Response of Ocular Surface Epithelium to Corneal Wounding in Retinol-Deficient Rabbits

Mohamed El-Ghorab,* Antonio Capone, Jr.,† Barbara A. Underwood,* Diane Hatchell,‡ Judith Friend,† and Richard A. Thoft†

The purpose of this study was to explore the nature and time course of the functional effects of early retinol deficiency on the ocular surface epithelium. To this end, the conjunctival epithelial healing rate, mitotic rate, and goblet cell frequency were determined following experimental ocular surface epithelial wounding in 15 rabbits sustained on a retinol-deficient diet and in 12 pair-fed controls. The animals were sacrificed at 2, 7, and 14 days after wound closure. The mean serum retinol level (±SEM) prior to wounding was 6.0 ± 0.9 μg/dl for the group fed the deficient diet. The mean serum and liver retinol levels for this group following defect closure were 4.0 ± 0.5 μg/dl and 0 μg/g, respectively. These values are all significantly less than the analogous respective control values of 83.2 ± 2.4 μg/dl, 77.6 ± 2.3 μg/dl and 35.1 ± 3.0 μg/g (P < 0.001 for each pair, student t-test). In the retinol-deficient animals, the unwounded eyes had an abnormally high rate of conjunctival epithelial cell mitosis, the earliest ocular surface cellular abnormality detected. Hypermitosis of the unwounded corneal epithelium was also noted, though somewhat later than in the conjunctiva. Epithelial wound healing was delayed considerably in the retinol-deficient group, with only 33% of eyes in this group healed within the same time period as the controls (P < 0.05, Chi-square). Normal numbers of goblet cells were noted in the conjunctiva of retinol-deficient animals, despite at least 5, and up to 8 weeks of profoundly depleted retinol stores. Retinol deficiency appears to result in delayed healing of the conjunctival epithelium despite associated increased conjunctival and corneal epithelial mitosis. The nature of the abnormality in healing remains unclear. Invest Ophthalmol Vis Sci 29:1671–1676, 1988

Retinol deficiency is endemic in a number of developing countries and can result in significant ocular morbidity, including xerosis and keratomalacia.1–3 It is also a frequent and unrecognized problem among patients with chronic cholestatic liver disease,4 cystic fibrosis,5 and malabsorption syndromes.6–7 We recently noted that the reduction in goblet cell frequency which occurs in retinol deficiency8–10 is associated with a striking increase in the frequency of conjunctival epithelial cell mitosis,11 detectable prior to clinically apparent abnormalities of the conjunctiva such as Bitot’s spots or xerosis. Such changes in conjunctival mitosis and differentiation could be expected to be associated with abnormalities in corneal epithelial maintenance and repair. For example, the association between measles infection, ocular herpes virus infection, and severe xerophthalmia with keratomalacia in retinol-deficient children has been noted previously.12–14 Xerophthalmic rabbits have been shown to be more susceptible to corneal infection and keratomalacia following topical inoculation with Pseudomonas aeruginosa than pair-fed control animals at a similar level of protein calorie malnutrition.15 While the mechanism whereby retinol deficiency alters the pathogenicity of infectious agents is not completely understood, it seems reasonable to postulate that compromise of the integrity of the epithelial barrier may play a pivotal role in the apparent vulnerability of the ocular surface to infection.

The temporal relationship of change in mitosis and differentiation of the ocular surface epithelium to retinol deficiency is also unknown. Ocular cicatricial pemphigoid (OCP) is an ocular surface disorder also

From the *National Eye Institute, Bethesda, Maryland, the †Department of Ophthalmology, the Eye and Ear Institute and University of Pittsburgh, Pittsburgh, Pennsylvania, and the ‡Department of Ophthalmology, Duke University, Durham, North Carolina.

Supported in part by the United Nations University Fellowship Program, Research Grants R01 EY-06186 (DHH) and R01 EY-05279 (JF and RAT) from the National Eye Institute, National Institutes of Health, and an Unrestricted Grant to the Department of Ophthalmology, University of Pittsburgh from Research to Prevent Blindness, Inc.

Dr. El-Ghorab was a United Nations University Fellow at the National Eye Institute during the course of this study, and is currently located at the National Institute of Nutrition, Cairo, Egypt.

Dr. Hatchell was a Research to Prevent Blindness, Inc., William and Mary Greve International Research Scholar during the course of this study.

Submitted for publication: February 5, 1988; accepted April 18, 1988.

