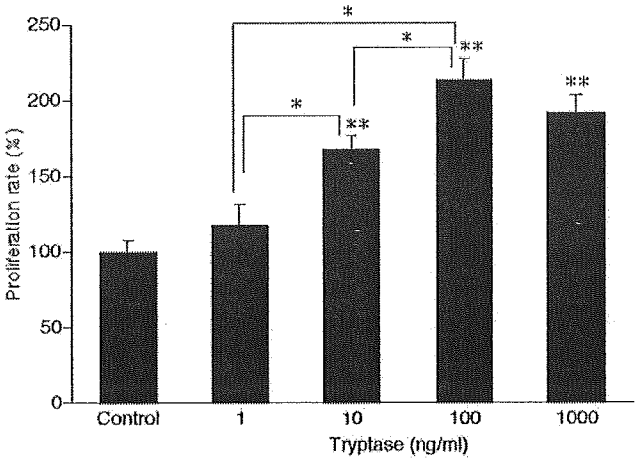

FIGURE 1. Proliferation of cultured conjunctival fibroblasts incubated with various concentration of tryptase. Tryptase increased the proliferation of conjunctival fibroblasts in a statistically significant and dose-dependent manner (*$P < 0.05$; **$P < 0.05$ compare to control). No further increase above 100 ng/mL was observed. The data are representative of experiments using fibroblasts from three different donors.

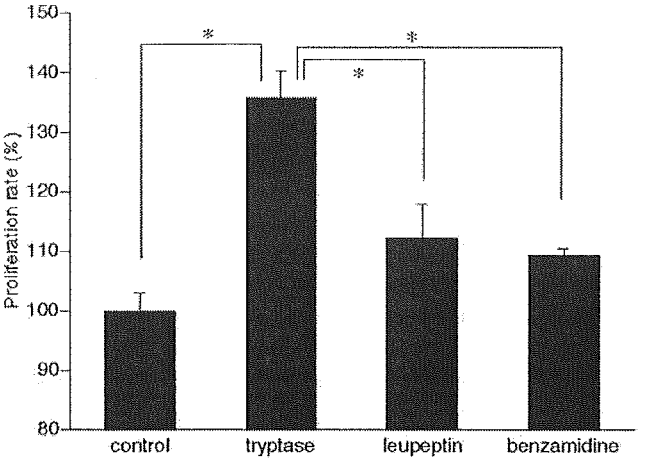

FIGURE 3. Effects of the protease inhibitors leupeptin and benzamidine on tryptase-induced conjunctival fibroblast proliferation. Both leupeptin (10^{-4} M) and benzamidine (10^{-4} M) significantly inhibited 100 ng/mL of tryptase-induced conjunctival fibroblast proliferation (*$P < 0.05$).

performed in triplicate, using fibroblasts from three different donors. Tryptase produced a statistically significant increase in conjunctival fibroblast proliferation in a dose-dependent manner, with maximum proliferation observed at a dose of 100 ng/mL ($P < 0.05$; Fig. 1).

Existence of PAR-2 on Conjunctival Fibroblasts

We investigated the existence of mRNA for the known tryptase receptor, PAR-2, in human conjunctival fibroblasts using RT-PCR. Primary cultured fibroblasts from three different donors were used for the assay. PAR-2 mRNA was detected in all specimens from three different donors (Fig. 2A).

Next, we examined the existence of PAR-2 protein on human conjunctival fibroblasts using Western blot analysis. Primary cultured fibroblasts from three different donors were used for the assay. PAR-2 protein was detected in the specimens from all three different donors (Fig. 2B).

Inhibition of Cell Proliferation by Tryptase Inhibitors

We next examined the inhibition of tryptase inhibitors on the proliferative activity of tryptase-stimulated conjunctival fibroblasts. Leupeptin and benzamidine are known to inhibit the catalytic activity of tryptase. After conjunctival fibroblasts were cultured in 96-well culture plates (5000 cells per well) for 24 hours in DMEM/F12 supplemented with FCS, the medium was replaced with serum-free DMEM/F12 containing 100 ng/mL of tryptase with or without inhibitors. The culture medium was preincubated with 100 ng/mL of tryptase, or 100 ng/mL of tryptase and 10^{-4} M of each inhibitor at 37.0°C for 1 hour before use. Both leupeptin and benzamidine significantly in-

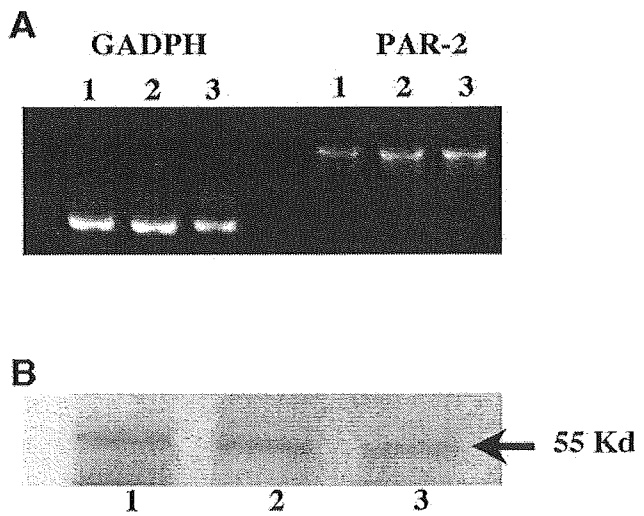

FIGURE 2. Existence of PAR-2 mRNA and protein on human conjunctival fibroblasts. (A) RT-PCR analysis shows PAR-2 mRNA expression in cultured conjunctival fibroblasts. (B) Western blot analysis shows the presence of a 55-kDa protein (PAR-2) in cultured conjunctival fibroblasts.

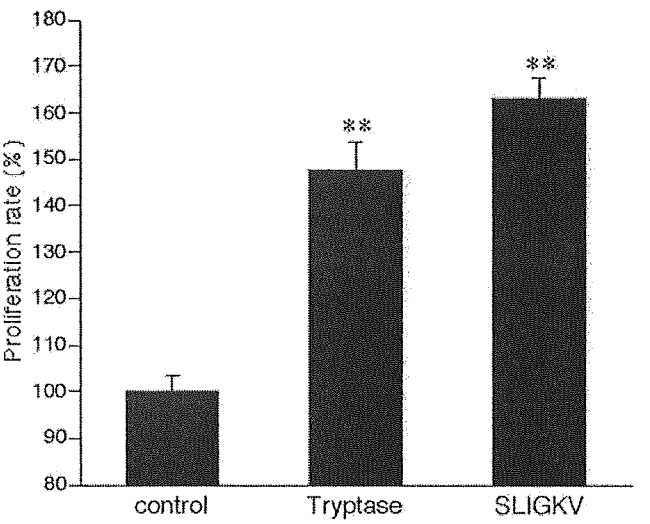

FIGURE 4. Effect of the PAR-2 agonist SLIGKV on conjunctival fibroblast proliferation. The PAR-2 agonist SLIGKV (10^{-4} M) mimicked 100 ng/mL of tryptase's effect and significantly increased the proliferation of conjunctival fibroblasts (*$P < 0.05$).

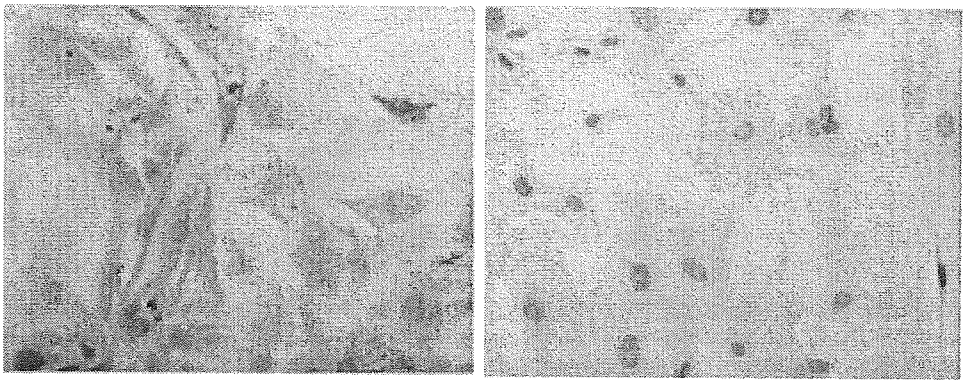

FIGURE 5. Immunoreactivity of PAR-2 receptor protein in cultured conjunctival fibroblasts. *Left*: immunohistochemistry shows that PAR-2 immunoreactivity was observed in cultured conjunctival fibroblasts. *Right*: isotype control.

hibited effect of tryptase on the proliferative activity of the cells (Fig. 3; $P < 0.05$).

The Effect of PAR-2 Agonist on Conjunctival Fibroblast Proliferation

Human PAR-2 is known to be activated by a synthetic peptide, SLIGKV. SLIGKV is designed based on the amino acid sequence of the tethered ligand, which directly binds to the body of PAR-2 without cleaving the N-terminal peptide, acting as an agonist for PAR-2.[14,15] SLIGKV mimicked the effect of tryptase (100 ng/mL of dose) and significantly increased the proliferative activity of cultured conjunctival fibroblasts (Fig. 4; $P < 0.05$).

Immunoreactivity of PAR-2 in Conjunctival Fibroblasts

We assessed the existence of PAR-2 receptor protein in conjunctival fibroblasts. In cultured conjunctival fibroblasts, PAR-2 immunoreactivity was observed except cell nucleus (Fig. 5). Because the anti-PAR-2 antibody, SAM-11 binds to the amino acid sequence 37-50, which is contained in the amino terminal of exodomain of human PAR-2, we strongly suspected that this staining pattern reflected PAR-2 localization on the cell membrane.

In the surgically excised papillae from patients with VKC, there were many tryptase-positive cells within the inflammatory cell infiltration site, whereas conjunctival tissues from normal volunteers contained no tryptase-positive cells. In both surgically excised papillae from patients with vernal keratoconjunctivitis and conjunctival tissue from normal control subjects, fibroblast-like cells with a flattened appearance were positively stained by the PAR-2 antibody (Fig. 6).

DISCUSSION

In this study, we showed that tryptase upregulated the proliferative activity of cultured conjunctival fibroblasts and that this stimulation was mediated by the known tryptase receptor, PAR-2. To the best of our knowledge, this is the first report indicating that conjunctival fibroblasts, in addition to fibroblasts derived from lung or dermal tissue, express PAR-2 and proliferate after stimulation with tryptase.

