead cells increased in both the more and the less complex populations after treatment (Table 3).

For pretreatment ev-DED patients, 80.9% ± 6.4% of the cells were G0/G1 phases of the cell cycle, which was higher than in healthy controls (76.9% ± 7.1%; Figs. 4, 5). In contrast, the percentages of cells in S and G2/M phases (3.2% ± 0.9% and 8.6 ± 3.4%, respectively) were significantly lower in pre-
Healthy controls 97.1 /H11006
in those patients with higher percentages of CD19
 fraction between intraepithelial CD3
 (0.037; Fig. 7A) and with immunologic parameters, reflecting
 itching score, almost reaching statistical significance (P,
 3.0%, respectively) were both significantly lower
 the patients. Treatment also decreased the number of T-helper
 propanolol resulted in improved symptoms for approximately 87% of
 live cells, more apoptotic and dead cells, and fewer proliferating
 stage in single samples of BC-collected conjunctival cells. The
 techniques is a useful tool for investigating the ocular surface
 epithelium.44 Ideally, it would be
 conjunctival cell reorganization such as B cells (P = 0.034; Fig.
 7B) and CD4/CD8 ratio (P = 0.047; Fig. 7C).

Correlations between FC Data and Clinical Signs and Symptoms

In pretreatment ev-DED patients, the CD4/CD8 ratio significantly decreased as the dryness score in the SODQ questionnaire increased (P = 0.049; Fig. 6A). Moreover, the percentage of dead cells in the less complex cell population increased with itching score, almost reaching statistical significance (P = 0.052; Fig. 6B). After treatment, there was an inverse correlation between intraepithelial CD3 \(^+\) T cells and SANDE intensity score (P = 0.036; Fig. 6C). In contrast, the intensity increased in those patients with higher percentages of CD19 \(^+\) B cells (P = 0.045; Fig. 6D).

Tear lysozyme content, a measure of tear production, was negatively correlated with cells in the G\(_2\)/M phases (P = 0.037; Fig. 7A) and with immunologic parameters, reflecting

**TABLE 2. Cell Lineage Analysis**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Epithelial (CD45(^+), high SS)</th>
<th>Leukocyte (CD45(^+), low SS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment DED</td>
<td>98.2 ± 0.9</td>
<td>1.8 ± 0.9</td>
</tr>
<tr>
<td>Posttreatment DED</td>
<td>98.5 ± 0.5</td>
<td>1.5 ± 0.5(^*)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>97.1 ± 1.5</td>
<td>2.9 ± 1.5(†)</td>
</tr>
</tbody>
</table>

\(^*\) P < 0.01, compared with healthy controls.

\(†\) P < 0.05, compared with pretreatment DED.

**DISCUSSION**

As we previously reported, the combined use of BC and FC techniques is a useful tool for investigating the ocular surface pathophysiology and determining the presence of cell infiltrates in the human conjunctival epithelium.44 Ideally, it would be better to analyze stromal cells, but the required biopsies in the DED patients would be too invasive and not acceptable.

In this study of ev-DED patients and healthy controls, we analyzed the cell lineage, viability, apoptosis, death, and cell cycle stage in single samples of BC-collected conjunctival cells. The procedure of BC itself is minimally invasive and caused no detectable harm to the patient. Although they constituted only a small proportion of the total cells recovered by BC, we determined the percentages of intraepithelial B cells, T-helper cells, T-cytotoxic cells, and NK cells. The healthy controls were slightly older than the ev-DED patients; however, this age difference was not high enough to be responsible for the observed immune differences between the two groups. Moreover, the observed values for the younger patients in our study were opposite those expected; thus, age apparently did not interfere with the results.

Although patients with mild to moderate ev-DED had the same proportion of total IELs as did healthy controls, they had fewer live cells, more apoptotic and dead cells, and fewer proliferating cells than the controls. Treatment with lid hygiene, unpreserved artificial tears, and a 3-week course of topical unpreserved steroids resulted in improved symptoms for approximately 87% of the patients. Treatment also decreased the number of T-helper cells while it increased the T-cytotoxic cells, thus decreasing the CD4/CD8 ratio. Treatment also increased the early apoptosis of
less complex cells and caused no changes in the stages of proliferating cells. The dexamethasone-associated decrease of CD4 cells and the increase of CD8 cells and apoptosis has been previously described not only in the mucosal immune system, but also in the peripheral blood of different animal models and in humans.

