Inhibition of Conjunctival Transdifferentiation by Topical Retinoids

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During the healing of a total corneal epithelial defect extending beyond the limbus, conjunctival transdifferentiation can be inhibited by corneal vascularization as evidenced by the lack of morphological transformation of the conjunctival epithelium into a cornea-like epithelium and the persistence of goblet cells on the corneal surface. We speculated that corneal vascularization might play a causative role in inhibiting conjunctival transdifferentiation, and examined the hypothesis that vitamin A or retinoids might be one of the blood-borne factors in modulating this process. To test this hypothesis, we created total corneal epithelial defects extending 3 mm beyond the limbus in rabbits using n-heptanol, and segregated the resultant corneas into nonvascularized and vascularized groups. After re-epithelization, both groups received topical 0.1% Etretinate (Roche-Hoffmann, Nutley, NJ) or 13-cis retinonic acid in corn oil three times a day for 8 weeks. Controls received corn oil only. The extent of transdifferentiation was analyzed by assaying goblet cell density and distribution using flat-mount preparations and Alcian blue and periodic acid-Schiff stains (Fisher Scientific Co., Fair Lawn, NJ) and by conventional histology. Topical retinoid application inhibited conjunctival transdifferentiation in nonvascularized corneas to the same extent as that caused by corneal vascularization, suggesting that vitamin A is an important blood-borne factor for goblet cell maintenance. Its relative deficiency in the normal avascular cornea may explain why conjunctival transdifferentiation occurs. Invest Ophthalmol Vis Sci 28:538-542, 1987

When a total corneal epithelial defect extends beyond the limbus, the epithelial source for wound healing is derived from the surrounding conjunctiva. During this healing process, the migrating conjunctival epithelium undergoes serial stages of morphological transformation into a cornea-like epithelium with the loss of goblet cells.1-3 This process, termed conjunctival transdifferentiation, occurs when the involved cornea has minimal vascularization.1-3

The modulating mechanism remains unexplained. When corneal vascularization is present, conjunctival transdifferentiation is either inhibited1,3-5 or reversed,6 as evidenced by the persistence of goblet cells. These studies indicate that corneal vascularization may be a causative factor in inhibiting conjunctival transdifferentiation. Therefore, the responsible blood-borne factor should meet the following criteria: (1) it should be present in the systemic vascular circulation in a high concentration but in a comparatively low or negligible concentration in extravascular spaces away from vessels, and (2) it should be capable of maintaining goblet cell differentiation. Because of the relative deficiency of this factor in the normal avascular corneal stroma after removing the corneal epithelium, goblet cells disappear and conjunctival transdifferentiation occurs. The conjunctival epithelium morphologically changes into a cornea-like tissue.

A possible candidate for inhibiting conjunctival transdifferentiation by maintaining the presence of goblet cells is vitamin A or retinoids. Retinoids are lipid-soluble vitamins present primarily in serum and epithelial tissues such as the liver and retina, which have higher concentrations of retinol-binding protein (RBP). Under normal conditions, the conjunctival and scleral blood vessels in the limbus region are the major source of vitamin A for the cornea when the distribution of RBP is analyzed,7 or when the blood-borne horseradish peroxidase tracer is studied.8 Retinoids are essential for epithelial growth and differentiation.9,10 Numerous studies, both in vivo and in vitro, have demonstrated that deficiency of this vitamin can convert secretory epithelium to squamous (squamous metaplasia), and a vitamin excess can convert a stratified squamous epithelium to a secretory epithelium (mucous metaplasia).9,10 In this study, we tested the
hypothesis that topical retinoids can inhibit conjunctival transdifferentiation in nonvascularized corneas by maintaining goblet cell differentiation.

Materials and Methods

Rabbit Model of Conjunctival Transdifferentiation

The model of conjunctival transdifferentiation was created in New Zealand albino rabbits in a manner similar to a previous method using n-heptanol originally described by Cintron et al. Corneas that healed within 6 days were excluded because the resultant epithelium amly have originated from corneal remnants. The remaining corneas were screened on the 10th day after wounding for epithelial defects and corneal vascularization. Those healed without epithelial defects could then be classified into two major groups: the nonvascularized (non-V) group, which included corneas with vessels extending less than 2 mm into the cornea, and the vascularized (Vas) group, with vessels extending more than 2 mm beyond the limbus into the cornea and involving more than three quadrants of the cornea. The occurrence of these two corneal types was haphazard, unpredictable, and possible related to the severity of the injury. Corneas that had not healed completely by day 10 were rescreened on day 12. A total of 50 rabbits were used in this experiment, including 70 nonvascularized corneas and 22 vascularized corneas; 8 corneas were excluded because of vascularization occurring in only part of the cornea. Our study conformed to the ARVO Resolution on the Use of Animals in Research.

Topical Application of Retinoids

Upon re-epithelialization, both non-V and Vas corneas received topical applications three times a day for 8 weeks of 13-cis retinoic acid (RO 4-3780, Isotretinoin) or an aromatic retinoid (ethyl-all-trans-9-[4-methoxy-2,3,6-trimethylphenyl]-3,7-dimethyl-2,4,6,8-nonatetraenoate, RO 10-9359, Etretinate) in a final concentration of 0.1% (w/v) in corn oil. Controls received corn oil only. Three or four corneas were obtained from each experimental group on days 7, 17, 27, 43, and 57 after re-epithelialization by killing the rabbits with an overdose of intravenous phenobarbital.

Tissue Preparation For Morphological Study and Goblet-Cell Analysis

After killing the rabbits, we removed the corneas with a 1–2 mm scleral rim, using a razor blade and scissors. The corneal button was bisected through the vertical meridian into nasal and temporal halves. One half was assigned to routine histological study and the other to goblet cell analysis.