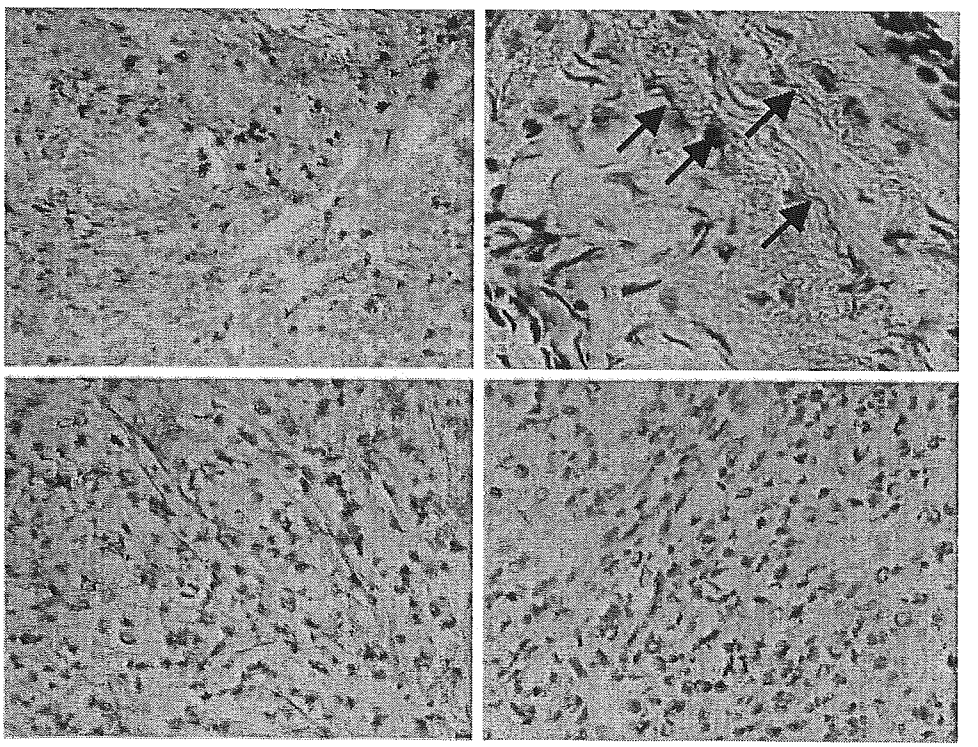

FIGURE 6. Immunoreactivity of human mast cell tryptase and PAR-2 receptor protein in conjunctival fibroblasts from surgically excised papillae. *Top left*: immunohistochemistry shows tryptase immunoreactive cells are observed within the area with inflammatory cell infiltration in the papillae from VKC specimens. *Top right*: PAR-2 immunoreactive stromal fibroblasts (*arrows*) are observed in conjunctival tissues from normal volunteers. *Bottom left*: PAR-2 immunoreactivity is seen in spindle-shaped cells, thought to be fibroblasts, in surgically excised papillary specimens from patients with VKC. *Bottom right*: isotype control shows no immunoreaction.

Tryptase is mainly released by mast cells. Mast cells activated by IgE binding to FcεRI play a critical role in the early phase of allergic inflammation, releasing preformed mediators like histamine, chymase, cathepsin-G, and mast-cell–specific carboxypeptidase A. Human mast cells produce α and β forms of tryptase. α-Tryptase is constitutively produced, while β-tryptase is stored in the secretory granules and released by exocytosis.[11] Our current results revealed that β-tryptase upregulated fibroblast proliferation, indicating that mast cells can affect the mitogenic acceleration of fibroblasts, when activated.

Like fibroblasts derived from lung or skin, conjunctival fibroblasts are known to produce various factors and affect other type of cells, including leukocytes. Previous investigation reported that conjunctival fibroblasts secreted Th-2 cytokines,[19] eotaxin,[20] procollagen type I and III,[21–23] MMPs,[23] and VEGF.[24] Solomon et al.[25] reported that conjunctival fibroblasts enhanced the survival and functional activity of eosinophils through IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor. Leonardi et al.[26] reported that histamine, which is accumulated in preformed granules in mast cells as well as tryptase, increases the proliferative and productive activity of conjunctival fibroblasts in patients with VKC. These facts may indicate a hypothesis that mast cells activate residential conjunctival stromal cells through the degranulation of preformed mediators, resulting in massive leukocyte infiltration and excess fibrovascular proliferation, causing giant papillary formation. Thus, mast cells may affect tissue remodeling[27] in allergic conjunctiva.

PAR-2 is known to be the sole receptor of tryptase. The current results show that the proliferative activity upregulated by tryptase was almost totally inhibited by leupeptin and benzamidine and that the PAR-2 antagonist peptide SLIGKV completely mimicked the effect of tryptase. These findings suggest that the local administration of a PAR-2-blocking peptide may effectively inhibit fibroblast proliferation in conjunctival tissue in vivo. We also demonstrated the existence of PAR-2 mRNA and protein on cultured conjunctival fibroblasts derived from normal volunteers, as well as on stromal fibroblasts in giant papillae from patients with VKC. We speculate that under allergic conditions, when Th-2 cytokines or proinflammatory factors are abundant, PAR-2 expression on conjunctival stromal cells may be upregulated. A quantitative assessment of PAR-2 expression on conjunctival fibroblasts would provide interesting data.

In conclusion, tryptase upregulates conjunctival fibroblast proliferation, and this effect appeared to be mediated via the PAR-2 receptor. Mast cells may play an important role in giant papillary formation during late-phase allergic conjunctivitis by means of releasing tryptase. Inhibitors of tryptase or peptides that block PAR-2 on the surfaces of fibroblasts may offer therapeutic benefits by selectively preventing the activation of fibroblasts and consequently the formation of giant papillae, in patients with allergic conjunctivitis. Further investigations are required in this field.

Acknowledgments

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Ocular Surface and MUC5AC Alterations in Atopic Patients with Corneal Shield Ulcers

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ABSTRACT *Purpose*: To describe MUC5AC alterations and the ocular surface disorder in atopic patients with or without corneal ulcers. *Methods*: Atopic patients' eyes were divided into two groups according to the presence and absence of corneal ulceration. The subjects underwent corneal sensitivity measurements, Schirmer test, tear film break-up time (BUT), fluorescein and Rose Bengal staining of the ocular surface and conjunctival impression cytology and brush cytology. Impression cytology samples underwent PAS and immunohistochemical staining for MUC5AC. Brush cytology specimens underwent evaluation for inflammatory cell expression and quantitative real-time PCR for MUC5AC mRNA expression. The differences related to the tear function and ocular surface examination parameters between patients with and without corneal ulceration and healthy control subjects were studied. In addition, the differences of the study parameters related to ocular surface epithelial health and inflammatory status between patient eyes with atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC) were investigated. *Results*: The mean corneal sensitivity and BUT values were significantly lower in atopic patients with corneal ulcers, compared to patients without ulcers and controls ($p < 0.001$). Brush cytology specimens from patients with corneal ulcers revealed significantly higher expression of inflammatory cells compared to patients without ulcers and controls ($p < 0.001$). Impression cytology samples from eyes with corneal ulcers showed significant squamous metaplasia and reduction in goblet cell density compared to eyes without ulcers and eyes of control subjects. The mean squamous metaplasia grade was significantly higher in eyes with AKC compared to eyes with VKC ($p < 0.02$). The mean goblet cell density was significantly lower in eyes with AKC compared to eyes with VKC ($p < 0.01$). Specimens from eyes with corneal ulcers showed PAS positive mucin pickup and did not stain positive for MUC5AC. MUC5AC mRNA expression was significantly lower in eyes with corneal ulcers compared to eyes without ulcers and eyes of control subjects. MUC5AC mRNA expression was also significantly lower in eyes with AKC compared to eyes with VKC. *Conclusions*: Ocular surface inflammation, tear film instability, and decreased conjunctival MUC5AC mRNA expression were thought to be important in the pathogenesis of noninfectious corneal shield ulcers in atopic ocular surface disease.

KEYWORDS atopy; conjunctival squamous metaplasia; corneal sensitivity; corneal ulcer; tear film; impression cytology; goblet cell

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INTRODUCTION

Atopic diseases such as bronchial asthma, allergic rhinitis, atopic dermatitis, and ocular allergic disorders are among the most frequent conditions experienced by allergic patients. Recent studies indicate that the lifetime prevalence rates for these atopic diseases in children and adolescents vary between 24% and 45%.[1] Allergic conjunctivitis, an atopic ocular disease, is one of the most common problems encountered in these patients.

Ocular allergies have been classified into four categories; seasonal or perennial allergic conjunctivitis, contact lens associated giant papillary conjunctivitis, vernal keratoconjunctivitis, and atopic keratoconjunctivitis.[2] The ocular allergic disease results from a complex interplay of several immunological and inflammatory pathways. A unifying characteristic of all forms of ocular allergy is the presence of eosinophils, a major inflammatory cell in the IgE-mediated late-phase reaction. Leukocytes are also known to be recruited in ocular allergies.[3] The ocular inflammatory process and release of allergic mediators onto the ocular surface and tear film are thought to be responsible for a wide range of clinical corneal manifestations including superficial punctate keratitis, macroerosions, corneal ulceration, plaque formation, corneal neovascularization, and lipid infiltration.[4]

We previously reported that the ocular surface disorder in atopic states was characterized by tear film instability, goblet cell loss, and conjunctival squamous metaplasia. In that study, we also reported that reduction in goblet cell counts related to tear instability significantly.[5] Indeed, goblet cells are thought to secrete the main source of tear mucin, namely, mucin MUC5AC, the secretion of which is limited only to conjunctival goblet cells that are interspersed among the stratified cells in the conjunctival epithelium.[6] Ocular surface mucins are believed to provide a barrier to prevent pathogens and particulate matter from entering the ocular surface epithelium and, through their heavy O-linked glycosylation, maintain hydration of the ocular surface and tear stability.[6] Our previous report stressed the need for further studies investigating the changes of ocular mucin expression in atopic patients trying to explain the relation of such changes to the ocular surface disorders. An increased understanding of such changes at the ocular surface, including alterations at the cellular level in the conjunctiva, may help explain the pathogenesis and the subsequent clinical appearance of atopic ocular allergies, which may be potentially blinding. Therefore, we performed Schirmer test, tear film break-up time, corneal sensitivity measurements, fluorescein and Rose Bengal staining of the ocular surface, conjunctival impression and brush cytology, immunohistochemistry staining of the impression cytology samples with MUC5AC antibodies, and quantitative real-time (RT)-PCR of the brush cytology samples for MUC5AC mRNA expression in atopic patients with or without corneal ulcers and compared the results with those of normal control subjects. In addition, the differences of the study parameters related to ocular surface epithelial health and inflammatory status between patient eyes with atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC) were studied.