Reprint requests: Richard A. Thoft, MD, Eye and Ear Institute, 203 Lothrop Street, Pittsburgh, PA 15213.
characterized by conjunctival epithelial hypermitosis, a low goblet cell frequency, and, in its advanced stages, keratinization of ocular surface epithelial cells. Further understanding of the chain of events responsible for the surface abnormalities in retinol deficiency may serve to enhance our understanding of the pathophysiology of other disorders characterized by epithelial hypermitosis, such as OCP.

To explore the nature and time course of the functional effects of early retinol deficiency on the ocular surface epithelium, the ocular surface epithelial healing rate, mitotic rate and goblet cell frequency were determined following experimental corneal and conjunctival epithelial wounding in rabbits sustained on a retinol-deficient diet, and in control animals paired fed a retinol-supplemented diet.

Materials and Methods

Animal Preparation

All investigations described in this report conform to the ARVO Resolution on the Use of Animals in Research.

Following gestation, lactating New Zealand albino rabbits were randomly divided into two groups. One group was maintained on a retinol-deficient diet, fed ad libitum as previously described. The control dams were given the same diet base supplemented with retinyl palmitate. Each dam was housed with an equal number of pups. After weaning at 4 to 8 weeks, the control weanlings were continued on the retinol-supplemented diet, while those from the other group were maintained on the same retinol-deficient diet their mothers had received during the lactating period. Food intake and body weight were monitored every 3 days, with intake of the control group restricted to that of the vitamin-deficient group (pair feeding). Unfortunately, at week 19 the retinol-deficient group inadvertently received a retinol-supplemented diet. This was corrected by week 21.

Animals were examined weekly for evidence of clinical ocular findings. No evidence of inflammation or discharge was noted at any point during the course of the study. Serum retinol levels were determined monthly, just prior to wounding and at the time of sacrifice using the method described by Bieri et al. Liver retinol stores were determined at the time of sacrifice by the method described by Olson. Mean retinol levels of the two groups were compared using student t-test.

Epithelial Wounding

At week 29, epithelial defects were created in one randomly selected eye of each of the 15 animals from the group fed the deficient diet, and similarly in one eye of each of the 12 controls. Following appropriate intramuscular anaesthesia consisting of 1 cc (25 mg) of chlorpromazine hydrochloride followed by 2 cc (200 mg) of ketamine hydrochloride, topical proparacaine was applied to each eye and proptosis was induced. The whole corneal epithelium plus 2 mm of limbal and bulbar conjunctiva were removed by application of n-heptanol-soaked filter paper discs, as described previously. The area was gently rubbed with a cotton-tipped applicator and rinsed with sterile saline. The resultant defect was stained with diluted Richardson’s stain to confirm complete epithelial removal. A 1:10 dilution was used in this study, as previous work has shown that repeated applications of undiluted Richardson’s stain inhibits corneal epithelial wound healing in rabbits. Erythromycin ophthalmic ointment was then applied. Animals were divided for serial sacrificing at 2, 7 and 14 days following defect closure.

Determination of Healing Rates

All eyes were examined and photographed immediately after wounding and daily thereafter until defect closure. The photographs were digitized and healing rates (HR) were determined using the IS2000 digital image processing system (PAR Microsystems, New Hartford, NY).

Histology and Autoradiography

Corneal-scleral-conjunctival preparations were incubated in 2 ml of tissue culture medium (Dulbecco’s Modified Eagles Medium) with 10 μCi/ml of tritiated thymidine (New England Nuclear, Boston, MA, 20 μCi/mmol) at 37°C in an air:CO₂ (95%:5%) water-jacketed incubator for 2.5 hr, followed by 0.5 hr incubation in isotope-free medium. Samples were then fixed in 10% buffered formalin and 7 μm histological sections were prepared. Sections were stained with the periodic acid Schiff (PAS) reaction or hematoxylin and eosin (H&E). Other sections used for autoradiography were dipped in periodic acid, rinsed, dipped in Kodak NTB2 emulsion and then stored for 14 days at -20°C. They were developed with Kodak D-19 developer, fixed with Kodak fixer and stained with Schiff’s reagent and hematoxylin.

Morphological Analysis

Autoradiographic sections from each eye were counted in a masked fashion by the same technician. The mitotic rate (MR) was determined by counting the number of epithelial cells across the cornea or in the conjunctiva which incorporated tritiated thymi-
Table 1. Retinol levels ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Serum (ng/dl)</th>
<th>Liver (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 weeks</td>
<td>29 weeks*</td>
</tr>
<tr>
<td>Control (n=12)</td>
<td>77.2 ± 1.7</td>
<td>83.2 ± 2.4</td>
</tr>
<tr>
<td>Retinol-deficient (n=15)</td>
<td>14.2 ± 1.4</td>
<td>6.0 ± 0.9</td>
</tr>
</tbody>
</table>

The results of serum and liver retinol level determinations for control and retinol-deficient animals. Values are given as averages bracketed by the standard error of the mean. Control values are all statistically significantly different from those of the corresponding retinol-deficient group to \( P < 0.001 \), by student t-test.

