The Role of Oxidative Stress and Inflammation in Conjunctivochalasis

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PURPOSE. To investigate the status of oxidative stress and histopathologic alterations in patients with conjunctivochalasis and compare the findings with those in healthy control subjects.

METHODS. Eleven patients (n = 20 eyes) with Yokoi grade 3 conjunctivochalasis and 11 health control subjects (n = 22 eyes) were prospectively recruited. ELISA for tear hexanoylated lysine (HEL) and inflammatory cytokines, tear film break-up time tests, Schirmer test measurements, and fluorescein and rose bengal vital staining were performed. Conjunctival specimens obtained during surgery for conjunctivochalasis and cataract underwent immunohistochemical staining for HEL + 8-OHdG, MMP-3, and MMP-9, and positively stained cells were counted. Transmission electron microscopy was also performed, with staining for elastic fibers in the conjunctival stroma.

RESULTS. The mean tear stability and vital staining scores were significantly worse in the conjunctivochalasis patients than in the control subjects. The tear HEL and tear cytokine levels showed significantly higher values in eyes with conjunctivochalasis. IL-1β and IL-6 levels showed a significant correlation with corneal epithelial damage. IL-1β and TNFα showed a significant correlation with 8-OHdG-stained cell counts. Specimens from patients with conjunctivochalasis revealed a significantly higher number of cells positively stained for HEL, 8-OHdG, MMP-3, and MMP-9 than did specimens from age- and sex-matched control subjects. Transmission electron microscopy showed decreased intercellular cohesiveness, with the conjunctival stroma showing an accumulation of elastic fibers.

CONCLUSIONS. Lipid and DNA oxidative stress were present in the conjunctiva. Increased tear inflammation seemed to coexist with loss of conjunctival epithelial cohesiveness and increased collagenolytic activity, which may explain the conjunctival laxity observed in patients with conjunctivochalasis. (Invest Ophthalmol Vis Sci. 2010;51:1994–2002) DOI:10.1167/iovs.09-4130

Conjunctivochalasis (CCh) is defined as redundant, non-edematous conjunctiva typically located between the eyelid and the lower eyelid. It commonly occurs in the elderly and can cause intermittent epiphora, ocular irritation, and subconjunctival hemorrhage. Although age-related and inflammatory changes and mechanical friction from blinking eyelids have been implicated as potential causes, the etiology of conjunctivochalasis remains largely unknown. Hughes2 considered the formation of conjunctival folds to be a senile change. Abnormalities in conjunctival extracellular components such as degeneration of elastic fibers or increased collagenolytic activity have also been reported. Indeed, Meller et al.1,3 suggested that if elastic degeneration were the main histopathologic event, the ocular surface inflammation might stimulate collagenolytic action that may be linked to elastotic degeneration of the conjunctiva. However, they emphasized the need for detailed histologic and histochemical studies. We have reported that in superoxide dismutase-1 enzyme–knockout mice, increased reactive oxygen species leads to severe ocular surface, lacrimal gland, and tear film inflammation with dry eye and concomitant oxidative damage to cellular DNA and lipids. This mouse model compared to age- and sex-matched wild-type mice was reported by us to be a good tool for the study of the role of oxidative stress and inflammation in age-related dry eye syndromes in humans (Wakamatsu TH, et al. JOVS 2007; 48:ARVO E-Abstract 1919). A recent report related to skin changes in the same mouse model suggested decreases in collagen and elastin content via cellular oxidative damage with skin thinning and wrinkles.4 In cases of skin aging and relevant inflammatory events in humans, oxidative stress has been shown to promote inflammation by activating the redox-sensitive transcription factor, nuclear factor-κB (NF-κB) which, in turn, triggers generation of proinflammatory cytokines and chemokines, and hence, inflammation.5,6 One of the earliest detectable responses of the aging skin cells is the activation of multiple cytokine receptors, including those of TNF-α, interleukin (IL)-1, and epidermal growth factor (EGF). Activation of cell surface cytokine and growth factor receptors results in the recruitment of adaptor proteins and activation of multiple MAP kinase pathways.7–9 MAP kinase activation has been reported to result in the induction of transcription factor AP-1, which...
regulates the expression of many genes involved in the regulation of cellular growth and differentiation. AP-1 is known to closely regulate the transcription of several metalloproteinases, including MMP-9 (gelatinase-b), which degrades collagen fragments generated by collagenases, and MMP-3 (stromelysin-1), which degrades basement membrane-type collagen.10-12

