Immunological impression cytology of the conjunctival epithelium in patients with thyroid orbitopathy-related dry eye

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ABSTRACT. Thyroid orbitopathy (TO) is an autoimmune disease that is complicated by ocular surface disorders, leading to discomfort. Dry eye is very prevalent in patients with TO. Recent studies on the pathogenesis of dry eye have focused on the inflammatory process, and some supporting evidence has been discovered. Because TO is a disorder of autoimmune origin, we assumed that the association between TO and dry eye is related to inflammation. Inflammation of the ocular surface in TO-related dry eye has not been well studied. In this study, we assessed cellular inflammation of the ocular surface and the cytokine profiles in patients with TO-related dry eye. Conjunctival impression cytology (CIC) was assessed with an immunofluorescent
assay. TO-related dry eye was diagnosed by using the Schirmer test, tear break-up time, thyroid function, and clinical signs. CIC was combined with immunological staining of interleukin-1α (IL-1α), IL-1β, and IL-6. The immunological impression cytology (IC) grade was compared to the clinical activity score of TO. All TO patients with dry eye were positive for IL-1α, IL-1β, and IL-6. However, the normal controls were also positive for IL-1α. A trend was observed between the clinical inflammatory score and immunological IC grade. This study was the first to delineate the immunological IC of TO-related dry eye. Our study aimed to investigate the pathogenesis of dry eye in TO. Our findings suggest that the conjunctival cytokines IL-1α, IL-1β, and IL-6 may play a role. The results of this study will be useful for future studies of additional inflammatory cytokines, and the levels of these cytokines could be used as an outcome to assess the efficacy of treatment, such as anti-cytokine or immunosuppression therapy, in patients with TO-related dry eye or other ocular surface inflammatory disorders.

Key words: Thyroid orbitopathy; Conjunctival impression cytology; Dry eye

INTRODUCTION

Thyroid orbitopathy (TO) is an autoimmune disease involving visual disturbances and orbital manifestations, which can cause severe proptosis, dry eyes, strabismus, and optic neuropathy (Bothun et al., 2009). The presence of dry eyes is the most frequent complaint in TO patients with ocular surface disorders in association with corneal exposure secondary to exophthalmos and lagophthalmos (Weetman, 1991; Burch and Wartofsky, 1993; Bartley et al., 1996; Boulos and Hardy, 2004). For visualization of the dry spots on the conjunctiva and cornea, both lissamine green and rose bengal, which have excellent staining ability, are preferable to fluorescein. The staining intensity scores for both of these stains were significantly high in TO-related dry eye, pointing to the presence of drying epithelial cells in patients with early and late TO (Stern et al., 2002).

Dry eye is an inflammatory disorder that affects the ocular surface, and proinflammatory cytokines have been increasingly suggested as possible mediators of lacrimal gland insufficiency (Zoukhri 2006). A previous study suggested that secretion of proinflammatory cytokines and matrix metalloproteinases (MMPs) by corneal epithelial cells promotes the development of ocular surface inflammation in dry eye syndrome (Boulos and Hardy, 2004), as desiccating stress stimulates the expression of MMP-9, interleukin-1α (IL-1α), IL-1β, and tumor necrosis factor-alpha (TNF-α) in the corneal epithelium (Boulos and Hardy, 2004). Hyperosmolarity in dry eyes induced the release of IL-1β, IL-6, TNF-α, MMP-9, and IL-8 from corneal and conjunctival epithelial cells (Luo et al., 2005; Cavet et al., 2010).

The tear fluid in dry eyes has been shown to contain increased levels of proinflammatory cytokines, such as IL-1 (both IL-1α and mature IL-1β) and decreased levels of inactive IL-1β precursor (Boulos and Hardy, 2004). The conjunctival epithelium appears to be the source of the increased IL-1 detected in the tear fluid of patients with dry eyes (Boulos and Hardy, 2004). These results suggest that IL-1, and possibly other cytokines, may play important roles in the
pathogenesis of keratoconjunctivitis sicca or dry eye (Barton et al., 1998; Narayanan et al., 2008).

IL-1β can induce nitric oxide (NO) synthesis, which is related to cell death, in several secretory epithelial cell types (Beauregard and Brandt, 2003; Beauregard et al., 2003). In menopausal women, dry eye is related to a sex steroid deficiency and consequent fluid-secreting cell damage (Beauregard and Brandt, 2004). Estrogen was shown to increase the expression of IL-1β, IL-6, and IL-8 in corneal epithelial cells, and this hormone may play an etiologic role in the ocular surface inflammation related to dry eye (Suzuki and Sullivan, 2005).

