

Original article

The differences of tear function and ocular surface findings in patients with atopic keratoconjunctivitis and vernal keratoconjunctivitis

Background: The pathogenesis of the ocular surface disease in atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC) has not been fully understood. We tried to clarify the differences in the ocular surface status in patients with AKC, VKC, and healthy control subjects.

Methods: Twenty-four eyes of 12 AKC patients, 12 eyes of six VKC patients, and 20 eyes of 10 normal control subjects were studied. The subjects underwent corneal sensitivity measurements, Schirmer test, tear film break-up time (BUT), vital staining of the ocular surface, conjunctival impression and brush cytology. Impression cytology samples underwent periodic acid Schiff staining for goblet cell density, squamous metaplasia grading, and immunohistochemical staining for MUC1, 2, 4, and 5AC. Brush cytology specimens underwent staining for inflammatory cell counting and Real Time PCR for MUC1, 2, 4, and 5AC mRNA expression.

Results: The mean BUT, corneal sensitivity, and conjunctival goblet cell density values in AKC patients were significantly lower compared with VKC patients and control subjects. The squamous metaplasia grades in eyes with AKC were significantly higher compared to eyes with VKC and controls. The inflammatory cell response in brush cytology specimens was different between patients with AKC and VKC. Eyes with AKC showed significantly higher MUC1, 2 and 4 and lower MUC5AC mRNA expression compared to eyes with VKC.

Conclusions: Differences of the infiltrates, higher level of tear instability, lower corneal sensitivity, up-regulation of MUC1, 2, and 4, and down regulation of MUC5AC were important differential features of the ocular surface disease in AKC compared with VKC.

Y. Hu^{1,2,3}, Y. Matsumoto¹,
M. Dogru^{1,3,4}, N. Okada¹,
A. Igarashi^{1,4}, K. Fukagawa¹,
K. Tsubota^{1,4}, H. Fujishima¹

¹Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan; ²Department of Ophthalmology, Xinhua Hospital affiliated to Medical School of Jiaotong University, Shanghai, China; ³J & J Ocular Surface and Visual Optics Department, Keio University School of Medicine, Tokyo, Japan; ⁴Department of Ophthalmology, Tokyo Dental College, Chiba, Japan

Key words: atopic keratoconjunctivitis; brush cytology; impression cytology; ocular surface mucin; vernal keratoconjunctivitis.

Dr Murat Dogru
Department of Ophthalmology
Keio University School of Medicine
Shinanomachi 35
Shinjuku-ku
Tokyo 160-8582
Japan

Accepted for publication 3 April 2007

Atopic diseases are present in 5–20% of the general population (1). The lifetime prevalence rates for the atopic diseases in children and adolescents vary between 24% and 45%. Allergic conjunctivitis is one of the most common problems encountered in these patients. Atopic keratoconjunctivitis (AKC), and vernal keratoconjunctivitis (VKC) are two main types of allergic conjunctivitis which can affect the physiology and function of ocular surface and may cause significant complications leading to loss of vision (2). Atopic keratoconjunctivitis is a bilateral chronic hypersensitivity disease of the ocular surface associated with systemic atopic dermatitis (AD), characterized by conjunctival and corneal lesions of variable severity (3). The ocular inflammatory process which release cytokines and chemical mediators onto the ocular surface and tear film are thought to be responsible for a wide variety of clinical manifestations, such as lipid infiltration, superficial punctate keratitis, macroerosions, corneal ulcerations, and neovascularization.

Vernal keratoconjunctivitis is a recurrent chronic disease of children and adolescents characterized by ocular surface inflammation and corneal lesions of variable severity. More than 60% of VKC cases have many relapses of the disease in a year. In many patients, the disease becomes perennial after the first few years. In 23% of the cases, the disease is perennial without any remission in symptoms from the beginning (4). Giant papillae on the upper tarsal conjunctiva and gelatinous limbal infiltrates are the characteristic features of two clinical forms of VKC (5).

Pathogenesis of allergic conjunctival disorders has not been fully understood and differences of ocular surface and tear film status between AKC, VKC and healthy control subjects have not been fully investigated. An increased understanding of the alterations of the ocular surface at the cellular level may help explain the pathogenesis and the subsequent clinical appearance of the atopic ocular allergies which may be potentially blinding.

Therefore, we carried out Schirmer test, tear film break-up time (BUT), ocular surface staining, corneal sensitivity measurements, conjunctival impression and brush cytology, and quantitative Real Time PCR of the brush cytology samples for MUC1,2,4, and 5AC mRNA expression in eyes with AKC and VKC, and compared the results with those of healthy control subjects to enable further insight into the underlying mechanisms of the disease process.

Methods

Subjects

Twenty-four eyes of 12 AKC cases complicated with AD (11 males, one female; age range: 6–34 years; average ocular disease duration: 12.4 years) as well as 12 eyes of six patients (four males, two females; age range: 6–13 years; average ocular disease duration: 5.4 years) with VKC were studied. 20 eyes of 10 subjects (eight males, two females; age range: 9–33 years) who did not have any history of contact lens use, ocular surgery, ocular or systemic disease, or drug that would alter the ocular surface were studied as control group.

The diagnosis of AD was confirmed by the dermatology department in the patients who had pruritus, typical flexural lichenification, popular eruptions, and tendency toward chronically relapsing dermatitis. All patients were diagnosed by two ophthalmology specialists (H.F. and K.F.) who also were allergy specialists.