MATERIALS AND METHODS

Subjects and Examinations

Forty-two eyes of 23 atopic patients (17 males; 6 females) aged between 6 and 41 years (mean: 24.8 years) as well as 40 eyes of 20 normal subjects aged from 12 to 42 years (mean: 24.6 years; 15 males, 5 females) were recruited from the Ocular Allergy Subspecialty Clinic of the Department of Ophthalmology, Tokyo Dental College, Ichikawa Hospital. All patients had moderate to severe atopic disease that was treated with systemic steroids, psychiatric counseling, or desensitization therapy at least once during the course of their disease. All patients also had radioallergosorbent tests (RAST) and scratch tests to confirm their allergic status. None of the patients with atopy had a history of Stevens-Johnson syndrome, chemical, thermal, or radiation injury; or any other systemic disorder or underwent any ocular surgery that would create an ocular surface problem. Patients who were recalcitrant to the same treatment regimen prescribed at their referral centers including topical 0.025% ketotifen fumarate q.i.d. and topical 0.01% betamethasone q.i.d. for 2 weeks were included in this study. No patient was being treated with cytotoxic immunosuppressants, systemic steroids, antibiotics, or prostaglandin inhibitors at the time of inclusion into the study. Routine ophthalmic examinations consisted of best corrected visual acuity (B.C.V.A.) measurements, slit-lamp examination, and anterior segment photography. At slit-lamp examination, particular attention was paid to lid margins, tarsal and bulbar

conjunctiva, and cornea. The patients and control subjects underwent ocular surface examinations including corneal sensitivity measurements, tear film break-up time (BUT), fluorescein and Rose Bengal staining, Schirmer test, conjunctival impression cytology, and brush cytology. All examinations were performed by the same researcher (M.D.) In addition, swabs obtained from the margin and base of the ulcers were sent for cultures and microbiologic examination to rule out the presence of an infectious process. An informed consent about the procedures as well as permission from the Ethical Committee of Tokyo Dental College was obtained. This study adhered to the tenets of the Declaration of Helsinki. The control subjects did not have any history of ocular or systemic disease or a history of drug or contact lens use that would alter the ocular surface as well. Corneal lesions involving at least one-third of the corneal stroma were termed as corneal ulcer in this study. Patients were divided into two groups as those with and without corneal ulcers, and the differences of the study parameters between the two groups and with the controls were also investigated. In addition, the differences of the study parameters related to ocular surface epithelial health and inflammatory status between patient eyes with atopic keratoconjunctivitis and vernal keratoconjunctivitis and with the controls were studied.

Corneal Sensitivity Measurements

Measurement of corneal sensitivity was performed using a Cochet-Bonnet aesthesiometer. The measurements were begun with the nylon filament fully extended. The tip of the nylon filament was applied perpendicularly to the surface of the cornea making certain not to touch the eyelashes and was pushed until the fiber's first visible bending. The length of the fiber was gradually decreased until a blink reflex was observed. The length was recorded in units of millimeter. Measurements were taken from 5 points including central, superior, inferior, nasal, and temporal cornea and the mean of the measurements was recorded as the corneal sensitivity reading of that eye. A corneal sensitivity measurement of less than 50 mm was regarded as low corneal sensitivity in this study.[7,8]

Tear Function Tests and Ocular Surface Staining

The standard tear film break-up time measurement was performed. The ocular surface was examined by the double vital staining method. Two microliters of preservative-free combination of 1% Rose Bengal and 1% fluorescein dye was instilled in the conjunctival sac. The dye mixture was introduced to the conjunctival sac with minimal stimulation by a micropippette and were undetected by the patients. The subjects were then instructed to blink several times for a few seconds to ensure adequate mixing of the dye. The interval between the last complete blink and the appearance of the first corneal black spot in the stained tear film was measured three times, and the mean value of the measurements was calculated. A BUT value of less than 10 s was considered abnormal.[9] Fluorescein and Rose Bengal staining of the cornea was also noted and scored. Fluorescein staining scores ranged between 0 and 9 points. A score greater than 3 points was regarded as abnormal. The Rose Bengal staining scores of the ocular surface ranged between 0 and 9 points. Any score above 3 points was regarded as abnormal.[10]

For further evaluation of tears, the standard Schirmer test with topical anesthesia (0.4% oxybuprocaine chloride) was performed. The standardized strips of filter paper (Alcon Inc., Fort Worth, TX, USA) were placed in the lateral canthus away from the cornea and left in place for 5 min with the eyes closed. Readings were reported in millimeters of wetting for 5 min. A reading of less than 5 mm was referred to as dry eye.[9]

Conjunctival Impression Cytology

The impression cytology specimens were obtained after administration of topical anesthesia with 0.4% oxybuprocaine. Two seperate strips of cellulose acetate filter paper (Millipore HAWP 304, Bedford, MA, USA) that were soaked in distilled water for a few hours and dried at room temperature were applied on adjacent areas of the medial upper palpebral conjunctiva, close to each other, pressed gently by a glass rod, and then removed. The specimens were then fixed with formaldehyde. One of the specimens was stained with periodic acid–Schiff (PAS), dehydrated in ascending grades of ethanol and then with xylol, and finally coverslipped. The other specimen underwent immunohistochemical staining with MUC5AC antibodies. The quantitative studies of conjunctival goblet cells and squamous metaplasia of conjunctival epithelial cells were conducted by taking photographs using a calibrated grid under a light microscope at a magnification of 400×. We photographed five non-overlapping areas of each sample

selected at random and averaged the outcomes for a single sample score. The goblet cell densities were reported as cells per square millimeter with standard deviations. The specimens were also assigned a grade of conjunctival epithelial squamous metaplasia according to Nelson's grading scheme.[11] The same researcher who was masked to whom the samples came from evaluated the specimens for goblet cell counts, squamous metaplasia grades, and mucin pickup.

Immunohistochemistry Staining

The impression cytology specimens for immunohistochemistry staining were placed in plastic fenestrated embedding cassettes (Murazumi, Osaka, Japan) and then immersed in glass jars containing 10 mM of sodium citrate buffer (pH 6.0) and were subjected to microwave treatment at 600 W for 15 min for antigen activation. The specimens were then washed in phosphate-buffered saline (PBS) twice for 5 min. The specimens were placed on slides and blocked in PBS with horse serum albumin for 20 min. The primary mouse monoclonal antibody to MUC5AC at a dilution of 1:100 (Cosmo Bio, Tokyo, Japan) as suggested by the distributor was applied for 1 hr at room temperature in a moist chamber, and then the slides were rinsed with PBS for 10 min. Samples were then processed by SAB-PO (M) kit (Nichirei, Tokyo, Japan) protocol (DAB-peroxide staining). The specimens were treated with antimouse secondary antibodies for 30 min afterward, washed with PBS for 5 min. Finally, the specimens were placed in the plastic embedding cassettes again for counterstaining with hematoxylin, dehydrated in ascending grades of ethanol and then with xylol, and coverslipped for light microscopic examination. For isotope controls, primary antibody was replaced with mouse IgG1 (Sigma, St. Louisi, MO, USA). The evaluation of specimens under light microscopy for presence of positive immunohistochemical staining was also performed in a masked fashion.

Conjunctival Brush Cytology

The brush cytology specimens were obtained after administration of topical anesthesia with 0.4% oxybuprocaine. Two adjacent areas of central upper palpebral conjunctiva which did not undergo impression cytology were used for sampling. Conjunctiva was scraped seven times with the Cytobrush-S (Medscand AB, Malmo, Sweden), the examiner holding the brush 2 cm away from the brush end, applying a gentle pressure to the conjunctiva. Care was taken not to touch any part of the conjunctival surface to undergo impression cytology during brush cytology. After sampling, the brushes were immediately placed in 1 ml of Hank's buffered solution, and the containers were shaken in order to detach the cells from the brush. One of the samples was reserved for Giemsa staining for the assessment of conjunctival inflammatory cell numbers. The suspended cells were collected using the Millipore filter technique employing filters with 8-μm pore size. The filters were fixed in 95% ethyl alcohol and stained using Giemsa staining. The other brush cytology samples were transferred to 1.5-ml Eppendorf tubes immediately and centrifuged at 4°C of 10,000 rpm for 3 min. The supernatants were discarded and replaced with 1 ml Isogen solution and stored at −20°C for real-time quantitative RT-PCR.

Quantitative RT-PCR for Relative MUC5AC Expression

RNA was extracted from Isogen samples. Quantitative RT-PCR was performed according to the manufacturer's instructions (Applied Biosystems, Weiterstadt, Germany). cDNA (10 ng) was amplified in 25 μl final volume, in the presence of 1.25 μl of the following "Assay by Design" oligonucleotides (MUC5AC and GAPDH; Applied Biosystems). Test gene primer and probe sets were optimized for concentration, amplification efficiency, and faithful coamplification with housekeeper gene primer and probe sets, the latter including GAPDH. Quantitative RT-PCR was set up in 96-well plates using the above reagents and Taq Man master mix and as indicated by optimization data and it was run on 7700 ABI thermal cyclers (Applied Biosystems). The thermal profile consisted 50°C for 2 min, 95°C for 10 min, followed by 40 cyclesof 94°C for 15 s and 60°C 1 min. Real-time data were acquired and analyzed using Sequence Detection System Software (Applied Biosystems) with manual adjustment of the baseline and threshold parameters. Relative expression levels were determined using cycle threshold values and the Compared Ct method to adjust for coamplified housekeeper gene levels, amplification cycle rates, and the reference expression level of control samples.[12,13]

Statistical Analysis

Data were processed using Stat View software (Abacus Concepts, Inc., San Diego, CA, USA). The unpaired

t test was used for the analyses of nonparametric values. A probability level less than 5% was considered statistically significant. The analysis of categorized data was performed by the ANOVA test with the probability level set at 5% for statistical significance.

