In Vivo Evaluation of Superior Limbic Keratoconjunctivitis Using Laser Scanning Confocal Microscopy and Conjunctival Impression Cytology

Takashi Kojima,¹,² Yukihiro Matsumoto,¹ Osama M. A. Ibrahim,¹ Enrique Adan Sato,¹ Murat Dogru,¹ and Kazuo Tsubota²

PURPOSE. To investigate the cytologic findings of superior bulbar conjunctiva in superior limbic keratoconjunctivitis (SLK) using laser scanning confocal microscopy and impression cytology in a prospective controlled study.

Methods. Twenty-one eyes of 11 SLK patients (9 women, 2 men; mean age, 49.3 ± 17.9 years) and 18 eyes of 9 control subjects (6 women, 3 men; mean age, 46.4 ± 8.7 years) underwent tear function tests including vital stainings, Schirmer test, tear clearance test, digital confocal laser scanning microscopy, and conjunctival impression cytology. After confocal microscopy and impression cytology images were obtained, the mean individual epithelial cell area (MIECA), nucleocytoplasmic (N/C) ratio, and inflammatory cell density were analyzed. The correlation between confocal microscopy and impression cytology parameters was investigated.

Results. The MIECA of SLK patients and control subjects in confocal microscopy was 786.54 ± 463.88 m² and 311.50 ± 78.30 m², respectively. The mean N/C ratio was 0.356 ± 0.090 and 0.490 ± 0.038, respectively. The MIECA and N/C ratio in impression cytology showed significant correlation with the corresponding confocal microscopy parameters (MIECA, P = 0.0028; N/C, P = 0.0051). The inflammatory cell density in confocal microscopy significantly correlated with superior bulbar conjunctival Rose-Bengal scores (P = 0.0264).

Conclusions. Laser scanning confocal microscopy seems to be an efficient noninvasive tool in the evaluation of phenotypic alterations of the conjunctival epithelium in SLK and may serve as an alternative for impression cytology. N/C ratio and inflammatory cell density appear to be two new promising parameters of in vivo confocal microscopy in the assessment of ocular surface disease in SLK. (Invest Ophthalmol Vis Sci. 2010;51: 3986–3992) DOI:10.1167/iovs.09-4932

Superior limbic keratoconjunctivitis (SLK) is an ocular surface disease of unknown etiology characterized by marked inflammation of the upper bulbar conjunctiva and by fine punctate staining of the upper limbal cornea and the adjacent bulbar conjunctiva. Patients report various symptoms such as foreign body sensation, burning sensation, photophobia, and pain.

To clarify the pathophysiological mechanisms of this disease, histopathologic evaluation of SLK has been carried out and reported by several investigators. Using the biopsy samples of bulbar conjunctiva, Theodore showed keratinization and acanthosis, dyskeratosis, and balloon degeneration of nuclei. The acanthotic degeneration of epithelial cells is indicated by condensed nuclear chromatin whose appearance is highly characteristic (snakelike chromatin) and cytoplasmic glycogen overload. Decreased goblet cell density was reported in affected tissues compared with normal subjects.

Impression cytology examines the ocular surface epithelium with application of cellulose acetate filter material to the ocular surface to remove the superficial layers of the epithelium. The technique is easy to perform and can be used to observe ocular surface epithelial cell changes over time. Impression cytology has been used for many ocular surface diseases, including dry eye, atopic keratoconjunctivitis, ocular pemphigoid, and SLK.

Confocal microscopy is an emerging, noninvasive technology that evaluates tissue structure and cell phenotype in vivo and is useful as a supplementary diagnostic tool for the assessment of histopathologic processes in many ocular surface diseases and anterior-segment disorders, including in vivo examination of the cornea, bulbar and palpebral conjunctiva, and meibomian glands.

Squamous metaplasia of the conjunctival epithelium has been reported as an integral part of many ocular surface diseases. Change in the extent of squamous metaplasia has been reported to be useful in the evaluation of treatment responses in dry eye syndromes.

Our purpose in the present study was to evaluate and characterize the findings of superior bulbar conjunctiva in SLK using laser scanning confocal microscopy and to compare the confocal microscopy parameters with impression cytology findings. We also evaluated whether confocal microscopy examination could be an alternative for impression cytology in the assessment of SLK.

