Pro- and Anti-inflammatory Forms of Interleukin-1 in the Tear Fluid and Conjunctiva of Patients with Dry-Eye Disease

Abraham Solomon,[1] *Dilek Dursun,*[1] *Zuguo Liu,*[1,2] *Yuhuan Xie,*[1,2] *Angelo Macri,*[1] *and Stephen C. Pflugfelder*[1]

Purpose. To compare the expression of the pro- and anti-inflammatory forms of interleukin (IL)-1 in the tear fluid and conjunctival epithelium of normal eyes and those with dry-eye disease.

Methods. The concentrations of IL-1α, IL-1β (precursor and mature forms), and IL-1 receptor antagonist (IL-1Ra) were measured by ELISA in tear fluid samples obtained from normal individuals and patients with dry eye who had rosacea-associated meibomian gland disease (MGD) or Sjögren's syndrome (SS) aqueous tear deficiency (ATD). These cytokines were also measured in normal tear fluid before and after nasal stimulation to induce reflex tearing. The relative expression of these cytokines was evaluated in conjunctival impression cytology specimens and conjunctival biopsy tissue obtained from normal subjects and SS ATD–affected patients using immunofluorescent staining. Matrix metalloproteinase (MMP)-9 concentration and activity in the tear fluid were evaluated with gelatin zymography and with an MMP-9 activity assay kit, respectively.

Results. Compared with normal subjects, the concentration of IL-1α and mature IL-1β in the tear fluid was increased, and the concentration of precursor IL-1β was decreased in patients with MGD ($P < 0.05$, $P = 0.02$, and $P < 0.01$, respectively) and SS ATD ($P < 0.001$, $P = 0.02$, and $P < 0.001$, respectively). There was no significant change in the concentration of IL-1α, precursor IL-1β, and IL-1Ra in reflex tear fluid, indicating that the lacrimal glands may secrete these cytokines. The activity of MMP-9, a physiological activator of IL-1β, was significantly elevated in the tear fluid of both dry-eye groups compared with normal subjects. A strong positive correlation was observed between the intensity of corneal fluorescein staining and the tear fluid IL-1α concentration ($r^2 = 0.17$, $P < 0.02$) and the mature-to-precursor IL-1β ratio ($r^2 = 0.46$, $P < 0.001$). Positive immunofluorescent staining for IL-1α, mature IL-1β, and IL-1Ra was observed in a significantly greater percentage of conjunctival cytology specimens from eyes with SS ATD than in those from normal eyes ($P < 0.01$ for IL-1α, $P < 0.009$ for mature IL-1β, and $P < 0.05$ for IL-1Ra).

Conclusions. Dry-eye disease is accompanied by an increase in the proinflammatory forms of IL-1 (IL-1α and mature IL-1β) and a decrease in the biologically inactive precursor IL-1β in tear fluid. Increased protease activity on the ocular surface may be one mechanism by which precursor IL-1β is cleaved to the mature, biologically active form. The conjunctival epithelium appears to be one source of the increased concentration of IL-1 in the tear fluid of patients with dry-eye disease. These results suggest that IL-1 may play a key role in the pathogenesis of keratoconjunctivitis sicca. (*Invest Ophthalmol Vis Sci.* 2001; 42:2283–2292)

There is increasing evidence that dry eye is accompanied by subclinical ocular surface inflammation. The evidence for this inflammation includes increased expression of immune activation markers, such as HLA-DR, intercellular adhesion molecule (ICAM)-1, and CD-40, by the conjunctival epithelium and infiltration of the conjunctiva by inflammatory cells.[1–7] The importance of inflammation in the pathogenesis of dry eye is underscored by reports that the signs and symptoms of dry eye markedly improve with anti-inflammatory therapies such as glucocorticosteroids and cyclosporin.[8,9]