Radioallergosorbent tests and scratch tests were also carried out in all patients. Skin prick tests were performed with allergen extracts of rye grass, *Dermatophagoides pteronyssinus* (Der p) (house dust mite), *Phleum pratense* (timothy) pollen, cedar tree pollen, cat dander, dog dander, penicillium, rice, tuna, salmon, shrimp, cedar cheese, milk (SRL, Tokyo, Japan) by applying a drop of allergens to the forearm and then puncturing the skin with a prick needle. Histamine was used as a positive control (1 mg/ml) and the diluent, buffered saline containing 0.03% human serum albumin, as a negative control. A wheal and flare reaction, with a diameter of > 2 mm and occurring within 15 min from the application of the allergen, indicated a positive allergic response. Venous peripheral blood (20 ml) was collected from all patients and stored at –20°C for subsequent assaying of total IgE. All measurements were performed with commercially available radioimmunoassays (SRL).

Patients who had AD, symptoms of allergic conjunctivitis without seasonal aggravation, presenting with small and middle-sized conjunctival papillae and keratopathy, were diagnosed as having AKC. A diagnosis of VKC was based on its typical clinical presentation, including presence of tarsal or limbal papillae, itching, photophobia and tearing. None of the patients 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. This research followed the tenets of the Declaration of Helsinki. Informed consent from all subjects and permission from the Ethical Committee of Tokyo Dental and Keio University, School of Medicine, were obtained about the procedures and possible consequences of the study. As ethic board committees did not allow a wash-out period in subjects with an active disease process to study the naïve ocular surface status, only 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 4 weeks were included in this study. No patient of AKC or VKC was being treated with cytotoxic immunosuppressants, topical/systemic cyclosporine, systemic steroids or prostaglandin inhibitors within 6 months prior to the time of inclusion into the study. All examinations were performed by the same researcher (M.D).

The subjects underwent tear function and ocular surface examinations including corneal sensitivity measurements, Schirmer test, tear film BUT, fluorescein and Rose Bengal staining of the ocular surface, conjunctival impression cytology and brush cytology. Impression cytology samples underwent periodic acid Schiff (PAS) staining to observe the grade of epithelial squamous metaplasia and evaluate the goblet cell density. Samples also underwent immunohistochemical staining with MUC1, 2, 4, and 5AC antibodies to confirm their presence on the ocular surface. Brush cytology specimens from upper tarsal conjunctiva underwent Diff-Quik staining for inflammatory cell counting and quantitative RT-PCR for MUC1, 2, 4, and 5AC mRNA expression.

Corneal sensitivity measurements

Measurement of corneal sensitivity was performed using a Cochet-Bonnet aesthesiometer as described previously (6). A corneal sensitivity measurement of < 50 mm was regarded as low corneal sensitivity in this study.

Tear function tests and ocular surface staining

The standard tear film BUT measurement was performed. The ocular surface was examined by the double vital staining method. Two microliter of preservative-free combination of 1% Rose Bengal and 1% fluorescein dye was instilled in the conjunctival sac as previously reported (7–9). 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 < 10 s was considered abnormal. Fluorescein and Rose Bengal staining of the ocular surface was also noted and scored. Fluorescein and Rose Bengal staining scores range between 0 and 9 points. Any score above 3 points was regarded as abnormal. For further evaluation of tears, the standard Schirmer test 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 recorded in millimeters of wetting for 5 min. A reading of < 5 mm was referred to as aqueous deficiency.

Conjunctival impression cytology

After administration of topical anesthesia with 0.4% oxybuprocaine, five 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 the upper nasal and temporal palpebral conjunctiva, pressed gently by a glass rod, and then removed, fixed in formaldehyde. One of the specimens was stained with PAS, dehydrated in ascending grades of ethanol and then with xylol, and finally coverslipped. Other specimens underwent immunohistochemical staining with MUC1, 2, 4, and 5AC antibodies as reported previously (10). One of the specimens allocated to immunohistochemical staining to confirm the presence of mucin staining was cut into two halves, one-half reserved for negative control antibody staining observations. The quantitative studies of conjunctival goblet cells and squamous metaplasia of

conjunctival epithelial cells were conducted under a light microscope at a magnification of 400 $\times$. We photographed five nonoverlapping 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 pick up.

Immunohistochemical staining

The impression cytology specimens for immunohistochemical 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, placed on slides and blocked in PBS with rabbit or horse serum albumin according to later used primary antibodies for 20 min. Then primary mouse monoclonal MUC 1 and 2 antibodies at a dilution of 1 : 10 (PROGEN Biotechnik GmbH, Heidelberg, Germany), the primary goat polyclonal MUC 4 antibody at a dilution of 1 : 10 (Santa Cruz Biotechnology, Inc., CA, USA), and the primary mouse monoclonal MUC 5AC antibody at a dilution of 1 : 50 (Cosmo Bio, Tokyo, Japan) were applied for 1 h at room temperature in a moist chamber. After rinsed with PBS for 10 min, the specimens were treated with anti-goat/anti-mouse secondary antibodies for 30 min and rinsed again. Samples were then processed by SAB-PO (M) kit (Nichirei, Tokyo, Japan) protocol (DAB-peroxide staining). Finally, the specimens were placed in the plastic embedding cassettes again for counter staining with hematoxylin, dehydrated in ascending grades of ethanol and then transferred to glass bottles with xylol, and coverslipped for light microscopic examination. For isotype controls, primary antibody was replaced with goat/mouse IgG1 (Sigma, St Louis, 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

After administration of topical anesthesia with 0.4% oxybuprocaine, two adjacent areas of central upper palpebral conjunctiva were each scraped seven times with the Cytobrush- S (Medscand AB, Sweden) as reported previously (10). The examiner held the brush 2 cm away from the brush end and applied gentle pressure to the conjunctiva. After sampling, the brushes were immediately soaked in 1 ml of Hank's buffered solution, and the containers were shaken to detach the cells from the brush. The suspended cells were collected using the Millipore filter technique employing filters with 8 μ m pore size. One of the samples underwent Diff-Quik staining for the assessment of conjunctival inflammatory cell numbers. To be able to delineate the differences in the numbers of inflammatory cells, the epithelial cell counts were ignored. Only the inflammatory cells were counted from 10 nonoverlapping fields and the means were calculated for purposes of this study. Neutrophil, lymphocytes, and eosinophil percentages were then compared between eyes with AKC and VKC and healthy control eyes. The other brush cytology samples were transferred to 1.5 ml Eppendorf tubes immediately and centrifuged at 4°C 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 PCR.