Patients and Methods
Twenty-one eyes of 11 consecutive SLK patients (8 women, 3 men) aged 28 to 73 years (mean, 49.3 ± 17.9 years) were recruited from the Department of Ophthalmology at Keio University School of Medicine from April 2007 through May 2009. Eighteen normal eyes of nine age-matched volunteers (6 women, 3 men; mean, 46.4 ± 8.7 years) were registered as controls. Five patients were not receiving any treatment at the time of first examination. Referral patients who were receiving the same preservative-free artificial tear drops and 0.3% hyaluronic acid eyedrops with the same frequency of instillations for at least 1 month were recruited in this study. One patient receiving 20%...
Bengal staining of the ocular surface was scored according to the criteria proposed by van Bijsterveld.22,23 To evaluate the local changes in the superior bulbar conjunctiva under the eyelids (mild staining), 1 point; confluent, dense staining associated with nonstaining areas in the superior bulbar conjunctiva, 2 points; confluent, dense staining in the superior bulbar conjunctiva, 3 points. Fluorescein staining was scored according to the protocol described by Shimmura et al.24 Briefly, the cornea was divided into three equal areas of upper, middle, and inferior corneal compartments. Each compartment was graded on a scale of 0 points (no staining) to 3 points (intense staining). A fluorescein staining score above 1 point was considered abnormal (maximum, 9 points).

### Tear Quantity and Clearance Evaluation

To evaluate the tear functions, Schirmer test without anesthesia, tear clearance rate, and tear break-up time were measured. Briefly, Schirmer test without anesthesia and tear clearance test were performed 5 minutes after instillation of 2 μL fluorescein 1% into the conjunctival sac. The sterilized Schirmer strip (Showa Yakuhin Kako Co. Ltd., Tokyo, Japan) was placed for another 5 minutes. The length of the wet portion was measured. A value <5 mm was considered abnormal. The intensity of its staining was compared with the standard strip colors and graded as 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, and 1/256. The tear clearance rate was determined by the rate at which the color of the fluorescein dye was diluted.

### Tear Film Stability Evaluation

Standard tear film break-up time (BUT) was measured after instillation of 2 μL of 1% fluorescein preservative-free solution in the conjunctival sac with a micropipette. Patients were instructed to blink several times for a few seconds to ensure adequate mixing of the dye. The interval between the last complete blink and 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 <5 seconds was considered abnormal.

### Impression Cytology

Impression cytology specimens were obtained after administration of topical anesthesia with 0.4% oxybuprocaine. Strips of cellulose acetate filter paper (HAWP 01300; Millipore, Bedford, MA) soaked in distilled water for a few hours and dried at room temperature were applied on the superior bulbar conjunctiva adjacent to the corneal limbus, pressed gently by a forceps, and then removed. Specimens were fixed with autologous serum eyedrop treatment was also included in this study. One SLK patient underwent cataract surgery in the right eye 15 years ago. None of the other patients underwent thermocauterization or previous ocular surgery. The protocol of this study was approved by the institutional review board, and the study procedures conformed to the ethical principles for research involving human subjects as outlined in the Declaration of Helsinki. Informed consent was obtained from all subjects after explanation of the nature and possible consequences of taking part in the study.

### Ocular Surface Vital Staining

The ocular surface was examined with the use of fluorescein and Rose Bengal staining.22 Briefly, 2 μL preservative-free 1% Rose Bengal and 2 μL of 1% fluorescein dye was instilled in the conjunctival sac. Rose Bengal staining of the ocular surface was scored according to the criteria proposed by van Bijsterveld.22,23 To evaluate the local changes of conjunctiva, we defined the Rose Bengal score at the superior bulbar conjunctiva as the superior Rose Bengal score (S-RB score). The minimum and maximum S-RB staining scores for superior bulbar conjunctiva were 0 to 3 points. The S-RB score was graded as follows: absence of any staining, 0 points; nonconfluent, scarce/trace Rose-Bengal stain-

### Confocal Microscopy Evaluation for SLK Patients

Confocal microscopy was used to evaluate the conjunctival epithelial cell images in confocal microscopy (A–C) and impression cytology (D, E). (A, D) Eyes of control subjects. (B, C, E) Confocal microscopy and impression cytology findings of eyes of SLK patients. In patients with SLK, marked enlargement of cell size with pyknotic nuclei was observed (B, E). In some areas, sloughing of superficial conjunctival epithelium was observed (C). Arrowheads: goblet cells with glycogen overload.