Our group has previously evaluated the levels of the inflammatory cytokines that are capable of modulating the expression of these inflammatory markers and of stimulating leukocyte chemotaxis onto the ocular surface of dry eyes. Our studies showed that the levels of RNAs encoding a number of different inflammatory cytokines, including interleukin (IL)-1, -6, and -8 and TNF-α, were elevated in the conjunctival epithelium of patients with Sjögren's syndrome (SS) keratoconjunctivitis sicca (KCS) compared with normal subjects.[10] The levels of IL-1 and -8 RNA are directly correlated with the intensity of corneal fluorescein staining and are inversely correlated with conjunctival goblet cell density. In a subsequent study, we found that the concentration of matrix metalloproteinase (MMP)-9, the principal MMP enzyme produced by the corneal epithelium and a key factor in the pathogenesis of sterile corneal ulceration, increases as tear clearance decreases.[11] The mechanism by which these inflammatory and matrix-degrading factors are upregulated in dry-eye disease has not been established.

The proinflammatory cytokine IL-1 is an important mediator of inflammation and immunity.[12] IL-1 has been implicated in the pathogenesis of human inflammatory diseases, such as septic shock, rheumatoid arthritis, and periodontitis,[13–15] as well as the corneal and ocular surface diseases rosacea, bullous keratopathy, keratoconus, and sterile corneal ulceration.[11,16–17] Both proinflammatory forms of IL-1 (IL-1α and -1β) are multifunctional cytokines that in general produce similar biological effects, although these may vary among different cell types and organ systems.[19,20] IL-1 is a potent inducer of other inflammatory cytokines such as IL-6 and -8, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF).[21,22] It also stimulates production of MMP enzymes by epithelial and inflammatory cells.[23] Both IL-1α and -1β are

From the [1]Ocular Surface and Tear Center, Bascom Palmer Eye Institute, Department of Ophthalmology, University of Miami School of Medicine, Florida; and the [2]Zhongshan Ophthalmic Center, Sun Yat-sen University of Medical Sciences, Guangzhou, China.

Supported in part by Fight-for-Sight Postdoctoral Fellowship PD98025; Grant EY11915, National Eye Institute, Bethesda, Maryland; an unrestricted grant from Research to Prevent Blindness; and the Drs. David and Maureen Smith Ocular Surface and Tear Research Fund.

Submitted for publication February 2, 2001; revised April 20, 2001; accepted May 15, 2001.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Stephen C. Pflugfelder, Cullen Eye Institute, 6565 Fannin, NC 205, Houston, TX 77030. stevenp@bcm.tmc.edu

Table 1. Demographic Characteristics of Study Patients

Group	*n*	Gender	Age	Age Range
Normal 1	10	6 women, 4 men	32 ± 6.6*	24–44
Normal 2	7	4 women, 3 men	46 ± 8.6†	35–57
SS ATD	9	8 women, 3 men	68 ± 9.1	53–80
MGD	13	7 women, 6 men	55 ± 16.3	25–73

Age is expressed as mean years ± SD.
* Normal 1 vs SS ATD $P < 0.001$; Normal 1 vs MGD $P = 0.0004$.
† Normal 2 vs SS ATD $P = 0.001$; Normal 2 vs MGD $P = 0.36$.

synthesized as precursor proteins with a molecular mass of approximately 33 kDa.[19] The precursor and the mature 17-kDa forms of IL-1α are both biologically active.[19] In contrast, the precursor form of IL-1β possesses minimal biological activity and requires cleavage to the 17-kDa mature form to become active.[19] This conversion occurs within cells by IL-1β–converting enzyme (also known as ICE or caspase 1) and in the extracellular environment by a number of proteases, including leukocyte elastase, granzyme A, and MMP-2 and -9.[12,24,25] Among a number of different MMPs evaluated, MMP-9 was found to be the most efficient activator of precursor IL-1β.[25]