Real time quantitative PCR for MUC1, 2, 4, and 5AC mRNA 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 μ l final volume in the presence of 1.25 μ l of the following 'Assay by Design' oligonucleotides (MUC1, 2, 4, 5AC, 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 TaqMan master mix, and was run on 7700 ABI thermal cyclers (Applied Biosystems). The thermal profile consisted of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 94°C for 15 s, and 60°C for 1 min. Data were acquired and analyzed using Sequence Detection System Software (Applied Biosystems) with manual adjustment of the baseline and threshold parameters. The expression levels of mRNA for MUC1, 2, and 4 were normalized by the median expression of a housekeeping gene (GAPDH). Relative expression levels of MUC5AC 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 GRAPH PAD software (InStat, San Diego, CA, USA). The analysis of data was performed ANOVA test. A probability level of <5% was considered statistically significance.

Results

Patient characteristics and slit lamp findings

All patients with AKC had active severe AD with pruritus, typical flexural lichenification, papular eruptions, and tendency toward chronically relapsing dermatitis. Personal or family history of atopic disease was present and confirmed by the consulting dermatologist in all cases at the time of examination. All patients with AKC and 80% of patients with VKC had positive skin reactivity to multiple allergens. The most frequent sensitizing allergens in patients with AKC were Der p (89%), cedar tree pollen (78%), and *P. pratense* pollen (67%), whereas cedar tree pollen (80%), Der p (60%), and *P. pratense* pollen (60%) were the most common sensitizing allergen in patients with VKC. Eighty-nine percent of the patients with AKC had an elevation of serum IgE level (mean: 10060 IU/ml, range: 140–49730 IU/ml), whereas 80% of the patients with VKC had an elevation of serum IgE level (mean: 1180 IU/ml, range: 42.6–2600 IU/ml). The average duration of AD was 12.4 years and the average age was 21 years. Seven out of 12 patients with AKC (58%) had the onset of disease at preteen years. All patients with VKC suffered from tarsal form of the disease, had allergic history like asthma or allergic rhinitis and seasonal recurrence of

keratoconjunctivitis characterized by intense itching, tearing, mucus secretion, photophobia. The average duration of VKC was 5.4 years and the average age was 10 years. All patients with AKC and VKC complained of allergic and dry eye symptomatology, including itchiness, redness, foreign body sensation, and irritation.

By slit lamp observation, all patients with active AKC had conjunctival injection, chemosis, and mild to moderate papillary hypertrophy without any giant proliferative papillary lesions. All eyes with AKC also had diffuse subconjunctival fibrosis, while all eyes with VKC showed upper tarsal giant papillae without an evidence of subconjunctival fibrosis or gelatinous limbal infiltration.

Tear function examinations

All patients with AKC and VKC had BUT values < 10 s (3.1 ± 1.6 and 4.5 ± 1.0 s, respectively), whereas none of the control eyes had a low BUT value (11.4 ± 1.0 s). The mean BUT value for eyes with AKC was significantly lower than control eyes, and the mean BUT value of AKC fared significantly worse than that of VKC as shown in Fig. 1 ($P < 0.01$). All eyes of patients with AKC, VKC, and control subjects had normal Schirmer test values (13.5 ± 2.5 , 25.3 ± 8.1 , and 18.5 ± 3.0 mm, respectively). The mean tear quantity of eyes with VKC was significantly higher compared with AKC and healthy control subjects as shown in Fig. 2 ($P < 0.01$) while the mean tear quantity in eyes with AKC was significantly lower than the controls.

Ocular surface vital staining

The mean fluorescein and Rose Bengal staining scores of eyes with AKC and VKC were significantly higher than those of the control group which were 0.1 ± 0.3 and 0.06 ± 0.25 points, respectively ($P < 0.01$) as shown in Fig. 3. Atopic keratoconjunctivitis group showed comparatively higher fluorescein and Rose Bengal staining scores of 4.9 ± 3.2 and 4.5 ± 3.3 points compared with the values in the VKC group which were 4.2 ± 2.5 and 3.6 ± 2.8 points, respectively.

Corneal sensitivity

The mean corneal sensitivities were significantly lower in eyes with AKC compared to eyes with VKC and healthy control subjects, which were 37.5 ± 5 , 45.5 ± 2.7 , and 60 ± 0 mm, respectively as shown in Fig. 4 ($P < 0.01$).

Conjunctival impression cytology

Conjunctival imprints from all eyes contained sheets of conjunctival epithelial cells with variable amount of goblet cells and mucin pick-up.

Squamous metaplasia

The mean squamous metaplasia grade was significantly higher in eyes with AKC (2.4 ± 0.5) and VKC (1.5 ± 0.6) compared with control subjects (0.06 ± 0.5) as shown in Table 1 ($P < 0.01$). The mean squamous metaplasia grade in eyes with AKC was significantly higher compared to eyes with VKC ($P < 0.05$).