* During wound healing.
† 30 to 32 weeks (ie, 2, 7 and 14 days following defect closure).
‡ Not detectable.

dine (S-phase cells) in 2.5 hr. The results are expressed as labeled cells per 100 basal corneal or conjunctival epithelial cells per 2.5 hr.

Goblet cells, which stained with PAS, were counted in the conjunctiva. Goblet cell frequency (GCF) is expressed as goblet cells per 100 basal conjunctival epithelial cells. Final results are expressed as percent averages bracketed by the standard errors of the mean, with the stated n for all MR and GCF data. The \( P \) values were calculated using one-way analysis of variance with a posteriori multiple comparisons by Gabriel’s Sums of Simultaneous Squares Test.

**Results**

**Vitamin A Status**

By 12 weeks postpartum, serum retinol levels in the group fed the retinol-deficient diet uniformly measured less than 20 µg/dl. Those from the control group ranged from 70 to 80 µg/dl. Serum levels in the experimental group remained below 20 µg/dl for 8 weeks prior to the inadvertent feeding of the retinol-supplemented diet during weeks 19 and 20. Levels attained during those 2 weeks are unknown. By the time epithelial defects were created at week 29, however, serum levels in the group maintained on the retinol-deficient diet had averaged well under 20 µg/dl for at least 4 weeks, and were significantly different from those of the control animals from week 25 until sacrifice (Table 1) \( P < 0.001 \). Liver retinol determinations performed at sacrifice were within normal limits for controls (normal value = greater than 20 µg/g). In contrast, liver retinol was not detectable in the retinol-deficient diet group \( P < 0.001 \).

**Number of Defects Healed and Healing Rates**

All defects in the controls healed within 8 days (Fig. 1). Epithelial wound healing was delayed considerably in the retinol-deficient group: only 33% (5/15) of these eyes healed within the same time period \( P < 0.01 \) for day 6 to day 9, Chi-square). All 15 eyes in the retinol-deficient group healed within 11 days post-wounding. Differences in the degree of defect closure in the retinol-deficient animals as compared to the pair-fed controls (n = 12) were statistically significant from days 2–7 \( P < 0.05 \), student t-test.

**Mitotic Rates and Goblet Cell Frequency**

The corneal and conjunctival epithelial MRs for the unwounded and wounded eyes from each group

![Fig. 1. Percentage of healed defects as a function of time. The percentage of eyes with healed corneal epithelial defects is plotted against the number of days post-wounding. The striped bars represent the retinol-deficient animals (n = 15), the open bars the pair-fed controls (n = 12). Differences between the two groups were statistically significant to \( P < 0.01 \) for days 6–9 (Chi-square).](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933368/)

![Fig. 2. Ocular surface wound healing as a function of time. The remaining ocular surface epithelial defect area relative to that of the initial defect is plotted against the number of days post-wounding. The open circles represent the retinol-deficient animals (n = 15), the closed circles the pair-fed controls (n = 12). Data are expressed as the mean percentage of initial wound area bracketed by the standard error of the mean. Differences between the two groups were statistically significant to \( P < 0.05 \) for days 2–7 (student t-test).](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933368/)
Group. It was therefore possible to average these corneas or conjunctivas of the unwounded control eyes from the same group.

There were no significant differences in average MRs at different days in controls was four times that in the unwounded control eyes from the retinol-deficient group had increased to a level that was statistically significantly different relative to the unwounded controls (P < 0.01).

The conjunctival epithelial MRs of the wounded control eyes were not significantly different at any stage from those of their unwounded fellows. However, mitotic activity in the conjunctiva of the unwounded retinol-deficient animals was significantly higher than in the unwounded controls at all times (P < 0.05), but there were no statistically significant differences between wounded and unwounded eyes in the retinol-deficient animals.

Goblet cells were present in normal numbers in the conjunctiva of animals fed the retinol-deficient diet (Table 4). Although the mean conjunctival GCF following wound closure appeared higher than that in the respective unwounded fellow eyes in both the normal and retinol-deficient groups, these values were not statistically significantly different.

