Effects of Azithromycin on Gene Expression Profiles of Proinflammatory and Anti-inflammatory Mediators in the Eyelid Margin and Conjunctiva of Patients With Meibomian Gland Disease

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IMPORTANCE Topical application of azithromycin suppresses expression of proinflammatory mediators while restoring transforming growth factor β1 (TGF-β1) levels as evaluated by eyelid margin and conjunctival impression cytology.

OBJECTIVE To explore the effects of azithromycin therapy on expression of proinflammatory and anti-inflammatory mediators in meibomian gland disease (MGD).

DESIGN, SETTING, AND PARTICIPANTS Case-control study performed in a clinic setting from August 17, 2010, to December 31, 2010. Sixteen patients with posterior blepharitis and conjunctival inflammation due to MGD were treated with azithromycin, 1%, drops for 4 weeks. Impression cytology of the lower eyelid margin and tarsal conjunctiva to measure cytokine expression by quantitative real-time polymerase chain reaction as well as tear collection to measure matrix metalloproteinase 9 (MMP-9) activity were performed once in 8 asymptomatic healthy control participants and 5 times in the 16 symptomatic patients (every 2 weeks for 8 weeks), before, during, and after azithromycin treatment.

EXPOSURE Azithromycin, 1%, drops for 4 weeks.

MAIN OUTCOMES AND MEASURES Cytokine expression in the eyelid margin and conjunctiva, and MMP-9 activity in tears.

RESULTS Compared with a 1-time measurement of 8 healthy participants, among 16 symptomatic patients, the mean (SD; 95% CI) fold change of expression of proinflammatory mediators interleukin-1β (IL-1β), IL-8, and MMP-9 increased to 13.26 (4.33; 11.14-15.38; \( P < .001 \)), 9.38 (3.37; 7.73-11.03; \( P < .001 \)), and 13.49 (4.92; 11.08-15.90; \( P < .001 \)), respectively, in conjunctival cells and to 11.75 (3.96; 9.81-13.69; \( P < .001 \)), 9.31 (3.28; 7.70-10.92; \( P < .001 \)), and 11.52 (3.50; 9.81-13.24; \( P < .001 \)), respectively, in the eyelid margin of patients with MGD. In contrast, the mean (SD; 96% CI) fold change of expression of TGF-β1 messenger RNA (mRNA) decreased to 0.58 (0.25; 0.46-0.70; \( P = .02 \)) and 0.63 (0.14; 0.56-0.70; \( P = .02 \)) in conjunctival and eyelid margin cells, respectively, of patients with MGD. Azithromycin, 1%, caused a change in the expression pattern of these mediators toward normal levels during 4 weeks of treatment. Levels of IL-1β, IL-8, and MMP-9 mRNA remained suppressed, although they rebounded toward pretreatment values 4 weeks after azithromycin withdrawal. Expression of TGF-β1 increased during treatment and remained at levels similar to the healthy controls after drug withdrawal. Change in tear MMP-9 activity was similar to the pattern of MMP-9 transcripts.

CONCLUSIONS AND RELEVANCE While the study did not control for potential confounding factors over time independent of the intervention that may have contributed to the results, topical azithromycin suppressed expression of proinflammatory mediators and increased expression of TGF-β1 to normal levels. Increased TGF-β1 expression may contribute to the anti-inflammatory activity of azithromycin in MGD.
Blepharitis is one of the most common ocular surface disorders. The prevalence of this condition has ranged from 12% to 47% in previously reported surveys.\(^1,2\) Blepharitis can be broadly categorized into anterior and posterior, with the former involving an infectious and inflammatory condition of the external lamella of the eyelids and eyelashes, and the latter having atrophy and/or obstruction of the meibomian glands that is often accompanied by tear dysfunction and eyelid and conjunctival inflammation.\(^3\) Posterior blepharitis or meibomian gland disease (MGD) is the most prevalent type of blepharitis.