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**TABLE 1. Patient Demographics and Tear Function Tests Results**

<table>
<thead>
<tr>
<th></th>
<th>SLK Patients</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyes, n</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Patients, n</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2 (18.2)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>Female</td>
<td>9 (82.8)</td>
<td>6 (66.6)</td>
</tr>
<tr>
<td>Mean age, y</td>
<td>49.3 ± 17.3</td>
<td>46.4 ± 8.4</td>
</tr>
<tr>
<td>Tear function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schirmer I test, mm</td>
<td>8.3 ± 9.04</td>
<td>21.12 ± 11.82</td>
</tr>
<tr>
<td>1/Tear clearance rate</td>
<td>47.47 ± 40.48</td>
<td>87.5 ± 41.6</td>
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<tr>
<td>Ocular surface evaluation score, points</td>
<td></td>
<td></td>
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<tr>
<td>Fluorescein</td>
<td>1.27 ± 0.70</td>
<td>0.12 ± 0.35</td>
</tr>
<tr>
<td>Rose Bengal</td>
<td>0.73 ± 1.10</td>
<td>0.35 ± 0.70</td>
</tr>
<tr>
<td>S-Rose Bengal</td>
<td>2.40 ± 0.74</td>
<td>0.05 ± 0.24</td>
</tr>
</tbody>
</table>

Data represent mean ± SD unless otherwise indicated. S-Rose Bengal, Rose Bengal score at superior bulbar conjunctiva.

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**FIGURE 1.** Representative conjunctival epithelial cell images in confocal microscopy (A–C) and impression cytology (D, E). (A, D) Eyes of control subjects. (B, C, E) Confocal microscopy and impression cytology findings of eyes of SLK patients. In patients with SLK, marked enlargement of cell size with pyknotic nuclei was observed (B, E). In some areas, sloughing of superficial conjunctival epithelium was observed (C). Arrowheads: goblet cells with glycogen overload.
10% formaldehyde, stained with periodic acid-Schiff, dehydrated in ascending grades of ethanol and then xylene, and finally coverslipped. Quantitative studies of conjunctival epithelial cells were conducted by taking photographs with a calibrated grid under a light microscope at a magnification of 200×. We photographed 10 different areas of each sample selected at random. In this study, we used confocal microscopy and impression cytology to evaluate the squamous metaplasia based on diagnostic parameters such as the mean individual epithelial cell area (MIECA) and nucleocytoplasmic (N/C) ratio. The MIECA and N/C ratio were calculated using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html), and the outcomes were averaged.

In Vivo Laser Scanning Confocal Microscopy

Before impression cytology, in vivo laser confocal microscopy was performed on all subjects with a new-generation confocal microscope (Rostock corneal software version 1.2 of the Heidelberg Retina Tomograph II Rostock Cornea Module [HRTII-RCM]; Heidelberg Engineering GmbH, Dossenheim, Germany). After topical anesthesia with 0.4% oxybuprocaine, the subject’s chin was placed on the chin rest. The superior conjunctiva was scanned while moving the applanating lens from the limbal area toward the fornix with minute vertical movements. Ten sequences, each containing 100 frames, were taken in each eye. Ten nonoverlapping frames with the best resolution were selected from each sequence. Subepithelial inflammatory cell density was measured. Calculation of all parameters was performed using ImageJ software. The length of a single confocal microscopy examination session was approximately 10 minutes. None of the subjects reported discomfort, and no adverse effects were observed after examination in this series.

We performed in vivo confocal microscopy of a 5 mm × 5 mm area just above the superior 12 o’clock corneal limbus, followed by impression cytology over the same area with a 5 mm × 5 mm filter paper (Millipore).

We measured BUT first and followed this with vital stainings 3 minutes later. Fifteen minutes after the vital stainings, Schirmer and tear clearance tests were performed. Confocal microscopy examination was performed afterward, followed by impression cytology of the same superior bulbar conjunctival area. Clinical examinations including tear function tests and vital stainings were performed by one examiner (TK). In vivo confocal microscopy and impression cytology examinations were also performed by one researcher (MD), who was masked to whether the subject was an SLK patient or a control subject.

Statistical Analysis

Pearson’s correlation analysis was performed to analyze the correlation between impression cytology and confocal microscopy parameters. Similarly, Pearson’s correlation analysis was performed to analyze the correlation between inflammatory cell density and S-RB score. Age and sex differences were studied by χ² analysis. P < 0.05 was considered statistically significant. Statistical software (InStat; GraphPad Software Inc., San Diego, CA) was used for these analyses.
RESULTS

The demographics of patients and control subjects are shown in Table 1. In the SLK group, tear functions, including mean Schirmer and tear clearance rate values, were significantly worse than in the control group (Schirmer, \( P = 0.0003 \); tear clearance test, \( P = 0.0024 \)). Ocular surface vital staining scores, including mean fluorescein and S-Rose Bengal scores, were also significantly worse in SLK group than in the control group (fluorescein score, \( P = 0.0002 \); S-Rose Bengal score, \( P < 0.0001 \)). Mean Rose Bengal scores between the SLK and control groups were not statistically different (\( P = 0.138 \)).

