Molecular Vision 2007; 13:1379-89 <http://www.molvis.org/molvis/v13/a152/>
Received 22 March 2007 | Accepted 6 August 2007 | Published 10 August 2007

Conjunctival in vivo confocal scanning laser microscopy in patients with atopic keratoconjunctivitis

Yiqian Hu,[1,2,3] Enrique Sato Adan,[1] Yukihiro Matsumoto,[1] Murat Dogru,[1,3,4] Kazumi Fukagawa,[1] Yoji Takano,[5] Kazuo Tsubota,[1] Hiroshi Fujishima[1]

(The first two authors contributed equally to this publication)

[1]Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan; [2]Department of Ophthalmology, Xinhua Hospital affiliated to Medical School of Jiaotong University, Shanghai, China; [3]Johnson & Johnson Ocular Surface and Visual Optics Department, Keio University School of Medicine, Tokyo, Japan; [4]Department of Ophthalmology, Tokyo Dental College, Chiba, Japan; [5]Kawasaki Municipal Hospital, Dept. Ophthalmology, Kawasaki, Japan

Purpose: To elucidate the morphological alterations of the conjunctiva in atopic keratoconjunctivitis (AKC) using the new generation Heidelberg Retina Tomograph II (HRT II)/Rostock Cornea Module confocal microscope in a prospective controlled study.

Methods: Sixteen eyes from AKC patients (eight males, mean age: 20.3±5.9 years) were treated with 0.05% topical cyclosporine A (CsA) in addition to topical steroid and anti-allergic eye drops and 12 eyes from patients with AKC were treated using topical steroids and anti-allergic drops (six males, mean age: 22.2±10.0 year). These two groups, as well as 14 eyes from normal subjects (six males, one female, average age 30.4±6.8 years) were studied. All subjects underwent corneal sensitivity measurements, the Schirmer test, tear film break-up time (BUT), fluorescein, Rose Bengal staining of the ocular surface, and confocal laser scanning microscopy of the tarsal palpebral conjunctiva. The density of conjunctival inflammatory infiltrates was calculated. Morphological characteristics of the papillary lesions were also studied.

Results: Corneal sensitivity, tear stability, and vital staining scores were significantly worse in patients with AKC compared to control subjects ($p<0.01$). Eyes of AKC patients using CsA showed a significantly lower density of inflammatory infiltrates compared to eyes on topical steroid and anti-allergic drops. Conjunctival inflammatory cell density showed a negative correlation with tear stability and corneal sensitivity and a positive correlation with the vital staining scores. Papillary lesions revealed remarkable fibrosis in patients using CsA.

Conclusions: Confocal scanning laser microscopy was an efficient and a noninvasive tool for the quantitative assessment of the conjunctival inflammation and evaluation of pathological alterations in the papillary lesions and their relation to the ocular surface disease in patients with AKC.

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 24-45% [1]. Atopic keratoconjunctivitis (AKC) is a bilateral chronic hypersensitivity disease of the ocular surface associated with systemic atopic dermatitis (AD). The ocular inflammatory process and the release of allergic mediators onto the ocular surface and tear film are thought to be responsible for a wide range of clinical corneal and conjunctival manifestations including superficial punctate keratitis, macroerosions, corneal ulceration, plaque formation, dry eyes, corneal neovascularization, lipid infiltration, conjunctival papillary hypertrophy, cicatrization, and symblepharon [2].

Correspondence to: Associate Professor Dr. Murat Dogru, J & J Department of Ocular Surface and Visual Optics, Keio University School of Medicine, Shinanomachi 35, Shinjuku-ku, Tokyo 160-8582, Japan; Phone: 81-3-5363-3820; FAX: 81-3-3358-5961; email: muratodooru@yahoo.com

To our knowledge, in vivo alterations of the conjunctiva by confocal laser scanning in patients with AKC have not been reported previously.

An increased understanding of the conjunctival alterations at the cellular level and the relation with tear functions may help explain the pathogenesis and the subsequent clinical appearance of the atopic ocular allergies, which may potentially cause blindness. To that end, we carried out confocal scanning laser microscopy on the tarsal conjunctiva in AKC patients. We also performed tear function and ocular surface examinations including Schirmer test, tear film break-up time (BUT), vital stainings, and corneal sensitivity measurements then compared the results with those of healthy control subjects to be able to provide further insight into the underlying mechanisms of the disease process in AKC.

METHODS

Subjects: Sixteen eyes from AKC patients (eight males, mean age: 20.3±5.9 years) were studied. These eyes were refractory to a treatment protocol of unpreserved topical 0.025% ketotifen fumarate four times a day (q.i.d.) and unpreserved topical

0.01% betamethasone q.i.d. for eight weeks and were then additionally prescribed unpreserved 0.05% topical cyclosporine A (CsA) eye drops q.i.d. for another eight weeks. Fourteen eyes from normal subjects (six males, one female, average age 30.4±6.8 years) were studied as well as 12 eyes from patients with AKC who were also refractory to the same treatment regimen of unpreserved topical 0.025% ketotifen fumarate q.i.d. and unpreserved topical 0.01% betamethasone q.i.d. for eight weeks and who did not consent to topical CsA use (six males, mean age: 22.2±10.0 years). Patients who strictly adhered to their treatment regimens including the frequency of instillations during the aforementioned periods were then referred to the Allergy and Ocular Surface/Visual Optics research group for examination of tear functions and ocular surface health parameters. All tear function and ocular surface examinations were performed by the same researcher (M.D.). Confocal scanning laser microscopy examinations were performed by the same researcher (E.S.). All AKC patients had active AD. AD patients (diagnosis confirmed with positive radioallergosorbent tests (RAST) and scratch tests) who had symptoms of allergic conjunctivitis without seasonal aggravation and were presenting with conjunctival papillae and keratopathy were diagnosed as having AKC. Skin prick tests were performed with allergen extracts of rye grass, *Dermatophesagoides pteronyssinus* (house dust mite), *Phleum pratense* (timothy) pollen, cedar tree pollen, cat dander, dog dander, penicillium, rice, tuna, salmon, shrimp, chedar 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, was used 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. Those patients who had a history of Stevens-Johnson syndrome, chemical, thermal, or radiation injury, keratoconus, ocular or systemic disease other than AKC, a history of ocular surgery, or contact lens or drug use that would alter the ocular surface were excluded.

As the ethics board committee did not allow a wash-out period in subjects with an active disease process to study the naïve ocular surface status, patients being treated with nonpreserved 0.05% topical CsA eye drops q.i.d. plus topical 0.025% ketotifen fumarate q.i.d. plus topical 0.01% betamethasone q.i.d. for eight weeks or patients being treated with topical 0.025% ketotifen fumarate q.i.d. plus topical 0.01% betamethasone q.i.d. for eight weeks were included in this study. No patients were being treated with systemic steroids, prostaglandin inhibitors, or systemic immunosuppressants at the time of inclusion in the study.