It was shown that that cytokines in tear fluid could interfere with the synthesis of membrane-associated mucins (MUC-1, MUC-4, and MUC-16) in corneal epithelial cells (Jones et al., 1998; Blalock et al., 2008; Albertsmeyer et al., 2010), which can be clinically observed by rose bengal staining on the corneal surface.

In dry eye, upregulated expression of human leukocyte antigen-antigen D related (HLA-DR), which is induced by inflammation in epithelial cells, and apoptosis-related markers in conjunctival epithelial cells has been demonstrated (Brignole et al., 2000, 2001). Some researchers found increased levels of human beta-defensin-2 (hBD-2), which is induced by IL-1β and TNF-α, in the conjunctival epithelium of patients with moderate dry eye (Boulos and Hardy, 2004). In addition, decreased levels of IL-6 were noted in the conjunctival epithelium of patients with moderate to severe dry eye treated with 0.05% topical ophthalmic cyclosporine for 6 months (Turner et al., 2000). Next-generation cyclosporine A, which showed 4-fold inhibition of T-cell proliferation and proinflammatory cytokine release compared to those in the controls, was safe and effective in early clinical trials (Boulos and Hardy, 2004). Therefore, the conjunctival epithelium may be one of the sources of cytokine production.

Our hypothesis is that patients with TO-related dry eye have strong inflammation of the ocular surface. To test this hypothesis, we assessed ocular surface inflammation by calculating the inflammatory index using CIC and IFA. Knowledge of the cytokines expressed in different clinical situations will provide more clues about the pathogenesis of TO-related dry eye, a testable model for future research, and potential treatment targets.

MATERIAL AND METHODS

Human subjects

All the study patients were enrolled from the Outpatient Department of Ophthalmology at Kaohsiung Medical University Hospital. Patients with autoimmune disorders, conjunctivitis, or dry eye were excluded from the normal control group. The study was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUH-IRB-960252). The ethics IRBs approved the consent procedure. Written informed consent was obtained from all study participants, and consent was obtained from the guardians of minors enrolled in this study.

Diagnosis of TO

TO was diagnosed as hyperthyroidism with clinical eye signs, including proptosis, eyelid retraction, and restriction of ocular motility. Orbital inflammation was graded as the sum of clinical scores, including congestion (0 for negative, 1 for positive), conjunctival chemosis (0 for negative, 1 for positive, and 2 for overhanging chemosis), eyelid swelling (0 for negative, 1 for positive, and 2 for overhanging swelling), erythema (0 for negative, 1 for
positive), the presence of ocular motility pain (0 for negative, 1 for positive), and retrobulbar pressure (0 for negative, 1 for positive).

**Diagnosis of dry eye**

To diagnose dry eye, patients with TO complaining of dry eyes or observed to be congested with low tear meniscus, were assessed using Schirmer’s test and tear break-up time (BUT).

**Schirmer test**

The test was performed after installation of topical anesthetic eye drops, followed by lightly blotting of the residual fluid in the inferior fornix. A Schirmer test paper strip (5 mm wide, 35 mm long) was placed at the junction of the middle and lateral thirds of the lower eyelid of both eyes to minimize irritation to the cornea. The eyes were then closed to limit the effect of blinking. Aqueous tear production was quantified by measuring the amount of wetting. Less than 5 mm of wetting is highly suggestive of aqueous tear deficiency, while 5-10 mm is equivocal (Hikichi et al., 1995; Shrestha et al., 2011; Beckman et al., 2015; Courtin et al., 2016).

**Measurement of tear BUT**

Tear BUT was determined by instilling fluorescein into the inferior cul-de-sac and then evaluating precorneal tear film stability. It was measured prior to instillation of any eye drops and before the eyelids were manipulated in any way. The examiner moistened a fluorescein strip with sterile saline and applied it to the tarsal conjunctiva. After several blinks, the tear film was examined under a slit lamp with a blue filter, and the patient was asked not to blink. The timing of the appearance of the first randomly distributed dry spot of tear film is the tear BUT. A BUT of less than 10 s is considered abnormal (Brasche et al., 2001; Beckman et al., 2015; Courtin et al., 2016).