RESULTS

Patient Characteristics and Slit-Lamp Findings

There were no statistically significant differences between the patient and control groups regarding age and gender characteristics. Seventeen patients (73.9%) had active atopic dermatitis, and six patients had both bronchial asthma and allergic rhinitis as their atopic disease at the time of inclusion into the study. All patients had active allergic ocular disease as evidenced by conjunctival injection, chemosis, papillary hypertrophy, tearing, and mucus discharge. Ten eyes (23.8%) had vernal and 32 eyes (76.2%) had atopic keratoconjunctivitis. The mean ages for patients with VKC and AKC were 11.2 ± 3 years and 20.6 ± 3 years, respectively ($p = 0.038$). Nine eyes had corneal shield ulcers without evidence of an infectious infiltration at slit lamp examination in this study. Microbiological investigation also did not reveal evidence of an infectious process in these patients. The mean age of the six patients (4 males; 2 females) with corneal ulcers was 23.8 years (range: 9 years to 34 years) and the mean age of patients without ulcers (12 males, 5 females) was 25.6 years (range: 6 years to 41 years). There were no age or gender related differences between the patients with and without corneal ulcers and the control subjects. All patients complained of allergic and dry eye symptomatology, including itchiness, redness, grittiness, tiredness, foreign body sensation, discomfort, and irritation. None of the patients or the controls had meibomian gland disease or blepharitis at slit-lamp examination. Anterior segment photographs of the palpebral conjunctiva, cornea and fluorescein stained ocular surface of a representative atopic patient without corneal ulceration are shown in Figures 1A–1C. Likewise, anterior segment photographs of the palpebral conjunctiva and cornea of a representative atopic patient with corneal ulceration are shown in Figures 2A–2B.

Corneal Sensitivity

Eight out of 9 eyes (88.8%) with corneal ulcers had a low corneal sensitivity. Eleven out of 33 eyes (33.3%) without corneal ulcers had a low corneal sensitivity value below 50 mm. On the other hand, none of the control eyes had a low corneal sensitivity measurement. The mean corneal sensitivity value in eyes with ulcers was 32.5 ± 20.0 mm and was significantly lower than in eyes without ulcers and eyes of control subjects ($p = 0.026$) (Fig. 3).

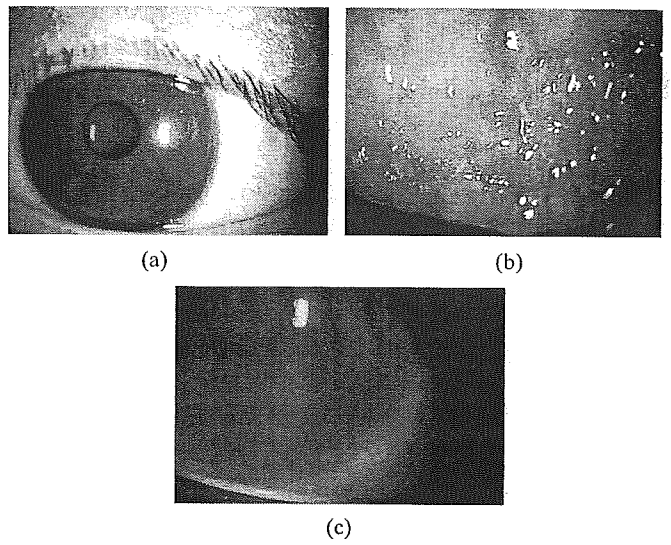


FIGURE 1 (A) Anterior segment photograph of a 14-year-old atopic male boy without corneal ulceration. Corneal sensitivity, 60 mm; Schirmer test, 15 mm, BUT, 7 s. (B) Note the upper tarsal conjunctival papillary hypertrophy, injection and chemosis. (C) Slit-lamp photograph of corneal fluorescein staining. Fluorescein stain score was 3 points.

Tear Function Examinations

All eyes with and without corneal ulcers had a low BUT value measuring less than 10 s. None of the control eyes had a low BUT value. The mean BUT score in eyes with corneal ulcers was significantly lower than in eyes without corneal ulcers and eyes of healthy control

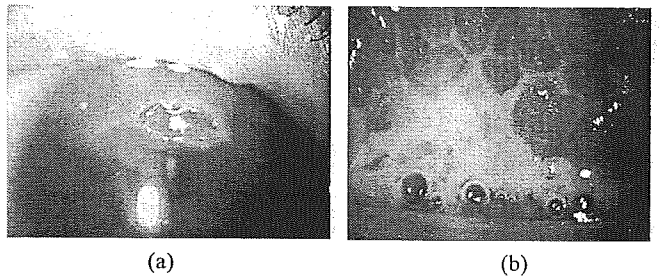
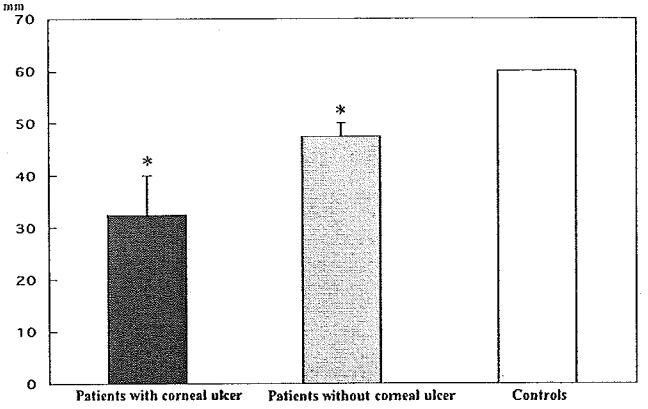


FIGURE 2 (A) Anterior segment photograph of a 9-year-old atopic male boy with corneal ulceration. Corneal sensitivity, 25 mm; Schirmer test, 25 mm; BUT, 1 s. Note the upper corneal ulceration. (B) Note the upper tarsal conjunctival papillary hypertrophy, injection, chemosis, and the copious mucus discharge.



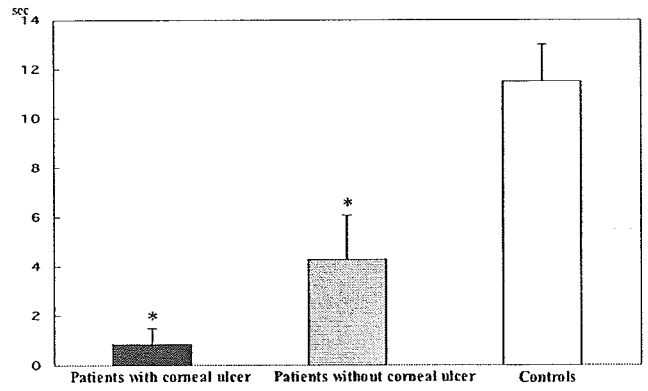
* P=0.026 ANOVA test

FIGURE 3 Comparison of corneal sensitivity between patient and control eyes.

subjects as shown in Figure 4 ($p < 0.001$). The mean Schirmer test scores were 15 ± 4 mm, 17 ± 3 mm, and 16 ± 2.5 mm in eyes with and without corneal ulcers and in eyes of control subjects-respectively. The differences were not statistically significant ($p = 0.68$).

Ocular Surface Vital Staining

Nine out of 42 eyes (21.4%) had corneal ulcers in this study. The mean size of the corneal ulcers was 4.6 mm × 4.2 mm. Ulcers were located superiorly in 4 eyes and centrally in five eyes. Ulcers involved one-third of the corneal stroma in depth as assessed by slit-lamp biomicroscopy. Twenty out of 33 eyes (60.6%) without ulcers had a fluorescein staining score exceeding 3 points. Likewise, 25 out of 33 eyes (78.7%) without ulcers had a Rose Bengal staining score exceeding 3 points. The mean fluorescein and Rose Bengal staining scores in the control eyes were 0.5 ± 0.5 and 1.0 ± 0.25 points. The mean fluorescein and Rose-Bengal staining scores in eyes without ulcers were 5.47 ± 3.2 points and 4.7 ± 0.5 points, respectively, and were significantly higher than the staining scores in the healthy control eyes ($p = 0.001$).



* P<0.001 ANOVA test

FIGURE 4 Comparison of tear film break-up time between patient and control eyes.

Conjunctival Impression Cytology

Conjunctival imprints from all eyes contained sheets of conjunctival epithelial cells, variable amount of goblet cells, and mucin pickup. Imprints from eyes with corneal ulcers revealed both neutrophils and eosinophils, with predominance of neutrophils as observed under light microscopy.

Squamous Metaplasia

The average grades of squamous metaplasia in the control eyes as well as eyes with and without corneal ulcers were 0.08 ± 0.29, 2.35 ± 0.79, and 1.52 ± 0.84, respectively. The differences were statistically significant as shown in Table 1 ($p < 0.001$). The mean squamous metaplasia grade was significantly higher in eyes with AKC compared to eyes with VKC as shown in Table 2 ($p < 0.01$).

Goblet Cell Density

The goblet cell densities were lower than 1000 cells/mm^2 in all atopic eyes, whereas none of the control eyes had a goblet cell count less than 1000 cells/mm^2. The average goblet cell density was significantly lower in eyes with ulcers compared to the control eyes and eyes without ulcers as demonstrated in Table 1 ($p < 0.01$). The mean goblet cell density was significantly lower in eyes with AKC compared to eyes with VKC as shown in Table 2 ($p < 0.01$).

TABLE 1 Comparison of Impression Cytology Parameters Between Patient and Control Eyes

	Corneal ulcer (+)	Corneal ulcer (–)	Controls
Squamous metaplasia (Nelson's)	2.35 ± 0.79*	1.22 ± 0.84*	0.08 ± 0.29
Goblet cell density (cells/mm^2)	204 ± 226*	712 ± 527*	1726 ± 583

*$p < 0.001$, ANOVA test.

TABLE 2 **Comparison of Impression Cytology Parameters Between Controls and Patients with AKC and VKC**

	AKC	VKC	Controls
Squamous metaplasia (Nelson's)	2.2 ± 0.46*	0.79 ± 0.5*	0.08 ± 0.29
Goblet cell density (cells/mm^2)	167 ± 94*	1052 ± 358*	1726 ± 583

*$p < 0.001$ ANOVA test.

