In Vivo Confocal Evaluation of the Ocular Surface Morpho-Functional Unit in Dry Eye


ABSTRACT

Purpose. To study, by a new, integrated, laser scanning confocal microscopy approach, the ocular surface morpho-functional unit in patients with primary Sjogren syndrome (SSI), non–Sjogren syndrome dry eye (non-SSDE), and meibomian gland disease (MGD).

Methods. Patients and age- and sex-matched control subjects (N = 60; 15 each) were consecutively enrolled in a prospective case-control study. Laser scanning confocal microscopy was used to obtain simultaneous optical sampling of the ocular surface components: cornea, bulbar and tarsal conjunctiva, MGs, and eyelid margin.

Results. For all superficial epithelia, except eyelid margins, there were reduced cell densities in each group compared with that in controls (p < 0.001). The lowest cell densities were in the SSI group (p < 0.001). Eyelid margin superficial cell density was decreased only in MGD (p < 0.001). Basal epithelial cell density at the corneal apex was increased in both SSI and non-SSDE compared with that in controls (p < 0.01). In the conjunctiva, it was decreased in each group compared with that in controls (p < 0.01). Subbasal dendritic cell density was significantly increased in both SSI and MGD compared with that in controls (p < 0.01). Conjunctival inflammatory cell density and MG inflammation were increased in each group compared with those in controls (p < 0.001), with the highest values in SSI. Subbasal nerve plexi had fewer fibers and higher bead density in each group compared with those in controls (p < 0.001). There was increased tortuosity in both SSI and MGD (p < 0.001). Patients with MGD had the lowest MG acinar density, the largest diameter of acini and acinar orifices, and the highest secretion reflectivity (p < 0.001).

Conclusions. Laser scanning confocal microscopy can provide an in vivo, noninvasive, high-resolution overview of the ocular surface morpho-functional unit. This confocal integrated approach may be useful in both research and clinical settings. (Optom Vis Sci 2013;90:576–586)

Key Words: in vivo confocal microscopy, dry eye, cornea, conjunctiva, inflammation, ocular surface, Sjogren syndrome, meibomian glands, imaging

Dry eye is a multifactorial disease of the tears and the ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability. It is accompanied by increased tear film osmolarity and inflammation and has the potential to damage the ocular surface.1,2 Dry eye is recognized as a disturbance of the ocular surface morpho-functional unit, an integrated system composed of the tear film, lacrimal glands, cornea, conjunctiva, meibomian glands (MGs), lids, and the sensory and motor nerves that connect them.1 The corneal nerves help maintain the functional integrity of the ocular surface by releasing trophic substances that promote corneal epithelial homeostasis and by activating brain stem circuits that stimulate reflex tear production and blinking.3 Changes in the corneal subbasal plexus fibers can alter the complex regulation of the ocular surface morpho-functional unit. Such changes can compromise various components such as blinking, tear reflex, and trophism of the epithelial cells, thus contributing to increases in a vicious cycle of hypotearing, phlogosis, and cell and nerve damage.4 This wide variety of pathogenic and clinical elements could necessitate different therapeutic approaches.

Laser scanning confocal microscopy (LSCM) is an emerging noninvasive technology that is a useful supplementary diagnostic tool for quantitative histopathologic assessment of ocular surface inflammation,5–8 innervation,5,9–11 and tissue damage.5 It allows the in vivo examination of the cornea,12 the eyelid margin,13–15
and the tarsal and bulbar conjunctivae without significantly affecting the ocular surface steady state. In this study, we used LSCM to obtain an integrated evaluation of the whole ocular surface morpho-functional unit in patients with dry eye.

METHODS

Patients

This study adhered to the tenets of the Declaration of Helsinki, and all of the subjects provided written informed consent before examination. We consecutively enrolled 15 patients with primary Sjogren syndrome (SSI; 11 women and 4 men; mean ± SD age, 52.1 ± 15.4 years; range, 24 to 70 years), 15 patients with non–Sjogren syndrome dry eye (non-SSDE; 10 women and 5 men; mean ± SD age, 56.3 ± 9.8 years; range, 32 to 70 years), 15 patients with MG disease (MGD; 10 women and 5 men; mean ± SD age, 55.3 ± 7.3 years; range, 40 to 65 years), and 15 healthy control subjects (10 women and 5 men; mean ± SD age, 45.2 ± 15.9 years; range, 25 to 69 years). Patients with SSI were recruited at the Referral Centre for Systemic Autoimmune Diseases of Fondazione Cà Granda IRCCS, Ospedale Maggiore Policlinico, and the diagnosis was made according to the American-European Consensus Group criteria. Patients with non-SSDE, MGD, and control subjects were recruited at the Center for Clinical and Experimental Research of Ocular Surface Disease, Fondazione Cà Granda IRCCS, Ospedale Maggiore Policlinico. The diagnosis of non-SSDE was made according to the International Dry Eye Workshop. The diagnosis of MGD was made according to the criteria reported by Matsumoto et al. Briefly, MGs were transilluminated using a fiber-optic device for meibography of the lower eyelid. The degree of MG dropout was scored as follows: grade 0, no gland dropout; grade 1, gland dropout in more than half of the inferior tarsus; and grade 2, gland dropout in more than half of the inferior tarsus.