**Immunofluorescent cytokine staining of CIC**

CIC was performed as follows. With the patient in the supine position, 0.5% proparacaine (Alcaine) was instilled into the conjunctival sac. Then, the excess eye drops pooled in the conjunctival sac were gently swabbed. Cellulose acetate filter paper (Millipore membrane) was prepared (3 x 3 mm). An impression of the four quadrants (temporal, upper, nasal, and lower) of the bulbar conjunctiva was obtained. One corner of the filter paper was grasped with blunt smooth-ended forceps, and the paper was gently pressed to stick it to the conjunctival surface. The filter paper was kept on the conjunctival surface for 3-5 s, and then peeled off. The paper was then applied to a clean glass slide coated with gelatin and the impression was transferred by applying gentle pressure. After fixation with 95% ethanol and 1% formalin, the slides were stained with hematoxylin-eosin and observed under a light microscope at low and high power (Boulos and Hardy, 2004).

The expression of IL-1 (IL-1\(\alpha\), precursor and mature IL-1\(\beta\)) and IL-6 was evaluated in the CIC specimens. The membranes were stored at -80°C until processing. They were fixed with cold methanol for 10 min, and then blocked with phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) for 20 min to block nonspecific staining. The primary antibodies against IL-1\(\alpha\), precursor and mature IL-1\(\beta\), and IL-6 were used. The membrane
was incubated with the antibodies for 1 h at room temperature in a moist chamber. Then, the membranes were rinsed with PBS three times, and incubated with FITC-conjugated secondary antibody for 1 h. Cytology specimens obtained from the inferior bulbar conjunctiva treated only with the secondary antibody served as a negative control. Membranes were washed with PBS three times, placed on a glass microscope slide, and then covered with non-fade mounting medium and a glass coverslip. Specimens were examined with a fluorescent microscope and photographed. The staining was scored as negative if the fluorescence intensity was less than or equal to that of the secondary antibody control. The staining was scored as positive if the intensity was greater than that of the control. Staining intensity was independently evaluated by two observers. To establish an experimental model of IFA, we applied these procedures to cultured human retinal pigment epithelial (RPE) cells obtained from American Type Culture Collection (ATCC). The human RPE cells were infected herpes simplex virus-1 (HSV-1) pre-IFA. The infected human RPE cells showed bright green fluorescence under a fluorescent microscope. In contrast, mock-infected human RPE cells showed no such fluorescence.

Statistical analyses

The chi-square test was used for statistical comparison of CIC IFA expression between the TO-related dry eye and normal control groups. A P value less than 0.05 was considered statistically significant.

RESULTS

The study included 39 TO patients with dry eye (12 males and 27 females). The mean age of the TO patient group was 48.5 years (range, 17-70 years). The control group included 30 participants (6 males and 24 females). The mean age of the control group was 38.5 years (range, 22-64 years).

The brightness of fluorescence in the immunological CIC staining in TO patients with dry eye and the controls is shown in Figure 1. The immunofluorescence was graded on a scale from 0 to 3 according to intensity (Figure 2). The negative control, which was only treated with the secondary antibody, showed only trace immunofluorescence (Figure 2A) and was graded as 0. Grade 1+ showed faint fluorescence (Figure 2B), grade 2+ showed bright fluorescence (Figure 2C), and grade 3+ showed diffuse, shiny fluorescence (Figure 2D). The fluorescence intensity greater than or equal to 1+ was considered positive.

IL-1α IFA expression was assessed in 16 TO patients with dry eye, including 3 males and 13 females (Table 1), and was graded as 0, 1+, 2+, and 3+ in 3, 3, 8, and 2 TO patients with dry eye, respectively. The positive rate (1+ or higher) was 81.25% (3 grade 1+, 8 grade 2+, and 2 grade 3+). IL-1α IFA expression was also assessed in 15 control patients, including 4 males and 11 females. The positive rate was 86.67% (Table 1).

IL-1β IFA expression was assessed in 13 patients TO patients with dry eye, including 5 males and 8 females (Table 2), and was graded as 0, 1+, 2+, and 3+ in 3, 4, 6, and 0 patients, respectively. The positive rate was 76.92% (4 grade 1+ and 6 grade 2+). IL-1β IFA expression was also assessed in 15 control patients, including 2 males and 13 females, and the positive rate was only 13.33% (Table 2).

IL-6 IFA expression was assessed in 7 TO patients with dry eye, including 1 male and 6 females (Table 3), and the positive rate was 85.71% (4 Grade 1+ and 2 Grade 2+).
Figure 1. Immunofluorescence staining of IL-1β from immunological CIC in patients with TO-related dry eye and normal controls. IL-1β IFA expression: A. B. IL-1β IFA expression in patients with TO-related dry eye. The arrows indicate conjunctival cells with bright green fluorescence. C. D. IL-1β IFA expression in normal control subjects. The arrows indicate conjunctival cells with faint fluorescence compared to A and B.