The resultant loss of cellular differentiation and proliferation and the presence of collagen degradation have been used as possible explanations for wrinkles and laxity of skin with aging. Conjunctivochalasis has been reported to be one of the age-related diseases of the eye.13

Our observation of the presence of oxidative stress in age-related dry eye animal models and its possible association with inflammation, together with the observations on skin laxity related to oxidative stress and inflammation, first stimulated us to investigate whether oxidative stress is present in an age-related disorder we frequently encounter in our dry eye practice—namely, conjunctival laxity. We have reported increased tear film inflammation in patients with conjunctivochalasis.14

We know of no studies in which the researchers looked into the association of inflammation and oxidative stress in conjunctival laxity, and we found it of interest to investigate whether changes similar to skin laxity could be observed in conjunctivochalasis. First of all, a PubMed and Medline search using the key words “conjunctival laxity,” “conjunctivochalasis,” “inflammation,” and “oxidative stress” revealed no studies in the literature of whether oxidative stress is a feature of conjunctivochalasis. Thus, we were motivated to perform further investigation in this unexplored territory.

In this study, we investigated the presence of oxidative stress markers on the ocular surface of subjects with conjunctivochalasis: especially 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, and Nε-hexanoyllysine (HEL), a marker of lipid peroxidation. We examined the status of inflammatory cytokines in the tear film and the histopathologic alterations in conjunctival samples of patients undergoing conjunctival resection surgery and compared the results with those in age and sex-matched healthy control subjects.

**METHODS**

**Subjects**

Twenty eyes of 11 patients (6 women, 5 men; mean age, 72.4 ± 6.9 years) with Yokoi grade 3 conjunctivochalasis and 22 eyes of 11 healthy control subjects (6 women, 5 men; mean age: 72.4 ± 4.5 years) who were attending the dry eye subspecialty outpatient clinic were examined in this prospective study. Risks and benefits were fully explained, and written informed consent was obtained from all subjects, in accordance with the Declaration of Helsinki. All dry eye specialty examinations and biopsy/surgical procedures were reviewed and approved by the ethics board. All eyes underwent tear sampling and tear film function and ocular surface examinations. Subjects who wore contact lenses or who had lid conjunctival disorders, meibomian gland disease, blepharitis, a history of recent ocular surgery, systemic or topical drug use, or other systemic or ocular diseases with known association with ocular surface disease were excluded from the study.

Subjects with definite dry eye, as classified by the Japanese Dry Eye Diagnostic Criteria (dry eye-related symptoms in addition to at least three points of ocular surface staining and Schirmer test or tear break-up time [BUT] ≤ 5) were also excluded. The Yokoi classification was used to grade conjunctivochalasis severity as follows: grade 0, no conjunctivochalasis; grade 1, no conjunctivochalasis with natural blinking; chalasis aggravated after forced blinking (chalasis height less than the height of the tear meniscus); grade 2, chalasis apparent without forced blinking, height less than height of the tear meniscus; and grade 3, chalasis higher than the tear meniscus height, apparent without forced blinking.15 Subjects with grade 3 conjunctivochalasis associated with recurrent subconjunctival hemorrhage and severe dry eye symptoms, such as grittiness, pain, or irritation, were recommended for conjunctival resection surgery. Inferior conjunctival tissue samples were obtained from seven eyes of seven patients during the conjunctival resection surgery (three eyes of three female patients; four eyes of four male patients; mean age: 73.7 ± 7.3 years) and from six age and sex-matched normal subjects during cataract extraction surgery (mean age, 76.8 ± 3.5 years). Conjunctival resections for conjunctivochalasis were all performed by the same surgeon (MK). Conjunctival resection samples from cataract surgery were provided by Ishida Eye Clinic. Tissue biopsy procedures (tarcimal and conjunctival) for investigational purposes in patients with dry eyes and control subjects (only from those who provide informed consent) were approved by Keio University Internal Review Board (Ethics Board Approval 16-5-1). The control samples during cataract surgery were also obtained from the inferior conjunctiva as 3 × 3-mm samples so that the tissues could be fairly compared. Before surgery, the control and conjunctivochalasis subjects also underwent the same tear sampling, tear film, and ocular surface examinations.