Exclusion criteria for all groups included lymphoma, AIDS, diabetes mellitus, Stevens-Johnson syndrome, or chemical, thermal, or radiation injury. In addition, subjects with any other systemic/ocular disorder or any systemic or topical treatments (except artificial tears), ongoing or performed in the previous 3 months, with known effect on the ocular surface were excluded. Finally, patients who underwent any ocular surgery or had contact lens use that created an ocular surface problem were also excluded.

Clinical Evaluation

An accurate medical history was drawn up for each participant in the study, and all patients completed the Ocular Surface Disease Index (OSDI), the questionnaire for a standardized evaluation of dry eye–related symptoms. Each subject underwent a thorough ophthalmic evaluation, including biomicroscopic examination of the ocular adnexa and anterior segment. Fluorescein tear film breakup time (BUT), corneal staining with fluorescein, and bulbar conjunctival staining with lissamine green were also performed. Ocular surface staining was scored according to the Collaborative Longitudinal Evaluation of Keratoconus (CLEK) scheme. Tear secretion was evaluated by the Schirmer test with oxybuprocaine chlorohydrate 0.4%.

Assessment of obstruction in the MG orifices was conducted by applying digital pressure on the upper tarsus, and the degree of ease in expressing meibomian secretion (meibum) was evaluated semiquantitatively as follows: grade 0, clear meibum that was easily expressed; grade 1, cloudy meibum that was expressed with mild pressure; grade 2, cloudy meibum that was expressed with more than moderate pressure; grade 3, meibum that could not be expressed even with hard pressure.

Both eyes were examined in all subjects. For statistical analysis, the eye with the highest fluorescein staining score was selected. In case of equal scores for the two eyes, the discriminating criteria considered were, by order of relevance, the conjunctival staining and the tear BUT.

Laser Scanning Confocal Microscopy

Image Acquisition

Laser scanning confocal microscopy was performed on all subjects with a new-generation confocal microscope, the HRT II Corneal Rostock Module (Heidelberg Engineering, Dossenheim, Germany), the characteristics of which have already been published. Before each examination, a drop of oxybuprocaine chlorohydrate 0.4% and an ophthalmic gel (polyacrylic gel 0.2%) were separately instilled in the conjunctival fornix. After the lower eyelid was partly everted, the center of the Tomo-Cap was applanated onto the center of the eyelid margin, halfway between the inner and outer canthi. The instrument focus was manually set while in the acquisition modality “Section Mode.” Focusing started at the most superficial tissues and continued down to the deepest ones visualized with a satisfactory resolution. This procedure was repeated on the nasal and temporal eyelid margins. The lower eyelid was then totally everted, and the images of tarsal conjunctiva were acquired by repeating the same procedure as the eyelid margin. To study the nasal and temporal bulbar conjunctiva, the patient was asked to look to the right and left sides for each eye. The center of the Tomo-Cap was applanated onto the center of bulbar conjunctival area, halfway between the limbus and the canthus.

To study corneal structure, the patient was asked to fixate on a point in front of him or her. Images of the cornea were taken approximately at corneal apex. Ten images were taken every 10 μm of depth. Other images were taken midway through the cornea in an attempt to manually resolve the different structures that appeared at those depths.

The two-dimensional image sizes were 384 × 384 pixels, with 400 × 400 μm field of view. The length of a single LSCM examination session was approximately 10 to 12 minutes.

Image Analysis

For each variable examined, a single masked investigator analyzed three randomized, nonoverlapping, high-quality digital images. Within the largest available regions of interest, we manually marked and quantified the cellular densities of the superficial and basal epithelium of the cornea, eyelid margin, and tarsal and bulbar conjunctivas (Fig. 1). The calculations were performed automatically with the Cell Count software (Heidelberg Engineering, Dossenheim, Germany).