Confocal microscopy in patients with SLK revealed that superficial epithelial cells were enlarged and had pyknotic nuclei. Areas of superficial epithelial cell loss were observed in all patients. Basal cells showed no changes compared with control subjects. Marked conjunctival infiltration with polymorphs and dendritic cells was observed in patients with SLK. Subconjunctival tissues could not be evaluated effectively because of high brightness. Impression cytology also showed similar changes in relation to cell sizes and nuclei in the SLK group. None of the impression cytology specimens in the SLK patients in this series displayed goblet cells, whereas occasional to numerous goblet cells were encountered in the control subjects. Representative images from SLK patients and control subjects are shown in Figure 1. Representative corresponding Nelson’s grades of conjunctival squamous metaplasia in confocal microscopy images and impression cytology specimens are shown in Figure 2.

Correlation between Confocal and Impression Cytology Observations in Relation to the Mean N/C Ratio Assessment

Mean N/C ratios with confocal microscopy and impression cytology observations in the SLK group were 0.356 ± 0.090 and 0.301 ± 0.044, respectively. Similarly, mean N/C ratios in the control group were 0.490 ± 0.038 and 0.515 ± 0.034 with confocal microscopy and impression cytology observations, respectively. A significant correlation in relation to the mean N/C ratios was found between confocal microscopy and impression cytology methods in SLK patients (\( r^2 = 0.442; P = 0.0125 \)). Similarly, a significant correlation was observed between the two methods with respect to the mean N/C ratio in the control group (\( r^2 = 0.480; P = 0.0014 \); Fig. 3).

Bland-Altman plot did not show significant differences in mean N/C values between impression cytology and confocal microscopic examinations in both control and SLK subjects (control, \( P = 0.185 \); SLK, \( P = 0.051 \); Fig. 4).

Correlation between Confocal Microscopy and Impression Cytology Observations in Relation to the Mean MIECA Assessment

The mean MIECA evaluated by the confocal microscopy and impression cytology observations in the SLK group was...
Similarly, the mean MIECA evaluated by confocal microscopy and impression cytology observations in the control group was 311.50 ± 78.30 μm² and 293.50 ± 87.30 μm², respectively. A significant correlation in relation to the mean MIECA value was found between confocal microscopy and impression cytology methods in SLK patients \( r^2 = 0.542; P = 0.0028 \). Similarly, a significant correlation was observed in the control group \( r^2 = 0.254; P = 0.033; \text{Fig. 5} \). There was no significant difference in relation to mean MIECA values assessed by impression cytology and confocal microscopy in the control subjects \( P = 0.173; \text{Fig. 6} \). However, the impression cytology value in the SLK group was significantly greater than the confocal microscopy value \( P = 0.007 \). Bland-Altman plot showed no agreement between the two parameters in relation to the MIECA assessment.

Conjunctival Subepithelial Inflammatory Cell Density Assessed by Confocal Microscopy

Mean inflammatory cell densities in the control subjects and SLK patients were 75.3 ± 37.8 and 439.5 ± 240.3 cells/mm², respectively (Fig. 7). There was a significant difference in relation to inflammatory cell densities between the control subjects and the SLK patients \( t = -3.62; P < 0.0001 \). Representative images are shown in Figure 7. Inflammatory cell density significantly correlated with the S-RB score \( r^2 = 0.186; P = 0.026 \) and Schirmer test value \( r^2 = 0.192; P = 0.042 \).

DISCUSSION

Squamous metaplasia has been reported in various ocular surface diseases such as dry eye syndrome, Stevens-Johnson syndrome, ocular pemphigoid, and alkali injury-related ocular surface disease. SLK is one of the ocular surface diseases associated with squamous metaplasia. In impression cytology, decrease in N/C ratio, increased cell area, decreased expression of ocular surface mucins by both goblet and non-goblet cells, snake-like chromatins, and pyknosis have been reported to be associated with squamous metaplasia.\(^2\) The severity of dry eye and the degree of squamous metaplasia have been reported to be closely associated in patients with dry eye.\(^2\) Nelson and Tseng et al.\(^2\) proposed grading systems using impression cytology samples widely used in clinical practice.