The apparent contradiction of dexamethasone treatment altering all immune parameters in the opposite direction from healthy subjects is well documented. Long-term posttreatment assay will be required to determine whether these parameters return to healthy levels over time.

To our knowledge, this is the first exhaustive study that investigates IEL subpopulations within the eye-associated lymphoid tissue. The encountered IEL subsets are different from those described in the gut epithelia. Moreover, gut IELs are present in both the small and the large intestine, but their frequencies vary within the gut areas, suggesting a specialized anatomic and divergent immune functionality. More studies must be conducted to elucidate the functional significance of the differences between gut and eye IELs.

Finally, the viability, the proliferative capacity, and the proportion of large complex cells to small, less complex cells may also serve as diagnostic tools of this particular DED subgroup. Further follow-up of this series, together with more patients, are necessary to confirm these observations.

As we reported previously, there was a small population of inflammatory CD45 cells in the conjunctival epithelium of the healthy controls. This is consistent with previous suggestions that a nonpathologic, diffuse lymphoid presence is a regular component of the human conjunctival ocular surface. Surprisingly, patients with mild to moderate ev-DED had significantly fewer CD45 cells than did controls. Based on impression cytology studies, this reduction in conjunctival IELs has also been reported in keratoconjunctivitis sicca patients. The reduction of IELs could be attributed to an increase of lymphocytes within the stroma and not the epithelium. In addition, there may be a reduction of healthy regulatory cells in disease conditions that were not analyzed by the antibodies used in this study. Finally, the loss of CD45 cells could be the result of apoptosis as...
occurs in the epithelial cells during tissue reorganization associated with the inflammatory DED environment.11,26 No studies using BC samples have been published that describe the conjunctival inflammatory status of ev-DED patients. In aqueous-deficient DED, which is usually more severe, the con-

**TABLE 3.** Flow Cytometric Analysis of Viability and Apoptosis

<table>
<thead>
<tr>
<th>Total Cells</th>
<th>Less Complex Cells (%)</th>
<th>More Complex Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>Live</td>
<td>Dead</td>
</tr>
<tr>
<td>Origin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>85.4 ± 3.2</td>
<td>21.7 ± 6.3</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>41.6 ± 4.9</td>
<td>23.6 ± 9.16</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>51.4 ± 1.70</td>
<td>35.7 ± 18.90</td>
</tr>
</tbody>
</table>

*P < 0.05, compared with posttreatment DED. †P < 0.01, ‡P < 0.001, and §P < 0.005, compared with healthy controls.

**FIGURE 4.** FC dot plots of cell cycle analysis of conjunctival cells recovered by BC from a representative DED patient. (A) SS versus FS dot plot of conjunctival cells shows that only cells of homogeneous size and complexity (gate C: 77% of cell sample) were analyzed for DNA content. (B) Linear fluorescence histogram of PI gated on the homogeneous population shows different phases of the cell cycle. D: 80.9% of the cell population consisted of 2n cells in G0 and G1 phases. G: 3.5% of the cell population was in S-phase. N: 9.3% of the cell population consisted of 4n cells in the G2 and M phases.

**FIGURE 5.** Cell cycle analysis in conjunctival cells obtained by BC from DED patients (pretreatment and posttreatment) and healthy controls.

*P < 0.001, †P < 0.05, ‡P < 0.01 compared with pretreatment DED.
junctival epithelium sampled by impression cytology exhibits increased numbers of inflammatory markers compared with normal eyes.\textsuperscript{17,22,24} Similar to our own findings, Baudouin et al.\textsuperscript{23} reported a significantly lower percentage of CD45-expressing cells in aqueous-deficient DED patients than in healthy controls. They attributed that finding to the conjunctival impression cytology technique that collects only the more superficial conjunctival cells, unlike BC, which collects deeper cells.\textsuperscript{43,60,61} Conjunctival impression cytology excludes basal cells of the epithelium and the stroma, whereas theoretically there are higher proportions of proinflammatory lymphocytes, such as Th1 and Th17 cells, secreting the corresponding cytokines.\textsuperscript{57} We believe, therefore, that the severity and type of DED, evaporative versus aqueous deficient, may account for regional differences both in the type of IEL infiltration and in the expression of inflammation markers by the surrounding epithelium.