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FIGURE 1.
Epithelia of the ocular surface imaged using LSCM. Superficial (A) and basal (B) epithelium of the cornea; superficial (C) and basal (D) epithelium of the eyelid margin; superficial (E) and basal (F) epithelium of tarsal conjunctiva; superficial (G) and basal (H) epithelium of bulbar conjunctiva.
FIGURE 2.
LSCM signs of inflammation: SSI patient compared with healthy subject. SSI patient showed increased density of corneal subepithelial dendritic cells (B vs. A), activated keratocytes (D, arrows vs. C), presumed conjunctival PMN cells (F vs. E), inhomogeneous appearance of MG interstice (H vs. G).
For MG measurements in the nasal, middle, and temporal regions of the lower eyelid margin, we manually marked the acinar units within each 400 × 400–μm frame. We measured the largest diameter of each acinus, and, using the Cell Count software, we determined the density of the units in each region.20 Finally, the mean density was determined as the average of the densities in the nasal, middle, and temporal regions of the lower eyelid margin. The mean MG density in the tarsal conjunctiva was determined in the same way. We also measured the diameter of the glandular orifices after manually marking the longest axis of each.14 We graded the meibum secretion reflectivity and the inhomogeneous appearance of interstices and walls of acinar units using grading scales previously described.14 For the lower tarsal conjunctiva, we used the Cell Count software to calculate the density of the hyperreflective polymorphonuclear cells18 ([PMNs] Fig. 2) that were manually marked inside each 400 × 400–μm frame.

For the cornea, we evaluated the number of nerve fibers of the subbasal plexus in each frame, the density of anterior and posterior stromal cells (Fig. 3), dendritic cells (Fig. 4A), and activated keratocytes (Fig. 4B). Each was manually marked inside the 400 × 400–μm frames and calculated automatically with the Cell Count software. Subbasal nerve tortuosity was graded (0–4) by comparison with the reference images, according to the method proposed by Oliveira-Soto and Efron.23 We also counted the number of beadlike formations per 100 μm of nerve fiber (Fig. 3).11

Statistical Analysis

All data were calculated as means ± SDs. For nonparametric variables, the Kruskal-Wallis test was applied to test the statistical differences among the four groups. For parametric variables, the analysis of variance (ANOVA) was applied to test the statistical differences among the four groups. The Mann-Whitney U test was for post hoc testing of nonparametric variables, and the least significant difference (LSD) test was used for post hoc testing of parametric variables. The variables quantified with grading scales, for example, secretion reflectivity, inhomogeneous appearance of acinar unit interstices and wall, and tortuosity of subbasal plexi, were treated as nonparametric variables. The minimum criterion for tests of significance was p < 0.01. The statistical analyses were conducted with commercial software (SPSS for Windows, version 12.0; SPSS Sciences, Chicago, IL).

RESULTS

Clinical Data

The OSDI score was higher in all dry eye groups compared with that in the controls, 3.00 ± 2.00 (p < 0.001, LSD; Table 1). The corneal and conjunctival staining were higher in SSI, 3.61 ± 1.24 and 4.15 ± 1.02, respectively, than that in non-SSDE and MGD (p < 0.001, LSD). Tear BUT was lower in all dry eye groups compared with that in the controls, 10.53 ± 1.98 seconds (p < 0.001, LSD). The BUT was also lower in SSI and MGD compared with that in non-SSDE (p < 0.01, LSD). The Schirmer test results in all of the dry eye groups were lower than those in the controls, 12.64 ± 4.02 mm per 5 minutes (p < 0.001, p < 0.001, p < 0.01, respectively, LSD). The SSI Schirmer rate was also lower than that in non-SSDE and MGD (p < 0.01, p < 0.001, respectively, LSD), and the non-SSDE rate was lower than the MGD rate (p < 0.01, LSD).

LSCM Data

Cell Density

The eyelid margin superficial epithelial cell density was lower in MGD subjects, 982.00 ± 301.00 cell/mm² than the controls and non-SSDE subjects (p < 0.001, LSD; Table 2). Tarsal superficial

![Figure 3](image-url)

LSCM appearance of subbasal plexus of an SSI patient. Nerve fibers show increased tortuosity (A) and evident beadlike formations (B, arrows).
The epithelial cell density was lowest in SSI patients, 990 ± 167 cells/mm² (Table 2). It increased successively in MGD and non-SSDE patients and was highest in control subjects, 2381.00 ± 267.00 cells/mm² (p < 0.001 for all pairwise comparisons except non-SSDE vs. MGD; Table 2). The cell density of the bulbar conjunctival superficial epithelium was significantly lower in all groups than that in the controls, and it was lower in SSI than that in MGD (Table 2). The superficial corneal epithelium density was significantly lower in all groups compared with that in the controls, and it was lower in SSI than either non-SSDE or MGD (p < 0.001, LSD).