### Discussion

The HR, MR and GCF data for the wounded and unwounded animals maintained on the retinol-supplemented diet are comparable to previously reported values in normal rabbits.²⁵⁻²⁷

The mean serum retinol levels in rabbits maintained on the retinol-deficient diet were well below 20 μg/dl for at least 4 weeks prior to wounding. The cut-off point of 20 μg/dl is cited since the clinical findings of retinol deficiency in humans are rarely seen when serum values are above this level.²⁸ Liver retinol determinations were performed at sacrifice in recognition of the fact that serum levels may not accurately reflect physiologic stores. Liver retinol was not detectable at any stage in the group maintained on the retinol-deficient diet, confirming that these animals were indeed retinol-deficient.

In a recent study, we noted that the conjunctival GCF was higher than normal for at least 28 days following closure of ocular surface wounds which included removal of total corneal, limbal, and 2-3 mm of bulbar conjunctival epithelium.²⁶ Though this trend was also apparent in the current series, the

### Table 2. Corneal epithelial mitotic rate ± SEM

<table>
<thead>
<tr>
<th>Days after closure</th>
<th>Control Unwounded</th>
<th>Wounded</th>
<th>Retinol-deficient Unwounded</th>
<th>Wounded</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.4 ± 0.3 (4)</td>
<td>9.2 ± 2.0 (4)</td>
<td>2.2 ± 0.3 (4)</td>
<td>7.7 ± 2.5 (4)</td>
</tr>
<tr>
<td>7</td>
<td>2.3 ± 1.7 (2)</td>
<td>5.6 ± 1.1 (3)</td>
<td>3.2 ± 0.5 (4)</td>
<td>4.8 ± 1.1 (4)</td>
</tr>
<tr>
<td>14</td>
<td>2.1 ± 0.2 (3)</td>
<td>3.3 ± 2.0 (3)</td>
<td>7.0 ± 0.7 (7)</td>
<td>6.9 ± 0.7 (7)</td>
</tr>
<tr>
<td></td>
<td>2.3 ± 0.7 (9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Corneal epithelial mitotic rates expressed as mitoses per 100 basal epithelial cells per 2.5 hr in both wounded and unwounded control and retinol-deficient animals. Values are given as averages bracketed by the standard error of the mean. The number of eyes within each group is stated within parentheses.

### Table 3. Conjunctival epithelial mitotic rate ± SEM

<table>
<thead>
<tr>
<th>Days after closure</th>
<th>Control Unwounded</th>
<th>Wounded</th>
<th>Retinol-deficient Unwounded</th>
<th>Wounded</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.4 ± 0.2 (4)</td>
<td>0.8 ± 0.2 (3)</td>
<td>3.1 ± 0.5 (3)</td>
<td>2.0 ± 1.3 (3)</td>
</tr>
<tr>
<td>7</td>
<td>0.2 (2)</td>
<td>0.6 ± 0.3 (3)</td>
<td>3.3 ± 1.5 (3)</td>
<td>2.6 ± 0.7 (4)</td>
</tr>
<tr>
<td>14</td>
<td>0.5 ± 0.4 (2)</td>
<td>0.3 ± 0.3 (3)</td>
<td>2.7 ± 1.5 (6)</td>
<td>4.4 ± 1.5 (5)</td>
</tr>
<tr>
<td></td>
<td>0.3 ± 0.1 (8)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conjunctival epithelial mitotic rates expressed as mitoses per 100 basal epithelial cells per 2.5 hr in both wounded and unwounded control and retinol-deficient animals. Values are given as averages bracketed by the standard error of the mean. The number of eyes within each group is stated within parentheses.

### Table 4. Goblet cell frequencies ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Control Unwounded</th>
<th>Wounded</th>
<th>Retinol-deficient Unwounded</th>
<th>Wounded</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>6.5 ± 3.3 (7)</td>
<td>12.5 ± 4.3 (9)</td>
<td>8.8 ± 2.8 (13)</td>
<td>14.9 ± 3.4 (12)</td>
</tr>
</tbody>
</table>

 Conjunctival goblet cell frequency expressed as goblet cells per 100 basal conjunctival epithelial cells in both wounded and unwounded control and retinol-deficient animals. Values are given as averages bracketed by the standard error of the mean. The number of eyes within each group is stated within parentheses.
GCF's were not sufficiently different to permit the claim of statistical significance. As discussed elsewhere, this underscores the primary problem with interpretation of data gleaned from conjunctival biopsies; there is a great deal of regional variation in conjunctival GCF. Impression cytology, a newer technique for taking conjunctival surface specimens, has been used successfully in both rabbits and humans. The main advantage of the method is that larger fields of cells can be evaluated with relative ease. This noninvasive technique may prove to be the preferred method for ocular surface GCF evaluation, although it has been difficult to standardize and reproduce in our hands.

