An In Vivo Confocal Microscopy and Impression Cytology Analysis of Goblet Cells in Patients with Chemical Burns

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PURPOSE. To evaluate goblet cell density (GCD) on conjunctiva and cornea in patients with ocular chemical burns by in vivo laser scanning confocal microscopy (LSCM) and impression cytology (IC) and to explore the correlation between two methods.

METHODS. Fifty-four patients (58 eyes) with chemical burn were enrolled in the study. LSCM was applied to identify the goblet cells on conjunctiva and cornea under in vivo conditions, and GCD was analyzed with the customized software. Impression cytology was then performed, and the biopsy specimens were stained to visualize goblet cells in vitro and to measure the density. Statistical software was used to analyze the correlation between GCD taken by two methods.

RESULTS. Conjunctival goblet cells could be discriminated in 55 eyes and 57 eyes by in vivo LSCM and IC. They could be identified on the cornea in nine eyes and eight eyes by two methods. The positive rate of two methods had no significant difference. GCDs on conjunctiva measured by in vivo LSCM and IC were 136 ± 79 cells/mm² and 121 ± 66 cells/mm². Median GCDs on cornea detected by two methods were 30 cells/mm² and 23 cells/mm², respectively. A significant positive correlation was found between the GCDs on conjunctiva measured by these two methods as well as the GCDs on cornea.

CONCLUSIONS. GCD decreased in patients with chemical burns. A positive correlation was found between GCD measured by in vivo LSCM and IC after chemical burns. In vivo LSCM was a promising device to study goblet cells in vivo under pathologic conditions. (Invest Ophthalmol Vis Sci. 2010;51:1397–1400) DOI:10.1167/iovs.09-3886

Ocular chemical burn is one of the most common ocular injuries, especially in the developing countries.1,2 The severity of ocular injury after a chemical exposure is closely related to the contact surface area and the degree of penetration. Severe chemical burns, either alkaline or acid, not only cause limbal stem cell deficiency, but also damage corneal and conjunctival epithelium and stroma, including goblet cells. Goblet cells are an important indicator in the follow-up of chemical burn because of two reasons. On the one hand, the detection of goblet cells on the cornea implies the conjunctivalization of corneal epithelium and limbal stem cell deficiency.3 On the other hand, the reduction or even absence of mucus in the tear because of damaged conjunctival goblet cells compromises the proper dispersion of precorneal tear film, leading to keratoconjunctivitis sicca, one of the most commonly seen long-term complications after chemical burns.4,5

Impression cytology (IC) used to be the standard technique to investigate goblet cells loss in ocular surface disease, including chemical injuries and dry eye.6 With periodic acid-Schiff (PAS) staining, goblet cells are easily recognized on the IC specimen, and their density can be calculated. Previous researchers obtained imprints of the surface of the conjunctiva with an absorbent filter.7,8 However, lack of standardization limits the potential application of conjunctival IC, which was attributed to the variety of filter paper used.9 Meanwhile, frequent sampling in the same patient led to the aggravation of the already seriously damaged ocular surface.10 For these reasons, it is unsuitable to apply IC to evaluate the degree of goblet cell loss and to study its evolution in patients with severe chemical burns.

The opportunity to study goblet cells in a simple and rapid manner has expanded dramatically. Recently, with the application of in vivo laser scanning confocal microscopy (LSCM) on the human conjunctiva, a noninvasive, real-time, in vivo microscopic examination that provided images with high resolution and contrast opened up a promising new method to investigate goblet cells. Kobayashi and Messmer11,12 first demonstrated goblet cell morphology with the use of confocal microscopy. Thereafter, confocal microscopy was increasingly popularized in the in vivo study on conjunctival changes under various pathologic conditions.13–15

To the best of our knowledge, few reports were available on the correlation of goblet cell density (GCD) detected by confocal microscopy and IC. The purpose of the present study was to explore the correlation between GCD measured by in vivo LSCM and IC in patients with ocular chemical burns.

SUBJECTS AND METHODS

Subjects

This study was conducted in compliance with informed consent regulations and the Declaration of Helsinki. Fifty-four patients (58 eyes) with chemical burn were included in the study; the average age was 38.8 ± 13.5 years (range, 10–62 years). Apart from four female pa-
tients (5 eyes), the rest (53 eyes) were male. Eleven cases (12 eyes) were acid burn; the other (46 eyes) were alkali burn. All patients were evaluated according to the Roper-Hall classification. Twenty-six cases (27 eyes) were classified as grade 2, 15 cases (16 eyes) as grade 3, and 13 cases (15 eyes) as grade 4. The median duration after injury was 10 months (range, 1–216 months).