The tarsal basal epithelium density was significantly lower in SSI, non-SSDE, and MGD than that in the controls, 4044 ± 661 cells/mm² (Table 2). The density in SSI, 3062 ± 506 cells/mm², was lower than that in non-SSDE (p < 0.01, LSD).

The bulbar conjunctival basal epithelium density was lower in SSI, non-SSDE, and MGD than that in the controls, 5164 ± 328 cells/mm² (p < 0.001, p < 0.001, p < 0.01, respectively, LSD; Table 2). The density in SSI, 3616 ± 272 cells/mm², was less than that in non-SSDE (p < 0.001, LSD). The basal corneal epithelium density was higher in SSI and non-SSDE than in the
controls, 6304 ± 265 cells/mm² (p < 0.001, p < 0.01, respectively, LSD; Table 2). The density in SSI was also greater than in non-SSDE and MGD (p < 0.01 and p < 0.001, respectively, LSD; Table 2).

Anterior corneal stroma density was higher in all groups than in controls and in SSI compared with those in non-SSDE and MGD (p < 0.001, LSD; Table 2). However, there were no differences in the posterior corneal stromal cell densities among any of the groups.

### TABLE 1.

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>SSI (n = 15)</th>
<th>Non-SSDE (n = 15)</th>
<th>MGD (n = 15)</th>
<th>C (n = 15)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OSDI</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>36.00 ± 15.00</td>
<td>27.00 ± 18.00</td>
<td>32.00 ± 13.00</td>
<td>3.00 ± 2.00</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Corneal staining</td>
<td>3.61 ± 1.24</td>
<td>1.47 ± 0.84</td>
<td>1.62 ± 0.71</td>
<td>nd</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Conjunctival staining</td>
<td>4.15 ± 1.02</td>
<td>1.88 ± 0.94</td>
<td>2.30 ± 1.06</td>
<td>nd</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>BUT, sec</td>
<td>3.24 ± 2.10</td>
<td>6.13 ± 1.74</td>
<td>3.87 ± 2.59</td>
<td>10.53 ± 1.98</td>
<td>&lt;0.001§</td>
</tr>
<tr>
<td>Shimmer, mm per 5 min</td>
<td>3.18 ± 2.39</td>
<td>6.33 ± 3.61</td>
<td>9.57 ± 4.25</td>
<td>12.64 ± 4.02</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*SSI versus non-SSDE, not significant; SSI versus MGD, not significant; SSI versus C, p < 0.001; non-SSDE versus MGD, not significant; non-SSDE versus C, p < 0.001; MGD versus C, p < 0.001 (LSD post hoc test).
†SSI versus non-SSDE, p < 0.001; SSI versus MGD, p < 0.001; non-SSDE versus MGD, not significant (LSD post hoc test).
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§SSI versus non-SSDE, p < 0.01; SSI versus MGD, not significant; SSI versus C, p < 0.001; non-SSDE versus MGD, p < 0.01; non-SSDE versus C, p < 0.001; MGD versus C, p < 0.001 (LSD post hoc test).
||SSI versus non-SSDE, p < 0.01; SSI versus MGD, p < 0.001; SSI versus C, p < 0.001; non-SSDE versus MGD, p < 0.01; non-SSDE versus C, p < 0.001; MGD versus C, p < 0.01 (LSD post hoc test).
SSI, primary Sjogren syndrome; non-SSDE, non-Sjogren syndrome dry eye; MGD, meibomian gland dysfunction; C, control; p, p values were obtained by analysis of variance; nd, not done.