Goblet cell density

The goblet cell densities were lower than 1000 cells/mm² in all eyes with AKC and VKC whereas none of the control eyes had a goblet cell count < 1000 cells/mm². The average goblet cell density was significantly lower in eyes with AKC (285 ± 264 cells/mm²) compared to eyes with VKC (569 ± 477 cells/mm², $P < 0.05$) and controls (1778 ± 486 cells/mm², $P < 0.01$), as shown in Table 1.

Immunohistochemical staining

Immunohistochemical stains of the conjunctival imprints from patient eyes confirmed presence of MUC1, 2, 4, and 5AC and epithelial cells with variable degrees of squamous metaplasia. Isotope control stainings for all subjects showed absence of nonspecific staining.

Conjunctival brush cytology

By Diff-Quik staining, all brush cytology samples showed numerous epithelial cells. All eyes with AKC and VKC had significantly higher counts of neutrophils, lymphocytes, and eosinophils while no inflammatory cells were found in normal control eyes ($P < 0.01$). The mean percentage of lymphocytes was significantly higher in eyes with AKC ($12 \pm 6\%$) whereas the mean percentage of eosinophils ($16 \pm 7\%$) was significantly higher in eyes with VKC ($P < 0.01$), and no significant difference of the percentage of neutrophils was found between AKC and VKC (Fig. 5).

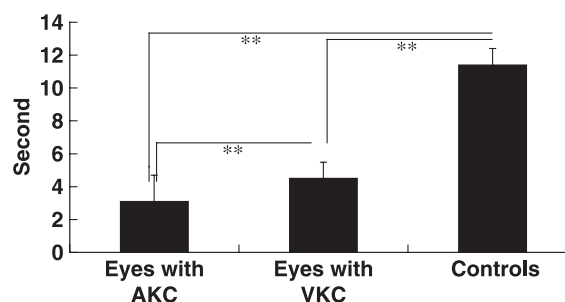


Figure 1. Comparison of break-up time (BUT) values among atopic keratoconjunctivitis (AKC), vernal keratoconjunctivitis (VKC), and control eyes. Statistical significance is denoted by $**P < 0.01$.

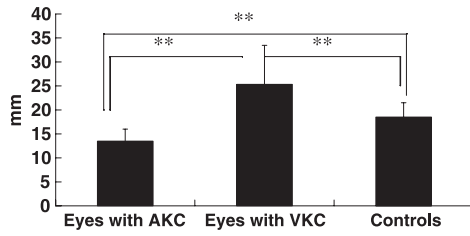


Figure 2. Comparison of Schirmer test value among atopic keratoconjunctivitis (AKC), vernal keratoconjunctivitis (VKC), and control eyes. Statistical significance is denoted by $**P < 0.01$.

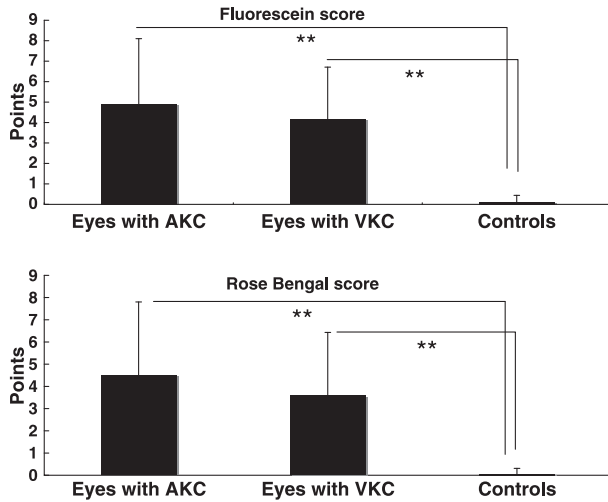


Figure 3. Comparison of fluorescein and Rose Bengal staining scores among atopic keratoconjunctivitis (AKC), vernal keratoconjunctivitis (VKC), and control eyes. Statistical significance is denoted by $**P < 0.01$.

Quantitative RT-PCR

For MUC 1, 2, and 4 mRNA expressions, there was a significant increase in number of copies/ng RNA (GAPDH) from eyes of AKC patients (MUC1: 12422 ± 4109 copies/ng, MUC2: 18 ± 7 copies/ng, MUC4: 3096 ± 850 copies/ng) compared with samples obtained from healthy controls (MUC1: 5728 ± 2740 copies/ng, MUC2: 6 ± 2 copies/ng, MUC4: 904 ± 20 copies/ng), and eyes with VKC (MUC1: 3503 ± 1525 copies/ng, MUC2: 8 ± 7 copies/ng, MUC4: 735 ± 86 copies/ng) ($P < 0.01$). The relative expression of MUC5AC mRNA in eyes with AKC and VKC was significantly down-regulated compared with healthy control eyes ($P < 0.01$). The relative MUC5AC mRNA expression in AKC was also significantly lower compared to eyes with VKC as shown in Fig. 6 ($P < 0.05$).

Discussion

In this study, we examined the tear functions and ocular surface mucin expressions in 24 eyes of 12 AKC patients,

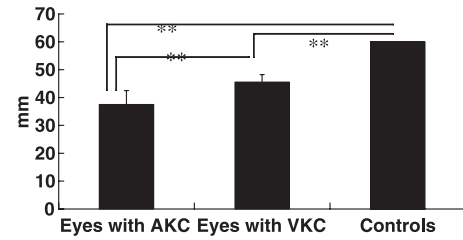


Figure 4. Comparison of corneal sensitivity. Statistical significance is denoted by $**P < 0.01$.