**In Vivo Laser Scanning Confocal Microscopy**

The Heidelberg retina tomograph (HRTII)/Rostock cornea module (RCM) (Heidelberg Engineering GmbH, Dossenheim, Germany) was used in this study, with a 60× water-immersion objective lens (Olympus Europa GmbH, Hamburg, Germany) and a 670-nm diode laser as a light source, allowing a scanning area of 384 × 384 μm² with lateral and vertical resolutions of both 1 μm and a magnification up to 800 times. Before the examination, one drop of 0.4% oxybuprocaine hydrochloride (Benoxil; Santen Pharmaceutical, Osaka, Japan) was applied to the lower conjunctival sac. The patient was required to place the chin and forehead firmly on the headrest of the microscope and to fixate a target. The central cornea of the injured eye was examined, and images were recorded at one point along the z-axis using the single scan mode. Then the examination was similarly performed on superior, inferior, nasal, and temporal bulbar conjunctiva with the examination point at 3 mm outside the limbus.

All images were subsequently checked by a masked observer, and the images with goblet cells identifiable were selected and encoded randomly. At each examination point, three images were selected to count the number of goblet cells (Cell Count Software; Heidelberg Engineering GmbH) in manual mode and to calculate the average GCD of each examination point. The conjunctival GCD was determined by further averaging the GCD measured at both nasal and temporal bulbar conjunctiva. As for the cornea, the average value of the specimen on the cornea was considered as corneal GCD. All conjunctival impression cytology specimens were obtained and evaluated in a masked fashion by the same technician.

**Impression Cytology**

Impression cytology was performed after LSCM examination. Techniques for impression cytology specimen collection, preparation, and examination have been described previously.16 In brief, the nitrocellulose filter strips (3 × 3 mm) with a pore size of 0.22 mm were soaked in distilled water for 3 to 4 hours and dried at room temperature before use. After topical anesthesia with the administration of 0.5% proparacaine (Alcaine; Alcon Inc., Irvine, CA), strips were placed on the surface of the central cornea and the nasal and temporal interpalpebral bulbar conjunctiva, where in vivo LSCM was performed and then were pressed gently by a glass rod. Excess tears in the inferior conjunctival fornix were gently absorbed with cotton wipes before the nitrocellulose strip was peeled off. The specimens were immediately fixed with 95% ethanol and stained with PAS.7 Under the microscopy, goblet cells were stained red by PAS. The mean GCD of each specimen was determined by averaging the total number of goblet cells in three consecutive visual fields of high magnification (×200). The mean conjunctival GCD of each enrolled eye was then calculated by further averaging the GCD measured at both nasal and temporal bulbar conjunctiva. The average value of the specimen on the cornea was considered as corneal GCD. All conjunctival impression cytology specimens were obtained and evaluated in a masked fashion by the same technician.

**Statistical Analysis**

Statistical analysis was performed (SPSS 13.0 for Windows; SPSS Inc., Chicago, IL). First a χ² test was performed to compare the positive rate of two methods of identifying goblet cells. Then the data about GCD were analyzed to confirm whether they conformed to Gaussian distribution. For those with Gaussian distribution, the data were analyzed with paired-samples t-tests to compare the GCD measured by LSCM and IC. For those with nonnormal distribution, the data were analyzed with the nonparametric Kruskal-Wallis test. To determine the correlation of the GCD measured by two methods, nonparametric Spearman correlation was performed. P < 0.05 was considered statistically significant.

**RESULTS**

**Morphology of Goblet Cells**

In present study, the goblet cells in the in vivo confocal microscopic image were characterized as large to giant hyperreflective oval cells with hyporeflective nuclei, significantly larger than the surrounding epithelial cells, and were gathered in groups or mainly dispersed throughout the epithelium (Figs. 1a, 2a). Many Langerhans cells and inflammatory cells could be found in the surrounding tissue. Goblet cells were easily distinguished by impression cytology because they were stained pink with PAS staining, whereas the nuclei of surrounding epithelial cells were stained blue (Figs. 1b, 2b).

**Positive Rate of Identifying Goblet Cells**

Conjunctival goblet cells could be discriminated in 55 eyes and 57 eyes by in vivo LSCM and IC, respectively, with the positivity rates of 93.2% and 98.3%. Goblet cells could be identified on the cornea in nine eyes and eight eyes by two methods, with the positivity rates of 15.5% and 13.8%. Statistical analysis showed no significant difference between these two methods (χ² = 0.069 and 0.984, P = 0.793 and 0.618, respectively).

**Correlation between Conjunctival GCD Measured by In Vivo LSCM and IC**

The data on conjunctival GCD were in conformity to Gaussian distribution. Average GCD measured by in vivo LSCM and IC
was $136 \pm 79$ cells/mm$^2$ and $121 \pm 66$ cells/mm$^2$. The difference had no statistical significance ($t = 0.789; P = 0.430$). A significant positive correlation was found between GCD measured by these two methods ($\rho = 0.929; P = 0.000$), as shown on the scatterplot graph (Fig. 3).