**Tear Staining and Examination**

**Ocular Vital Staining Scores and Tear Film BUT.** The ocular surface was initially stained with 2 μL of 1% fluorescein solution instilled into the conjunctival sac with a micropipette. The patient was instructed to blink several times for a few seconds to ensure adequate mixing of the dye. The tear (T) BUT was determined to be the interval between the last complete blink and the appearance of the first corneal black spot in the stained tear film. The TBUT was measured three times and the mean value of the measurements was calculated. Two microliters of a preservative-free solution of 1% rose bengal was then instilled into the conjunctival sac with a micropipette. Rose bengal staining of the ocular surface was scored according to the criteria proposed by van Bijsterveld,16 and fluorescein staining of the cornea was scored according to the protocol described by Shimamura et al.17 Both the fluorescein and rose bengal staining scores ranged between 0 and 9 points.

**Schirmer I Test.** A standard Schirmer I test without topical anesthesia was performed. A sterilized strip of filter paper (Showa Yakuhin Kako Co., Ltd., Tokyo, Japan) was placed in the lateral canthus away from the cornea and left in place for 5 minutes. Readings were recorded in millimeters (mm) of wetting after 5 minutes.

**Enzyme-Linked Immunosorbent Assays**

**Measurement of HEL.** Tears were collected with 2-μL glass capillary tubes. All the tear samples were obtained from the lateral canthus, parallel to the ocular surface, without stimulating reflex tearing. After collection, the tears were immediately stored at −80°C. A commercially available HEL ELISA (JaICA, Shizuoka, Japan) was used to determine the tear HEL concentration, as reported previously.18 We then investigated the correlation between tear HEL levels and the number of cells positively stained with HEL in conjunctival resection specimens.

**Measurement of Tear Cytokine Levels.** Tear samples were taken from each eye with 10-μL glass capillary tubes in the same manner as just described. After collection, the tears were centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatants were removed and stored at −80°C. A human inflammation kit (Cytometric Bead Array; BD Biosciences, San Diego, CA) was used in combination with flow cytometry (FACSCalibur; BD Biosciences) to quantitatively measure IL-12, IL-6, IL-8, IL-10, TNF-α, and IFN-1β, according to the manufacturer’s instructions and as previously reported.19,20 The data acquired from the flow cytometer were processed (CBA Analysis Software; BD Biosciences). We then investigated the correlation between tear cytokine levels and the number of cells positively stained with HEL and 8-OHdG in conjunctival resection specimens.
Immunohistochemical Staining for Oxidative Stress Markers and the Matrix Metalloproteinases MMP-3 and -9