Table 1. Comparison of impression cytology parameters between controls and patients with atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC)

	Squamous metaplasia (Nelson's)	Goblet cell density (cells/mm ²)
AKC	$2.4 \pm 0.5^{* **}$	$285 \pm 264^{* **}$
VKC	$1.5 \pm 0.6^{* **}$	$569 \pm 477^{* **}$
Control	0.1 ± 0.5	1778 ± 486

* $P < 0.01$, compared with control group, ANOVA test.

** $P < 0.05$, compared within AKC and VKC group, ANOVA test.

12 eyes of six VKC patients, and 20 eyes of 10 healthy normal controls. All patients with AKC and 80% of patients with VKC had positive skin reactivity to multiple allergens. The most frequent sensitizing allergens in our patients were Der p, cedar tree pollen, and *P. pratense* pollen. Eighty-nine percent of the patients with AKC had an elevation of serum IgE level whereas 80% of the patients with VKC had an elevation of serum IgE level. The sensitizing allergens were similar with other parts of the world when compared with large scale study results from Italy, Sweden, and UK (14–16). The mean IgE levels in our study were higher compared with the results of an epidemiological study from Italy (17).

The corneal sensitivity was significantly lower in eyes with AKC compared to eyes with VKC and healthy control subjects. Decreased corneal sensitivity is thought to be associated with corneal epithelial and/or stromal disease (18). As described by Dogru et al., (10) the loss or decrease of trophic effects of corneal nerves because of primary or secondary inflammatory events with progression of atopic eye disease may play an important role in the worsening of the atopic ocular surface disease. Although the mechanism of corneal nerve injury in AKC and VKC is still not clear, worse corneal sensitivity in eyes with AKC may be explained by persistently active atopic skin disease in childhood in our patients which has been reported to be associated with significant general inflammation (19, 20). Indeed, all eyes with AKC had comparably more severe ocular surface disease as evidenced by diffuse subconjunctival fibrosis, partial limbal deficiency, and higher incidence of corneal opacity and vascularization.

Because we believed that decreased corneal sensitivity might have adversely affected tear functions and ocular surface status, we tried to provide more evidence by

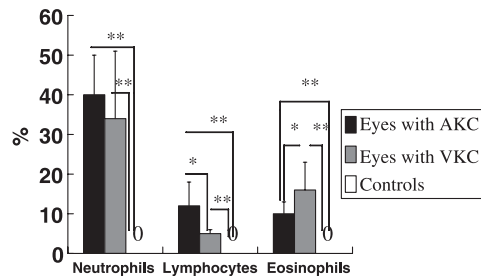


Figure 5. Comparison of percentage of inflammatory cells between controls and eyes with atopic keratoconjunctivitis (AKC), vernal keratoconjunctivitis (VKC). Statistical significance is denoted by $**P < 0.01$.

performing tear function tests and ocular surface vital staining scores.

A previous ocular surface disease study in 362 patients with severe AD and AKC revealed lid eczema and superficial punctate keratopathy as the dominant features of the ocular disease. In that study, tear function tests showed a tear instability in 62.4% of the eyes (21). In our study, although none of the patients showed aqueous deficiency type of dry eyes and all patients had normal Schirmer test values, all patients with AKC and VKC reported dry eye symptomatology and had low BUT scores. This finding suggested a BUT deficient dry eye status with positive symptomatology as described by Lemp et al., (22). The mean BUT value was significantly lower in AKC patients compared to patients with VKC, suggesting a higher grade of tear instability. To investigate whether the higher grade of tear instability in AKC coexisted with a more severe ocular surface disease, we carried out impression cytology and ocular surface vital stainings.

Conjunctival squamous metaplasia grades and goblet cell densities have been reported to reflect the ocular surface health status. Goblet cell numbers < 1000 cells/mm² have also been reported to be associated with severe ocular surface disease (23). In our study, the squamous metaplasia grades were indeed significantly higher in eyes with AKC compared to eyes with VKC. Likewise, goblet cell densities were significantly lower in eyes with AKC compared to eyes with VKC and healthy control subjects. These findings were consistent with a previous study of ours which showed higher grades of squamous metaplasia and lower goblet cell densities in eyes with AKC compared to eyes with VKC and healthy control subjects (10). The impression cytology parameters worsened with progression of the ocular surface disease process to corneal ulceration in that study. Another study comparing the severity of the ocular surface epithelial disease in patients with AKC revealed significantly worse squamous metaplasia grades and goblet cell densities in patients with significant epithelial disease which was described as a fluorescein and Rose Bengal score exceeding 4 points (24). In our current study, both vital staining scores were worse in eyes with AKC compared to eyes with VKC and healthy control subjects, suggesting a relatively more severe ocular surface disease state in eyes with AKC. To provide more clues in relation to differences of these two ocular surface diseases between AKC and VKC, we carried out brush cytology analysis.

All specimens from patients with AKC and VKC contained numerous inflammatory cells in contrast to healthy control eyes which did not show any inflammation. We believe that the high-grade inflammation in eyes with AKC and VKC resulted in depletion of goblet cells which are reported to be very sensitive to inflammation and undergo apoptosis during inflammatory states (23).