IL-1Ra is a cytokine that inhibits the activities of the proinflammatory forms of IL-1 by competitively binding to the type 1 IL-1 receptor.[26] Administration of IL-1Ra has been found to be clinically beneficial in the treatment of arthritis and prevention of corneal transplant rejection in experimental models.[14,27] Both proinflammatory forms (IL-1α and -1β) and the anti-inflammatory form (IL-1Ra) of IL-1 have been detected in the human corneal epithelium.[28–31] IL-1Ra has also been detected in human conjunctival epithelial cells.[32] IL-1α and -1β have also been detected in human tear fluid.[11,33]

We hypothesized that increased concentration and/or activity of IL-1 could be an initiating factor for the observed ocular surface immunopathology of dry eye. This study was designed to test this hypothesis by comparing the concentrations of IL-1α, inactive precursor and active mature IL-1β and IL-1Ra in tear fluid samples obtained from patients with dry-eye disease who had rosacea-associated meibomian gland disease (MGD) or Sjögren's syndrome (SS) aqueous tear deficiency (ATD) and normal asymptomatic subjects. The relative levels of expression of the IL-1 family of cytokines in the conjunctival epithelium of normal subjects and patients with SS ATD, the dry-eye condition that has been reported to involve the most severe KCS,[34] were compared using immunofluorescent staining. The activity of MMP-9, a protease that activates precursor IL-1β in the extracellular environment was also evaluated. Finally, the tear fluid concentrations of lactoferrin, a protein secreted by the lacrimal glands into human tears that inhibits the formation of reactive oxygen species that can damage cells and promote production and release of IL-1 was measured.[35,36]

Materials and Methods

Materials

Rabbit anti-human polyclonal antibodies specific for IL-1α, the precursor and mature forms of IL-1β, and recombinant precursor and mature human IL-1β and ELISA kits for the precursor and mature forms of IL-1β were purchased from Cistron (Pine Brook, NJ); ELISA kits for IL-1α and IL-1Ra and recombinant human IL-1α and IL1-Ra and polyclonal antisera for IL-1Ra from R&D Systems (Minneapolis, MN); FITC-conjugated secondary antibodies from Caltag Laboratories (Burlingame, CA); and Texas red-conjugated secondary antibodies from Molecular Probes (Eugene, OR).

Patient Selection

This study was conducted according to a protocol approved by the University of Miami School of Medicine Institutional Review Board and in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from participants after the nature and possible consequences of the study were explained.

Tear fluid samples were obtained from 9 patients with primary SS ATD, 1 patient with non-SS ATD, 13 patients with rosacea-associated MGD, and 17 normal subjects. The demographic characteristics of these patients are presented in Table 1. One group of normal subjects consisted of six women and four men who had no history of eye disease or ocular surgery, did not use eye drops, and had no ocular irritation symptoms. All the subjects had a Schirmer 1 test score greater than 15 mm, normal meibomian glands, and no corneal fluorescein staining. This group was used for evaluating tear proteins in unstimulated tear fluid. A second group of asymptomatic normal subjects (four women and three men) who met the same criteria was used for evaluating the effects of reflex tearing on the concentration of the IL-1 family of cytokines in tear fluid. All normal subjects were recruited from the employees of the Bascom Palmer Eye Institute.

The SS group consisted of eight women and one man. Diagnosis of primary SS was based on criteria proposed by Fox et al.[37] and included the following: (1) a Schirmer 1 test score of 5 mm or less in at least one eye, (2) interpalpebral zone fluorescein dye staining of the conjunctiva

Table 2. Concentrations of IL-1 Family Cytokines in Unstimulated Tear Fluid Obtained from Normal Subjects and Patients with MGD and SS ATD