Oxidative stress-induced lipid peroxidation was assessed by immunohistochemical detection of HEL protein adducts. Oxidative DNA damage was investigated by immunohistochemical staining of 8-OHdG. The avidin-biotin-peroxidase complex (ABC) method was used for immunostaining. Tissue samples were fixed overnight in Bouin’s solution and processed for paraffin embedding. Sections (5 μm) were cut from paraffin wax blocks, mounted on precoated glass slides, deparaffinized, and rehydrated. Antigen retrieval was achieved by microwaving the sections in 10 mmol/L sodium citrate buffer for 5 minutes and then cooling for 20 minutes. Nonspecific background staining was blocked by treating the sections with normal horse serum (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. The sections were then incubated for 90 minutes with the following monoclonal mouse primary antibodies: anti-8-OHdG (dilution factor: 1:10; N45.1; JaICA) and anti-HEL (dilution factor: 1:10, 5H4; JaICA). For the negative control primary antibodies: anti-8-OHdG (dilution factor: 1:10; N45.1; JaICA) and anti-HEL (dilution factor: 1:10; 5H4; JaICA). For the negative control experiments, the primary antibody was replaced with mouse IgG1, (MOPC-21; Sigma-Aldrich, St. Louis, MO). Endogenous peroxidase activity was blocked with 3.0% H2O2 in methanol for 3 minutes. The sections were incubated for 30 minutes with biotin-labeled horse anti-mouse IgG serum (Vector Laboratories), followed by avidin-biotin-alkaline phosphatase complex (Vector Laboratories) for 30 minutes. The sections were washed in PBS buffer, developed in prepared 3,3'-diaminobenzidine (DAB) chromogen solution, lightly counterstained with hematoxylin, dehydrated, and mounted. For MMP-3 and -9 immunostaining, after deparaffinization of the specimens, microwave antigen retrieval was performed for 20 minutes. To block nonspecific background staining, we treated the sections with normal horse serum (Vector Laboratories) for 60 minutes at room temperature. The sections were then incubated for 90 minutes with the following monoclonal mouse primary antibodies: anti-MMP-3 (dilution factor: 1:50; CosmoBio, Tokyo, Japan), and anti-MMP-9 (dilution factor: 1:50; CosmoBio). Endogenous peroxidase activity was blocked using 3.0% H2O2 in methanol for 3 minutes. The sections were incubated for 30 minutes with biotin-labeled horse anti-mouse IgG serum (Vector Laboratories), followed by avidin-biotin-alkaline phosphatase complex (Vector Laboratories) for 30 minutes. The sections were washed in PBS buffer, developed in prepared DAB chromogen solution, lightly counterstained with hematoxylin, dehydrated, and mounted.

Quantification of Cellular Oxidative Damage and MMP-3 and -9 Staining

All sections were evaluated with light microscopy at 20× magnification for the presence of positive immunohistochemical staining for 8-OHdG, HEL, MMP-3, and MMP-9. The number of positively stained conjunctival epithelial cells and stromal fibroblasts was counted in three nonoverlapping areas by two examiners, in a masked fashion. The total cell counts were determined from each examiner and averaged.

Verhoeff–Van Gieson Staining for Conjunctival Elastic Fibers

Conjunctival resection specimens were deparaffinized and hydrated with distilled water and stained in Verhoeff’s solution for 1 hour. The tissues were then rinsed in tap water with three changes and treated with 2% ferric chloride for 1 minute. The samples were checked microscopically for black elastic fiber staining and gray background. We repeated 2% ferric chloride treatment and tap water rinses as necessary for adequate demonstration. The tissue samples were treated with 5% sodium thiosulfate for 1 minute, washed in running tap water for 5 minutes, and counterstained in Van Gieson’s solution for 5 minutes. The specimens were dehydrated in ascending grades of alcohol, cleared in xylene, and coverslipped with a synthetic mounting medium.

Transmission Electron Microscopy of the Conjunctival Samples

For transmission electron microscopy, the specimens were immediately fixed with 2.5% glutaraldehyde solution in 0.2 M phosphate buffer (pH 7.4) for 48 hours at 4°C and washed with 0.1 M phosphate buffer. The samples were then postfixed in 1% osmium tetroxide, dehydrated in a series of ethanol and propylene oxide, and embedded in epoxy resin. The semithin sections (1-μm) were stained with methylene blue. Then, ultrathin specimens were sectioned with a microtome (LKB, Gaithersburg, MD) with a diamond knife. Sections in the range of gray to silver were collected on 200-mesh grids, stained with uranyl acetate and lead citrate, and examined by electron microscope (model 1200 EXII; JEOL, Tokyo, Japan). Ultrathin specimens also underwent elastic fiber staining, to study the status of elastic fibers in the conjunctival stroma.