A conventional slit-lamp microscopic examination was performed. Severity of conjunctival injection was graded on a four-point scale as described previously [3]. Briefly, the absence of an injection was graded as zero and mild injection was graded as one point. Moderate injection with edema of the palpebral conjunctiva and hazy view of the deep tarsal vessels was given two points. Severe injection obscuring visualization of the deep tarsal vessels was graded as three points. The subjects then underwent tear function and ocular surface examinations including corneal sensitivity measurements, tear film break-up time (BUT), fluorescein and Rose Bengal staining of the ocular surface, Schirmer test I, and confocal scanning laser microscopy of the upper palpebral conjunctiva. Confocal images before and after topical cyclosporine were available in one patient. This research followed the tenets of the Declaration of Helsinki. Informed consents from all subjects and permission for the ocular surface examinations from the Ethical Committee of Keio University School of Medicine were obtained.

Corneal sensitivity measurements: Measurements of corneal sensitivity were performed using a Cochet-Bonnet aesthesiometer as described previously [4]. The examination was initiated by a 60 mm length of nylon filament perpendicularly applied to the central cornea and continued by shortening it by 5 mm until the patient felt the contact of the filament. A corneal sensitivity measurement of less than 50 mm was regarded as low corneal sensitivity in this study.

Tear function tests and ocular surface vital staining: The standard tear film break-up time measurement was performed. The ocular surface was examined by the double vital staining method. Two μl of preservative-free combination of 1% Rose Bengal and 1% fluorescein dye were instilled into the conjunctival sac as previously reported [5,6]. 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. Fluorescein and Rose Bengal stainings of the ocular surface were also noted and scored. Both fluorescein and Rose Bengal staining scores ranged 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) were placed in the lateral canthus away from the cornea and left in place for five min with the eyes closed [7]. Readings were recorded in millimeters of wetting for five min. A reading of less than 5 mm was referred as an aqueous deficiency.

Confocal scanning laser microscopy: In vivo confocal scanning laser microscopy (CSLM) was performed on all subjects with a new generation confocal microscope, the Rostock Corneal Software Version 1.2 of the Heidelberg Retina Tomograph II (RCM/HRT II; Heidelberg Engineering GmbH, Dossenheim, Germany). After the administration of topical anesthesia with 0.4% oxybuprocaine, the subject's chin was placed in the chin rest. The objective of the microscope was an immersion lens (magnification 63C; Zeiss, Oberkochen, Germany) covered by a polymethylmethacrylate cap (Tomo-Cap; Heidelberg Engineering GmbH). Comfort gel (Bausch&Lomb, GmbH, Berlin, Germany) was used as a coupling agent between the applanating lens cap and the conjunctiva. By adjusting the controller, the center of the Tomo-Cap was applanated onto the center of the upper palpebral conjunctiva, and in vivo digital images of the conjunctiva were visualized directly on the computer screen. When the first superficial cells were seen, the digital micrometer gauge was set

at zero. By pressing on the foot pedal, sequence images were recorded by a charge-coupled device (CCD) color camera (five frames/s) while gradually moving the focal plane forward into the conjunctival stroma.

The laser source employed in the Heidelberg Retina Tomograph II/Rostock Corneal Module is a diode laser with a wavelength of 670 nm. Two-dimensional images consisted of 384x384 picture elements, covering an area of 400 µm by 400 µm. Transversal field of view was captured using the "400 FOV" field lens. Digital resolution was quoted as 1 µm/pixel at transversal and 2 µm/pixel at longitudinal by the manufacturer.

Conjunctival image analysis: At least three sequences (100 images per sequence) of conjunctival CSLM images were taken for each eye. The morphological characteristics in different layers of the conjunctiva in patients with AKC were observed and compared with those of normal eyes. The three best focused images from the conjunctival epithelium were selected for inflammatory cell counting. After selecting three random nonoverlapping frames of 0.02 mm^2 on each image, the cells were manually marked inside the frame, and the inflammatory cell densities were calculated automatically by the software installed in the machine. Selected images were randomly presented to a masked observer for analysis.

Statistical analysis: Data were processed using the Instat, GraphPad software version InStat 3.0 (San Diego, CA). The Kruskal-Wallis test was used to compare the parameters between the AKC subjects and the normal controls. The Spearman rank correlation analysis was used to determine the correlation between the conjunctival inflammatory cell densi-

Table 1. Patient characteristics and examination findings

Number	Patient	Gender	Age (years)	Treatment	Conjunctival injection grade	Schirmer (mm)	BUT (s)	FS (pts)	RB (pts)	Confocal ICD (cells/mm^2)
1	O.Y. RE	M	11	TS+TAA+CsA	Severe (G3)	15	6	9	6	1781
	LE					27	6	9	6	1781
2	O.K. RE	M	13	TS+TAA+CsA	Moderate (G2)	12	6	9	9	1143
	LE					35	6	9	9	1140
3	K.M. RE	M	27	TS+TAA+CsA	Moderate (G2)	5	2	9	4	1281
	LE					5	3	6	3	250
4	K.J. RE	M	25	TS+TAA+CsA	Moderate (G2)	2	3	9	4	556
	LE					2	3	5	4	431
5	M.S. RE	M	20	TS+TAA+CsA	Moderate (G2)	7	6	9	6	NA
	LE					9	5	0	0	264
6	F.M. RE	M	23	TS+TAA+CsA	Moderate (G2)	12	2	0	0	437
	LE					12	3	1	1	875
7	M.T. RE	M	27	TS+TAA+CsA	Moderate (G2)	6	3	9	9	543
	LE					9	2	9	9	150
8	Y.T. RE	M	17	TS+TAA+CsA	Moderate (G2)	13	5	2	0	750
	LE					25	5	3	0	587
9	Y.K. RE	M	33	TS+TAA	Severe (G3)	24	8	9	0	868
	LE					20	8	9	0	650
10	S.A. RE	M	22	TS+TAA	Severe (G3)	16	2	9	9	2058
	LE					16	3	9	9	2218
11	S.T. RE	M	13	TS+TAA	Severe (G3)	7	0	9	9	1450
	LE					13	1	9	9	1446
12	M.T. RE	M	27	TS+TAA	Severe (G3)	6	3	9	9	1600
	LE					9	2	9	9	1296
13	Y.J. RE	M	37	TS+TAA	Severe (G3)	22	1	9	9	1460
	LE					22	1	9	9	1436
14	K.T. RE	M	9	TS+TAA	Severe (G3)	25	0	9	9	1800
	LE					25	0	9	9	1096

The table demonstrates the gender, age, and treatment protocols assigned for each patient, the results of tear function tests, ocular surface vital stainings, conjunctival inflammatory cell densities (as determined by confocal microscopy), and conjunctival injection grades assessed by slit lamp microscopy. Note the comparatively higher conjunctival injection grades, lower BUT values, higher vital staining scores and higher inflammatory cell numbers in patients not using CsA. RE: right eye; LE: left eye; TS: topical steroid; TAA: topical antiallergic; CsA: topical cyclosporine; BUT: tear film break-up time; FS: fluorescein score; RB: Rose Bengal score; ICD: inflammatory cell density; G: grade.

ties and corneal sensitivity as well as between the tear quantity, tear stability, and ocular surface vital stainings. A probability level of less than 5% was considered statistically significant.