Cytokine	Normal (n = 10)	MGD (n = 13)	SS (n = 9)	P
IL-1α	43.1 ± 24	253.7 ± 90	443.3 ± 128.5	*<0.05 †<0.001
Precursor IL-1β	379.2 ± 73	54.6 ± 16	21.2 ± 10	*<0.01 †<0.0001
Mature IL-1β	29.8 ± 10	187.7 ± 72	80.9 ± 22	*0.02 †0.02
Precursor-mature IL-1β	19.1 ± 5	1.17 ± 0.7	0.35 ± 0.2	*†0.0001
IL-1 Ra ($\times 10^5$)	2.95 ± 1.27	9.40 ± 2.54	23.96 ± 12.35	*†NS
IL-1 Ra/IL-1α ($\times 10^3$)	27.86 ± 20.57	6.95 ± 6.6	5.01 ± 6.28	*0.012 †0.007
IL-1 Ra/mature IL-1β ($\times 10^3$)	40.5 ± 18.39	14.28 ± 42.59	70.29 ± 44.15	*0.0016 †NS

Data are expressed as mean picograms per milliliter.
* Normal vs MGD.

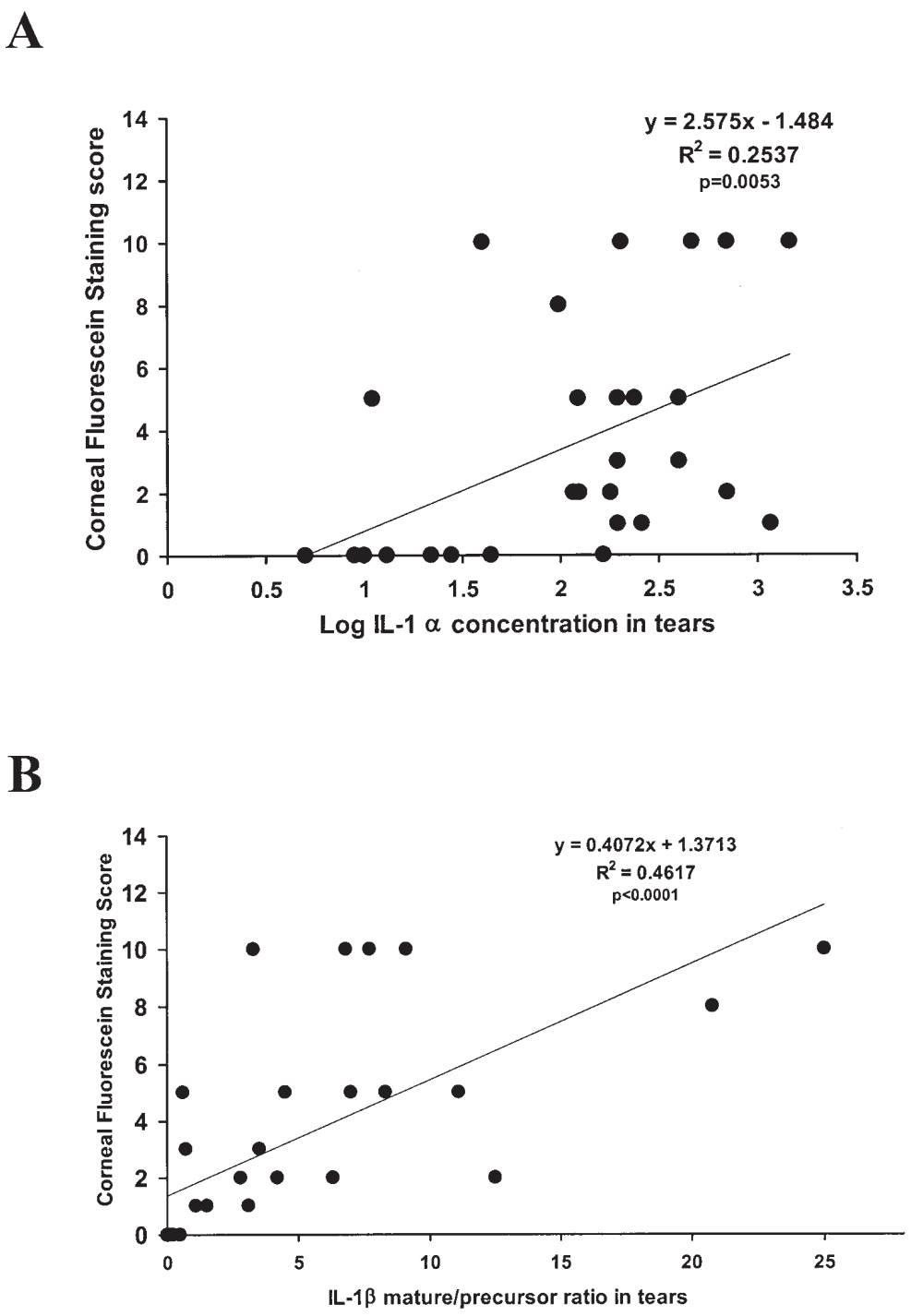

FIGURE 1. (**A**) Correlation between IL-1α concentration in tears and corneal fluorescein staining scores. (**B**) Correlation between mature-precursor IL-1β ratio in tears and corneal fluorescein staining scores.

and cornea, (3) xerostomia, (4) elevated serum autoantibody titers (antinuclear antibody and/or rheumatoid factor titer $\geq$1:160), and (5) symptoms of moderate to severe ocular irritation.[37] The patient with non-SS ATD was a 67-year-old woman who had a Schirmer 1 test score of 4 mm in both eyes and interpalpebral corneal and conjunctival staining, but did not have xerostomia or serum autoantibodies.

The MGD group consisted of seven women and six men with the chief symptoms of ocular irritation and/or redness that was diagnosed as ocular rosacea with MGD, according to previously reported criteria.[38] Corneal fluorescein staining was graded using a previously reported method.[39] The cornea was examined under blue-light illumination 2 minutes after instillation of 5 μl 2% fluorescein into the tear film. The intensity of the corneal fluorescein staining was graded in each of a standardized four-point scale (0, no staining; 1, mild; 2, moderate; and 3, intense). The range of staining scores was 0 to 12. Patients with dry eye were excluded if they had any clinical signs of external ocular infection, including staphylococcal blepharitis or bacterial conjunctivitis.