The mean resting conjunctival epithelial MR in the retinol-deficient animals was uniformly higher than in the controls at all times measured. Though not entirely analogous to the high conjunctival MR seen in Indian children with retinol-deficiency (15 ± 1.2%), this increase in resting conjunctival epithelial mitotic activity seems to corroborate the clinical observation. The mean corneal epithelial MR was also elevated in the unwounded retinol-deficient eyes at 14 days, suggesting that this hypermitosis may be a general feature.

We previously demonstrated that the conjunctival epithelium participates in the healing of acute corneal epithelial wounds, as reflected by a 10-fold increase in peripheral conjunctival epithelial MR 1 day after removal of the corneal, limbal, and 2 mm of bulbar conjunctival epithelium. By 3 days after wounding, the conjunctival epithelial MR returns to normal. In the present study, we observed no increase in conjunctival epithelial mitosis in control or retinol-deficient animals from 7 to 22 days following the creation of similar defects. In view of our earlier work, any alteration in conjunctival epithelial mitotic activity in the present study is likely to have occurred prior to the earliest date of sacrifice, ie, 7 days post-wounding.

One goal of this study was to assess the effects of early retinol deficiency on the healing capacity of the ocular surface epithelium. To our knowledge, this is the first time epithelial healing rates of corneal defects have been measured in vivo at a time when the conjunctiva is known to have been in a hypermitotic state. Paradoxically, healing was slowed. We recently noted that profound suppression of mitosis by topical 5-fluorouracil (5-FU) had no effect on average rate of healing of 8 mm central corneal epithelial defects in normal rabbits. This is consistent with in vitro demonstrations that neither stimulation of mitosis by EGF nor suppression of EGF-stimulated mitosis by 5-FU had any effect on rate of closure of 6 mm defects, which heal primarily by cell sliding.

Taken together, these findings suggest that, while it may serve to reestablish normal corneal morphology and function after healing, cellular proliferation is not a sine qua non for successful defect closure—even when the defect is quite large. This is not surprising if one considers that the conjunctiva occupies over 90% of the ocular surface, providing a more than adequate reserve for closure of sizeable defects by migration alone if necessary.

Topical retinoic acid has been shown to stimulate the healing rate of corneal epithelial wounds in normal rabbits. Evidence that [3H]-retinol injected into the anterior chamber of rabbits following corneal wounding is taken up by the corneal epithelium and concentrated in migrating cells along the wound edge would lead one to expect compromise of the reparative capacity of the ocular surface epithelium in retinol-deficient animals, as noted in this study. The mechanism by which retinoic acid influences epithelial migration is not completely understood. Retinoic acid stimulates glycoprotein synthesis in the corneal epithelium as well as attachment of epidermal cells in vitro. Continued glycoprotein synthesis is known to be necessary for sustained corneal epithelial migration following wounding. Retinol-dependent modulation of cell membrane glycoprotein synthesis may be the mechanism whereby topically applied retinoids and retinol deficiency exert their respective effects on the process of ocular surface epithelial defect closure.

It has long been known that retinol is essential for normal growth and differentiation of epithelial cells. There has been considerable speculation in recent years as to the relationship of change in mitosis and differentiation of the ocular surface epithelium to retinol deficiency. Sommer noted that punctate corneal epithelial keratopathy, the earliest corneal change seen in clinical retinol deficiency, precedes clinical conjunctival abnormalities such as Bitot's spots or xerosis. We recently reported that the reduction in conjunctival goblet cell frequency which occurs in retinol deficiency is associated with epithelial hypermitosis. This finding is consistent with the work of Kinoshita and colleagues, who found that a high rate of ocular surface mitosis is associated with a loss of goblet cells from epithelium in the process of transdifferentiation of conjunctiva to cornea. In the current study, goblet cells in normal numbers were noted in the conjunctiva of animals fed a retinol-deficient diet, despite at least 5 and up to 8 weeks of profoundly depleted hepatic retinol stores and an elevated conjunctival MR. These findings demonstrate that delayed conjunctival epithelial healing, associated with conjunctival and corneal epithelial hypermitosis, occur prior to the reduction in conjunctival GCF in retinol deficiency.
Key words: ocular surface epithelium, retinol deficiency, healing rate, mitotic rate, goblet cell frequency

Acknowledgments

The authors would like to acknowledge the technical contribution of Mr. Jean-Paul Vergnes in the determination of ocular surface mitotic activity and goblet cell frequency.

References