RESULTS

Patient characteristics and slit lamp findings: All patients with AKC had active severe AD with pruritus, typical flexural lichenification, papular eruptions, and a tendency toward chronically relapsing dermatitis. Personal or family history of atopic disease and "positive" immediate skin reactivity were present in all cases at the time of examination. All patients with AKC had positive skin reactivity to multiple allergens. The most frequent sensitizing allergens in patients with AKC were *Dermatophagoides pteronyssinus*, cedar tree pollen, and *Phleum pratense* pollen. All patients with AKC complained of allergic and dry eye symptomatology, including itchiness, redness, dryness, foreign body sensation, and irritation.

Upon slit lamp observation, all patients had active atopic keratoconjunctivitis as evidenced by conjunctival injection, chemosis, and papillary hypertrophy. All patients except one receiving additional CsA had moderate conjunctival injection (Grade 2). The other six patients not receiving topical CsA had severe conjunctival injection (Table 1). Patient characteristics and findings of examinations are summarized in Table 1.

Tear function examinations: The mean BUT value in eyes of AKC patients with or without topical CsA use was significantly lower than the control eyes ($p<0.05$). All eyes of patients with AKC and the control subjects had normal Schirmer test values without statistically significant differences ($p>0.05$) as shown in Figure 1A,B.

Ocular surface vital stainings: The mean fluorescein and Rose Bengal staining scores in eyes of AKC patients with or

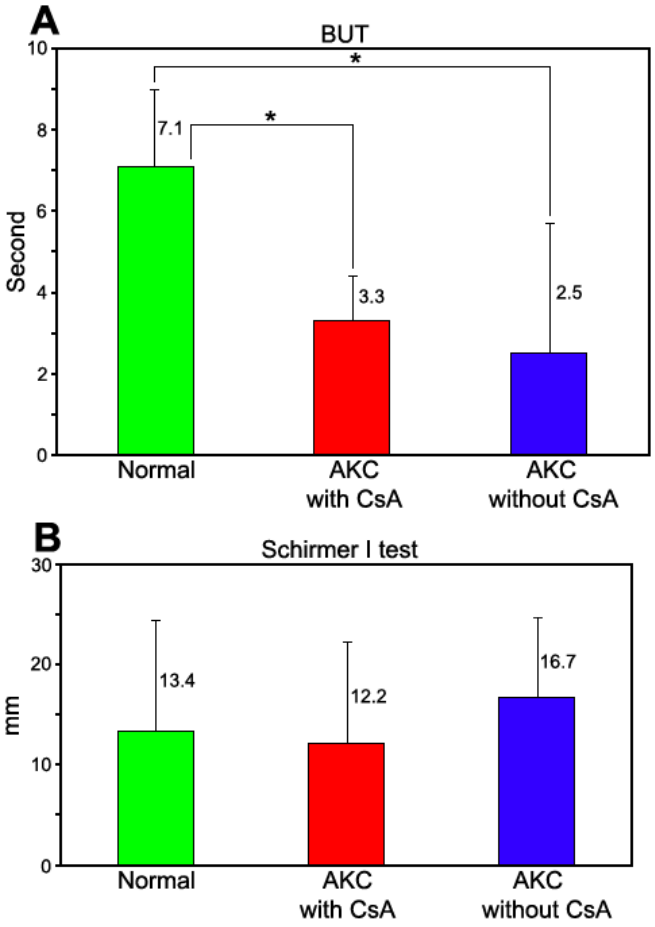

Figure 1. Comparison of tear functions in atopic keratoconjunctivitis patients and normal controls. **A**: Note the statistically significant tear instability in eyes of AKC patients compared to healthy control subjects. The asterisk denotes that $p<0.05$. **B**: Note the absence of statistically significant differences between the eyes of patients with AKC and healthy control subjects.

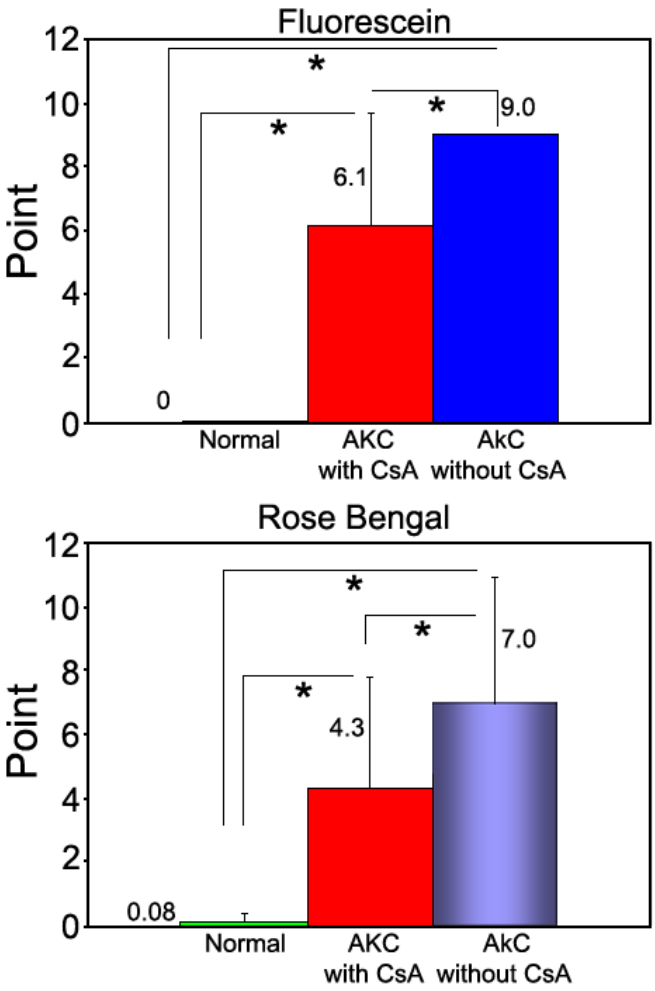

Figure 2. Comparison of vital staining scores in atopic keratoconjunctivitis patients and normal controls. Note the statistically significant higher vital staining scores in eyes of patients with AKC compared to controls. Fluorescein and Rose- Bengal scores were also significantly higher in eyes of patients using only topical steroids and anti-allergic eye drops compared to patients using an additional treatment of topical CsA. The asterisk denotes that $p<0.05$. AKC with CsA: Atopic keratoconjunctivitis patients using topical CsA in addition to topical antiallergic and steroid eye drops; AKC without CsA: Atopic keratoconjunctivitis patients using topical antiallergic and steroid eye drops only.

without topical CsA were significantly higher than those of the control group. The mean fluorescein and Rose Bengal staining scores in eyes of AKC patients with topical CsA were significantly lower than the mean vital staining scores of AKC patients without topical CsA ($p<0.05$), as shown in Figure 2.

Corneal sensitivity: Mean corneal sensitivity was significantly lower in the eyes of AKC patients with or without topical CsA use than in the eyes of healthy control subjects as shown in Figure 3 ($p<0.05$).

Confocal scanning laser microscopy findings-inflammatory cell densities: Conjunctival inflammatory cell densities in eyes of AKC patients with topical CsA and without topical CsA as well as in normal control eyes were 696±468 cells/mm^2, 1448±803 cells/mm^2, and 394±158 cells/mm^2, respectively.

The mean conjunctival inflammatory cell density was significantly lower in the controls and in the eyes with AKC using topical CsA than in the AKC eyes without CsA drops as shown in Figure 4 ($p<0.05$).