Tear Fluid Collection and Sample Extraction

For experiments comparing the concentrations of proteins in unstimulated tear fluid, a tear fluid sample was collected from the inferior tear meniscus of both eyes, causing the least irritation possible, using a preweighed polyester wick (Transorb rods; American Filtrona, Richmond, VA) to obtain the sample, as previously described.[11,40] The

Table 3. Poststimulation Tear Fluid Concentrations of IL-1α, Precursor IL-1β, and IL-1Ra in Seven Normal Subjects

Cytokine	Prestimulation	Poststimulation 1 (1 minute)	Poststimulation 2 (5 minutes)	*P**
IL-1α	53.6 ± 26.8†	35.2 ± 10.0	108.7 ± 48.6	NS
Precursor IL-1β	712 ± 395‡	92.1 ± 68	384.7 ± 188	NS
IL-1 Ra ($\times 10^4$)	23.81 ± 11.25§	22.13 ± 81.37	113.67 ± 73.65	NS

Data are expressed as mean picograms per milliliter.

* NS = no significant within group differences by ANOVA and no significant differences between the prestimulation concentration and either poststimulation concentration or between the 1- and 5-minute poststimulation concentrations, by the Wilcoxon test.

† $P = 0.01$ compared with MGD; $P = 0.006$ compared with SS ATD (Table 2).

‡ $P = 0.03$ compared with MGD; $P = 0.05$ compared with SS ATD (Table 2).

§ $P = 0.01$ compared with MGD; $P = 0.04$ compared with SS ATD (Table 2).

immediately after tear collection (model GA110 scale; Ohaus, Florham Park, NJ). Wicks were then placed into the end of a micropipette tip located within a 0.5-ml tube (Eppendorf, Fremont, CA) as described by Jones et al.[40] For experiments evaluating the concentrations of IL-1 cytokines in reflex tear fluid, an unstimulated tear fluid sample was collected, and reflex tearing was induced by placing a dry cotton-tipped applicator under the middle nasal turbinate, advancing it until the nasal membrane of the ethmoid sinus was reached, then rotating it for 1 minute as previously described.[41] Tear fluid was then collected from the ipsilateral eye 1 and 5 minutes after stimulation.

