Goblet cell counts have been used to evaluate the suitability of conjunctiva as a source of ocular surface epithelial cells. However, since tear mucin content can be determined without tissue excision, it seemed that the concentration of those compounds might be a useful indicator of conjunctival vitality. To test the extent to which aqueous tear composition reflects conjunctival goblet cell frequency, goblet cell frequency and aqueous tear mucin content were measured after alkali injury in rabbits. Mild alkali injury (0.1 N NaOH for 30 sec) caused a transient but substantial decrease in goblet cells (to 25% of normal at day 7) with a return to normal by six weeks. Tear mucin content was decreased to a lesser degree, from a normal value of 6.4 ± 0.47 nmol oligosaccharide per µl (n = 10) to a minimal value of 4.7 ± 0.64 (n = 7) (73% of normal) at day 7, returning to normal 4 weeks after injury. Thus, the direction of the change was the same, but the magnitudes were quite different. These results suggest that conjunctival goblet cell frequency is not accurately reflected by aqueous tear mucin content, and, therefore, that tear mucin content cannot be used directly as an indicator of conjunctival health. Invest Ophthalmol Vis Sci 24:612-618, 1983

A plethora of reports has documented the effect of alkali burns on various aspects of corneal epithelial and stromal metabolism and morphology in rabbits, but relatively little attention has been paid to the effects of such burns on the conjunctiva. Recent observations suggest that peripheral corneal and/or conjunctival epithelium may play an important role in corneal epithelial maintenance and integrity, implying that changes in the conjunctiva may eventually become manifest on the corneal surface. In injured eyes, for example, where the corneal and limbal epithelia are completely destroyed, there is no question that the conjunctiva resurfaces the cornea. Even in the absence of total epithelial loss, there is evidence that there is an ongoing centripetal motion of cells from the periphery towards the center of the cornea, as evidenced by central movement of epithelial dots in postkeratoplasty eyes, by attenuation of a central corneal epithelium isolated from the peripheral epithelium by a glue ring, and by replacement of donor epithelium by host epithelium in rabbit keratoplasties. Although it is not proven, the implication of those studies is that conjunctival epithelium may be involved in corneal epithelial maintenance even in normal eyes.

It is also important to understand the effects of injury on the conjunctiva if one proposes to use such conjunctiva to resurface damaged corneas, since such conjunctiva serves as the source of healthy epithelial surface cells. Conjunctival transplantation is now used in the treatment of several kinds of ocular surface disease. Currently, its use is limited to autografts in patients with unilateral disease because it is assumed that only undamaged conjunctiva can be used for grafting. Verification of the usefulness of previously injured conjunctiva for corneal resurfacing might increase the number of patients for whom conjunctival grafting is possible.

Previous studies have suggested that goblet cell frequency is a useful indicator for the health of the conjunctiva. The presence of goblet cells may reflect a degree of differentiation or maturation of the conjunctiva which in turn reflects the presence of healthy tissue. However, it is not always possible or desirable to take conjunctival biopsies. Since tear mucin is believed to be secreted primarily by conjunctival goblet cells, tear mucin content might be a useful assay to determine conjunctival differentiation.
The work to be reported here, therefore, measured conjunctival goblet cell frequency and aqueous tear mucin, glucose and protein content after alkali injury in rabbits to (1) evaluate the response of the conjunctiva to chemical injury, and (2) evaluate the extent to which aqueous tear composition reflects conjunctival goblet cell content and, therefore, whether it is a useful indicator of conjunctival differentiation.

Materials and Methods

Animal Preparation and Tissue Sampling

Alkali burns: Rabbits, weighing 2.5–3.5 kg, were used for all experiments. Anesthesia was induced by intramuscular chlorpromazine-ketamine hydrochloride-pentobarbital intraperitoneal injection. The eyelids were opened and a Grade I (Hughes' classification) burn produced by putting 0.1 N sodium hydroxide onto the cornea and into the cul-de-sac. The eyelids were held open and moved slightly to ensure the alkali covered the entire ocular surface epithelium. After 30 sec, the alkali was washed out of the eye with a 1-min wash of 0.9% sodium chloride. In some cases, the nictitating membrane was removed, and the alkali was applied 1 week later after the eye was healed and noninflamed. Burns were made in a total of 66 eyes in 47 rabbits. Topical antibiotic ointment was applied daily until the eye was healed and noninflamed. Burns were classified as follows: Grade I (Hughes classification) in each eye over the 6-week period.