Although posterior blepharitis and meibomian gland dysfunction can develop in inflammatory conditions such as rosacea or atopic dermatitis, the etiology of most cases of posterior blepharitis remains to be determined.\(^4\) Inflammation induced by bacteria or microbial products has been implicated in the pathogenesis. Clinical improvement in symptoms and signs of meibomian disease has been observed following treatment with oral tetracyclines (doxycycline, tetracycline) and azithromycin.\(^5\) Tetracycline and azithromycin antibiotics have also been shown to have anti-inflammatory properties.\(^6,7\) A decrease in the level of the inflammatory protease matrix metalloproteinase 9 (MMP-9) in tears has been reported after 1 month of oral doxycycline therapy for MGD.\(^10\) Azithromycin was previously reported to inhibit production of inflammatory cytokines (interleukin 1β [IL-1β], IL-8) and MMPs (MMP-1, MMP-3, and MMP-9) by cultured human corneal epithelial cells stimulated with toll-like receptor agonists.\(^8\) However, to our knowledge, the effects of topically applied azithromycin on expression of inflammatory mediators in the eyelid and conjunctiva of patients with MGD have not been evaluated. Using impression cytology to obtain cells from the surface of the eyelid margin and conjunctiva, this study characterized the gene expression profiles of proinflammatory and anti-inflammatory mediators in samples from patients with MGD and further evaluated the anti-inflammatory properties of topical azithromycin application on the expression of these mediators in the eyelid margin and conjunctiva as well as its effects on tear MMP-9 activity.

### Methods

**Patients and Healthy Participants**
After receiving informed consent, 16 patients with untreated MGD, diagnosed by a single ophthalmologist (S.C.P.) at Alkek Eye Center, Houston, Texas, and 8 asymptomatic healthy individuals were recruited for study. Criteria for diagnosis of MGD included plugging of at least 4 of the central 10 meibomian glands on the lower eyelid, at least grade 2 (moderate) eyelid margin injection, and at least grade 1 (mild) erythema of the inferior bulbar and palpebral conjunctiva. Injection and erythema were graded on a scale of 0 to 4 using standardized photographic standards, with 4 being the most severe. Individuals were excluded if they were using any other topical treatment other than warm eyelid compresses and massage. The healthy controls had no signs of MGD and no conjunctival inflammation. Patients with MGD received topical treatment with a US Food and Drug Administration–approved azithromycin, 1%, ophthalmic suspension, 1 drop twice a day for 2 days followed by 1 drop per day in the evening for 28 days in both eyes. Participants did not use any other treatment throughout the study period. The study was conducted between August 17, 2010, and December 31, 2010. The clinical protocol was approved by the Baylor College of Medicine Institutional Review Board and adhered to the tenets of the Declaration of Helsinki and the Association for Research in Vision and Ophthalmology statement on human subjects.

**Eyelid Margin and Conjunctival Impression Cytology**
After administration of topical anesthesia with proparacaine hydrochloride, 0.5%, a 2 × 6-mm or 3 × 4-mm sterile cellulose acetate filter membrane (Supor 450 Gridded; Pall Life Sciences) was placed on the center of the lower eyelid margin or on the inferior bulbar conjunctiva, respectively, and the surface of the membrane was firmly rubbed over its entire length 3 times with blunt tying forceps. The membrane was then removed with the forceps, immediately placed into a tube containing 350 μL of lysis buffer (Qiagen), and stored at −80°C until total RNA was extracted.

**RNA Extraction, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction**
Total RNA was extracted with the RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions, measured with a NanoDrop spectrophotometer (ND-1000; Thermo Scientific), and stored at −80°C. First-strand complementary DNA (cDNA) was synthesized by reverse transcription from 100 ng of total RNA using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) as previously described.\(^12,13\) Quantitative real-time polymerase chain reaction was performed using a Mx3005P system (Stratagene) with a 20-μL reaction volume containing 5 μL of cDNA, 1 μL of gene expression assay mix, and 10 μL of TaqMan gene expression master mix (Applied Biosystems by Life Technologies). TaqMan gene expression assays were used to evaluate glyceraldehyde-3-phosphate dehydrogenase (Assay ID Hs99999905_m1), IL-1β (Assay ID Hs99999905_m1), IL-1β (Assay ID Hs99999905_m1), and IL-1β (Assay ID Hs99999905_m1).
Hs00174097_m1), IL-8 (Assay ID Hs00174103_m1), MMP-9 (Assay ID Hs00234579_m1), and transforming growth factor β1 (TGF-β1; Assay ID Hs99999918_m1). The thermocycler parameters were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. An nontemplate control was included to evaluate DNA contamination. The results were analyzed by the comparative threshold cycle method and normalized by glyceraldehyde-3-phosphate dehydrogenase as an internal control.\textsuperscript{14}