Correlation between corneal sensitivities, tear functions, vital staining scores, and conjunctival inflammatory cell density: By Spearman correlation analysis, we found significant positive correlations between inflammatory cell densities, conjunctival injection grades, and vital staining scores. A negative correlation was observed between tear film break-up time, corneal sensitivity, and inflammatory cell densities of the conjunctiva. The Spearman correlation indices and the p value of significance are shown in Table 2.

Morphological observations of the conjunctival epithelium and papillary formations: We could identify either neutrophils (12-15 μ) or dendritic cells in the confocal scan images of all subjects.

No papillary formations were observed in the control subjects. A few neutrophils dispersed in the conjunctival epithelium could be observed in all controls. No high reflections suggesting edema or opacification were noted in the normal control eyes. Figure 5A,B shows anterior segment photographs and confocal scanning microscopy findings of the upper palpebral conjunctiva from a control subject.

Confocal images from AKC eyes without topical CsA demonstrated extensive inflammation consisting of neurophils on the surface of the papillary formations. Deeper images in the papillary formations showed hyperreflective edematous areas and lacunae with neutrophil infiltrates. Figure 6A-C show anterior segment photographs and confocal scanning microscopy findings of the upper palpebral conjunctiva from an AKC patient using only unpreserved topical 0.025% ketotifen fumarate q.i.d. and unpreserved topical 0.01% betamethasone q.i.d.

Confocal images from AKC eyes with topical CsA demonstrated less inflammation on the surface of the papillary formations. Deeper images in the papillary formations showed fibrosis and vascular neoformations with few inflammatory infiltrates. Figure 7A-C show anterior segment photographs and confocal scanning microscopy findings of the upper palpebral conjunctiva from an AKC patient on topical CsA. The Schirmer test score at the time of the examination of this subject was 25 mm. The tear film break up time was 5 s and confocal inflammatory cell density was 587 cells/mm^2. Confocal images from another representative AKC patient on topical CsA demonstrated less inflammation on the surface of the papillary formations. Deeper sections revealed extensive fibrosis with lacunar spaces displaying no inflammatory infiltrates. The Schirmer test score was 2 mm. The tear film break up time was 3 s and the confocal inflammatory cell density was 556 cells/mm^2 (Figure 7D-F). Confocal scans from another representative patient using topical CsA demonstrated less inflammation on the edges of the surface of the papillary formations and dendritic cells. The Schirmer test score was 5 mm. The tear film break up time was 3 s and the confocal inflammatory cell density was 250 cells/mm^2 (Figure 7H-J). Figure 8A,B show the improvements in the injection and edema of the tarsal conjunctiva and the regression of papillary formations in a patient with AKC who received eight weeks of topical CsA drops in addition to the anti-allergic and steroid eye drops. Figure 8C,D show the improvements in the corneal

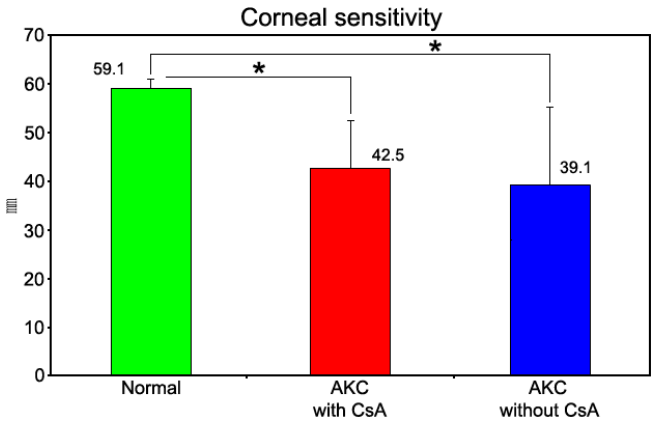

Figure 3. Comparison of corneal sensitivity scores in atopic keratoconjunctivitis patients and normal controls. Note the statistically lower corneal sensitivity values in eyes with AKC compared to control eyes. The asterisk denotes that $p<0.05$.

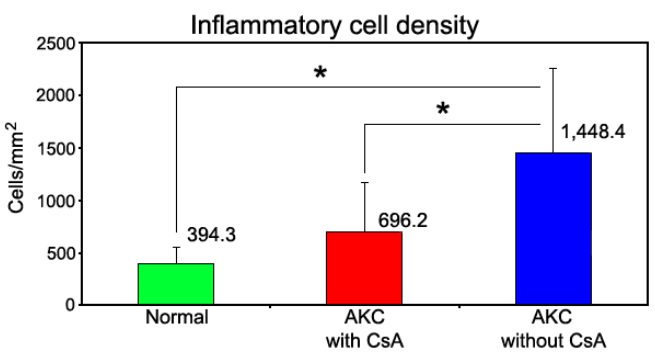

Figure 4. Comparison of conjunctival inflammatory cell densities in atopic keratoconjunctivitis patients and normal controls. Note the higher densities of conjunctival inflammatory cells in eyes with AKC compared to healthy control eyes. The asterisk denotes that $p<0.05$.

epithelial surface with CsA treatment. Figure 8E,F show the dramatic decrease in the conjunctival inflammatory cell infiltrates on the surface of the papillary formations and the fibrotic response within the papillary formations. The pre- and post-treatment Schirmer test values were 15 mm and 12 mm OD, respectively. The tear film break-up times before and after topical CsA treatment were 1 s and 5 s. The confocal inflammatory cell densities before and after treatment were 1781 cells/mm^2 and 95 cells/mm^2, respectively.

DISCUSSION

Histopathologically, the tarsal conjunctival changes in AKC have been reported as hyperplasia of the connective tissue, proliferative and degenerative changes in the epithelium, and prominent infiltration of the epithelium and substantia propria consisting of eosinophils, lymphocytes, mast cells, macrophages, basophils, plasma cells, and dendritic cells. One of the most spectacular events in AKC is the overgrowth of the conjunctival connective tissue forming gelatinous, sessile papillae. In the deep layers of the conjunctiva, the collagen fibers form a fibrous structure within the papilla. A proliferation of capillaries and vascular neoformations provides vascular support to the papillae. Hyaline degeneration of the conjunctival stroma has also been observed in the papillary lesions [8]. Invasive techniques such as brush cytology and conjunctival biopsies have been the source of such invaluable information in many studies.

The Confocal Rostock Scanning Laser Microscope equipped with the Rostock Cornea Module is a new technology enabling rapid, noninvasive, high resolution microstructural in vivo clinical examination of the human cornea and conjunctiva [9]. The device has been applied to the investigation of the morphology of normal human ocular surface and pathological alterations in dry eyes, diabetes, acanthamoeba keratitis, infectious corneal ulcers, herpetic keratitis, keratoconus, aging, contact lens wear, and after refractive surgical procedures [10-19]. To the best of our knowledge, in vivo alterations of the conjunctiva by confocal laser scanning in patients with AKC have not been previously reported.