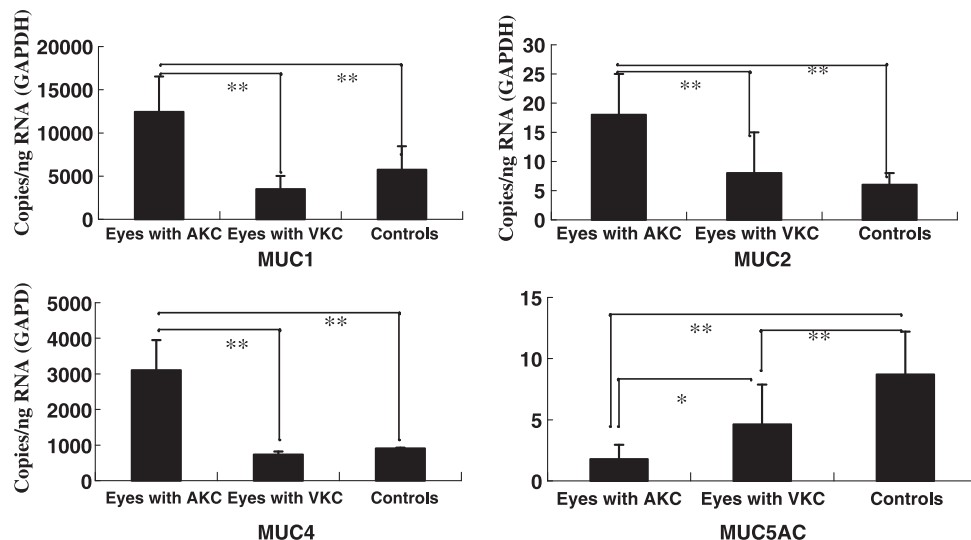


Figure 6. Comparison of MUC1,2,4, and 5AC mRNA expression levels between atopic keratoconjunctivitis (AKC), vernal keratoconjunctivitis (VKC), and controls. Statistical significance is denoted by $*P < 0.05$; $**P < 0.01$.

Eyes with AKC had significantly higher lymphocytes with relatively higher neutrophils compared to eyes with VKC, whereas eyes with VKC had a significantly higher number of eosinophils. This disparity may also provide explanation for the differences in the ocular surface disease status between patients with AKC and VKC. We also believed that the longer duration of the active ocular and skin disease process in AKC compared to VKC can also explain the aforementioned differences in ocular surface status.

Although tear levels of IL-4 and IL-5 were not measured in this study, it has been reported that their levels showed distinctive dissociative inflammatory patterns in patients with AKC and VKC. Atopic keratoconjunctivitis patients showed higher levels of tear IL-4, which was shown to have correlation with the severity of AD, while VKC patients showed higher levels of tear IL-5 (25). The differences in the levels of these TH2 cytokines, IL-4 and IL-5, between AKC and VKC may also be responsible for the phenotypic differences of the ocular surface between AKC and VKC.

Mucous production in allergy appears to be dependant on TH2 lymphocytes and their related cytokines (26). Indeed IL-4, IL-9, and IL-13 have been shown to cause goblet cell metaplasia and change expression patterns of MUC2 and MUC5AC *in vitro* and *in vivo* in respiratory areas (27–31). Kunert et al. (32) reported significant decrease of filled goblet cells and decrease of MUC5AC and MUC4 mRNA gene expression in a mouse model of allergic conjunctivitis. The level of mucin gene expression in human ocular surface allergy especially the difference between AKC and VKC has not been studied previously. In this study, consistent with goblet cell densities which were significantly lower in patients with AKC compared with VKC, the major goblet cell derived mucin expression, i.e. MUC5AC mRNA, was significantly lower in subjects with AKC compared to patients with VKC and healthy controls subjects. MUC1 and MUC4 mRNA levels tended to be lower in VKC compared with controls without statistical significance. To our surprise, however, we found that MUC1, 2, 4 mRNA expressions were significantly upregulated in eyes with AKC compared with VKC and to controls.

We believe that lower MUC5AC level in AKC compared with VKC was a descriptive feature of the severity of the ocular surface disease. Another descriptive feature of the ocular surface disease in AKC was the over-expression of epithelial mucins MUC1, 2, and 4, relative to MUC5AC expression. We also believe that over-expression of these mucins resulted from the ocular surface defense response which tried to protect the ailing ocular surface epithelium in AKC. These findings provide further explanation to our observations in a previous study where we found consistent negative MUC5AC and positive PAS staining in AKC patients with very severe ocular surface disease and corneal

ulcers, suggesting release of epithelial mucins, such as MUC1, 2, and 4 instead of MUC5AC (10). However over-expressions of MUC1, 2, and 4 instead of the most abundant and most important ocular surface mucin (33, 34), MUC5AC, were not associated with better tear stability, better vital staining scores, or impression cytology parameters. Although none of the patients with AKC had giant proliferative papillary lesions in this study, it would be interesting to study the mucin expression differences between patients with proliferative and nonproliferative variety of AKC and patients with VKC.

Although no studies exist in the literature, we believe that there may be a definite ratio and balance between goblet cell and ocular surface epithelial cell mucin expressions in healthy subjects. Any perturbation of this balance because of inflammatory processes may determine the extent of the severity of the ocular surface disease. Two studies in the literature describe goblet cell hyperplasia and proliferation in patients with AKC contrary to our findings. It might be that goblet cell hyperplasia is the result of direct stimulation by the chemical mediators of inflammation released during the immediate hypersensitivity response, and that the persistence of the inflammatory process may lead to goblet cell depletion, which might explain the differences of goblet cell findings between these previous studies and ours (35, 36).