**Tear MMP-9 Activity Assay**

Total MMP-9 enzyme activity was measured with an MMP-9 activity assay kit (GE Biosciences) as previously reported.\textsuperscript{15} One microliter of unstimulated tears was collected with a glass capillary tube from the central inferior tear meniscus of the left eye and was eluted into a tube containing 9 μL of buffer (phosphate-buffered saline with 0.1% bovine serum albumin). In brief, 100 μL of each pro–MMP-9 standard (0.125-4 ng/mL), tears (10 μL of diluted tears and 90 μL of assay buffer), and assay buffer (for blanks) were incubated at 4°C overnight in wells of a microtitr plate precoated with anti–MMP-9 mouse monoclonal capture antibody. Plates were washed 3 times with 0.01M sodium phosphate buffer (pH 7.0) containing 0.05% Tween 20. To measure total MMP-9 activity, bound pro–MMP-9 was activated with 50 μL of 1mM p-aminophenylmercuric acetate in assay buffer at 37°C for 1.5 hours. Detection reagent (50 μL) was added to each well and samples were incubated at 37°C for 6 hours. Active MMP-9 was detected through its ability to activate a modified prodetection enzyme that subsequently cleaved its chromogenic peptide substrate. Absorbance was read at 405 nm in a microplate reader (Versamax; Molecular Devices). The activity of MMP-9 in a sample was determined by interpolation from a standard curve. Tear sample absorbance readings were multiplied by a dilution factor of 100.

**Statistical Analysis**

One-way analysis of variance was used to make comparisons among 3 or more groups, followed by Dunnett post hoc test, and \(t\) test was used to compare differences between 2 groups. GraphPad Prism version 6 statistical software (GraphPad Software Inc) was used for statistical analysis. \(P < .05\) was considered statistically significant.

**Results**

**Gene Expression Profiles of Proinflammatory and Anti-inflammatory Mediators by Conjunctival and Eyelid Margin Cells in Patients With MGD**

Impression cytology was performed in 16 patients with MGD (mean age, 54 years; range, 34-80 years) and 8 healthy control participants with no signs of MGD (mean age, 28 years; range, 25-27 years). The mean age of the MGD group was significantly older than the control group (\(P = .002\)). Participants tolerated impression cytology of the eyelid margin and conjunctiva well with minimal or no reported irritation and no adverse effects. As evaluated by quantitative real-time polymerase chain reaction, the mean (SD) messenger RNA (mRNA) expression of inflammatory cytokine IL-1β, chemokine IL-8, and matrix metalloproteinase 9 (MMP-9) increased to 13.26 (4.33) fold (95% CI, 11.14-15.38; \(P < .001\)), 9.38 (3.37) fold (95% CI, 7.73-11.03; \(P < .001\)), and 13.49 (4.92) fold (95% CI, 11.08-15.90; \(P < .001\)), respectively, in conjunctival cells from patients with MGD compared with the mRNA levels found in specimens from healthy controls (Figure 1A). Expression of TGF-β1 was lower (mean [SD], 0.58 [0.25] fold; 95% CI, 0.46-0.70; \(P = .02\)) in conjunctival cells of patients with MGD compared with healthy controls. Similar to the conjunctival cells, mean (SD) expression of IL-1β, IL-8, and MMP-9 transcripts in eyelid margin cells increased to 11.75
Azithromycin Inhibited Expression of Proinflammatory Mediators and Increased Expression of TGF-β1 in Patients With MGD