Table 2. Conjunctival inflammation, tear function, and ocular surface status

	Infiltrate density (CSLM)	
	Spearman correlation coefficient	p value
Conjunctival Injection Grade	0.6552	0.0002*
Shirmer I	0.059	0.778
BUT	-0.471	0.013*
Fluorescein scores	0.522	0.005*
Rose Bengal scores	0.416	0.031*
Corneal sensitivity	-0.439	0.028*

Note the significant positive correlations between conjunctival inflammatory cell densities and conjunctival injection grades, and ocular surface vital staining scores (the higher the inflammatory cell density, the higher the conjunctival injection grades and the vital staining scores). Note also the significant negative correlations between the conjunctival inflammatory cell densities, tear stability (BUT) and corneal sensitivity values (the higher the inflammatory cell density, the lower the the tear stability and the corneal sensitivity).

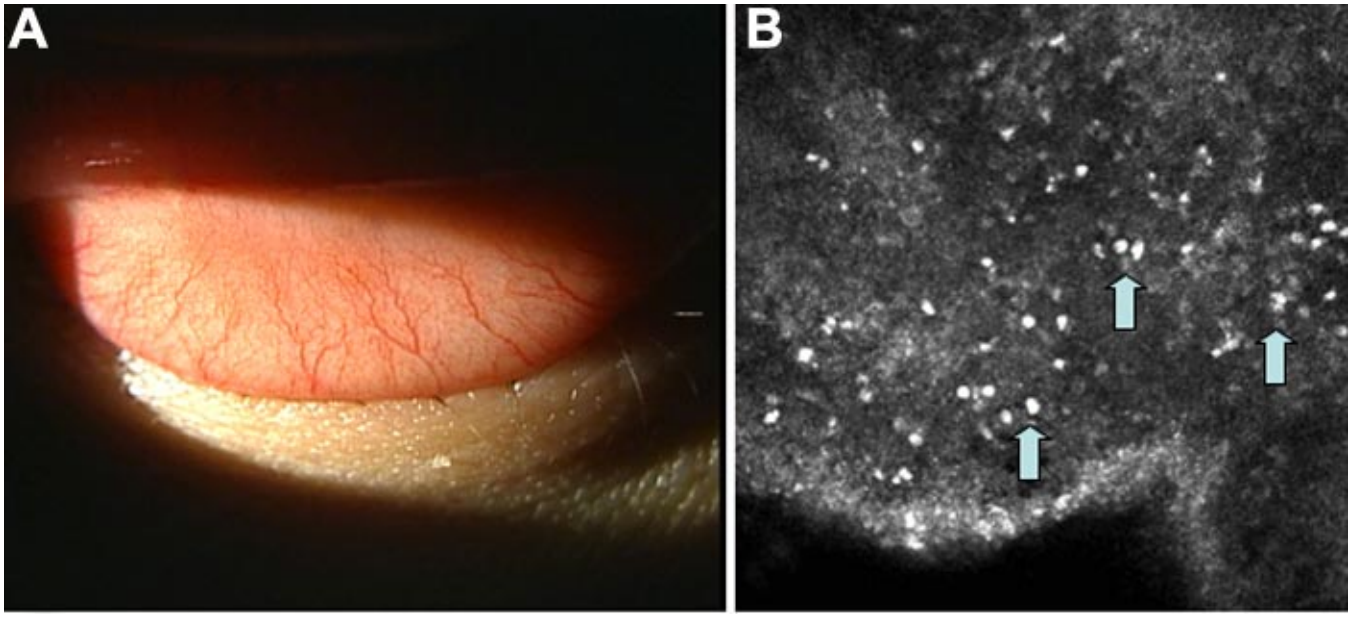

Figure 5. Conjuncival slit lamp photograph and confocal scan images from a normal control subject. Note the absence of papillary formations (**A**) and the few inflammatory cells dispersed in the conjunctical epithelium in the control subject (**B**). Note the absence of highly reflective areas suggesting fibrosis in the normal control eyes.

To be able to provide more clues for the status of the conjunctival epithelium in AKC and to evaluate the ocular surface effects of two different treatment protocols, we performed corneal sensitivity measurements, tear film break up time, Schirmer test, ocular surface vital stainings, and tarsal conjunctival confocal scanning laser microscopy in AKC patients receiving the same protocol of topical steroid and topical anti-allergic drops as well as in patients receiving the aforementioned protocol together with 0.05% topical cyclosporine q.i.d. Additionally, we also compared the results with healthy normal controls. We found better tear stability, ocular surface vital staining, and corneal sensitivity scores in the group receiving topical cyclosporin eye drops when compared to the group not using topical cyclosporin. Topical cyclosporin has been found to be more effective in alleviating signs and symptoms in severe refractory AKC than topical steroid treatments [3]. We attribute the better ocular surface features in the AKC group receiving topical cyclosporin to the suppression of ocular surface inflammation as evidenced by the lower inflammatory cell densities calculated from the confocal scan images. We found significant positive correlations between conjunctival inflammatory cell density and ocular surface vital stainings as well as significant negative correlations between corneal sensitivity, tear stability, and inflammatory cell density, all of which support the contention that conjunctival inflammation adversely affected tear functions and led to ocular surface epithelial disease. We previously reported that the tear film break up time was significantly related to conjunctival goblet cell density in patients with AKC [20]. Goblet cells are known to be very sensitive to inflammation and decrease with higher grades of inflammation [21]. Although we did not perform conjunctival impression cytology in this study, it is our belief that patients with higher inflammatory cell densities in confocal scans might have lower goblet cell densities, explaining the lower tear film break up time scores. Although weakly correlated, we found significantly lower break up time scores in patients with higher confocal inflammatory cell densities. Rose-Bengal dye has been reported to stain those areas of the ocular surface devoid of mucins, mainly MUC5AC, the goblet cell mucin which is believed to be important for the attainment of tear stability [22]. Conjunctival MUC5AC mRNA expression has been reported to decrease in AKC patients with severe conjunctival inflammation [23]. In this study, we found significantly higher Rose-Bengal scores in patients with higher confocal scan inflammatory cell densities. Finally, we had an important observation that corneal sensitivity was significantly reduced in eyes with higher confocal inflammatory cell densities. We recently reported that the density of corneal long nerve fibers and nerve branchings were significantly lower in AKC patients compared with healthy control subjects in confocal laser scans. By confocal laser microscopy, we demonstrated numerous inflammatory cells in close vicinity of or on the corneal subbasal and stromal nerves, which may explain the lower corneal sensitivity scores in patients with AKC and more severe ocular surface inflammation [24].

In a previous study by us, we noted extensive ocular surface inflammatory cell infiltration in AKC in brush cytology samples and also reported that not only the clinical tarsal conjunctival findings but also the inflammatory cell numbers in brush cytology specimens correlate with the severity of the corneal lesions in AKC [25,26]. Our confocal microscopy in vivo observations noninvasively reconfirmed the role of conjunctival inflammation in the ocular surface disease process and helped us to evaluate and compare the effects of different treatment protocols on the ocular surface in AKC.