However, the authors would like to remind the readership of the current study that all eyes in this series had received previous treatment with topical steroid and ketotifen fumarate which might at least in part have influenced the results of the current study. Although all patients with AKC and VKC received the same treatment with the same duration and frequency of instillations, steroids may up-regulate the steady state of MUC4 and MUC5AC mRNA expressions (37). The presence of preservatives in the topical formulations might also have influenced the tear function and ocular surface findings in the current study. Indeed, preservatives have been reported to induce dose dependent cytotoxicity on the conjunctivocorneal epithelium, disrupt the tear stability, various degrees of keratinization, ocular surface inflammation (38, 39). Although AKC and VKC patients usually have a history of several topical drug use at the time of first examination in many centers and recruitment of untreated naïve allergic conjunctivitis is a challenging issue, tear function, and mucin gene expression status of untreated naïve atopic ocular surfaces should be investigated in future studies. Topical cyclosporine has been reported to increase conjunctival goblet cell numbers in dry eye patients and goblet cell mucin production in canine dry eye models (40, 41). Further studies investigating the effects of topical cyclosporine on goblet cell density and preocular mucin expression in allergic conjunctivitis patients would provide invaluable information to the literature.

Another essential point that needs to be mentioned is the effect of duration of disease on the clinical ocular surface findings in AKC. Fifty-eight percent of the AKC patients in this study had the onset of the ocular disease at preteen years. Corneal sensitivity, tear stability, ocular surface vital staining and impression cytology parameters in-between patients with preteen onset AKC and VKC were not considerably different in this study (data not shown). However, these parameters were significantly worse in AKC patients with adult onset of the disease process compared with VKC patients and AKC patients with preteen onset of the disease (data not shown), suggesting that the pathogenetic mechanisms of the ocular surface disease process may be different between children and adults. The differences in these pathogenetic mechanisms may be more important than the duration of disease in determining the outcome of the severity of the ocular surface disease.

It is our belief that differences of the inflammatory processes, higher level of tear instability, and compar-

ably worse corneal sensitivity as well as over-expression of epithelial mucins, MUC1, 2, and 4 and down-regulation of MUC5AC were important differential features of the ocular surface disease in AKC. It remains the future goal of further prospective studies to determine how other ocular surface mucin genes respond to allergic ocular surface and tear film inflammation.

Acknowledgment

The authors have no proprietary interest in any of the products mentioned in this paper. Presented in part at the 29th Japan Cornea Congress, February 17–19, 2005, Tokushima, Japan. Presented at the 2006 ARVO Meeting, May, FortLauderdale, USA. This work is supported by research grants of the Japan Society for the Promotion of Science (JSPS Grant 02261 and 17591854). Dr Yiqian Hu is a research scholar of Japan China Sasakawa Medical Association.

References

- Albert DM, Jakobiec FA. Allergic and toxic reactions. In: Albert DM, Jakobiec FA, editors. Principles and practice of ophthalmology, 1st edn. Philadelphia, PA: WB Saunders, 1994:93–97.
- Bonini S, Lambiase A, Bonini S. Genetic and environmental factors in ocular allergy. In: Abelson MB, editor. Allergic diseases of the eye. Philadelphia, PA: W.B. Saunders, 2001:1–12.
- Casey R. Atopic keratoconjunctivitis. In: Abelson MB, editor. Allergic diseases of the eye. Philadelphia, PA: W.B. Saunders, 2001:137–143.
- Bonini S, Bonini S, Lambiase A, Marchi S, Pasqualetti P, Zuccaro O et al. Vernal keratoconjunctivitis revisited: a case series of 195 patients with long-term follow up. *Ophthalmology* 2000;**107**:1157–1163.
- Leonardi A, Smith ML, Secchi GA. Vernal keratoconjunctivitis. In: Abelson MB, editor. Allergic diseases of the eye. Philadelphia, PA: W.B. Saunders 2001:183–199.
- Dogru M, Yildiz M, Baykara M, Ozcetin H, Erturk H. Corneal sensitivity and ocular surface changes following preserved amniotic membrane transplantation for non-healing corneal ulcers. *Eye* 2003;**17**:139–148.
- Leibowitz HM, Villazon S. Corneal procedures. In: Leibowitz HM, Waring GO, editors. Corneal disorders. Philadelphia, PA: WB Saunders, 1998:983–984.
- Junko H. AD classification of fluorescein staining. In: Tano Y, Watanabe N, editors. Practical ophthalmology. Tokyo, Japan: Bunkodo Publishers, 1998:61.
- Toda I, Tsubota K. Practical double vital staining for ocular surface evaluation. *Cornea* 1993;**12**:366–367.
- Dogru M, Asano-Kato N, Tanaka M, Igarashi A, Shimmura S, Shimazaki J et al. Ocular surface and MUC5AC alterations in atopic patients with corneal shield ulcers. *Curr Eye Res* 2005;**30**:897–908.
- Nelson JD. Impression cytology. *Cornea* 1988;**7**:71–81.
- Di Nardo A, Vitiello A, Gallo RL. Cutting edge: mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide. *J Immunol* 2003;**170**:2274–2278.
- Razzaque MS, Foster CS, Ahmed AR. Role of collagen-binding heat shock protein 47 and transforming growth factor-beta1 in conjunctival scarring in ocular cicatricial pemphigoid. *Invest Ophthalmol Vis Sci* 2003;**44**:1616–1621.
- Dottorini ML, Bruni B, Peccini F, Bottini P, Pini L, Donato F et al. Skin prick-test reactivity to aeroallergens and allergic symptoms in an urban population of central Italy: a longitudinal study. *Clin Exp Allergy* 2007;**37**:188–196.
- Eriksson NE, Holmen A. Skin prick tests with standardized extracts of inhalant allergens in 7099 adult patients with asthma or rhinitis: cross-sensitizations and relationships to age, sex, month of birth and year of testing. *J Investig Allergol Clin Immunol* 1996;**6**:36–46.
- Arshad SH, Tariq SM, Matthews S, Hakim E. Sensitization to common allergens and its association with allergic disorders at age 4 years: a whole population birth cohort study. *Pediatrics* 2001;**108**:E33.
- Leonardi A, Busca F, Motterle L, Cavarzeran F, Fregona IA, Plebani M et al. Case series of 406 vernal keratoconjunctivitis patients: a demographic and epidemiological study. *Acta Ophthalmol Scand* 2006;**84**:406–410.
- Xavier YM, Avinoam BS. Corneal hypoesthesia. *Surv Ophthalmol* 1988;**33**:28–40.
- Carlsen KH, Halvorsen R, Pettersen M, Carlsen KC. Inflammation markers and symptom activity in children with bronchial asthma. Influence of atopy and eczema. *Pediatr Allergy Immunol* 1997;**8**:112–120.
- Neal P, Barney NP. Vernal and atopic keratoconjunctivitis. In: Krachmer JH, Mannis MJ, Holland EJ, editors. Cornea. Philadelphia, PA: Elsevier Mosby, 2005:667–674.
- Dogru M, Nakagawa N, Tetsumoto K, Katakami C, Yamamoto M. Ocular surface disease in atopic dermatitis. *Jpn J Ophthalmol* 1999;**43**:53–57.