Evidence of immune-based inflammation in the pathogenesis of DED has increased the use of anti-inflammatory agents to treat this ocular surface disease. Numerous studies have revealed that corticosteroids improve both signs and symptoms associated with dry eye.\textsuperscript{27,62} However, little is known about the effects of this treatment on epithelial cells or IELs infiltrating the conjunctiva. We found that corticosteroid treatment, in addition to lid hygiene and unpreserved artificial tears, induced a phenotypic change in the BC-recovered IELs from conjunctival epithelium: the percentage of CD4^+ T cells significantly decreased and, hence, the CD4/CD8 ratio also decreased. Similar results were reported by others using different anti-inflammatory agents.\textsuperscript{18,21} These findings demonstrate that the topical use of corticosteroids for the treatment of ev-DED may have a local immunoregulatory effect on conjunctival inflammation. It can inhibit the production of inflammatory mediators such as cytokines, chemokines, and metalloproteinases.\textsuperscript{63,64} Additionally, it can reduce the activation and proliferation of CD4^+ T cells, which are largely responsible for immune-based inflammation in DED.\textsuperscript{15,16,22,65}

We therefore believe that in spite of the interesting changes observed in the CD4/CD8 ratio in the conjunctival epithelium before and after treatment, this ratio cannot be offered as a disease activity or therapy biomarker. This possibility could be further elucidated by evaluating the CD4/CD8 ratio before (symptomatic) and after (asymptomatic) a treatment that does not involve drug application, such as after lid hygiene. In our experience, many patients do improve with only lid hygiene provided the disease is mild to moderate and if there is only an evaporative component. This was not the therapy decided in this particular study; we decided to maximize treatment by adding a short course of topical steroids (second step on the step-ladder approach) to increase the percentage of patients improving after only 2 months of therapy. Usually, therapy steps are applied more slowly; therefore, some patients need more time for improvement.

We found significantly increased apoptosis levels in the conjunctival epithelium of ev-DED patients compared with normal eyes. The double staining with annexin V and PI allowed us to distinguish not only between live, apoptotic, and dead cells but also between early and late apoptotic stages. Our findings are consistent with those of other reports of conjunctival apoptosis in DED.\textsuperscript{11,19,20} Proapoptotic cytokines, such as tumor necrosis factor (TNF)-\(\alpha\), interleukin (IL)-1\(\beta\), IL-8, and
others, are released by both epithelial and immune cells of the human conjunctiva in response to desiccating stress conditions. Indeed, a significant increase in the expression of pro-apoptotic markers such as Fas, FasL, CD40, and CD40L occurred in DED animal models and in patients with aqueous-deficient DED.

As we previously reported for healthy conjunctivae, two different cell populations were present in both ev-DED patients and healthy controls. The larger population consisted of larger, more complex (higher cytoplasmic granularity), and less viable cells, whereas the smaller population consisted of smaller, less complex, and more viable cells. According to recent confocal microscopy studies, we speculate that the more complex cells, which are generally less viable, consist of a variety of cells including epithelial cells located in the more superficial conjunctival layers, mature mucin-producing goblet cells located at the epithelial surface, immature goblet cells in the more basal layers of the epithelium, and dendritic Langerhans cells. The smaller, less complex (lower cytoplasmic granularity), and more viable cells were likely to consist of a mixture of more basal epithelial cells and IELs. These findings suggest that different cell types (e.g., epithelial cells, leukocytes, goblet cells) may be directly involved in apoptotic processes that play an important role in the physiopathology of ev-type DED, even in the absence of increased IELs.