Statistical Analysis

The Mann-Whitney test was used to compare the parameters between the conjunctivochalasis subjects and the normal control subjects. The correlation between tear HEL levels and the number of cells positively stained with HEL in conjunctival resection specimens as well as the correlation between tear cytokine levels and the number of cells positively stained with HEL and 8-OHdG were analyzed by using the Spearman correlation (all analyses: InStat ver. 3.0; GraphPad, San Diego, CA). P < 5% was considered statistically significant.

RESULTS

Tear Function and Ocular Surface Findings

All parameters examined, except Schirmer test, were significantly worse in the subjects with conjunctivochalasis than in the control subjects (P < 0.05). Results are summarized in Table 1.

Tear Film BUT. The mean TBUT was 3.1 ± 1.2 and 7.9 ± 1.8 in the conjunctivochalasis subjects and the control subjects, respectively. The difference was statistically significant (P < 0.05).

Ocular Surface Vital Staining Scores. The mean fluorescein scores were 1.5 ± 1.1 and 0.06 ± 0.2 and the mean rose bengal scores were 1.3 ± 1.4 and 0.08 ± 0.2 in the conjunctivochalasis and control subjects, respectively. The differences reached statistical significance for both parameters (P < 0.05).

Schirmer I Test. The mean Schirmer values were 10.6 ± 1.6 and 17.8 ± 10.8 mm in the conjunctivochalasis and control subjects, respectively, as shown in Table 1 (P > 0.05).

<table>
<thead>
<tr>
<th>Table 1. Tear Functions and Ocular Surface Findings in Patients with Grade 3 Conjunctivochalasis and Normal Control Subjects</th>
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<tr>
<td></td>
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<tr>
<td>CCh patients (n = 20 eyes)</td>
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<td>Normal control (n = 22 eyes)</td>
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* P < 0.05 Mann-Whitney test.
ELISA for Measurement of Tear Cytokine Levels. All tested cytokine concentrations were significantly higher in the eyes of patients with conjunctivochalasis than in the eyes of healthy control subjects, as shown in Figures 1A and 1B (P < 0.05). Tear IL-1β showed a significant correlation with corneal fluorescein staining scores, as well as conjunctival cells positively stained with HEL and 8-OHdG (Table 2). The tear IL-6 level also showed a significant correlation with corneal fluorescein staining scores and tended to correlate with HEL staining scores without statistical significance. A marginally significant correlation was observed between tear TNFα levels and conjunctival 8-OHdG staining counts (Table 2).

### Table 2. Correlation between Tear Inflammatory Cytokine Concentrations, Corneal Staining Scores, Conjunctival HEL and 8-OHdG Staining Counts

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Fluorescein scores</th>
<th>HEL counts</th>
<th>8-OHdG counts</th>
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<tr>
<td><strong>IL-1β</strong></td>
<td>Spearman Correlation Coefficient</td>
<td>0.62</td>
<td>0.67</td>
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<tr>
<td></td>
<td>P</td>
<td>0.04 *</td>
<td>0.03 *</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>Spearman Correlation Coefficient</td>
<td>0.61</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.040 *</td>
<td>0.050</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td>Spearman Correlation Coefficient</td>
<td>0.30</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.33</td>
<td>0.08</td>
</tr>
</tbody>
</table>
ELISA for Measurement of HEL. The tear HEL concentration was significantly higher in the eyes of the patients with conjunctivochalasis compared with that in the eyes of the healthy control subjects (Fig. 2; \( P < 0.05 \)). The tear HEL concentrations showed a significant correlation with the number of cells positively stained with HEL in conjunctival resection specimens \( (r = 0.65, P = 0.04) \).

Immunohistochemistry Staining and Quantification of Cellular Oxidative Stress

All conjunctival resection specimens from the patients with conjunctivochalasis revealed marked staining for HEL and 8-OHdG in the epithelium, conjunctival stroma, and endothelium of capillaries in the conjunctival stroma compared with that in specimens obtained during cataract surgery from the age- and sex-matched control subjects (Fig. 3). The average number of positively stained epithelial cells in the conjunctivochalasis subjects was 14.52 ± 6.11 and 14.14 ± 5.65 for the 8-OHdG and HEL antibodies, respectively. In the control group, the average number of positively stained conjunctival epithelial cells was 2.19 ± 0.97 and 1.96 ± 0.56 for 8-OHdG and HEL, respectively (Fig. 4).