Figure 2. Immunofluorescence grading (from 0 to 3+) based on fluorescence intensity. Grading of immunofluorescence (cytokine expression): A. grade 0, trace fluorescence; B. grade 1+, faint fluorescence; C. grade 2+, bright fluorescence; D. grade 3+, diffuse and shiny fluorescence.

Table 1. IL-1α IFA expression in patients with TO-related dry eye and normal controls.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Males (%)</th>
<th>Females (%)</th>
<th>Total (%)</th>
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<tr>
<td>Test group</td>
<td>0 1 (6.25%)</td>
<td>2 (12.5%)</td>
<td>3 (18.75%)</td>
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<tr>
<td></td>
<td>1+ 0 (0%)</td>
<td>3 (18.75%)</td>
<td>3 (18.75%)</td>
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<tr>
<td></td>
<td>2+ 1 (6.25%)</td>
<td>3 (18.75%)</td>
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<tr>
<td></td>
<td>3+ 1 (6.25%)</td>
<td>1 (6.25%)</td>
<td>2 (12.5%)</td>
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<tr>
<td>Control group</td>
<td>0 0 (0%)</td>
<td>2 (13.33%)</td>
<td>2 (13.33%)</td>
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<tr>
<td></td>
<td>1+ 4 (26.27%)</td>
<td>6 (40%)</td>
<td>10 (66.67%)</td>
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<tr>
<td></td>
<td>2+ 0 (0%)</td>
<td>3 (20%)</td>
<td>3 (20%)</td>
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<td></td>
<td>3+ 0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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Table 2. IL-1β IFA expression in patients with TO-related dry eye and normal controls.

<table>
<thead>
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<th>Females (%)</th>
<th>Total (%)</th>
</tr>
</thead>
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<td></td>
<td>1+ 2 (15.38%)</td>
<td>2 (15.38%)</td>
<td>4 (30.77%)</td>
</tr>
<tr>
<td></td>
<td>2+ 3 (23.08%)</td>
<td>3 (23.08%)</td>
<td>6 (46.15%)</td>
</tr>
<tr>
<td></td>
<td>3+ 0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Control group</td>
<td>0 0 (0%)</td>
<td>2 (13.33%)</td>
<td>2 (13.33%)</td>
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<td></td>
<td>1+ 0 (0%)</td>
<td>2 (13.33%)</td>
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<td></td>
<td>2+ 0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<td></td>
<td>3+ 0 (0%)</td>
<td>0 (0%)</td>
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The immunological CIC grading was combined with the clinical inflammation score. The CIC for IL-1α and IL-1β (Table 4) tended to show higher inflammation scores in patients with higher CIC grading.

We compared the IL-1α and IL-1β IFA expression between the TO and control groups. The positive rates of IL-1α IFA expression were not statistically significantly different between the two groups, but IL-1β IFA expression in the test group was significantly higher than that in the control group (Table 5).

The positive rates for IL-1α expression were similar between the TO and normal control groups; therefore, the difference was not significant (Table 5). In the test group, we did not collect cases with an inflammation score of 7-8 (Table 4).

**DISCUSSION**

T cells play a central role in cell-mediated immunity, which is involved in dry eye and TO (Förster and Kahaly, 1998; Gopinath et al., 2009). T helper (Th) cells are activated after being presented with major histocompatibility complex (MHC) class II molecules by antigen presenting cells (APCs), and then the Th cells divide and secrete cytokines that regulate the immune reaction. Th cells can assist in the maturation of B cells and the activation of both cytotoxic T cells and macrophages. After activation, Th cells can differentiate into Th-1, Th-2, Th-3, and Th-17 subtypes. Studies have shown that Th-1 and Th-17 cells are correlated with...
the pathogenesis of dry eye (Stern et al., 2005; Yoon et al., 2007; De Paiva et al., 2009; El Anan et al., 2009; Zheng et al., 2010).

IL-1 was first discovered in 1985. IL-1α is constitutively produced by epithelial cells and can be secreted by other cells, including fibroblasts, mast cells, endothelial cells, monocytes, etc., upon stimulation. Thus, IL-1α can be considered an epidermal cytokine. It has a central role in immune responses, and possesses metabolic, physiologic, and hematopoietic activities. IL-1α interacts with many other cytokines, and the synergism between TNF and IL-1α is most relevant, as they co-regulate some immune reactions, e.g., NO production and chemokine synthesis. In addition, IL-1α can induce IL-2 and IL-6 secretion.