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Tear samples: At 1 day and 1, 4, and 6 weeks after burns or in unburned normal eyes, aqueous tear samples were collected from the lower cul-de-sac of unanesthetized rabbit eyes using a 10-μl capillary pipette. No stimulators of tear flow were used. The 2–10 μl of fluid collected within 1–2 min were stored in the sealed capillary pipettes at -80 C pending analysis.

Conjunctival biopsies for goblet cell analysis: Rabbits were anesthetized as described above, except no topical proparacaine was used. One day and 1, 4, and 6 weeks after burns, or in unburned normal eyes, 2-3 mm diameter, approximately round, samples of conjunctiva near the limbus were excised using fine forceps and corneal scissors. Thus, four samples were taken from the four quadrants of the conjunctiva (upper and lower nasal, and upper and lower temporal) in each eye over the 6-week period.

Samples for goblet cell counts were also taken deep in the fornices, to confirm that the epithelium in that region was also injured by the alkali. Goblet cell counts in those regions were similar to those found around the limbus after injury.

Analyses

Goblet cell frequency: The conjunctival biopsy samples were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Seven-micron sections were stained with hematoxylin-esoin, or with the periodic acid-Schiff (PAS) reaction to demonstrate goblet cells.

Goblet cells were counted in PAS stained sections. The frequency was expressed as the number of goblet cells per 100 epithelial cells. A minimum of 200 epithelial cells was counted for each value.

Tears

Tear mucin and glucose: The amount of mucin-like glycoprotein in tears was determined by quantitation of the alkali-labile oligosaccharides in tears. This was done by specifically labeling N-acetylgalactosamine (the linkage sugar of mucin-like glycoprotein) with tritium, then determining the amount of those sugars present in tear samples by counting the radioactivity and comparing it to appropriate standards.

Briefly the method was as follows. Alkali treatment of mucin-containing samples with trinitated borohydride produced a tritium-labeled linkage amino sugar alcohol attached to oligosaccharide. Subsequent acid hydrolysis separated the labeled amino sugar alcohol from the oligosaccharide. The amino sugar alcohol was then isolated by binding to an AG 50W-X8 cation exchange column followed by elution with 1 M hydrochloric acid, and the amount of sugar quantitated by counting the radioactivity on a scintillation counter.

Free tear glucose was converted to sorbitol and labeled in this reduction procedure. Labeled sorbitol did not bind to the AG 50W-X8 column, but was eluted in water wash. Its amount was also determined by scintillation counting.

Counting for radioactivity was done in a toluene-triton X-100 scintillation cocktail using a Beckman LS 8100 counter. Correction for the efficiency of counting was based on the H number that the Beckman LS 8100 scintillation counter determines automatically for each sample.

The amount of free tear glucose and of N-acetylgalactosamine was determined by comparison to glucose standards. The amount of alkali-labile oligosaccharide, measured as N-acetylgalactosamine, was...
Fig. 1. Conjunctiva after alkali injury. One day after a 30-sec 0.1 N NaOH alkali burn, epithelium was absent from portions of the conjunctiva (A). In other areas, it was present as a single cell layer, with no goblet cells (B). At one day, the subepithelial tissue was edematous (A and B). One week after a 30-sec 0.1 N NaOH alkali burn, the epithelium covered the conjunctival surface, was of normal thickness, but had far fewer goblet cells than normal conjunctival epithelium (C). Six weeks after the alkali burn, the epithelium covering the conjunctiva was normal both in thickness and in goblet cell content, and the subconjunctival edema had subsided (D). (See also Fig. 3) (Epi = epithelium, Gc = goblet cell, bv = blood vessel) (periodic acid-Schiff reaction) (×103).
Distribution of Goblet Cells around the Rabbit Limbus

Fig. 2. Distribution of goblet cells around the rabbit limbus. Goblet cell frequency was the same in the upper and lower nasal and upper and lower temporal quadrants of the conjunctiva surrounding the limbus.

used as an estimate of the mucin content of the tears. That is expressed as nmol oligosaccharide per µl tears. The free tear glucose content of tears is expressed as mg per 100 ml.

Tear protein: Tear protein content was measured in 1-µl tear samples using the Coomassie Brilliant Blue procedure as modified for micro samples.20,21

Results

Appearance of Cornea and Conjunctiva

The clinical and histologic appearance of the conjunctival epithelium after Hughes' Grade 1 alkali injuries has been documented previously.8,18 One day following injury, there was a significant, but usually not total, loss of epithelium, edema, and invasion of inflammatory cells into the conjunctiva. Over the next weeks, the epithelium was re-established, and the inflammation subsided (Fig. 1).

Goblet Cell Frequency

The frequency of goblet cells in conjunctival epithelium near the limbus was the same in the upper and lower nasal, and upper and lower temporal quadrants (Fig. 2).