Noteworthy observations were made on the architecture and inflammation status of tarsal conjunctival papillae in in vivo confocal microscopy. We noted much more extensive inflammatory cell infiltrates on the surface of the papillae and in the lacunar spaces within the papillae along with stromal

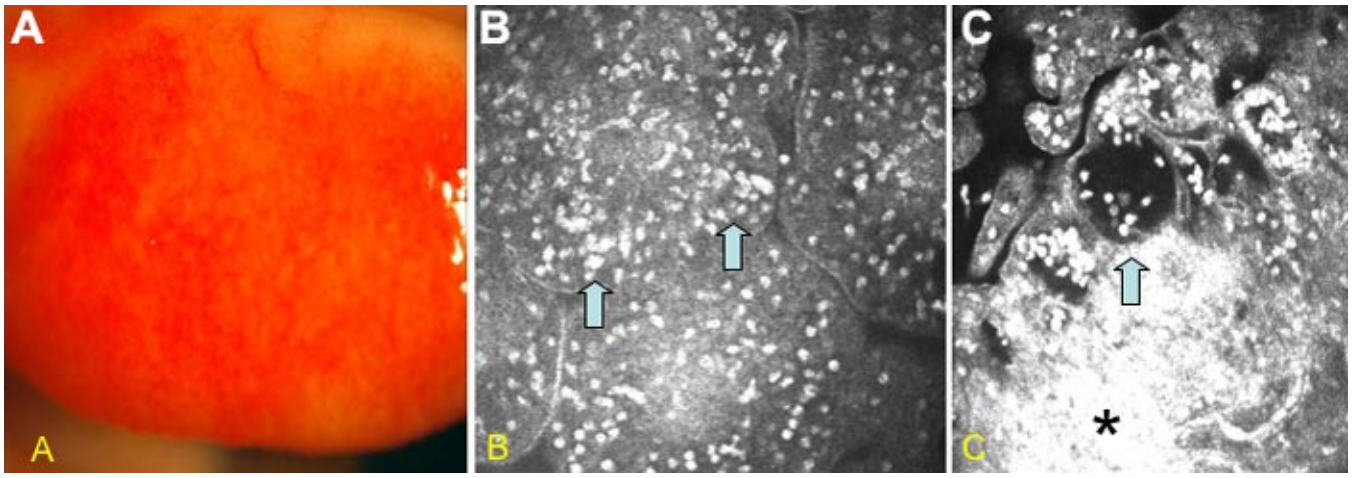

Figure 6. Conjunctival slit lamp photograph and confocal scan images from a patient with atopic keratoconjunctivitis on topical steroid and topical anti-allergic only. The slit lamp photograph reveals cherry red injection of the tarsal conjunctiva (**A**). Confocal images demonstrated extensive inflammation on the surface of the papillary formations (blue arrows; **B**). Deeper images in the papillary formations showed hyperreflective edematous areas (black asterisks) and lacunae with inflammatory infiltrates (blue arrow; **C**).

edema in AKC patients not using topical cyclosporin. Scanty infiltrates in the papillae, dendritic cells, vascular loops, and prominent fibrotic response around lacunar spaces were features observed in patients using topical cyclosporin. Lacunar morphology within the papillae may represent areas of collagen resorption during the remodeling processes or areas of stromal degeneration. It was our impression that none of the patients who were not using topical cyclosporin had a fibrotic papillary response and lacunar morphology to the extent we observed in the patients using topical cyclosporine, suggesting the efficacy of treatment in attaining involution of the papillary formations with in vivo evidence. Indeed, CsA has been reported to be very effective in preventing T cell and transcriptional activation of interleukin-2 and interleukin-4 genes in patients with organ transplantations, especially with kidney transplantation [27]. However, use of CsA is also known to be associated with renal fibrosis marked with extracellular matrix accumulation. It has been reported that epithelial-mesenchymal transition (EMT), a process where the epithelium loses the epithelial markers and functions and acquires a more fibroblastic phenotype, the myofibroblast, secretes profibrotic factors [27]. CsA has also been reported to inhibit cytokine-induced matrix metalloproteinase-9 in glomerular mesangial cells, resulting in further accumulation of extracellular matrix components [28]. CsA may be inducing EMT and fibrosis in the conjunctival epithelium of patients with AKC, an issue