Tears were extracted from the saturated wicks by centrifuging them at 12,000 rpm for 5 minutes within the pipette tip after adding a volume of buffer (50 mM Tris/HCl, 0.15 M NaCl, 10 mM $CaCl_2$, 0.005% Brij35, 0.02% sodium azide [pH 7.5]) 10 times greater than the original volume of the tear sample. The rods and pipette tips were carefully removed and the tear fluid aspirated. Tear fluid from both eyes was combined. This method resulted in a final tear dilution factor of 1:11 for the ELISA, gelatin zymography, and the MMP-9 activity assay. Tear samples were placed in numbered 500-μl tubes (Eppendorf) and stored at −80°C for 3 to 7 days until they were used.

Subjects with dry eye were not receiving any eye treatment other than nonpreserved artificial tears, and they were instructed not to instill any eye drops on the day the tear collection was performed. Tear collection was repeated on a separate day in all subjects, and both tear collections were performed in the morning. Tear fluid from one tear collection was used for cytokine ELISAs, and tear fluid from the second collection was used for the lactoferrin ELISA, gelatin zymography, and the MMP-9 activity assay.

IL-1α, -1β, and -1Ra and Lactoferrin ELISAs

The concentrations of IL-1 family cytokines were determined with commercial ELISA kits. For these assays, tear samples were diluted in ELISA buffer (supplied by the manufacturer) to a final volume of 100 to 200 μl. These assays were performed as recommended by the manufacturer. Tear lactoferrin concentration was measured with an immunoassay (Touch Tear MicroAssay System; Touch Scientific, Raleigh, NC).

Gelatin Zymography and MMP-9 Activity Assay

Gelatinase level in the tear fluid was measured by gelatin zymography, as previously described.[11] Diluted tear samples (all at a dilution of 1:11) were incubated with SDS-gel sample buffer for 30 minutes at room temperature and analyzed by electrophoresis on a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the proteins were renatured by removing SDS from the gel using two washes of 0.25% Triton X-100 (30 minutes per wash). This was followed by an 18-hour incubation at 37°C in the digestion buffer consisting of 50 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 10 mM $CaCl_2$, 2 μM $ZnSO_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.005% Brij35, and 0.02% sodium azide. After this incubation, the gel was briefly rinsed in distilled water and stained with 0.25% Coomassie brilliant blue R250 prepared in 40% isopropanol solution for 1 hour. The gel was destained with 7% acetic acid. Gelatinase activity in the gel was visible as a clear area in the blue background, indicating an area where the gelatin had been digested. The minimum sensitivity of this technique for detecting gelatinase B is 0.05 ng/lane. The molecular weight of gelatinases in the tear fluid was determined from molecular weight standards (prestained broad range standards; Bio-Rad, Hercules, CA) and 0.1 ng purified rabbit 92-kDa progelatinase B (Oncogene Research, Cambridge, MA) that were run in separate lanes on the gel. These gels were photographed with a camera (Polaroid, Cambridge, MA), and the photographs were scanned (Scan Jet 4C scanner; Hewlett-Packard, Palo Alto, CA) into a computer.