Alkali burns caused a transient decrease in the number of goblet cells in limbal conjunctiva relative to the number of epithelial cells from 7.7% to less than 3% within 24 hours. Within 4–6 weeks after injury, goblet cell frequency returned to normal (Figs. 1, 3). Similarly at 1 day and 1 week, there were reduced numbers of goblet cells in conjunctiva taken from the fornices.

Tear Analysis

Removal of the nictitating membrane significantly decreased mucin-oligosaccharide and protein content of the tears (Table 1), but had no effect on glucose content.

Alkali burns caused 3- to 12-fold, albeit transient, increases in tear glucose content, but the values were normal 4 weeks after injury. After 6 weeks, the glucose level in eyes with nictitating membranes was below normal, while that in eyes without such membranes was slightly elevated (Table 2).

Tear protein levels were increased one day after injury in those eyes without nictitating membranes, but returned promptly to normal and remained nor-

Conjunctival Goblet Cells After Total 30s Alkali Burn

(±SEM(n))

Fig. 3. Conjunctival goblet cells after 30-sec 0.1 N NaOH alkali burn. Goblet cell frequency in the conjunctiva was significantly below normal for 1 week after alkali injury, but was normal by 4–6 weeks after alkali burn.
Table 1. Rabbit tear glucose, protein, and mucin content*

<table>
<thead>
<tr>
<th>Tear component concentration</th>
<th>Normal</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/100 ml)</td>
<td>28 ± 6 (10)</td>
<td>18 ± 4 (21)</td>
</tr>
<tr>
<td>Protein (μg/μl)</td>
<td>17 ± 1 (10)</td>
<td>11 ± 1 (21)</td>
</tr>
<tr>
<td>Mucin oligosaccharide (nmol/μl)</td>
<td>6.4 ± 0.47 (10)</td>
<td>3.5 ± 0.24 (21)</td>
</tr>
</tbody>
</table>

* Values are given as averages ± the standard errors of the mean with the number of measurements in parentheses.† P values are given if P < 0.05 using the Student's t-test.

Table 3. Tear protein after alkali injury (μg/μl tears*)

<table>
<thead>
<tr>
<th>With nictitating membrane</th>
<th>Without nictitating membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>17 ± 1 (10)</td>
</tr>
<tr>
<td>After sodium hydroxide burn Day 1</td>
<td>19 ± 3 (8)</td>
</tr>
<tr>
<td>Day 7</td>
<td>16 ± 1 (7)</td>
</tr>
<tr>
<td>Day 28</td>
<td>23 ± 3 (4)</td>
</tr>
<tr>
<td>Day 42</td>
<td>16 ± 1 (4)</td>
</tr>
</tbody>
</table>

* Values are given as averages ± the standard errors of the mean with the number of determinations in parentheses.† When compared to the corresponding normal value using the Student's t-test, the value is significantly different at the 5% level.

Table 2. Tear glucose content after alkali injury (mg/100 ml*)

<table>
<thead>
<tr>
<th>Normal</th>
<th>With nictitating membrane</th>
<th>Without nictitating membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28 ± 6.3 (10)</td>
<td>18 ± 4.4 (21)</td>
</tr>
</tbody>
</table>

* Values are expressed as averages ± the standard errors of the mean with the number of determinations in parentheses.† When compared to the corresponding normal value using the Student's t-test, the value is significantly different at the 5% level.

Table 4. Tear mucin concentration after alkali injury (oligosaccharide-nmol/μl*)

<table>
<thead>
<tr>
<th>Normal</th>
<th>With nictitating membrane</th>
<th>Without nictitating membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.4 ± 0.47 (10)</td>
<td>3.5 ± 0.24 (21)</td>
</tr>
</tbody>
</table>

* Values are expressed as averages ± the standard errors of the mean with the number of determinations in parentheses.† When compared to the corresponding normal value using the Student's t-test, the value is significantly different at the 5% level.

Discussion

Tear glucose and protein content recover promptly from the alkali injury. The increases in those substances seen immediately after injury probably reflect only increased leakage through the large epithelial defect, which is present early. Within a few weeks, the glucose levels return to the expected low values. There is a large variability in the glucose values perhaps because this method measures all neutral sugars, not just glucose. The tear protein values are also normal23 within a few weeks of the alkali injury.