### TABLE 2.

<table>
<thead>
<tr>
<th>Cellular density</th>
<th>SSI (n = 15)</th>
<th>Non-SSDE (n = 15)</th>
<th>MGD (n = 15)</th>
<th>C (n = 15)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Superficial epithelium, cells/mm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyelid margin</td>
<td>1213 ± 249</td>
<td>1432 ± 340</td>
<td>982 ± 301</td>
<td>1623 ± 312</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Tarsal conjunctiva</td>
<td>990 ± 167</td>
<td>1433 ± 215</td>
<td>1312 ± 202</td>
<td>2381 ± 267</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Bulbar conjunctiva</td>
<td>984 ± 144</td>
<td>1403 ± 188</td>
<td>1351 ± 219</td>
<td>2136 ± 243</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>Cornea</td>
<td>941 ± 123</td>
<td>1506 ± 169</td>
<td>1345 ± 207</td>
<td>1902 ± 269</td>
<td>&lt;0.001§</td>
</tr>
<tr>
<td>Basal epithelium, cells/mm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyelid margin</td>
<td>4082 ± 386</td>
<td>4287 ± 418</td>
<td>4124 ± 431</td>
<td>4626 ± 405</td>
<td>Not significant</td>
</tr>
<tr>
<td>Tarsal conjunctiva</td>
<td>3062 ± 506</td>
<td>3604 ± 456</td>
<td>3177 ± 592</td>
<td>4044 ± 661</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Bulbar conjunctiva</td>
<td>3616 ± 272</td>
<td>4689 ± 292</td>
<td>4675 ± 341</td>
<td>5164 ± 328</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Cornea</td>
<td>6933 ± 325</td>
<td>6684 ± 231</td>
<td>6498 ± 202</td>
<td>6304 ± 265</td>
<td>&lt;0.001††</td>
</tr>
<tr>
<td></td>
<td>Corneal stroma, cells/mm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>1293 ± 97</td>
<td>1163 ± 83</td>
<td>1012 ± 88</td>
<td>863 ± 84</td>
<td>&lt;0.001‡‡</td>
</tr>
<tr>
<td>Posterior</td>
<td>866 ± 108</td>
<td>814 ± 98</td>
<td>789 ± 121</td>
<td>796 ± 115</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*SSI versus non-SSDE, p < 0.001; SSI versus MGD, p < 0.001; SSI versus C, p < 0.001; non-SSDE versus MGD, not significant; non-SSDE versus C, p < 0.001; MGD versus C, p < 0.001 (LSD post hoc test).
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SSI, Sjogren syndrome; non-SSDE, non-Sjogren syndrome dry eye; MGD, meibomian gland dysfunction; C, control; p, p values were obtained by analysis of variance.

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TABLE 3.
Presumed inflammatory confocal data

<table>
<thead>
<tr>
<th></th>
<th>SSI (n = 15)</th>
<th>Non-SSDE (n = 15)</th>
<th>MGD (n = 15)</th>
<th>C (n = 15)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneal dendritic cell density, cells/mm²</td>
<td>169 ± 48</td>
<td>56 ± 41</td>
<td>82 ± 38</td>
<td>53 ± 34</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Conjunctival polymorphonuclear cell density, cells/mm²</td>
<td>498 ± 106</td>
<td>177 ± 66</td>
<td>212 ± 83</td>
<td>87 ± 60</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Inhomogeneous appearance of MG Wall</td>
<td>3.0 ± 1.0</td>
<td>2.0 ± 0.9</td>
<td>2.5 ± 0.9</td>
<td>1.4 ± 0.5</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>Interstice</td>
<td>2.6 ± 0.6</td>
<td>1.4 ± 0.8</td>
<td>1.8 ± 0.8</td>
<td>1.0 ± 0.3</td>
<td>&lt;0.001§</td>
</tr>
<tr>
<td>Activated keratocyte density, cells/mm²</td>
<td>57.2 ± 23.3</td>
<td>53.4 ± 14.9</td>
<td>53.0 ± 14.7</td>
<td>28.8 ± 5.7</td>
<td>&lt;0.001¶</td>
</tr>
</tbody>
</table>

Values of p for corneal and conjunctival cell densities and for activated keratocytes were determined by analysis of variance; p values for MG appearance were determined by Kruskal-Wallis test.

*SSI versus non-SSDE, p < 0.001; SSI versus MGD, p < 0.001; SSI versus C, p < 0.001; non-SSDE versus MGD, p < 0.01; non-SSDE versus C, not significant; MGD versus C, p < 0.01 (LSD post hoc test).
†SSI versus non-SSDE, p < 0.001; SSI versus MGD, p < 0.001; SSI versus C, p < 0.001; non-SSDE versus MGD, not significant; non-SSDE versus C, p < 0.001; MGD versus C, p < 0.001 (LSD post hoc test).
‡SSI versus non-SSDE, not significant; SSI versus MGD, not significant; SSI versus C, p < 0.001; non-SSDE versus MGD, not significant; non-SSDE versus C, p < 0.001; MGD versus C, p < 0.001 (Mann-Whitney U test).
§SSI versus non-SSDE, p < 0.01; SSI versus MGD, p < 0.01; SSI versus C, p < 0.001; non-SSDE versus MGD, not significant; non-SSDE versus C, p < 0.001; MGD versus C, p < 0.001 (Mann-Whitney U test).
¶SSI versus non-SSDE, not significant; SSI versus MGD, not significant; SSI versus C, p < 0.001; non-SSDE versus MGD, not significant; non-SSDE versus C, p < 0.001; MGD versus C, p < 0.001 (LSD post hoc test).