More paradoxical were the findings of significantly increased early apoptosis in the less complex cells obtained from DED patients after topical steroid treatment. This effect would be more easily explained if patients had received prolonged topical treatment and eyedrops containing preservatives, as previous reports have suggested. However, the ev-DED patients who participated in this study received only a 3-week course of corticosteroids, and all drugs were preservative-free. Similar changes also occurred in patients with aqueous-deficient DED treated with topical cyclosporine A. That treatment significantly increased the APO2.7 proapoptotic marker expression in conjunctival epithelium, which presented clear signs of hyperplasia. Collectively, these results suggest that after treatment, conjunctival epithelial homeostasis is maintained by increased apoptosis of the injured epithelial tissue balanced by hyperplastic activity in the deeper epithelial layers (which most likely correspond to the niche of the less complex cell population present in the apoptosis assay). Clearly, further long-term studies are needed to monitor the effects of corticosteroid treatment on apoptotic pathways.

Cell proliferation of the conjunctival epithelium in dry eye has received little attention. We found that most BC-recovered conjunctival cells from ev-DED patients were in the G0/G1 phase, followed by the G2/M phase, and lastly by the S-phase. However, a completely altered conjunctival epithelium, as evidenced by the dysregulation of the cell cycle fractions, was present in ev-DED patients compared with normal conjunctiva. This pathologic feature has been shown by others to be strongly correlated with increased expression of apoptosis-inducing proteins such as CD40, CD40L, Fas, Fasl, and APO2.7 and proinflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α in dry eye. This suggests that ocular surface inflammation may decrease epithelial turnover, indeed helping to explain the epithelial damage seen clinically on fluorescein staining as corneal epithelial punctate keratopathy. Based on the Ki-67 cell proliferation marker, abnormal proliferation of the conjunctival epithelium was reported in patients with aqueous-deficient DED with or without Sjögren’s syndrome. However, these results are not comparable with our findings because of known discrepancies between the results of cell cycle determination by Ki-67 and DNA content. The Ki-67 protein is expressed during the G1, S, G2, and M phases of the cell cycle but not in the quiescent G0 phase. Measurement of DNA content by FC, as we performed, provides a more detailed description of the number and percentage of cells in the different phases of the cell cycle.

**Figure 7.** Correlation between immunologic parameters and clinical measurements of tear production in ev-DED patients. (A) Pretreatment G0/G1 cells, (B) posttreatment CD19+ B cells, and (C) CD4/CD8 ratio were negatively correlated with lysozyme levels. Spearman’s rho correlation coefficients were 0.459, 0.589, and 0.558 respectively. Dotted lines: 95% confidence bands.
Thus, the DNA content of the cells in the inflamed ocular surface may serve as a biomarker for the disease and the response to treatment. This warrants further investigation.

A wide variety of symptom questionnaires and diagnostic tests are often used to assess the presence, severity, or both of DED. However, evidence of inflammation in DED and poor correlation between symptoms and clinical tests, particularly in mild to moderate dry eye, have fostered the search for promising biological markers that are well correlated with dry eye symptoms and that accurately reflect ocular surface inflammation. We found no correlations of the global scores for the SODQ and SANDE questionnaires with any of the immunologic parameters assessed by FC. There was a significant association with the itching sensation score and the presence of apoptotic cells. Our results also showed that immunologic markers such as the CD4/CD8 ratio and infiltrating B-lymphocytes were inversely correlated with tear lysozyme levels. These results are in agreement with those showing negative correlations between the conjunctival infiltrate density and the tear film break up times. These findings provide further evidence that immune-based assessment of conjunctival inflammation by a combination of FC and BC techniques may well be correlated with subjective and objective clinical parameters and therefore may be of help in the diagnosis of DED.

In conclusion, the evaluation of conjunctival cells of DED patients by minimally invasive techniques can yield valuable differential information regarding viability, apoptosis, proliferative capacity, and immune response when compared with those of healthy controls. EV-DED decreases conjunctival epithelial cell viability and proliferative capacity, and symptomatic improvement after short-term therapy leads to a significant modification in the CD4/CD8 ratio. Therapeutic clinical trials using the parameters of epithelial viability and proliferative capacity would clarify their potential to be useful diagnostic biomarkers of DED disease.

References


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