PAS Staining and Immunohistochemical Staining with MUC5AC Antibodies

PAS staining of the conjunctival imprints from all control eyes showed positive staining of plum oval goblet cells and areas of mucin pickup with sheets of healthy conjunctival epithelial cells. A representative imprint from a 40-year-old healthy male subject is shown in Figure 5A. Staining with MUC5AC antibodies showed positive staining for goblet cells and secreted mucin in between the cells as shown in Figure 5B (same subject as in Fig. 5A) in all control eyes. Isotope control staining did not show positive staining of the goblet cells for MUC5AC in any of the samples (Fig. 5C). PAS staining of the conjunctival imprints from all atopic eyes without corneal ulcers showed positive staining for areas of mucin pickup and goblet cells. There were alternating areas with sheets of healthy conjunctival epithelial cells and conjunctival epithelial cells with variable degrees of squamous metaplasia. A representative imprint from a 14-year-old atopic male subject is shown in Figure 6A. Staining with MUC5AC antibodies showed positive staining for secreted mucin over sheets of epithelial cells with advanced squamous metaplasia as shown in Figures 6B–6C (same subject as in Fig. 6A) in all atopic eyes eyes without ulcers. PAS staining of the conjunctival imprints from eyes with corneal ulceration showed positive staining for the few goblet cells and areas of copious mucin pickup with sheets of conjunctival epithelial cells with various degrees of squamous metaplasia. A representative imprint from a 9-year-old atopic male subject with corneal ulcer is shown in Figures 7A and 7B. Staining with MUC5AC antibodies did not reveal

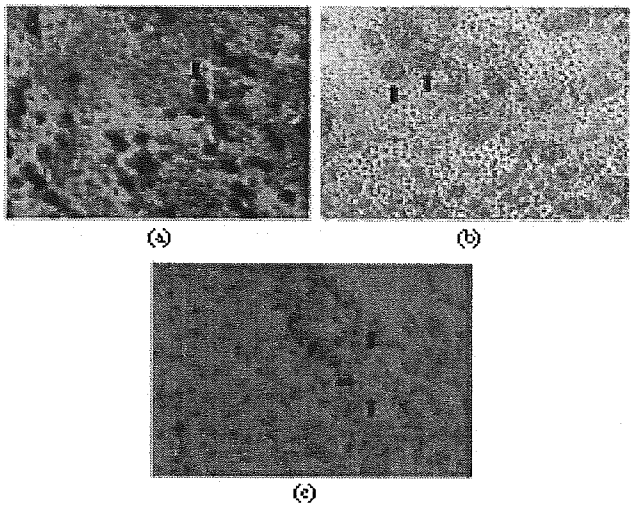


FIGURE 5 **(A) PAS staining of the impression cytology (IC) specimen from a representative 40-year-old male control subject. Black arrows indicate the goblet cells, and the pink arrow shows mucin pickup by the membrane. Note the sheets of healthy conjunctival epithelial cells in the background. Mean specimen squamous metaplasia grade: 0 (×200). Mean specimen goblet cell density: 2909 cells/mm^2. (B) Immune staining of the impression cytology specimen with MUC5AC antibodies. Black arrows indicate goblet cells with positive staining with the antibodies (×200). (C) Isotope control IC specimen. Black arrows indicate silhouettes of goblet cells that did not stain (×200).**

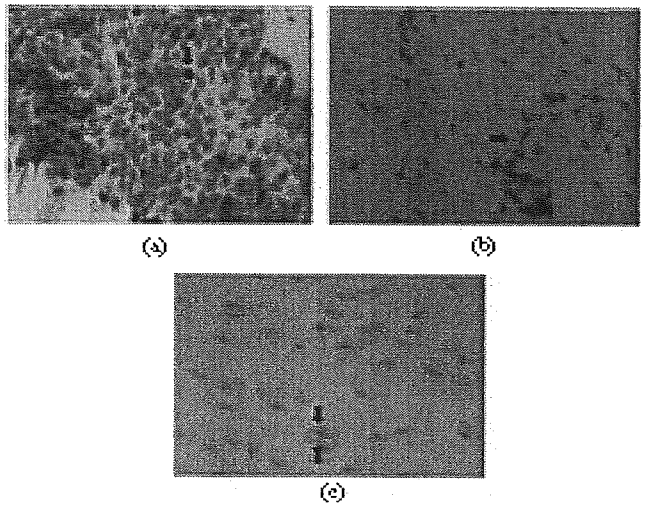


FIGURE 6 **(A) PAS staining of the impression cytology (IC) specimen from the representative 14-year-old atopic male subject with no corneal ulceration. Black arrow indicates the goblet cell, and the pink arrows show mucin pickup by the membrane (×200). Mean specimen squamous metaplasia grade: 1.33. Mean specimen goblet cell density: 545 cells/mm^2. (B) Immune staining of the impression cytology specimen with MUC5AC antibodies (×200). Black arrow indicates mucin pickup by the membrane with positive staining with MUC5AC antibodies. Note the conjunctival epithelial cells with advanced squamous metaplasia in the background. (C) Immune staining of the impression cytology specimen with MUC5AC antibodies (×200). Black arrows indicate a goblet cell with positive staining. Note the conjunctival epithelial cells with advanced squamous metaplasia and loss of cellular cohesion in the background.**

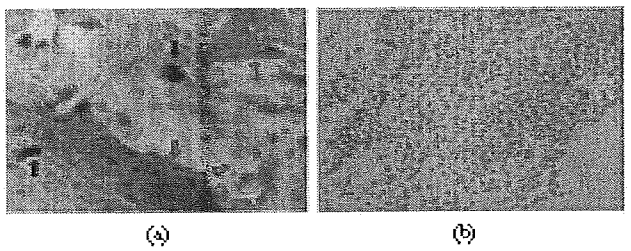


FIGURE 7 (A) (PAS) staining of the impression cytology (IC) specimen from the representative 9-year-old atopic male subject with corneal ulceration (×400). Black arrows indicate the goblet cells, and the pink arrows show mucin pickup by the membrane. Mean specimen squamous metaplasia grade: 2.03. Mean specimen goblet cell density: 60 cells/mm^2. (B) Immune staining of the impression cytology specimen with MUC5AC antibodies. Note the absence of positive staining with MUC5AC antibodies and advanced conjunctival squamous metaplasia and absence of goblet cells in the field (×200).

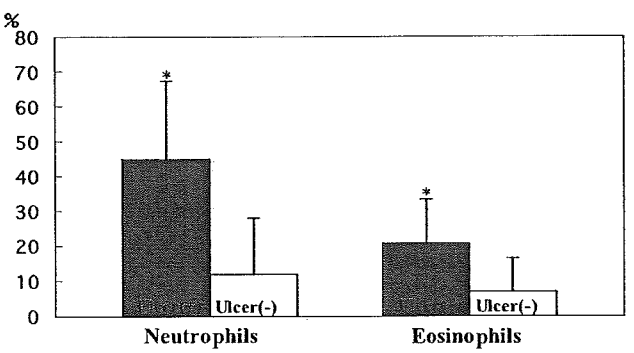
positive staining for goblet cells and secreted mucin in any of the samples in the eyes with corneal ulceration.

Conjunctival Brush Cytology

Giemsa staining of brush cytology samples showed significantly higher mean percentages of neutrophil and eosinophils in eyes with corneal ulcers compared to eyes without corneal ulceration as shown in Figure 8 ($p < 0.001$). Although there were no significant differences in relation to number of eosinophils between eyes with AKC and VKC, the mean percentage of neutrophils was significantly higher in eyes with VKC as shown in Figure 9 ($p < 0.001$). None of the control eyes revealed positive staining for neutrophils and eosinophils.

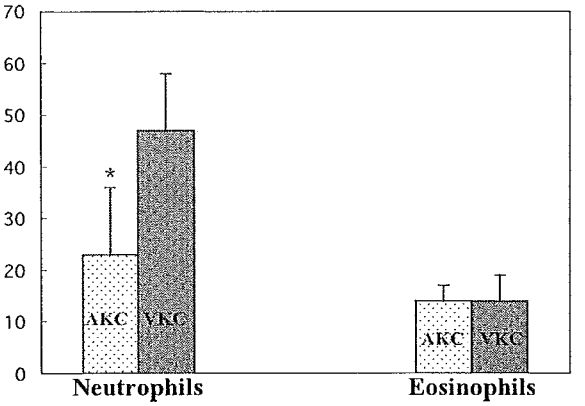
Quantitative RT-PCR

For MUC5AC mRNA, there was a significant increase in the samples from eyes of atopic patients without corneal ulcers relative to samples obtained from



*p<0.001 Mann Whitney test

FIGURE 8 Comparison of inflammatory cells in brush cytology samples in patients with and without corneal ulcers.



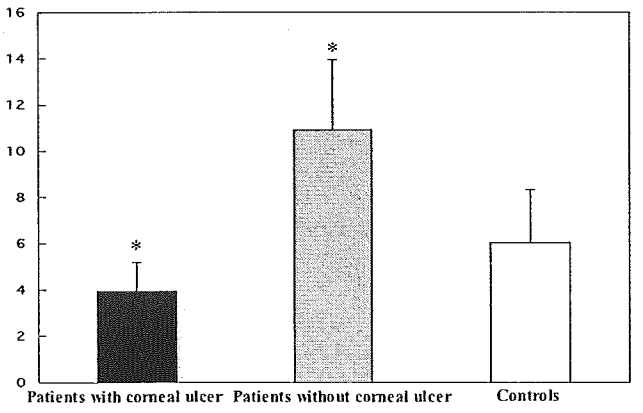
*p<0.001 Mann Whitney test

FIGURE 9 Comparison of inflammatory cells in brush cytology samples in patient eyes with AKC and VKC.

healthy control eyes. The level of MUC5AC mRNA was significantly reduced in the samples from eyes with corneal ulcers relative to samples obtained from control eyes and eyes of atopic patients without ulcers as shown in Figure 10 ($p = 0.029$). The level of MUC5AC mRNA was also significantly reduced in the samples from eyes with AKC relative to samples obtained from control eyes and eyes of VKC patients as shown in Figure 11 ($p < 0.01$).

DISCUSSION

Ocular allergic disease in atopic patients has been reported to be associated with eyelid eczema, blepharitis, meibomitis, keratoconus, keratoconjunctivitis, ptosis, trichiasis, cataract, madorosis, ocular herpes, and retinal detachment. The conjunctival involvement may vary from a normal looking conjunctiva to severe



* P=0.029 ANOVA test

FIGURE 10 Comparison of relative MUC5AC expression levels in real-time PCR between patient and control eyes.