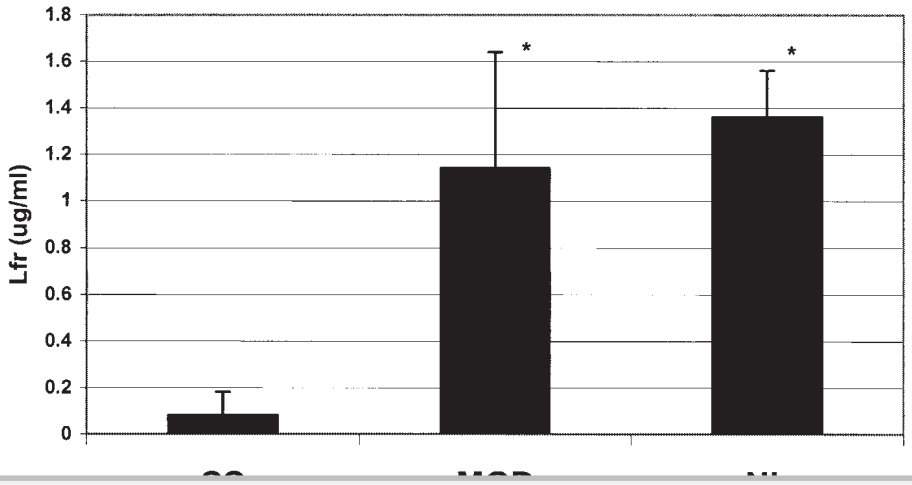

Figure 2. Lactoferrin concentration in tears from normal subjects, patients with MGD, and patients with SS ATD. *$P < 0.01$ compared

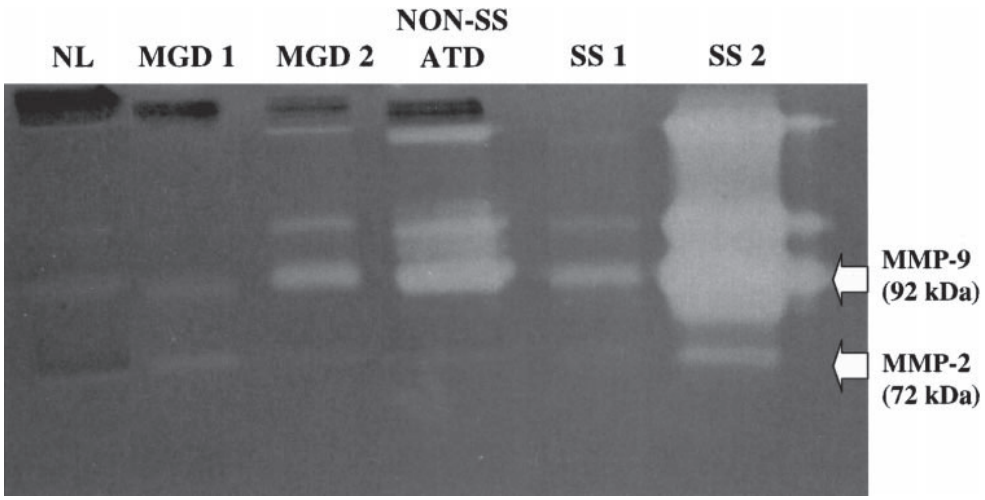

FIGURE 3. Gelatin zymogram of tear samples from a normal subject, two patients with MGD, a patient with non-SS ATD, and two patients with SS ATD. The 92-kDa pro-MMP-9 and 72-kDa pro-MMP-2 bands are marked. Sample SS2 (*right lane*) was obtained from a 75-year-old patient with SS who had a 30-year history of bilateral recurrent sterile corneal ulceration with perforation.

MMP-9 activity in tear fluid was measured with an MMP-9 activity assay system (Biotrak; Amersham Pharmacia Biotech, Piscataway, NJ), according to a previously published protocol.[42] This colorimetric assay captures MMP-9 in the tear fluid and measures its activity in cleaving a modified prodetection enzyme and the subsequent cleavage of its chromogenic peptide substrate.[43]

Immunofluorescent Staining of Conjunctival Impression Cytology and Conjunctival Biopsy Specimens

Impression Cytology. The expression of four forms of IL-1 (IL-1α, precursor and mature IL-1β, and IL-1Ra) was evaluated in conjunctival impression cytology specimens obtained from 6 ideal normal subjects and 16 patients with SS ATD.