Patients with MGD were topically treated with azithromycin, 1%, for 4 weeks. The proinflammatory profiles were evaluated every 2 weeks during treatment and for 1 month after treatment and were compared with levels obtained from a 1-time measurement of healthy participants. As shown in Figure 2A, the mean (SD) expression of IL-1β, IL-8, and MMP-9 transcripts in conjunctival cells decreased to 2.81 (1.58) fold (95% CI, 2.04-3.58; \( P < .001 \)), 4.20 (2.55) fold (95% CI, 2.95-5.45; \( P = .002 \)), and 3.90 (2.20) fold (95% CI, 2.82-4.98; \( P < .001 \)), respectively, after 2 weeks of azithromycin treatment, while the mean (SD) expression of TGF-β1 increased to slightly higher than the levels in healthy controls (1.27 [0.44] fold; 95% CI, 1.05-1.49; \( P = .002 \)) compared with pretreatment levels. After 4 weeks of azithromycin treatment, expression of IL-1β, IL-8, and MMP-9 decreased further or remained lower (IL-1β: mean [SD], 2.88 [2.64] fold; 95% CI, 1.59-4.17; \( P < .001 \); IL-8: mean [SD], 2.82 [2.20] fold; 95% CI, 1.74-3.90; \( P < .001 \); and MMP-9:
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Discussed in the conjunctival and eyelid margin cells. Distribution to the changes in expression of these inflammatory mediators were still lower than the pretreatment levels. Four weeks after azithromycin withdrawal, expression of IL-1β, IL-8, and MMP-9 remained lower than pretreatment levels. At 2 and 4 weeks after the drug was stopped, the mean (SD) TGF-β1 mRNA decreased slightly to 0.99 (0.38) fold (95% CI, 0.80-1.18; \( P = .01 \)) and 0.92 (0.44) fold (95% CI, 0.70-1.14; \( P = .01 \)), respectively, but remained higher than pretreatment levels.

As shown in Figure 2B, the expression patterns of IL-1β, IL-8, MMP-9, and TGF-β1 in eyelid margin cells showed a response to azithromycin similar to that in the conjunctival cells. The levels of IL-1β, IL-8, and MMP-9 decreased after azithromycin treatment for 2 and 4 weeks, and they remained lower after the drug was stopped. After azithromycin treatment, levels of TGF-β1 transcripts increased to slightly higher than those for the healthy controls and remained near the normal levels for 4 weeks after the drug was stopped. It should be noted that the study did not control for potential confounding factors over time independent of the intervention with azithromycin that may have contributed to the changes in expression of these inflammatory mediators in the conjunctival and eyelid margin cells.

**Azithromycin Suppressed Tear MMP-9 Activity in Patients With MGD**

To further investigate the anti-inflammatory activity of azithromycin, we evaluated MMP-9 activity in tears collected at 5 times from patients with MGD, before, during, and after azithromycin treatment. Compared with healthy controls, tear MMP-9 activity was 10-fold higher in tears obtained from patients with MGD. The mean (SD) tear MMP-9 activity decreased from 30.1 (12.6) ng/mL (95% CI, 23.93-36.27) to 9.4 (4.8) ng/mL (95% CI, 7.05-11.75; \( P < .001 \)) after azithromycin treatment for 2 weeks and 6.1 (3.9) ng/mL (95% CI, 4.19-8.01; \( P < .001 \)) after azithromycin treatment for 4 weeks. Interestingly, tear MMP-9 activity remained 70% lower than pretreatment levels for up to 4 weeks after cessation of azithromycin (Figure 3). As noted for the polymerase chain reaction results, the study did not control for potential confounding factors over time independent of the intervention with azithromycin that may have contributed to the changes in MMP-9 activity in tears.

**Discussion**

Meibomian gland disease is one of the most common ocular surface diseases. It can be associated with rosacea dermatitis and can cause conjunctival or corneal inflammation and tear instability. It is typically chronic, and the MGD workshop proposed treatment recommendations that included use of anti-inflammatory therapy. Our study revealed an interesting gene expression profile in the conjunctival and eyelid margin cells with elevated expression of proinflammatory mediators IL-1β, IL-8, and MMP-9 and reduced expression of anti-inflammatory cytokine TGF-β1 in patients with MGD compared with a younger healthy control group with no signs of MGD. We further demonstrated that the topical application of an azithromycin, 1%, ophthalmic solution altered this pattern by reversing the expression of these proinflammatory and anti-inflammatory mediators in patients with MGD. These findings suggest that the altered expression of these proinflammatory and anti-inflammatory mediators may be involved with the eyelid and ocular surface inflammation that develops in MGD. To our knowledge, the effects of azithromycin on expression of these mediators have not been previously evaluated in patients with MGD.