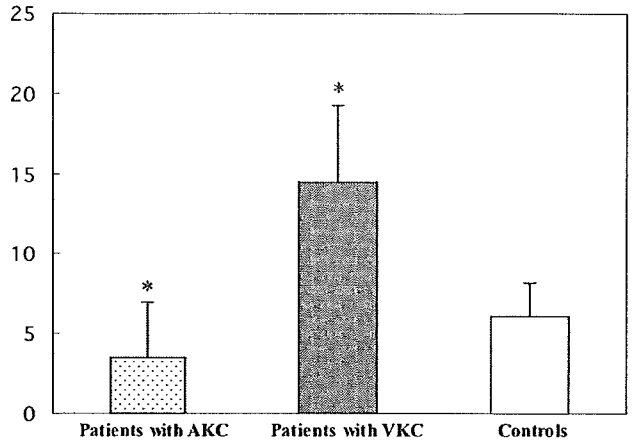


FIGURE 11 Comparison of conjunctival MUC5AC mRNA levels between patient eyes with AKC, VKC, and controls.

papillary hypertrophy with mucous discharge, subepithelial fibrosis, and symblepharon formation. Serious corneal complications are most uncommon, the earliest of which usually is a superficial punctate keratitis that may progress to corneal neovascularization, thinning, and perforation of the cornea.[14,15] We studied the allergic ocular surface disorder in 42 eyes of 23 patients with atopy by performing corneal sensitivity measurements, Schirmer test, tear film breakup time, impression and brush cytology, and also compared the results with those of normal control subjects.

Corneal sensitivity was significantly lower in atopic patients compared with controls. We think that decreased corneal sensitivity in our series of patients strongly implies corneal epithelial/stromal disease. Interestingly, we found that corneal sensitivity was further reduced in eyes with corneal ulcers compared to eyes without an ulcerative process. The mechanisms of these sensitivity changes during the course of atopic allergic eye disease are still not clear. We think that loss or decrease of trophic effects of corneal nerves due to primary or secondary events with progression of atopic eye disease should play an important role in the pathogenesis of the atopic ocular surface disease. Because it was our belief that reduction of corneal sensitivity should have adverse effects on the ocular surface and be able to provide some more clues, we went on with tear film examinations and ocular surface evaluation by fluorescein and Rose Bengal staining. Tear function examinations also revealed interesting findings. There were no aqueous deficient dry eyes in this study. Schirmer test values did not show any significant differences between eyes of patients and controls or between eyes with and without corneal ulceration. On the other hand, all patients reported dry eye symptomatology, and all patient eyes had low BUT scores. These findings suggest a BUT-deficient dry-eye state with positive symptomatology as described by Lemp.[16] Moreover, BUT values were significantly poorer in eyes with corneal ulcers, suggesting an increase of tear film instability with progression of the atopic ocular surface disease. We hypothesized that the reduction of BUT scores resulted from possible alterations of quality and/or quantity of mucin secretion by the diseased ocular surface epithelium, reduction of goblet cell numbers, or changes in the conjunctival nongoblet epithelial cells.[17–19] We also found that fluorescein and Rose Bengal scores were significantly higher in atopic eyes compared with the controls.

To provide more clues in relation to the tear function changes, we carried on with impression and brush cytologic analysis, which provided evidence that prominent squamous metaplasia and goblet cell loss existed in eyes of atopic patients but not in control subjects. The cytologic changes were more pronounced in eyes with corneal ulceration. We then attempted to look into the alterations of MUC5AC mRNA expression by quantitative RT-PCR because conjunctival goblet cells are thought to be the main source of tear mucin, and MUC5AC secretion is restricted to goblet cells. This mucin is thought to play an important role in increasing the tear film stability and the wetability of the ocular surface.[19] We found that despite the decrease of goblet cell counts in eyes of atopic patients compared with controls, MUC5AC mRNA expression was significantly upregulated in eyes without ulcers relative to healthy control eyes with significant downregulation in eyes with corneal ulcers. We believe that increased MUC5AC gene expression was a manifestation of an ocular surface defense response to compensate for the ailing ocular surface health with eventual downregulation due to progression of atopic allergic ocular surface disease. Immune staining for MUC5AC revealed presence of MUC5AC secretion by the ocular surface in both atopic eyes without ulcers and control eyes. Yet, despite PAS (+) staining results for mucin in all atopic eyes with corneal ulcers, immune staining with MUC5AC antibodies consistently revealed negative staining results. An explanation for this observation might be the decrease

in MUC5AC expression with possible upregulation of other alternative mucins to protect the ocular surface. Further studies identifying the concomitant alterations of mucins 1, 2, 4 and 16 in such eyes would provide essential and interesting information.

It should be remembered that the tear functions and mucin alterations reported in this study represent the findings from allergic ocular surfaces that did not respond to the same conventional treatment of 2 weeks prescribed at the referral centers. Tear function and mucin gene expression status of untreated atopic ocular surfaces and differences in the extent of changes in tear functions and mucin expression with different treatment modalities should be investigated in future studies.

An increase in the number of goblet cells with mucus overproduction is a common feature of allergic asthma.[20] A recent study associated this increase with an increased expression of MUC5AC mRNA in a murine model of allergic asthma.[21] In cases of allergic conjunctivitis, however, little information is available on mucin expression or goblet cell numbers. Two studies of goblet cells in a guinea pig model of allergic conjunctivitis demonstrated a decrease in conjunctival goblet cells, but there does not appear to be information on mucin gene expression relative to ocular allergic response.[22,23] A recent study by Kunert *et al.* in a mouse model of allergic conjunctivitis demonstrated that, in the conjunctiva of mice, repetitive application of allergens induces a reduction in the number of goblet cells and a decrease in Muc5AC and Muc4 mRNAs. In that study, after 48 hr, the goblet cell numbers returned to naive levels, and goblet cell mucin mRNA levels returned to above or within normal range. The study concluded that it remained to be determined how mucin gene regulation occured in allergic human ocular surface epithelia.[24] A PubMed and Medline search revealed that ours is the first report on the level of MUC5AC gene expression in human ocular surface allergy. Our observations on conjunctival neutrophil and eosinophil expression in brush cytology also provided some insight into the relationship between ocular surface inflammation and mucin gene expression. We found significantly higher numbers of eosinophils and neutrophils in eyes with corneal ulceration compared with eyes without an ulcerative process. We thought that an inverse relationship between the level of ocular surface inflammation and MUC5AC expression existed in atopic eyes with allergy; that is, the higher the degree of conjunctival inflammation the lower the MUC5AC gene expression.

Another interesting finding in this study was that eyes with AKC had significantly higher squamous metaplasia of the conjunctival epithelium and lesser numbers of goblet cells compared to eyes with VKC. The relative MUC5AC mRNA expression was also significantly reduced in eyes with AKC compared to eyes with VKC. Although there were no significant differences in relation to number of eosinophils between eyes with AKC and VKC, the mean percentage of neutrophils was significantly higher in eyes with VKC. The greater extent of conjunctival squamous metaplasia and goblet cell loss in eyes with AKC might be explained with the chronic nature of the disease process extending beyond puberty. The impact of the differences of the ocular surface inflammation on the ocular surface mucin production and epithelial status in AKC and VKC should be addressed in future studies employing immunohistochemical techniques to delineate the specific type of inflammatory cell infiltrates and keratinization.

Although not investigated in our study, increasing evidence from recent studies supports the concept that mucus production is directly affected by inflammatory cytokines. Mucus production appears to be independent of B cells and immunoglobulins but dependent on Th2 associated cytokines.[25] Initially, IL-4 was shown to cause goblet cell metaplasia and upregulation of mucins 2 and 5AC *in vitro* and *in vivo* in the airway.[26,27] Recently, the Th-2 associated cytokines IL-9 and IL-13 were demonstrated to be central mediators of allergic asthma and goblet cell metaplasia.[28,29] In patients with AKC and VKC, IL-4 and 5 have been shown to be increased in tears.[30] In addition, high levels of IL-4 were found by ELISA from conjunctival brush cytology samples of patients with allergic conjunctivitis.[31] Even though a majority of available publications related to mucin gene expression in allergy concentrate on cytokines, factors associated with neutrophils and eosinophils, as major inflammatory components, should also be considered in regard to ocular surface goblet cell population and mucin gene regulation. Indeed, goblet cells have been reported to be very sensitive to ocular surface inflammation, which leads to their depletion and subsequent detrimental effects on the ocular surface epithelia.[32] For instance, eosinophil-derived major basic and cationic proteins, neurotoxins, and collagenases have been shown to damage the corneal epithelium and basement membrane.[4,33–35] It

is our belief that higher level of ocular surface inflammation, tear instability, decreased corneal sensitivity, and decreased conjunctival MUC5AC mRNA expression are important in the pathogenesis of noninfectious corneal shield ulcers in atopic ocular surface disease. It remains the further goal of future studies to determine how other ocular surface mucin genes respond to allergic ocular surface inflammation. Such investigations will give insight into the underlying mechanisms of allergic conjunctivitis and the role of mucins in epithelial defense against allergens.

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Atopic Ocular Surface Disease

Implications on Tear Function and Ocular Surface Mucins

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Purpose: To describe tear function, mucin alterations, and ocular surface disorder in patients with atopic diseases.

Methods: Subjects underwent corneal sensitivity measurements, Schirmer test, tear film break-up time (BUT) assay, and fluorescein and rose Bengal staining of the ocular surface. Conjunctival impression cytology and brush cytology were also conducted. Impression cytology samples underwent PAS and immunohistochemical staining for MUC5AC. Brush cytology specimens underwent evaluation for inflammatory cell expression and RT-PCR for MUC5AC mRNA expression. Differences related to tear function and ocular surface examination parameters among patients with and without corneal ulceration and healthy control subjects were studied.

Results: Mean corneal sensitivity and BUT values were significantly lower in atopic patients with corneal ulcers compared with patients without ulcers and controls ($P < 0.001$). Brush cytology specimens from patients with corneal ulcers revealed significantly higher expression of inflammatory cells compared with patients without ulcers and controls ($P < 0.001$). Impression cytology samples from eyes with corneal ulcers showed significant squamous metaplasia and reduction of goblet cell density compared with eyes without ulcers and control subjects. Specimens from eyes with corneal ulcers showed PAS (+) mucin pick up and did not stain positive for MUC5AC. MUC5AC mRNA expression was significantly lower in eyes with corneal ulcers compared with in eyes without ulcers and control subjects.

Conclusions: Ocular surface inflammation, tear film instability, and decreased conjunctival MUC5AC mRNA expression are important in the pathogenesis of noninfectious corneal shield ulcers in atopic ocular surface disease.

Key Words: atopy, impression cytology, corneal sensitivity, corneal ulcer, tear film, goblet cell, conjunctival squamous metaplasia

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Bronchial asthma, allergic rhinitis, atopic dermatitis, and ocular allergic disorders are among the most frequent atopic conditions encountered in clinical practice. Recent studies indicate that the lifetime prevalence rates for these atopic diseases in children and adolescents vary at around 24%–45%.[1] Allergic conjunctivitis is one of the most common problems encountered in these individuals.