Inflammation

The corneal dendritic cell densities in SSI patients, 169 ± 48 cells/mm², and in MGD patients, 82 ± 38 cells/mm², were significantly higher than those in the controls (p < 0.001, p < 0.01, respectively, LSD; Table 3). The density in SSI patients was also higher than those in non-SSDE and MGD patients (p < 0.001, LSD), and the density in MGD patients was higher than that in non-SSDE patients (p < 0.01, LSD). The density of conjunctival PMNs in all dry eye groups was significantly higher than that in the controls, 5.8 ± 1.3 per frame (p < 0.001, LSD; Table 4). Furthermore, the density in SSI patients, 2.9 ± 0.8 per frame, was lower than those in non-SSDE and MGD patients. The tortuosity of these fibers was higher in SSI patients, 2.8 ± 0.8, and in MGD patients, 2.5 ± 0.9, than that in the controls (p < 0.001, Mann-Whitney U test). The tortuosity in SSI and MGD patients was also higher than that in non-SSDE patients; for MGD patients, it was higher than that in non-SSDE patients. There were more subbasal plexus fiber beadlike formations in all dry eye groups compared with that in the controls, 28.8 ± 5.7 cells/mm² (p < 0.001, LSD).

TABLE 4.
Corneal innervation

<table>
<thead>
<tr>
<th></th>
<th>SSI (n = 15)</th>
<th>Non-SSDE (n = 15)</th>
<th>MGD (n = 15)</th>
<th>C (n = 15)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subbasal plexus fiber number, numbers per frame</td>
<td>2.9 ± 0.8</td>
<td>3.9 ± 0.5</td>
<td>4.3 ± 1.1</td>
<td>5.8 ± 1.3</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Tortuosity</td>
<td>2.8 ± 0.8</td>
<td>1.5 ± 1.1</td>
<td>2.5 ± 0.9</td>
<td>1.2 ± 0.5</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Beadlike formations, numbers per 100 µm</td>
<td>304 ± 53</td>
<td>218 ± 49</td>
<td>242 ± 69</td>
<td>116 ± 39</td>
<td>&lt;0.001‡</td>
</tr>
</tbody>
</table>

Values of p for subbasal plexus fiber number and beadlike formations were obtained by ANOVA; p values for tortuosity were obtained by Kruskal-Wallis test.

*SSI versus non-SSDE, p < 0.001; SSI versus MGD, p < 0.001; SSI versus C, p < 0.001; non-SSDE versus MGD, not significant; non-SSDE versus C, p < 0.001; MGD versus C, p < 0.001 (LSD post hoc test).
†SSI versus non-SSDE, p < 0.001; SSI versus MGD, not significant; SSI versus C, p < 0.001; non-SSDE versus MGD, p < 0.01; non-SSDE versus C, not significant; MGD versus C, p < 0.001 (Mann-Whitney U test).
‡SSI versus non-SSDE, p < 0.01; SSI versus MGD, p < 0.01; SSI versus C, p < 0.001; non-SSDE versus MGD, not significant; non-SSDE versus C, p < 0.001; MGD versus C, p < 0.001 (LSD post hoc test).
TABLE 5.
Meibomian gland properties

<table>
<thead>
<tr>
<th></th>
<th>SSI (n = 15)</th>
<th>Non-SSDE (n = 15)</th>
<th>MGD (n = 15)</th>
<th>C (n = 15)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acini</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Density, acini/mm²</td>
<td>146 ± 62</td>
<td>128 ± 43</td>
<td>64 ± 33</td>
<td>121 ± 45</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Diameter, µm</td>
<td>46 ± 25</td>
<td>57 ± 28</td>
<td>94 ± 36</td>
<td>49 ± 12</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Orifice diameter, µm</td>
<td>29 ± 6</td>
<td>31 ± 4</td>
<td>48 ± 7</td>
<td>32 ± 5</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Secretion reflectivity</td>
<td>1.7 ± 0.6</td>
<td>1.5</td>
<td>3.3 ± 0.7</td>
<td>1.1 ± 0.7</td>
<td>&lt;0.001‡</td>
</tr>
</tbody>
</table>

Values of p for acini densities and diameters and for orifice diameters were obtained by ANOVA; p values for secretion reflectivity were obtained by Kruskal-Wallis test.