**Correlation between Corneal GCD Measured by In Vivo LSCM and IC**

The data on corneal GCD were in conformity to nonnormal distribution. The median GCD measured by in vivo LSCM and IC was 30 cells/mm$^2$ (range, 5–181 cells/mm$^2$) and 23 cells/mm$^2$ (range, 4–213 cells/mm$^2$), without significant difference ($Z = 1.444; P = 0.149$). A significant positive correlation was also found between GCD measured by these two methods ($\rho = 0.946; P = 0.000$), as shown on the scatterplot graph (Fig. 4).

**DISCUSSION**

The ocular surface is covered by two types of epithelium with distinct phenotypes. The conjunctival epithelium differs from the corneal epithelium in having mucin-secreting goblet cells intermixed with nongoblet epithelial cells. Goblet cells are essential for maintaining normal precorneal tear film and healthy ocular surface. Chemical burns partially or totally damage the ocular cells and tissue, including goblet cells, resulting in mucus deficiency and keratoconjunctivitis sicca.$^{4,5}$ Meanwhile, the destruction of limbal stem cells caused by chemical burns leads to conjunctival epithelial cell ingrowth with goblet cells onto the corneal surface, a hallmark representing limbal stem cell deficiency.$^{3}$ Therefore, the identification of goblet cells in patients with chemical burn is of great importance in evaluating the severity of injury and its prognosis.

Thus far, the classical way to visualize ocular surface goblet cells has been impression cytology.$^{17,18}$ The conjunctival GCD in healthy subjects was reported to range from 380 cells/mm$^2$ to 430 cells/mm$^2$ by many researchers.$^{5,6,8,19}$ However, there has been a controversial view that repeated sampling in the same site of conjunctiva every 4 days led to a localized reduction of GCD.$^{10}$ Furthermore, IC cannot provide the image of goblet cells instantly because it requires multiple procedures, such as fixation and staining. In contrast, in vivo LSCM produces the real-time image of goblet cells with high quality in a quick, simple, and noninvasive manner. Repetitive examination does not exert negative impact on the morphology or density of goblet cells. In addition, apart from interpalpebral bulbar conjunctiva, superior and inferior bulbar conjunctiva, especially those near the superior and inferior fornix, could be examined by in vivo LSCM, enhancing its sensitivity. The morphology of goblet cells in confocal microscopic images has been described as giant hyperreflective oval cells, gathering in groups or scattering among the epithelial cells.$^{11,12}$ Our previous research examined healthy subjects with in vivo LSCM and revealed an average GCD of $424 \pm 71$ cells/mm$^2$ on conjunctiva.
of subjects and peripheral sampling sites might be helpful to probably underestimated. Further study with a larger number not sampled in the present study, the detection rate of GCs was inadequate. Furthermore, because the peripheral cornea was primarily on the severity of injury, but the correlation between them could not be elucidated in the present study because the cell loss was the main reason for an unstable tear film and the consequent keratoconjunctivitis sicca, a common complication of chemical burn. Notably, the conjunctival GCD measured in this study was less than that in the Nelson\textsuperscript{5} report. Given that more than half the patients in this study were severely or moderately injured with burns classified as grade IV or grade III and that the patients examined by Nelson\textsuperscript{5} had mild burns, we presumed that the difference in the severity of injury to the eyes was the possible reason.

The presence of goblet cells on the corneal surface has been shown in many corneal diseases, manifesting the common denominator of limbal stem cell deficiency.\textsuperscript{3,20} However, there are few published studies of corneal GCD in patients with chemical burns. The present study revealed that goblet cells could be detected on the surface of cornea in 15.5\% injured eyes, and GCD ranged from 5 to 181 cells/mm\textsuperscript{2} with in vivo LSCM, similar to measurements by IC technique. It was presumed that the variation in GCD on the cornea depended primarily on the severity of injury, but the correlation between them could not be elucidated in the present study because the number of cases with goblet cells present on the cornea was inadequate. Furthermore, because the peripheral cornea was not sampled in the present study, the detection rate of GCs was probably underestimated. Further study with a larger number of subjects and peripheral sampling sites might be helpful to demonstrate this issue.

Spearman correlation analysis showed a positive correlation between the GCD measured by these two methods, indicating that the morphology and quantity of goblet cells could be identified not only by the classical IC technique but also by newly developed in vivo LSCM. Moreover, in vivo LSCM had many advantages over IC, as mentioned, and was capable of monitoring the evolution of the ocular surface after chemical burn and after evaluating the effect of therapeutic interventions.

In conclusion, the density of goblet cells decreased in patients with chemical burns. A positive correlation was found between conjunctival GCD measured by in vivo LSCM and IC after chemical burns. A similar positive correlation was found for corneal GCD measured by the two methods. In vivo LSCM is a promising device to study goblet cells in vivo under pathologic conditions.

References