Immunohistochemistry Staining and Quantification of MMP-3 and -9

All conjunctival resection specimens from the patients with conjunctivochalasis revealed marked staining for MMP-3 and -9, both in the epithelium and conjunctival stroma compared with that in specimens obtained during cataract surgery from the age- and sex-matched control subjects (Fig. 5). In the patients with conjunctivochalasis, the average number of conjunctival epithelial cells and stromal fibroblasts positively stained for MMP-3 and -9 were significantly higher than the cell counts calculated from the age- and sex-matched control conjunctival specimens obtained from the subjects undergoing cataract surgery (Fig. 6).

Verhoeff–Van Gieson Staining for Conjunctival Elastic Fibers

Elastic fibers in the specimens appeared blue-black to black, cellular nuclei appeared dark brown to black, collagen fibers stained red, and other tissue elements stained yellow. All conjunctival resection specimens from the patients with conjunctivochalasis revealed marked accumulation of elastic fibers compared with that in specimens obtained during cataract surgery from the age- and sex-matched control subjects. Representative staining is shown in Figure 7.

Transmission Electron Microscopy of the Conjunctival Samples

The conjunctival epithelial cells in specimens obtained during cataract surgery were compact, densely packed, and firmly adherent to one another, whereas the conjunctival epithelial cells from the patients with conjunctivochalasis were loosely...
adherent and displayed decreased intercellular cohesiveness. Some conjunctival epithelial cells from the patients with conjunctivochalasis showed signs of apoptosis, such as pyknotic nuclei or clumping of nuclear material, which was not observed in the specimens from the age- and sex-matched control subjects (Figs. 8A, 8B). The conjunctival stromas from the patients with conjunctivochalasis showed marked accumulation of elastic fibers compared with that in the control samples obtained during cataract surgery (Figs. 8C, 8D).

**DISCUSSION**

This preliminary research on conjunctival tissue samples obtained from conjunctivochalasis patients showed the presence of oxidative stress evidenced by a significantly higher number of cells positively stained for the lipid oxidation marker, HEL and the DNA oxidative damage marker 8-OHdG, compared with the cell counts in conjunctival samples of healthy age- and sex-matched individuals who underwent cataract surgery and consented to conjunctival tissue biopsy for investigational purposes.

Although we did not observe marked inflammatory cell infiltration in conjunctival tissue samples of patients with conjunctival laxity similar to observations made by Watanabe et al.\(^21\) we found increased levels of the proinflammatory cytokines TNF-\(\alpha\), IL-1\(\beta\), IL-6, IL-8, and IL-12 in tears of patients with conjunctivochalasis. It has been reported that silent inflammation originating from the vascular endothelium due to build up of reactive oxygen species and increased oxidative stress status may initiate NF-\(\kappa\)B pathway–mediated inflammation, with elevation of tissue cytokines without significant recruitment of inflammatory cells.\(^22\) We believe that the higher tear film inflammation observed in conjunctivochalasis may result from similar events or may be partly due to mechanical rubbing of the chalatic conjunctiva during blinking and ocular movements, resulting in release of cytokines into the tear film by the conjunctival epithelium or endothelia of conjunctival vessels. Although tear clearance was not measured in this cohort of
patients, delayed tear clearance is a well-known feature of conjunctivochalasis.\textsuperscript{23} It is possible that mechanical obstruction of the tear meniscus and puncta with delayed tear clearance amplified cytokine levels already present in the tears. Indeed, delayed tear clearance has been shown in previous studies to be associated with increased tear film and ocular surface inflammation.\textsuperscript{24}

How the increased tear film and ocular surface inflammation relates to the collagenolytic activity, presumed by Meller and Tseng\textsuperscript{1,3} to be one of the central events in the pathogenesis of conjunctival laxity, remains an unanswered question.