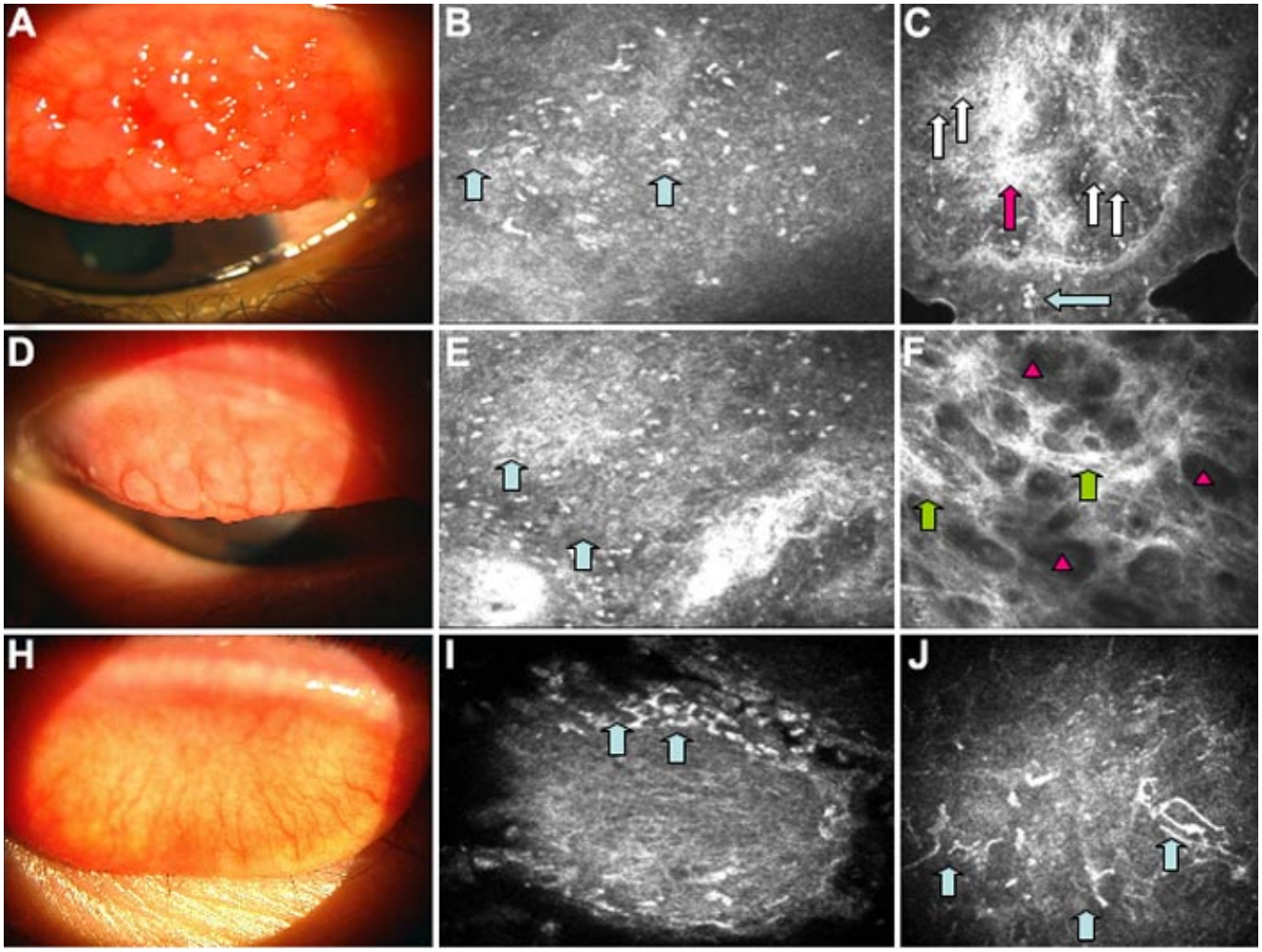

Figure 7. Conjunctival slit lamp photograph and confocal scan images from patients with atopic keratoconjunctivitis on topical cyclosporine. Upper row: **A**: The slit lamp photograph reveals injection of the tarsal conjunctiva and papillary hypertrophy. **B**: Confocal images demonstrated less inflammation on the surface of the papillary formations (blue arrows). **C**: Deeper images in the papillary formations showed fibrosis (pink arrow) and vascular neoformations (white arrows) with few inflammatory infiltrates (blue arrow). Middle row: This row shows the conjunctival slit lamp photograph (**D**) and confocal scan images from another patient with AKC on topical cyclosporine. **E**: Note the papillary hypertrophy and conjunctival hyperemia in the slit lamp photograph. Confocal images demonstrated less inflammation on the surface of the papillary formations (blue arrows). **F**: Deeper sections revealed extensive fibrosis(green arrows) with lacunar spaces displaying no inflammatory infiltrates (pink triangles). Lower row: Conjunctival slit lamp photograph and confocal scan images is shown from another patient with AKC. **H**: Note the conjunctival hyperemia in the slit lamp photograph. Confocal scans demonstrated lesser inflammation on the edges of the surface of the papillary formations (blue arrows; **I**) and dendritic cells (blue arrows; **J**).

that we have not found to be reported previously. Although CsA has been reported to be safe and efficient in dry eye trials [3], our in vivo confocal laser scan observations of subconjunctival fibrosis should be kept in mind and stimulate the physicians in the relevant specialties to closely follow up such patients for subconjunctival fibrotic changes.

In summary, we reported the first in vivo confocal scanning laser microscope study to elucidate the alterations of conjunctival morphology in AKC. Although the findings of this study may be limited by the number of subjects, it provides interesting new data regarding the architecture of the papillary formations in AKC and provides strong evidence for the

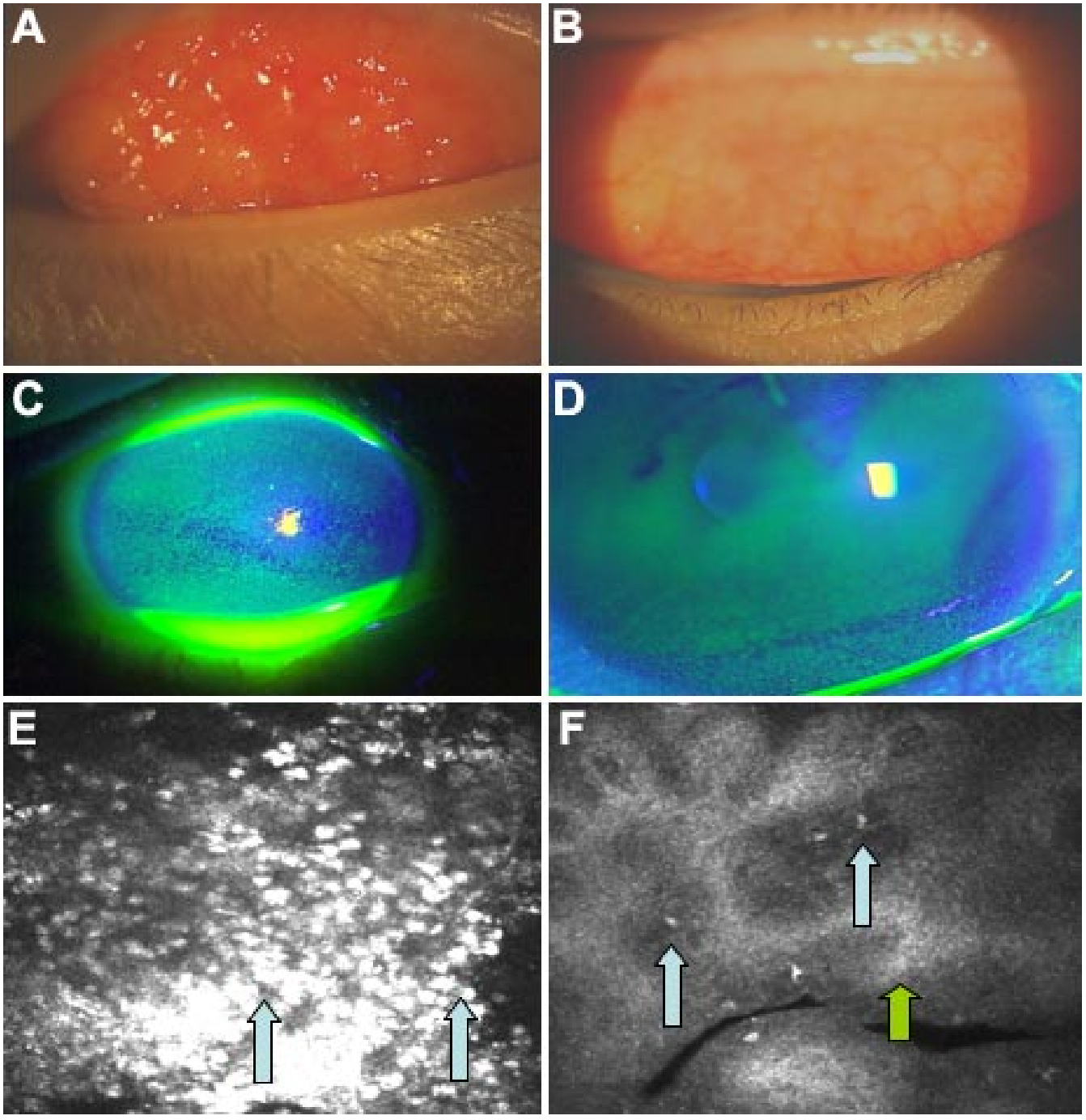

Figure 8. Pre- and post-treatment anterior segment photographs and confocal scan images from a patient with atopic keratoconjunctivitis using topical cyclosporine. **A** and **B** show the improvements in the injection and edema of the tarsal conjunctiva that is the regression of papillary formations in a patient with AKC (refractory to steroid and anti-allergic eye drop for eight weeks) who received eight weeks of topical cyclosporine drops in addition to the anti-allergic and steroid eye drops. **C** and **D** show the improvements in the corneal epithelial damage with cyclosporine treatment. **E** and **F** show the dramatic decrease in the conjunctival inflammatory cell infiltrates on the surface of the papillary formations (blue arrows) and the fibrotic response within the papillary formations (green arrow).

role of inflammation in the ocular surface disease process. Although AKC patients who usually have a history of various topical drug use at the time of first examination in many centers and of recruitment of untreated naïve allergic conjunctivitis or carrying out a wash out in a severe disease state present challenging issues, the tear functions, ocular surface status, and scanning confocal microscopy of untreated naïve atopic ocular surfaces should be investigated in future studies. Future studies may be directed at examining patients over time to observe whether the changes in the papillary lesions precede or follow the progression of disease. A time-wise observation of the pathological alterations in the papilla in future studies will increase our understanding about the development of papillary lesions.