Although both impression cytology and tissue biopsy have been traditionally used to study the process of squamous metaplasia and keratinization in conjunctival tissues and are very useful in the assessment of squamous metaplasia-related changes, both are uncomfortable techniques requiring the re-
moval of cells or tissue specimens. Although impression cytology is an effective, safe, and almost noninvasive technique, it has limitations. It can evaluate only the superficial layer of the conjunctival epithelium. It is sometimes difficult to evaluate the extent of inflammatory cell infiltration under or within the epithelium. It is also possible that there are cells that are not picked up by the cellulose acetate filter. On the other hand, confocal microscopy is almost noninvasive and can be used to follow the response to therapy and to evaluate it with frequent examinations.

Confocal microscopy has recently been reported to be an efficient tool in the assessment of corneal and conjunctival changes in patients with Sjogren syndrome (SS) and non-SS dry eyes. Both impression cytology and confocal microscopy effectively disclosed morphologic cellular alterations in SLK, including cellular enlargement, cell dropout, decreased cell cohesion, and shrinkage of nuclei. In this study, we devised new confocal microscopy parameters, namely MIECA and N/C ratio, which we hoped would serve as an alternative means of in vivo cytology and compared these parameters with the same parameters calculated from the impression cytology specimens.

When we compared the results of the newly devised parameters by these two methods, we found that N/C ratios and MIECA assessed by both methods showed a significant linear positive correlation both in patients with SLK and in healthy control subjects. Because we found a strong correlation between impression cytology and confocal microscopy findings in relation to the N/C ratio and MIECA, we believe that confocal microscopy will be very useful in the noninvasive assessment of ocular surface epithelium in dry eye syndromes. Further assessment of the N/C ratio on the compatibility of the two methods by Bland-Altman plots revealed that both techniques had acceptable agreement in relation to evaluation of the nucleocytoplasmic ratio in SLK patients and control subjects. However, in MIECA evaluation, Bland-Altman plot showed agreement between the two methods only in control subjects but not in SLK patients. There was correlation but lack of agreement between the two methods in relation to MIECA calculations in the SLK patients. We believe this discrepancy may result from cellular changes associated with fixation procedures used in impression cytology and from differences in the number of cells picked up by impression cytology in healthy control subjects and SLK patients. We presumed that in SLK patients, larger superficial cells with decreased cohesion before sloughing were readily picked up by filter papers in impression cytology compared with the relatively smaller epithelial cells with stronger cohesion. In vivo confocal microscopy should be able to visualize both cell types that explain the discrepancy observed in Bland-Altman plots. In relation to the N/C ratio, we observed a similar trend that the N/C ratio with confocal microscopy was higher than that assessed with impression cytology but did not find a statistically significant difference. We speculated that the same factors that affected the MIECA assessment, such as the fixation process, could have affected both nucleus and cytoplasm sizes equally and that the extent of the effect might possibly not have been evaluated efficiently when nucleus and cytoplasm sizes were expressed in ratios.

It should be noted that aging may affect the ocular surface epithelial phenotype. Future studies investigating such age-related morphologic changes of the conjunctival epithelium by in vivo confocal microscopy will provide invaluable information.
Apart from these parameters, inflammatory cell infiltrates consisting of polymorphs and dendritic cells could be easily viewed in in vivo confocal microscopy scans in SLK patients. Inflammatory cell density counts were not only significantly higher in SLK patients compared with control subjects, they also correlated significantly with S-RB scores. We did not encounter inflammatory cells in any of the impression cytology specimens. Assessment of the number and the types of inflammatory cells is one of the strengths of in vivo confocal microscopy compared with impression cytology.

Inflammatory cell density also correlated with tear volume in this study. The strength of such correlations must be addressed in future trials on a larger population.

In the present study, we observed two eyes with increased MIECA despite minimal Rose Bengal staining at the superior bulbar conjunctiva. This noteworthy observation suggests that it is possible to diagnose such subclinical SLK cases with increased MIECA but minimum vital staining using laser scanning confocal microscopy.

On the other hand, goblet cells may not be effectively assessed by in vivo confocal microscopy because of disagreement in relation to the appearance of goblet cells in the literature. Although some in vivo confocal microscopy studies reported goblet cells as oval, white bodies others reported them as dark round structures. Decreased goblet cell density remains one of the strengths of confocal microscopy. We observed numerous round, white cells in the eyes of healthy control subjects, whereas such cells were not observed in the eyes of SLK patients. Although these were presumably goblet cells, as also suggested by Efron et al. and Kobayashi et al. further improvements in resolution and image quality of in vivo confocal microscopy devices will allow sufficient visualization and quantification of goblet cells in the future.

In conclusion, confocal microscopy can serve as a tool of in vivo cytology by which N/C ratio and inflammatory cell density appear to be two new promising parameters in describing ocular surface health status in patients with SLK.

References