IL-1β is produced by activated macrophages as a proprotein that is proteolytically processed to its active form. IL-1β is important in inflammation, and it is involved in cell differentiation, proliferation, and apoptosis.

IL-6 is secreted by T cells and macrophages. It is also produced by muscle cells (myokine), adipocytes, and osteoblasts. It is important in trauma-induced inflammation and muscle contraction, and is an important mediator of fever during the acute phase response. In addition to its pro-inflammatory role, IL-6 also has anti-inflammatory effects, as an inhibitor of TNF-α and IL-1.

Inflammation has been implicated in both TO and dry eye. In our study, IL-1α, IL-1β, and IL-6 were elevated in the conjunctival epithelial cells of patients with TO-related dry eye. However, IL-1α was also expressed in the conjunctival epithelial cells of normal control patients. As IL-1α is constitutively expressed in epithelial cells, the high positive rate in the control group might be indicative of the increased permeability of epithelial cells after laboratory treatment. This study is the first to investigate the cytokine alterations that occur in the conjunctival epithelial cells of patients with TO-related dry eye. In our study, we detected IL-1α, IL-1β, and IL-6 expression in the conjunctival epithelial cells of patients with TO-related dry eye. However, others did not find a significant increase in IL-1β levels in CIC samples with moderate dry eye (Narayanan et al., 2006).

Anti-cytokine therapy has been proposed for the treatment of TO (Paridaens et al., 2005; Cawood et al., 2006; Garrity and Bahn, 2006). However, there has been no discussion of the cytokines involved in dry eye associated with TO. In our study, we enrolled TO patients with dry eye and surveyed the expression of various cytokines, including IL-1α, IL-1β, and IL-6, by CIC and IFA. Of the patients with TO-related dry eye, 76.92% were positive for IL-1β expression by immunofluorescence, compared to 13.33% of the control group. Patients with TO-related dry eye showed statistically significantly higher IL-1β expression in their conjunctival epithelium than normal patients. In contrast, similarly high positive immunofluorescent expression rates were noted for IL-1α (81.25%) and IL-6 (85.71%). This implies that inflammation may be involved in the pathogenesis of dry eye associated with TO. In addition, anti-inflammatory or anti-cytokine therapy may be effective for the treatment of such cases (Brignole et al., 2000; Pflugfelder, 2004; De Paiva et al., 2006; Cordero-Coma et al., 2007; Kymionis et al., 2008; Pflugfelder et al., 2008; Ridder, 2008; Goyal et al., 2009; Gürdal et al., 2010). Anti-TNF-α agents, cytokine receptor antibodies, and IFN-α2a are the routinely used biologics (Jap and Chee, 2008).

One difficulty we encountered in the study was that an uncertain number of cells had adhered to the Millipore membrane in the CIC. The number of cells was usually not sufficient for further surveys. To avoid this problem, we suggest drying the conjunctival surface as much as possible before pressing the membrane on the conjunctiva, to promote cell adhesion.
Moreover, preparing more samples may guarantee sufficient specimens for survey. In the beginning of our study, we also planned to detect the cytokines via CIC and reverse transcriptase polymerase chain reaction (RT-PCR). However, the insufficient quantity of cells led to RT-PCR failure. In addition, releasing the adhered cells from the Millipore membrane was usually not easy. Therefore, detection of cytokines via CIC and IFA was a more promising procedure.

Our study had some limitations. First, we did not collect IF-6 IFA data from the control group. Thus, we could not compare IF-6 levels between the TO and control groups. Second, due to the limited group size, we did not collect cases of TO without dry eye. Because TO and dry eye both show ocular surface inflammation, we would have liked to compare cytokine expression between TO with and without dry eye.

Additional trials with staining for more cytokines in CIC from TO patients are needed. Further studies of cytokine expression comparing TO with and without dry eye, dry eye in association with TO and other causes, and TO-related dry eye to ocular surface inflammatory disorders. These methods could also be used to evaluate the effectiveness of different treatment regimens for dry eye, such as artificial tears, topical steroid eye drops, and topical ophthalmic cyclosporine A as well as various combinations thereof.

These data show that there are changes in some inflammatory indicators in TO-related dry eye. These indicators may be promising targets for future studies focused on the treatment for TO.

In our study, the conjunctival epithelium of patients with TO-related dry eye showed highly positive expression of IL-1α, IL-1β, and IL-6. Therefore, we suggest that conjunctival cytokines play an important role in the pathogenesis of TO-related dry eye.

Conflicts of interest

The authors declare no conflict of interest.

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