Ocular allergies have been classified into 4 categories: seasonal or perennial allergic conjunctivitis, contact lens–associated giant papillary conjunctivitis, vernal keratoconjunctivitis (VKC), and atopic keratoconjunctivitis (AKC).[2] Ocular allergic disease results from a complex interplay of several immunologic and inflammatory pathways. However, a unifying characteristic of all forms of ocular allergy is the presence of eosinophils, major inflammatory cells in the IgE-mediated late-phase reaction. Furthermore, it is also known that leukocytes are recruited in ocular allergies.[3] The ocular inflammatory process and release of allergic mediators onto the ocular surface and tear film are thought to be responsible for a wide range of clinical corneal manifestations including superficial punctate keratitis, macroerosions, corneal ulceration, plaque formation, corneal neovascularization, and lipid infiltration.[4]

THE ROLE OF INFLAMMATION IN OCULAR ALLERGIC DISEASE

Eosinophils are thought to exacerbate the late-phase inflammatory response in immediate-type allergic reactions by releasing leukotrienes and highly cytotoxic proteins such as major basic protein (MBP) and eosinophilic cationic protein (ECP).[5] These proteins may cause a variety of corneal disorders including superficial punctate keratopathy and corneal ulcer in VKC and AKC[6–9]; the presence of eosinophils and deposition of ECP have been documented in conjunctival tissues and tears of patients with these 2 ocular allergies.[10–14] MBP deposits have also been observed in allergic corneal ulcers.[15] Furthermore, it has been shown that purified MBP and ECP reduce corneal epithelial cell viability and cause morphologic changes in vitro.[16] These findings strongly suggest that eosinophils play important roles in the pathogenesis of allergic corneal ulcer. However, the precise mechanisms by which eosinophils damage corneal tissue remain unclear.

Allergic reactions in the conjunctiva induce the release from inflammatory cells of various types of cytokines including proinflammatory cytokines and helper type 2 T cell (T_H2) cytokines. In tears of allergic patients, interleukin (IL)-4

and tumor necrosis factor (TNF)-α levels are significantly higher than in normal eyes.[17,18] These 2 cytokines are known to modulate various functions of fibroblasts such as eotaxin production and adhesion molecule expression.[19,20] Eotaxin is known to induce further recruitment of eosinophils,[21] and adhesion molecules are thought to play an important role in binding of eosinophils, which may stimulate these cells further to release inflammatory mediators.[22,23]

Recently, it has been demonstrated that CD11/18-dependent adhesion is a critical step in human eosinophil degranulation.[24,25] Eosinophils express all 4 members of the CD18 (β_2) leukocyte integrin family, CD11a–d, which allow them to bind to their ligands, ICAM-1–3.[26–31] Eosinophils also express the cell surface markers CD49d/CD29, which bind to the ligand VCAM-1.[32] In human lung fibroblasts, IL-4– and TNF-α–dependent expression of ICAM-1 and VCAM-1 and the influence of eosinophil-fibroblast adhesion on eosinophil degranulation have been reported.[33] However, it is still unclear how eosinophil-fibroblast interactions influence allergic corneal inflammation, in particular, the course of corneal ulcer formation. We believe that actual adhesion of eosinophils to corneal fibroblasts through ICAM-1 and/or VCAM-1 may induce subsequent activation and may contribute to the evolution of persistent allergic corneal ulcers.

We have found that among eosinophil adhesion-related genes, VCAM-1 and ICAM-1 are selectively expressed on fibroblasts activated with IL-4 and TNF-α (unpublished data). We have also noted that IL-5–primed eosinophils adhere to corneal fibroblasts treated with IL-4 and TNF-α, and thereby damage the fibroblasts. Anti-ICAM-1 antibody and anti-CAM-1 antibody inhibited eosinophil adherence to fibroblasts and hence fibroblast damage (unpublished data). Eosinophils can adhere to activated fibroblasts and induce subsequent fibroblast damage through these adhesion molecules. Eosinophil adhesion to fibroblasts may possibly contribute to the pathogenesis of severe persistent allergic corneal ulcers. In another study carried out by our allergy research team, the percentages of eosinophils and neutrophils in cell suspensions from VKC patients (n = 34) with corneal erosion or ulcer were found to be higher than those from subjects with clear corneas or superficial punctate keratopathy, and IL-8 concentrations in supernatant of samples significantly correlated with the percentage of neutrophils and eosinophils. Indeed, it has been reported that IL-8 has potent chemotactic activities for not only neutrophils but also activated eosinophils.[34]

Atopic Ocular Surface Disease: Tear Function Alterations and Changes of Ocular Mucins

In a previous study, we described the ocular surface disorders of 362 patients with severe active atopic dermatitis (AD) in whom routine ophthalmic examinations as well as tear film break-up time (BUT) and Schirmer tests were carried out.[35] Lid eczema (65.7%), AKC, and superficial punctate keratopathy (67.5%) were the dominant ocular diseases in these patients. Tear function tests showed BUT values of <10 seconds and Schirmer test values of <5 mm in 62.4% and 56.2% of the eyes, respectively. In our follow-up study, we aimed to clarify the pathogenesis of ocular surface disorders in 44 patients with AD.[36] A total of 22 normal control subjects

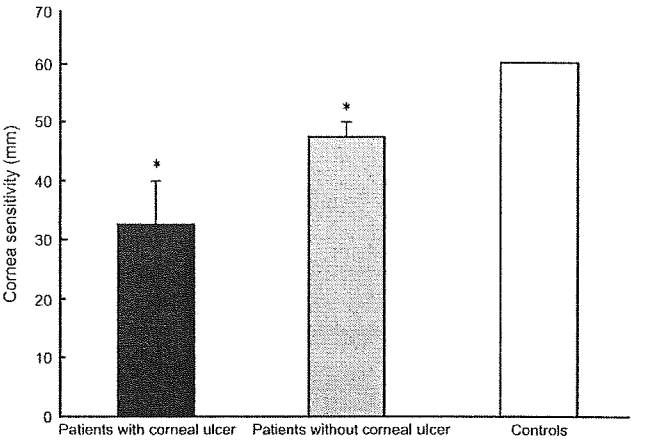

FIGURE 1. Comparison of corneal sensitivity between patient and control eyes. *$P < 0.05$ (Fisher exact test). Reprinted with permission from Dogru et al.[38]

were also included in the study protocol consisting of routine ophthalmic examinations, tear film BUT, Schirmer test, and conjunctival impression cytology. Patients and controls were compared for tear function parameters, goblet cell density, and conjunctival squamous metaplasia grade. The relation of duration and recurrences of AD to the ocular surface disorder also was investigated. Chronic allergic conjunctivitis with superficial punctate keratitis was the most frequent clinical presentation. BUT and Schirmer test values were significantly lower in patients with AD compared with those of control subjects. Impression cytology showed goblet cell loss and conjunctival squamous metaplasia, both of which correlated with the number of recurrences of AD rather than duration of disease. Facial atopy and AKC related to metaplasia of the ocular surface. Patients with reduced goblet cell density also showed low BUT levels. Based on these findings, we concluded that ocular surface disorder of AD is characterized by goblet cell loss and conjunctival squamous metaplasia, which seem to evolve independently of the duration of disease but to worsen with increased number of flare ups. Direct epithelial damage by allergic reaction and disorder of tear quality and

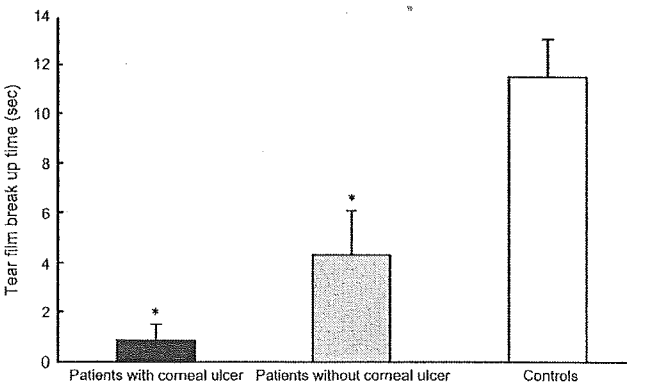

FIGURE 2. Comparison of tear film break-up time between patient and control eyes. *$P < 0.05$ (Fisher exact test). Reprinted with permission from Dogru et al.[38]

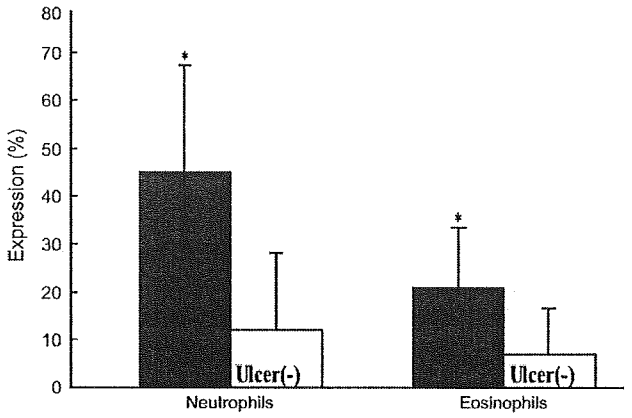

FIGURE 3. Comparison of inflammatory cell numbers in brush cytology samples in patients with (filled column) and without (open column) corneal ulcers. *$P < 0.05$ (Mann-Whitney U test). Reprinted with permission from Dogru et al.[38]

quantity were thought to be important in the progression of atopic ocular surface disease.