In rabbits, unlike humans,17 the goblet cell frequency is uniform around the limbus (Fig. 2), permitting a comparison of goblet cell frequency in the same animal over a period of weeks after alkali burn. By so doing, we have demonstrated a decrease followed by a prompt recovery of histologically identifiable goblet cells after alkali injury (Fig. 3). Conjunctival goblet cell frequency has been shown to be very sensitive to other abnormal conditions. For example, the number of these cells relative to the bulk of epithelial cells is severely decreased when conjunctiva is incubated in organ culture,16 when conjunctiva migrates across the cornea,10,24 and in a variety of pathologic conditions, eg, ocular pemphigoid,25,26 and Sjögren's syndrome.25 Some have also shown a decrease in goblet cells acutely following chemical injury in humans,25 but this is apparently not the case long after injury.27 In all these conditions, it is, of course, difficult to say whether the goblet cells are, in fact, absent, or whether they are simply unrecognized because of a lack of PAS, positive material.

Mal for the duration of the study (Table 3). In eyes that had retained the nictitating membrane, the tear protein remained normal throughout the 6 weeks after injury (Table 3).

Mucin concentration (nmol oligosaccharide per μl tears) in eyes with nictitating membranes was decreased initially by sodium hydroxide burns, but the decrease was only statistically significant for the first week (Table 4). Even at its lowest point, the mucin concentration in the tears of the alkali burned rabbits was approximately 73% of the normal value. Eyes without nictitating membranes showed no decrease in tear mucin content when the values were compared to that for unburned eyes without nictitating membranes.

Discussion

Tear glucose and protein content recover promptly from the alkali injury. The increases in those substances seen immediately after injury probably reflect only increased leakage through the large epithelial defect, which is present early. Within a few weeks, the glucose levels return to the expected low values.22 There is a large variability in the glucose values perhaps because this method measures all neutral sugars,
Recovery of goblet cells as shown in the present experiments has not been demonstrated previously in an experimental system. Clinically, recovery of goblet cells has been seen within 2–4 weeks after restoration of vitamin A levels in deficient patients. However, the mechanism in those cases would seem to be very different from that seen in our experiments. In vitamin A deficiency, the epithelium is abnormal, with keratinization, but there is no significant decrease in the number of cells. In that case, the goblet cell deficiency observed seems to be due to abnormal differentiation of the ocular surface cells, probably secondary to vitamin A deficiency. In the case of the experimental alkali injury reported here, on the other hand, reappearance of the goblet cells occurs only shortly after restoration of the epithelial cell mass implying that goblet cell appearance may be an advanced stage of ocular surface differentiation.

Despite the partial loss of goblet cells seen after the mild chemical injury to the conjunctiva, the tear mucin concentration remains at nearly normal levels throughout the experiment. Although there is correlation between goblet cell frequency and tear mucin content, substantial amounts of mucin are present even when goblet cell frequency is low (Fig. 4). This suggests that these aqueous tear samples do not include the undissolved mucin present on the ocular surface. Examination of human conjunctival mucin using a permeable membrane (Millipore) shows there is a significant amount of mucin on the ocular surface which can be collected on filters and stained with the PAS reaction indicating that this mucin is not dissolved in the aqueous tears. If the bulk of this undissolved mucin is derived from goblet cells, and only a small amount of mucin is dissolved in the aqueous tears, the relative independence of aqueous tear mucin and conjunctival goblet cell frequency is explicable. The relative contributions to ocular surface mucin from the lacrimal gland and mucin production by nongoblet cells remain undefined.

A lack of strict correlation between goblet cell frequency and aqueous tear mucin has also been shown in clinical experiments where disease conditions associated with markedly decreased or absent goblet cells (eg, ocular pemphigoid, keratoconjunctivitis sicca, and Stevens-Johnson syndrome) still had at least 60% of the normal tear mucin. The role of tear mucin in the structure and stability of the tear film has been described and even small mucin decreases may adversely affect tear stability, especially if they affect the mucus layer of the tear film. However, Holly et al were not able to demonstrate abnormalities in aqueous tear component surface activity sufficient to account for tear film instability in patients with ocular pemphigoid or mild keratoconjunctivitis, although they did demonstrate such abnormalities in tears from patients with Stevens-Johnson syndrome. Thus, the role of aqueous tear mucin in tear film stability remains unclear. These studies suggest that, at least in some diseases, there may be primary abnormalities of the ocular surface epithelium, not just problems related to tear film abnormalities. This report documents, for the first time, experimental confirmation of the fact that aqueous tear mucin content does not reflect accurately conjunctival goblet cell frequency, and, therefore, that aqueous tear mucin content is not a useful indicator of conjunctival differentiation or health.

Key words: ocular surface epithelium, conjunctiva, alkali burn, goblet cells, tear mucin

Acknowledgments

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References