Previously, Li et al.,\textsuperscript{25} reported that the MMP-1 and \textsuperscript{-3}, which are involved in connective tissue degradation and remodeling, are overexpressed and have enhanced activity in cultured conjunctivochalasis fibroblasts. Meller et al.,\textsuperscript{3} later suggested that this upregulation in MMPs may be due to the inflammatory cytokines IL-1\beta and TNF-\alpha, which have been reported to stimulate ROS production. Of interest, conjunctival tissues obtained during conjunctival resection surgery revealed significantly increased staining of the epithelium and conjunctival stromal fibroblasts with markers for oxidative lipid peroxidation and DNA damage as well as markers of collagenolytic activity, including MMP-3 and \textsuperscript{-9}, compared with findings in the control samples obtained during cataract surgery.

Among the lipid peroxidation markers we studied, HEL is known to be formed from an early byproduct of lipid peroxidation and is thus thought to be an important marker of early-stage oxidative damage.\textsuperscript{26} DNA bases are also very susceptible to ROS oxidation,\textsuperscript{27} and the predominant detectable oxidation product of DNA bases in vivo is 8-OHdG. We believe that the inflammation of the tear film, the presence of conjunctival oxidative stress evidenced by positive staining for markers of lipid peroxidation and DNA damage, and the increased collagenolytic activity evidenced by marked MMP-3 and \textsuperscript{-9} levels in the epithelium and stromal fibroblasts play a role in the pathogenesis of conjunctivochalasis and may relate to the clinical findings and histopathologic alterations, such as sparsely assembled collagen fibers, elastic fiber accumulation, and/or fragmentation. Indeed, Verhoeff staining and TEM observations by us showed marked accumulation of elastic fibers in tissue samples of the patients with conjunctival laxity compared with that in the age- and sex-matched control subjects. TEM also disclosed decreased conjunctival epithelial cell cohesion. It should be noted, however, that these preliminary observations, which we hope will stimulate further research,

\begin{figure}
\centering
\includegraphics[width=\textwidth]{FIGURE6.png}
\caption{Comparison of Cell Counts Positively Stained for MMP-3 and MMP-9 between Conjunctivochalasis Patients and Controls}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{FIGURE7.png}
\caption{Verhoeff–Van Gieson staining for conjunctival elastic fibers. Note the marked accumulation of elastic fibers in conjunctival resection specimens from the patients with conjunctivochalasis compared with that in specimens obtained during cataract surgery from age- and sex-matched control subjects.}
\end{figure}
should be backed up with biochemical and enzymatic verification in future studies.

In this study, we found significant correlations between tear IL-1β and −6 levels and corneal epithelial damage scores. Tear IL-1β concentration also correlated with cell counts that were observed to be positively stained with lipid peroxidation and DNA damage markers. Tear TNFα concentration correlated with positive staining for the DNA oxidative stress marker 8-OHdG. Such correlations were not observed for degradation of conjunctival stromal matrix (data not shown). We also noted a positive correlation between tear film peroxidation marker concentration and conjunctival tissue stain counts for the same marker, suggesting the presence of increased lipid peroxidation in tears and the ocular surface. Even though we observed positive correlations, these results should be interpreted with caution, in that they indicate no causal relationship between oxidative stress and inflammation, or between either of them and degradation of matrix, until our observations are repeated on a larger number of subjects in future studies. It is also a possibility that reflex tearing induced during tear collections may have influenced the tear cytokine measurements. Of clinical interest was our observation that we noted marked staining of vascular capillary endothelial cells for lipid and DNA oxidative stress markers in specimens from patients with conjunctivochalasis. Increased oxidative stress in vascular endothelium has been linked to vasculopathy and vascular incidents in the heart, brain, and kidneys. Apart from mechanical friction (with the conjunctiva being sandwiched between the lids during blinking) as a possible cause of recurrent subconjunctival bleeding episodes in our patients, oxidative stress–induced vasculopathy may be another explanation for conjunctivochalasis. Furthermore, time-wise changes in ocular surface and tear film inflammation and oxidative damage markers in patients with various grades of conjunctivochalasis would provide invaluable insight into the natural history and provide clues about the pathogenesis of conjunctivochalasis.

References