*SSI versus non-SSDE, not significant; SSI versus MGD, p < 0.001; SSI versus C, not significant; non-SSDE versus MGD, p < 0.001; non-SSDE versus C, not significant; MGD versus C, p < 0.001 (Mann-Whitney U test).
†SSI versus non-SSDE, not significant; SSI versus MGD, p = 0.001; SSI versus C, not significant; non-SSDE versus MGD, p = 0.001; non-SSDE versus C, not significant; MGD versus C, p = 0.001 (Mann-Whitney U test).
‡SSI versus non-SSDE, not significant; SSI versus MGD, p < 0.001; SSI versus C, not significant; non-SSDE versus MGD, p < 0.001; non-SSDE versus C, not significant; MGD versus C, p < 0.001 (Mann-Whitney U test).

116 ± 39. There were also more in SSI patients compared with those in non-SSDE and MGD patients (p < 0.001, LSD).

**Meibomian Gland**

The density of MG acinar units was lower in MGD patients, 64 ± 33 acini/mm², than those in SSI, non-SSDE, and control subjects (p < 0.001, LSD; Table 5). The diameters of the MG acini and orifices were higher in MGD patients, 94 ± 36 µm and 48 ± 7 µm, respectively, than those in SSI, non-SSDE, and control subjects (p < 0.001, LSD). The reflectivity of the secretion was also higher in MGD patients, 3.3 ± 0.7, than those in SSI, non-SSDE, and control subjects (p < 0.001, Mann-Whitney U test).

**DISCUSSION**

Laser scanning confocal microscopy is a new, emerging, noninvasive technology that is useful as a supplementary diagnostic tool for in vivo assessment of the histopathology of many ocular surface disorders. This technology enables new opportunities to develop integrated quantitative studies of all ocular surface structures. It is especially suited to the analysis of three pathogenic aspects of dry eye: tissue damage, inflammation, and alteration of corneal innervation.

In our study, we calculated the epithelial cell densities of all of the ocular surface structures (Fig. 1). The superficial epithelia of cornea and bulbar and tarsal conjunctivae had significantly lower cell densities in non-SSDE, MGD, and especially in SSI patients. These results can be readily interpreted as the consequence of ocular surface damage caused by mechanical, trophic, and inflammatory phenomena.

The basal epithelial cell density in the cornea was higher in SSI patients than those in non-SSDE, MGD, and control subjects, and it was also higher in non-SSDE patients than in the controls. These differences can be associated with a hyperproliferative stimulus to repair the damage of superficial epithelium, like that demonstrated in a dry eye animal model.24 The cell densities of the bulbar and tarsal conjunctival basal epithelia were not similarly increased in patients with dry eye. These data, according to Wakamatsu et al.,8 can be explained by the presence of inflammation that interferes with epithelial cellular turnover.

The cell density in the anterior corneal stroma was higher in all dry eye groups, particularly in SSI patients, compared with that in the controls. These data, according to Villani et al.,4 Benitez del Castillo et al.,25 and Mustonen et al.,26 may be the result of ocular surface inflammatory processes that determine a synchronous increase of apoptotic phenomena, proteolytic activity, and proliferative stimuli.

Several studies used corneal dendritic cells and PMNs as indices of inflammation6,27; however, we were not able to identify corneal PMNs, so we measured only dendritic cell density in the cornea. In the tarsal conjunctiva, others reported dendritic cells and PMNs8,18; however, we were able to measure only PMNs there. Although the density of corneal dendritic cells in non-SSDE patients was similar to that in the controls, the density in SSI and MGD subjects was greater than that in the controls. For all of the dry eye groups, the density of PMNs in the tarsal conjunctiva was greater than that in the controls. We also found that the density of activated keratocytes was higher in all of the dry eye groups compared with that in the controls. These stromal cells, termed in confocal reports but not histologically described, are hyperreflective and should be interpreted in a nonsingular way. They could be representative of several cases of normal stromal cells in which the light ray of the confocal microscope strikes an unusual angle. However, these findings are associated with corneal inflammation, as reported in patients with SS4,28 or Graves orbitopathy.29 We think that it could be assumed that these findings correspond to keratocytes in a particular state of metabolic activation, induced by proinflammatory cytokines, or to stromal bone marrow–derived cells.5