We have also reported that reduction of goblet cell counts is significantly related to tear instability.[36] Indeed, goblet cells are thought to secrete the main source of tear mucin, mucin MUC5AC, the secretion of which is limited only to conjunctival goblet cells that are interspersed among stratified cells in conjunctival epithelium.[37] Ocular surface mucins are believed to provide a barrier to prevent pathogens and particulate matter from entering the ocular surface epithelium and, through their heavy *O*-linked glycosylation, maintain hydration of the ocular surface and tear stability.[37] Our previous observations underlined the need for further studies investigating changes of ocular mucin expression in atopic patients so as to explain the relation of such changes to ocular surface disorders. Increased understanding of such changes at the ocular surface including alterations at the cellular level in the conjunctiva may help explain the pathogenesis and subsequent clinical appearance of atopic ocular allergies, which potentially may be blinding. Therefore, in a recent study, we performed Schirmer test, tear film BUT, corneal sensitivity measurements, fluorescein and rose Bengal staining of the ocular surface, conjunctival impression and brush cytology, immunohistochemical staining of the impression cytology samples with anti-MUC5AC antibodies, and RT-PCR of the brush cytology samples for MUC5AC mRNA expression in atopic patients with or without corneal ulcers and compared the results with those of normal control subjects.[38] Mean corneal sensitivity and BUT values were significantly lower in atopic patients with corneal ulcers compared with those in patients without ulcers and controls (Figs. 1 and 2). Brush cytology specimens from patients with corneal ulcers revealed significantly higher numbers of inflammatory cells compared with those from patients without ulcers and controls (Fig. 3). Impression cytology samples from eyes with corneal ulcers showed significant squamous metaplasia and reduction of goblet cell density compared with eyes without ulcers and eyes of control subjects (Table 1). Specimens from eyes of a representative 9-year-old male patient (Fig. 4A,B) with corneal ulcer showed PAS (+) mucin pick up and did not stain positive for MUC5AC (Fig. 5A,B). MUC5AC mRNA expression was significantly lower in eyes with corneal ulcers compared with that in eyes without ulcers and eyes of control subjects (Fig. 6). We found that despite the decrease in goblet cell counts in eyes of atopic patients compared with controls, MUC5AC mRNA expression was significantly up-regulated in eyes without ulcers relative to healthy control eyes with significant down-regulation in eyes with corneal ulcers. We believe that increased MUC5AC gene expression is a manifestation of an ocular surface defense response to compensate for the ailing ocular surface health with eventual down-regulation due to progression of atopic allergic ocular surface disease. Immune staining for MUC5AC revealed the presence of MUC5AC secretion by the ocular surface in both atopic eyes without ulcers and control eyes. However, despite PAS (+) staining results for mucin in all atopic eyes with corneal ulcers, immune staining with anti-MUC5AC antibodies consistently revealed negative staining results. An explanation for this observation might be the decrease of MUC5AC expression with possible up-regulation of alternative mucins to protect the ocular surface. Further studies identifying the concomitant alterations of mucins 1, 2, 4, and 16 in such eyes may provide essential and interesting information. Increases in the number of goblet cells with mucus overproduction are a common feature of allergic asthma.[39] A recent study associated this increase with increased expression of MUC5AC mRNA in a murine model of allergic asthma.[40] In cases of allergic conjunctivitis, however, little information on mucin expression and goblet cell numbers is available. Two studies of goblet cells in a guinea pig model of allergic conjunctivitis demonstrated decreases of conjunctival goblet cells, but there is no information on mucin gene expression relative to ocular allergic response.[41,42] A recent study by Kunert et al[43] in a mouse model of allergic conjunctivitis demonstrated that in mouse conjunctiva, repetitive application of allergens induces reduction in the number of goblet cells and decreases of MUC5AC and MUC4 mRNA. After 48 hours, goblet cell numbers returned to naive levels and goblet cell mucin mRNA levels returned to above or

TABLE 1. Comparison Between Impression Cytology Parameters in Patients and Controls

	Corneal Ulcer (+)	Corneal Ulcer (–)	Controls
Squamous metaplasia (Nelson's)	2.35 ± 0.79*	1.22 ± 0.84*	0.08 ± 0.29
Goblet cell density (cells/mm^2)	204 ± 226*	712 ± 527*	1726 ± 583

*$P < 0.05$ versus control (Fisher's exact test).

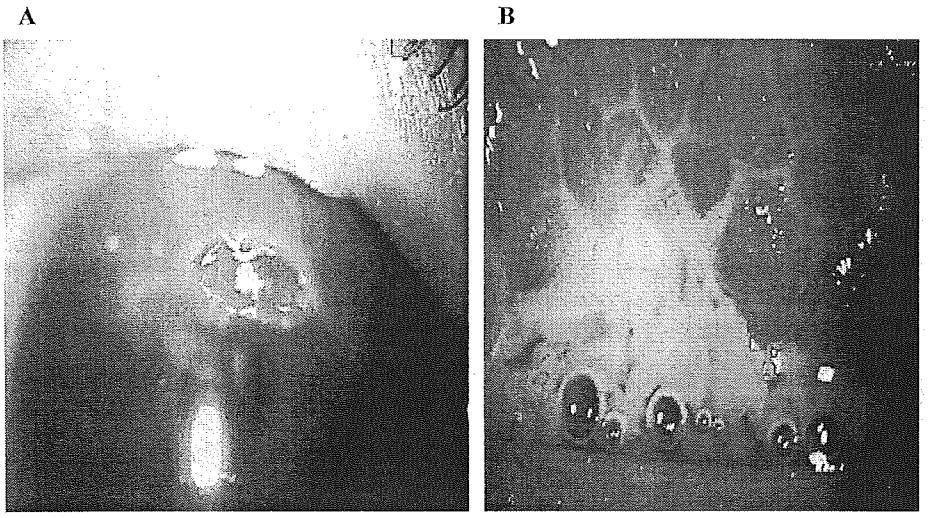

FIGURE 4. A, Anterior segment photograph of a 9-year-old atopic boy with corneal ulceration. Corneal sensitivity: 25 mm; Schirmer test: 25 mm; BUT: 1 second. Note the upper corneal ulceration. B, Note the upper tarsal conjunctival papillary hypertrophy, injection, chemosis, and the copious mucus discharge. Reprinted with permission from Dogru et al.[38]

within normal range. The study concluded that it remains to be determined how mucin gene regulation occurs in allergic human ocular surface epithelia. A PubMed and MEDLINE search revealed that ours is the first report on the level of MUC5AC gene expression in human ocular surface allergy. Our observations on conjunctival neutrophil and eosinophil expression in brush cytology also provide some insight into the relationship between ocular surface inflammation and mucin gene expression. We found significantly higher numbers of eosinophils and neutrophils in eyes with corneal ulceration compared with eyes without an ulcerative process. We believe that there is an inverse relationship between the level of ocular surface inflammation and MUC5AC expression in atopic eyes with allergy.

Increasing evidence from recent studies supports the concept that mucus production is directly affected by inflammatory cytokines. Mucus production seems to be independent of B cells and immunoglobulins but dependent on T_H2-associated cytokines.[44] Initially, IL-4 was shown to cause goblet cell metaplasia and up-regulation of MUC2 and MUC5AC both in vitro and in vivo in the airway.[45,46] Recently, the T_H2-associated cytokines IL-9 and IL-13 were demonstrated to be central mediators of allergic asthma and goblet cell metaplasia.[47,48] In patients with AKC and VKC, IL-4 and IL-5 are overexpressed in tears.[49] In addition, high levels of IL-4 have been noted in conjunctival brush cytology samples of patients with allergic conjunctivitis.[50] Even though most publications related to mucin gene expression in allergy focus on cytokines, factors associated with neutrophils and eosinophils as major inflammatory components should also be considered in regard to ocular surface goblet cell population and mucin gene regulation. Indeed, goblet cells have been reported to be very sensitive to ocular surface inflammation, which leads to their depletion and to subsequent detrimental effects on ocular surface epithelia.[51] For instance, eosinophil-derived major basic and cationic proteins, neurotoxins, and collagenases have been shown to damage corneal epithelium and basement membrane.[52–54] It is our belief that higher levels

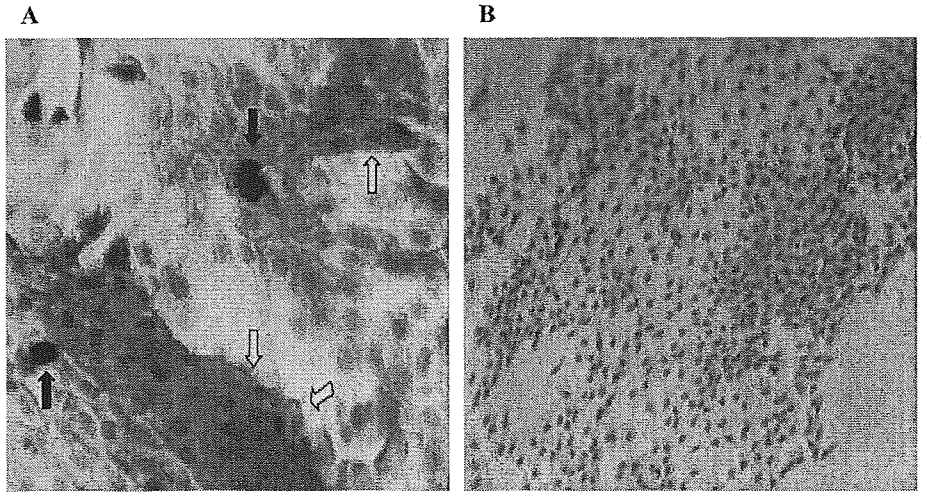

FIGURE 5. A, PAS staining of an impression cytology specimen from a representative 9-year-old atopic male subject with corneal ulceration (×400). Black arrows indicate goblet cells and gray arrows show mucin pick up by the membrane. Mean specimen squamous metaplasia grade: 2.03; mean specimen goblet cell density: 60 cells/mm^2. B, Immune staining of the impression cytology specimen with MUC5AC antibodies. Note the absence of positive staining (×200). Reprinted with permission from Dogru et al.[38]

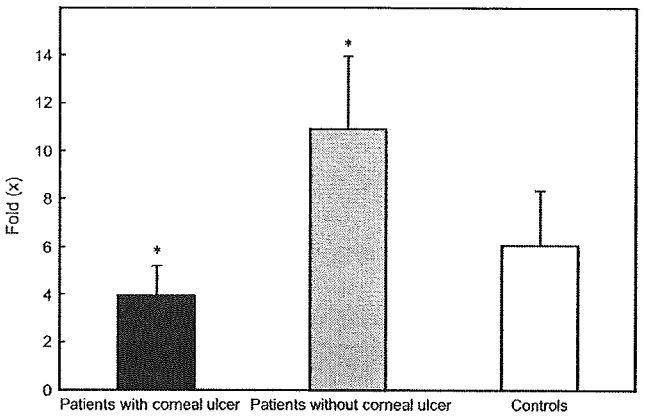

FIGURE 6. Comparison of relative MUC5AC expression levels in RT-PCR between patient and control eyes. $*P < 0.05$ (Fisher exact test). Reprinted with permission from Dogru et al.[38]

of ocular surface inflammation, tear instability, decreased corneal sensitivity, and decreased conjunctival MUC5AC mRNA expression are important in the pathogenesis of noninfectious corneal shield ulcers in atopic ocular surface disease.

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