ACKNOWLEDGEMENTS

The authors have no proprietary interest in any of the products mentioned in this paper. This work is supported by research grants of Johnson&Johnson Vision Care Company. Dr. Yiqian Hu is a research scholar of Japan China Sasakawa Medical Association.

REFERENCES

1. Mortz CG, Lauritsen JM, Bindslev-Jensen C, Andersen KE. Prevalence of atopic dermatitis, asthma, allergic rhinitis, and hand and contact dermatitis in adolescents. The Odense Adolescence Cohort Study on Atopic Diseases and Dermatitis. Br J Dermatol 2001; 144:523-32.
2. Bacon AS, Tuft SJ, Metz DM, McGill JI, Buckley RJ, Baddeley S, Lightman SL. The origin of keratopathy in chronic allergic eye disease: a histopathological study. Eye 1993; 7:21-5.
3. Akpek EK, Dart JK, Watson S, Christen W, Dursun D, Yoo S, O'Brien TP, Schein OD, Gottsch JD. A randomized trial of topical cyclosporin 0.05% in topical steroid-resistant atopic keratoconjunctivitis. Ophthalmology 2004; 111:476-82.
4. Dogru M, Yildiz M, Baykara M, Ozcetin H, Erturk H. Corneal sensitivity and ocular surface changes following preserved amniotic membrane transplantation for nonhealing corneal ulcers. Eye 2003; 17:139-48.
5. Junko H. AD classification of fluorescein staining. In: Tano Y, Watanabe N, editors. Practical Ophthalmology. Tokyo: Bunkodo Publishers; 1998. p. 61.
6. Toda I, Tsubota K. Practical double vital staining for ocular surface evaluation. Cornea 1993; 12:366-7.
7. Leibowitz HM, Villazon S. Corneal procedures. In: Leibowitz HM, Waring GO, editors. Corneal Disorders. Philadelphia: WB Saunders; 1998. p. 983-4.
8. Casey R. Atopic Keratoconjunctivitis. In: Abelson MB, editor. Allergic diseases of the eye. Philadelphia: WB Saunders; 2001. p. 137-143.
9. Guthoff RF, Stave J. In vivo micromorphology of the cornea: confocal microscopy principles and clinical applications. In: Reinhard T, Larkin F, editors. Essentials in ophthalmology: cornea and external eye disease. Berlin Heidelberg: Springer-Verlag; 2006. p. 173-208.
10. Benitez del Castillo JM, Wasfy MA, Fernandez C, Garcia-Sanchez J. An in vivo confocal masked study on corneal epithelium and subbasal nerves in patients with dry eye. Invest Ophthalmol Vis Sci 2004; 45:3030-5.
11. Rosenberg ME, Tervo TM, Immonen IJ, Muller LJ, Gronhagen-Riska C, Vesaluoma MH. Corneal structure and sensitivity in type 1 diabetes mellitus. Invest Ophthalmol Vis Sci 2000; 41:2915-21.
12. Pfister DR, Cameron JD, Krachmer JH, Holland EJ. Confocal microscopy findings of Acanthamoeba keratitis. Am J Ophthalmol 1996; 121:119-28.
13. Labbe A, Dupas B, Bensoussan L, Baudouin C. Bilateral infectious ulcers associated with atopic keratoconjunctivitis. Cornea 2006; 25:248-50.
14. Rosenberg ME, Tervo TM, Muller LJ, Moilanen JA, Vesaluoma MH. In vivo confocal microscopy after herpes keratitis. Cornea 2002; 21:265-9.
15. Patel DV, McGhee CN. Mapping the corneal sub-basal nerve plexus in keratoconus by in vivo laser scanning confocal microscopy. Invest Ophthalmol Vis Sci 2006; 47:1348-51.
16. Erie JC, McLaren JW, Hodge DO, Bourne WM. The effect of age on the corneal subbasal nerve plexus. Cornea 2005; 24:705-9.
17. Patel SV, McLaren JW, Hodge DO, Bourne WM. Confocal microscopy in vivo in corneas of long-term contact lens wearers. Invest Ophthalmol Vis Sci 2002; 43:995-1003.
18. Sonigo B, Iordanidou V, Chong-Sit D, Auclin F, Ancel JM, Labbe A, Baudouin C. In vivo corneal confocal microscopy comparison of intralase femtosecond laser and mechanical microkeratome for laser in situ keratomileusis. Invest Ophthalmol Vis Sci 2006; 47:2803-11.
19. Messmer EM, Mackert MJ, Zapp DM, Kampik A. In vivo confocal microscopy of normal conjunctiva and conjunctivitis. Cornea 2006; 25:781-8.
20. Dogru M, Katakami C, Nakagawa N, Tetsumoto K, Yamamoto M. Impression cytology in atopic dermatitis. Ophthalmology 1998; 105:1478-84.
21. Kinoshita S, Kiorpes TC, Friend J, Thoft RA. Goblet cell density in ocular surface disease. A better indicator than tear mucin. Arch Ophthalmol 1983; 101:1284-7.
22. Pflugfelder SC, Tseng SC, Yoshino K, Monroy D, Felix C, Reis BL. Correlation of goblet cell density and mucosal epithelial membrane mucin expression with rose bengal staining in patients with ocular irritation. Ophthalmology 1997; 104:223-35.
23. Dogru M, Asano-Kato N, Tanaka M, Igarashi A, Shimmura S, Shimazaki J, Okada N, Takano Y, Fukagawa K, Tsubota K, Fujishima H. Ocular surface and MUC5AC alterations in atopic patients with corneal shield ulcers. Curr Eye Res 2005; 30:897-908.
24. Hu Y, Dogru M, Sato EA, Fukagawa K, Matsumoto Y, Tsubota K, Fujishima H. In vivo investigation of the cornea in atopic keratoconjunctivitis patients using confocal laser scanning microscopy. ARVO Annual Meeting; 2007 May 6-10; Fort Lauderdale (FL).
25. Takano Y, Fukagawa K, Dogru M, Asano-Kato N, Tsubota K, Fujishima H. Inflammatory cells in brush cytology samples correlate with the severity of corneal lesions in atopic keratoconjunctivitis. Br J Ophthalmol 2004; 88:1504-5.
26. Tanaka M, Dogru M, Takano Y, Miyake-Kashima M, Asano-Kato N, Fukagawa K, Tsubota K, Fujishima H. The relation of conjunctival and corneal findings in severe ocular allergies. Cornea 2004; 23:464-7.
27. Slattery C, Campbell E, McMorrow T, Ryan MP. Cyclosporine A-induced renal fibrosis: a role for epithelial-mesenchymal transition. Am J Pathol 2005; 167:395-407.

28. Doller A, Akool el-S, Muller R, Gutwein P, Kurowski C, Pfeilschifter J, Eberhardt W. Molecular mechanisms of cyclosporin A inhibition of the cytokine-induced matrix metalloproteinase-9 in glomerular mesangial cells. J Am Soc Nephrol 2007; 18:581-92.

The print version of this article was created on 10 Aug 2007. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.