22. Lemp MA, Dohlman CH, Holly FJ. Corneal desiccation despite normal tear volume. *Ann Ophthalmol* 1970;**6**:258–284.
23. Kinoshita S, Kiorpes TC, Friend J, Thoft RA. Goblet cell density in ocular surface disease. *Arch Ophthalmol* 1983;**101**:1284–1287.
24. Dogru M, Okada N, Asano-kato N, Igarashi A, Fukagawa K, Shimazaki J et al. Alterations of the ocular surface epithelial mucins 1, 2, 4 and the tear functions in patients with atopic keratoconjunctivitis. *Clin Exp Allergy* 2006;**36**:1–10.
25. Uchio E, Ono SY, Ikezawa Z, Ohno S. Tear levels of interferon-gamma, interleukin(IL)-2, IL-4 and IL-5 in patients with vernal keratoconjunctivitis, atopic keratoconjunctivitis and allergic conjunctivitis. *Clin Exp Allergy* 2000;**30**:103–109.
26. Korsgren M, Erjefalt JS, Korsgren O, Sundler F, Persson CG. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J Exp Med* 1997;**185**:885–892.
27. Temann UA, Prasad B, Gallup MW, Basbaum C, Ho SB, Flavell RA et al. A novel role for murine IL-4 *in vivo*: induction of MUC5AC gene expression and mucin hypersecretion. *Am J Respir Cell Mol Biol* 1997;**16**:471–478.
28. Dabbagh K, Takeyama K, Lee HM, Ueki IF, Lausier JA, Nadel JA. IL-4 induces mucin gene expression and goblet cell metaplasia *in vitro* and *in vivo*. *J Immunol* 1999;**162**:6233–6237.
29. Longphre M, Li D, Gallup M, Drori E, Ordonez CL, Redman T et al. Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. *J Clin Invest* 1999;**104**:1375–1382.
30. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998;**282**:2258–2261.
31. Lee JJ, McGarry MP, Farmer SC, Denzler KL, Larson KA, Carrigen PE et al. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J Exp Med* 1997;**185**:2143–2156.
32. Kunert KS, Keane-Myers AM, Spurr-Michaud S, Tisdale AS, Gipson IK. Alteration in goblet cell numbers and mucin gene expression in a mouse model of allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 2001;**42**:2483–2489.
33. Ilene K, Gipson IK, Hori Y, Argueso P. Character of ocular surface mucins and their alteration in dry eye disease. *The Ocular Surface* 2004;**2**:131–145.
34. Ohashi Y, Dogru M, Tsubota K. Laboratory findings in tear fluid analysis. *Clin Chim Acta* 2006;**15**:369.
35. Roat MI, Ohji M, Hunt La VE, Thoft RA. Conjunctival epithelial cell hypermitosis and goblet cell hyperplasia in atopic keratoconjunctivitis. *Am J Ophthalmol* 1993;**116**:456–463.
36. Foster CS, Rice BA, Dutt JE. Immunopathology of atopic keratoconjunctivitis. *Ophthalmology* 1991;**98**:1190–1196.
37. Gollub EG, Waksman H, Goswami S, Marom Z. Mucin genes are regulated by estrogen and dexamethasone. *Biochem Biophys Res Commun* 1995;**217**:1006–1014.
38. Becquet F, Goldschild M, Moldovan MS, Ettaiche M, Gastaud P, Baudouin C. Histopathological effects of topical ophthalmic preservatives on rat corneal conjunctival surface. *Curr Eye Res* 1998;**17**:419–425.
39. Tomlinson A, Trees GR. Effect of preservatives in artificial tear solutions on tear film evaporation. *Ophthalmic Physiol Opt* 1991;**11**:48–52.
40. Kunert KS, Tisdale AS, Gipson IK. Goblet cell numbers and epithelial proliferation in the conjunctiva of patients with dry eye syndrome treated with cyclosporine. *Arch Ophthalmol* 2002;**120**:330–337.
41. Moore CP, McHugh JB, Thorne JG, Phillips TE. Effect of cyclosporine on conjunctival mucin in a canine keratoconjunctivitis sicca model. *Invest Ophthalmol Vis Sci* 2001;**42**:653–659.