We did not attempt to evaluate the density of goblet cells in the tarsal conjunctiva because there is no consensus among observers with regard to their confocal analysis. This lack of consensus might be caused by the misinterpretation of observed cells and/or the inability of LSCM to clearly discriminate between goblet and nongoblet cells.18

By LSCM, we were not able to identify periglandular and bulbar conjunctival dendritic cells like those that have been...
reported in previous studies.\textsuperscript{5,30} As a proxy for these cells, we semiquantitatively assessed the inhomogeneity of the MG interstices and wall, which varies with the level of inflammation.\textsuperscript{14,15} We found that this inhomogeneity was significantly higher in SSI patients than that in either non-SSDE or MGD patients. This is probably caused by the autoimmune pathogenesis of SSI that has an important interstitial inflammatory component. Based on the differences in densities of corneal dendritic cells, corneal activated keratocytes, tarsal PMNs, and inhomogeneity of the MG interstices and wall, all of the ocular surface structures in dry eye patients showed signs of inflammation, with the signs being especially evident in SSI patients.

There were significantly fewer subbasal plexus fibers in the cornea of each of the dry eye groups compared with those in the controls. This was particularly evident in SSI patients as reported by Villani et al.\textsuperscript{4} and Benitez del Castillo et al.\textsuperscript{31} This differs from the reports by Tuisku et al.\textsuperscript{10} and Zhang et al.\textsuperscript{32} who found an increase in the number of these fibers. The tortuosity of the fibers and the density of blood-like formations, associated with both neurodegeneration and growth factor-mediated neuroregeneration, seem to be the best parameters to study the subbasal plexus changes and damage.

Examination by LSCM showed a reduction of acinar density, increases of acinar and orifice diameters, and an increased secretion reflectivity in MGD compared with those in the other dry eye diseases and the controls. These alterations were associated with an increase in the meibum viscosity. This change could have resulted in obstruction of the glandular ducts that, in turn, could have caused the increases in diameters of the acini.

In summary, we compared LSCM findings in matched groups of SS, non-SSDE, MGD, and controls, applying lessons learned from research on single tissues with a new approach aimed to study the whole ocular surface in a single step. The ocular surface of dry eye patients showed several, different, and partially overlapping confocal changes. Our LSCM data allowed us to identify specific ocular surface abnormality patterns for each different type of dry eye. Primary Sjogren syndrome patients were characterized by the most severe and diffused signs of inflammation (increased corneal dendritic cells and hyperreflective keratocytes, conjunctival inflammatory cells, and inhomogeneous appearance of MGs interstice; Fig. 2), severe changes and damage of corneal innervation (reduced nerve fibers, increased tortuosity and density of endings; Fig. 3), and severe signs of tissue damage (reduced epithelial densities and increased stromal cells density). Meibomian gland disease patients showed the most severe abnormalities in MGs (decreased acinar density, increased diameter of acini and orifices, and increased reflectivity; Fig. 4). Non-SSDE patients presented confocal changes similar to those in SSI but less severe.

In clinical research, the chance to get simultaneous \textit{in vivo} high-resolution information on different tissues of the ocular surface provides us a new and ready-to-use tool to better understand the pathogenic processes, the behavior of resident and recruited cell populations, and the mutual roles of inflammation, neuropathy, and tissue damage in each pathological condition and disease phase. Important data on natural history and response to treatment might be provided by tracking changes over time. The potentially most exciting evolution of this approach is the attempt to move from bench to bedside, trying to expand the currently limited clinical applications of \textit{in vivo} confocal microscopy. At present, in our Ocular Surface Unit, confocal findings on inflammatory cells, MG morphology, and innervation have an ancillary but not insignificant role in clinical care.

In the near future, better standardization and evidence-based clinical validation of confocal data might help clinicians improve therapies, tailoring according to the idea “one ocular surface, one time, one treatment.”

In conclusion, LSCM allows an integrated high-resolution evaluation of the ocular surface, showing the overall behavior of the morpho-functional unit. Our confocal approach to the whole ocular surface might be particularly important in dry eye disease, actually recognized as a disease affecting an entire complex system.\textsuperscript{1} This approach may be useful to better understand the changes that occur over time in ocular surface components as a consequence of different pathogenic processes or different therapeutic approaches.

ACKNOWLEDGMENTS

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REFERENCES