Impression cytology was performed by lightly pressing a membrane (Biopore; Millipore, Bedford, MA) against the nasal, inferior, and temporal bulbar conjunctiva, 1 mm behind the limbus after instillation of topical anesthesia (0.5% proparacaine hydrochloride). The membranes were placed in a container and stored at −80°C until they were processed. Before staining, these membranes were fixed with cold methanol for 10 minutes and were then blocked for 20 minutes with PBS containing 2% fetal bovine serum (FBS) to prevent nonspecific staining. The cytology specimens obtained from the nasal and temporal bulbar conjunctiva were bisected, and one of the four primary antibodies (IL-1α, precursor and mature IL-1β and -1Ra) was applied to each membrane for 1 hour at room temperature in a moist chamber. Membranes were rinsed three times with PBS and incubated for 1 hour with FITC-conjugated secondary antibody. The cytology specimens obtained from the inferior bulbar conjunctiva was treated only with secondary antibody and served as a negative control. Membranes were washed three times with PBS, placed on a glass microscope slide, and covered with nonfade mounting medium (Fluoromount-G; Southern Biotechnology Associates, Birmingham, AL) and a glass coverslip. Specimens were examined and photographed with a microscope (Axiophot II; Nikon, Tokyo, Japan) using TMAX 400 film (Eastman Kodak, Rochester, NY). The fluorescein staining was visually graded negative if the staining was less than or equal to the secondary antibody control and positive if it was greater than the secondary antibody control by two independent observers.

Conjunctival Biopsy. Samples were taken from the superior or superotemporal bulbar conjunctiva of three normal subjects with Schirmer 1 test scores of 15 mm or more, no corneal fluorescein staining, and no lid or conjunctival inflammation, and three patients with SS KCS during cataract surgery. Tissue specimens were placed in DMEM (Life Technologies, Gaithersburg, MD) for transport and then were embedded in optimal cutting temperature (OCT) compound (Tissue Tek, Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C. Within 72 hours, serial 4- to 5-μm-thick sections were cut. Indirect immunofluorescence staining on tissue sections was performed by a previously reported technique,[44] using polyclonal anti-

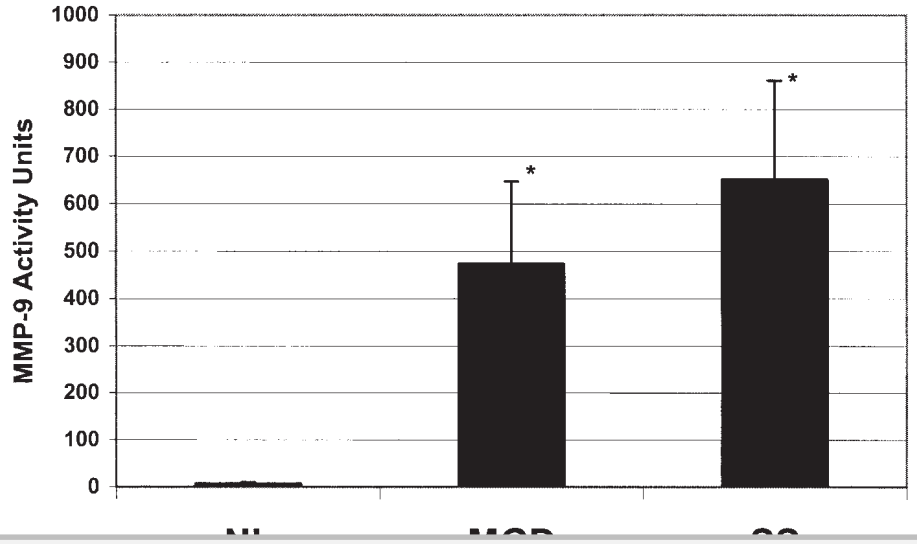

FIGURE 4. MMP-9 activity in tear samples of normal subjects, patients with MGD, and patients with SS ATD, measured by an MMP-9 activity assay. *$P < 0.001$ compared with normal

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.