Azithromycin is a macrolide antibiotic used to treat or prevent certain bacterial infections such as otitis media, tonsillitis, pharyngitis, sinusitis, pneumonia, bronchitis, periodontitis, urethritis, and chlamydial infection. Azithromycin has also been used topically to treat ocular surface infections including bacterial conjunctivitis. In addition to its antibiotic effects, azithromycin has been shown to have a variety of anti-inflammatory effects, particularly in the context of microbial infections. Our previous study showed that azithromycin suppressed zymosan-induced mRNA expression and protein production of proinflammatory cytokines (tumor necrosis factor α and IL-1β), chemokines (IL-6 and RANTES), and MMPs (MMP-1, MMP-3, and MMP-9) by human corneal epithelial cells and suggested the potential for using azithromycin...
Impression cytology has been widely used not only to facilitate the diagnosis of ocular surface disorders, including keratoconjunctivitis sicca, ocular surface squamous neoplasia, and ocular surface infections, but also to improve the understanding of pathogenesis of ocular surface diseases. Impression cytology has been successfully used to observe changes in cellular morphology and identify inflammatory markers. Some studies have also evaluated the expression of proinflammatory mediators in conjunctival impression cytology specimens. To our knowledge, this is the first study to assess the efficacy of topical azithromycin in blepharitis by evaluating the gene expression profiles of inflammatory mediators using eyelid margin and conjunctival impression cytology.

In this study, we performed impression cytology to obtain eyelid margin and conjunctival cells at 5 sequential visits for comparison of mRNA expression of inflammatory-associated mediators in patients with MGD before, during, and after topical azithromycin treatment compared with levels obtained from a 1-time measurement in healthy controls. The procedure was well tolerated with no adverse events. Interestingly, we observed that expression levels of major proinflammatory mediators IL-1β, IL-8, and MMP-9 were much higher in patients with MGD blepharoconjunctivitis than in healthy controls (P < .001). The elevated levels of those mediators gradually decreased (P < .001) during 4 weeks of azithromycin treatment. Expression of IL-1β, IL-8, and MMP-9 remained suppressed at the 4-week follow-up after drug withdrawal, although there was a slight rebound from their lowest point after 4 weeks of azithromycin treatment. The tear MMP-9 activity assay further confirmed higher MMP-9 activity in patients with MGD and the suppressive effects of topical azithromycin on this activity. These findings suggest that patients with MGD-associated eyelid and conjunctival inflammation may need intermittent pulse therapy with a topical anti-inflammatory agent such as azithromycin.

Surprisingly, we found that TGF-β1 expression was much lower in patients with MGD than in healthy controls and that it increased in the eyelid margin and conjunctiva during treatment with azithromycin. The TGF-β1 expression was still higher than in healthy controls 4 weeks after azithromycin withdrawal. Transforming growth factor β1 is a polypeptide member of the TGF-β cytokine superfamily. It was first identified in human platelets with a potential role in wound healing. Many types of cells secrete TGF-β, including human corneal and conjunctival epithelia as well as lacrimal gland acinar cells. The pivotal function of TGF-β in the immune system is to maintain tolerance via the regulation of lymphocyte proliferation, differentiation, and survival. It controls the initiation and resolution of inflammatory responses through the regulation of chemotaxis, activation, and survival of lymphocytes. The anti-inflammatory role of TGF-β1 has been recognized in different cell types by inhibiting proinflammatory cytokines including tumor necrosis factor α, IL-1, and interferon γ. In this study, we observed that TGF-β1 expression was lower in patients with MGD than in healthy controls but increased after treatment with azithromycin, suggesting that TGF-β1 may contribute to the clinical improvement that has been observed. Transforming growth factor β also has the potential to induce fibrosis, but this adverse effect has not been reported to occur with topical azithromycin therapy.

There are several limitations of this study. First, changes in levels of inflammatory mediators in the conjunctiva and eyelid margin are relative to a single measurement in a healthy control group. Second, there was no control group to determine whether there were confounding factors during the study period that could have influenced the expression of these inflammatory mediators in the conjunctival and eyelid margin cells independent of the treatment with azithromycin. A randomized clinical trial would be required to confirm the efficacy of azithromycin for treatment of the ocular surface inflammation in MGD.

Conclusions
Our findings demonstrate that impression cytology is a simple and noninvasive technique to sample eyelid margin and conjunctival cells in ocular surface diseases. Topical application of azithromycin suppresses the expression of proinflammatory mediators IL-1β, IL-8, and MMP-9 while increasing expression of TGF-β1.