Histological study was performed using 6 μm tissue sections stained with periodic acid-Schiff (PAS) reagent for goblet cells. Particular attention was directed to three zones of the cornea: central (C), mid-peripheral (M), and peripheral (P).

Goblet-cell analysis was performed as previously described. In brief, flat-mount preparations were made on each corneal half and then subjected to fixation and sequential staining with 0.5% Alcian blue (pH 2.5) and PAS reagent.

Morphological changes of goblet cells were studied by light microscopy under different magnifications. Attention was directed to the three zones (C, M, P). The topographical density of goblet cells could be counted at X400 magnification. The counting method was the same as described previously. The countings were performed by two persons in six equal zones designated as 1 to 6 from the central cornea to the periphery (Fig. 1). Each zone was 1.0–1.25 mm wide. The limbus was located approximately in the middle of zone 6. In each specimen, three countings were made radially through six zones of the three smaller sectors. In each zone, the mean of three countings was calculated and denoted as the measurement of that particular eye, of which the standard deviation was 3–8%. On any given sacrifice day, three or four eyes were counted similarly. The mean with its standard deviation of the sum of such measurements of the six zones from three or four eyes was reported as the relative goblet cell density. The relative goblet cell density was derived and used as an estimate for comparing the treated and control groups. The mean value of the measurements of each zone was used to prepare a schematic composite of the topographical distribution of goblet cell density.

Results

The goblet cell density of each zone (1–1.25 mm) was measured from the central cornea to the periphery (Fig. 1). The goblet cell densities of non-V controls peaked on day 7, gradually decreased centrifugally to the periphery, and became negligible after day 43 (Fig. 1A). Accompanying these density changes was the morphological transformation of the conjunctival epithelium into a cornea-like epithelium (data not shown). In contrast, the Vas controls maintained high goblet cell densities after day 7 and throughout the study (Fig. 1B) and adopted a conjunctiva-like epithelium containing goblet cells (data not shown). The goblet cell densities of non-V corneas receiving either 0.1% Etretinate or 13-cis retinoic acid were higher in each zone than their controls but were similar to Vas controls. Both Vas cornea groups receiving topical reti-
inoids showed a distribution of goblet cell densities similar to that of Vas controls but had more goblet cells (result not shown).

The relative goblet cell densities at each time point are shown in Figs. 2 and 3. Both non-V groups receiving either 13-cis retinoic acid or Etretinate maintained a high plateau density throughout the study (Fig. 2). Higher densities were achieved when the Vas group received topical retinoid applications (Fig. 3).

Histological sections of day 27 specimens confirmed the hypothesis that the conjunctiva-like epithelium containing goblet cells was maintained on non-V corneas when topical Etretinate or 13-cis retinoic acid was applied, similar to the finding in Vas controls (data not shown). On day 57, using flat-mount preparations and Alcian blue PAS staining, we observed that the goblet cell density on the non-V corneas receiving topical retinoids was similar or higher than that of Vas controls in C, M, P zones of the specimen (Fig. 4). Vas corneas receiving topical retinoids exhibited high goblet cell density during the entire study (results not shown).

**Discussion**

The experimental model of conjunctival transdifferentiation is ideal for exploring the mechanism that
modulates the cellular differentiation of conjunctival epithelium. Several studies have indicated that conjunctival transdifferentiation can be either inhibited\textsuperscript{1,3-5} or reversed\textsuperscript{6} by corneal neovascularization. These results strongly suggest that the persistence of goblet cells on the corneal surface is positively correlated with vascularization. This phenomenon is supported by our study in which the results of both non-V and Vas controls are consistent with those of our previous investigation.\textsuperscript{3} We have now further demonstrated that the conjunctiva-like epithelium containing goblet cells can be maintained on the non-V corneas by topically applying either of two retinoids, 13-cis retinoic acid or Etretinate. The resultant goblet cell densities of these corneas were comparable to or greater than those of Vas controls. These results support the hypothesis that vitamin A or retinoids may be one of the factors from blood circulation that can inhibit conjunctival transdifferentiation by maintaining goblet cell differentiation. Because of the relative deficiency of vitamin A in the normal avascular cornea,\textsuperscript{7,8} conjunctival transdifferentiation occurs.

We used the retinoids 13-cis retinoic acid and Etretinate rather than retinol because the latter requires the binding and uptake of RBP by target cells, but other retinoids may be able to enter cells without this step.\textsuperscript{12} Using retinoids circumvented the difficulty of preparing retinol-RBP complex for this experiment. We may have observed the action of topical retinoids in inhibiting conjunctival transdifferentiation, although this effect may not necessarily reflect the physiological action of circulating retinol or other natural retinoids.

Reservations exist concerning our hypothesis because topical retinoids maintained high goblet cell densities even in vascularized corneas, and we tested retinoids rather than retinol. Nonetheless, our study demonstrated that topical retinoids can maintain goblet cell differentiation even on avascular corneas.

Normal conjunctival epithelium contains goblet cells, which are primarily responsible for producing...
mucin, the innermost layer of the preocular tearfilm. Goblet cells and mucin production are crucial for ocular surface integrity. Clinically, loss of goblet cells and mucin deficiency are the early, if not the first, sign of squamous metaplasia, which is the common histopathologic feature of numerous ocular surface disorders such as xerophthalmia; various forms of cicatricial keratoconjunctivitis including ocular pemphigoid, Stevens-Johnson syndrome, and chemical burns; several forms of chronic keratoconjunctivitis; and sicca syndrome.\textsuperscript{13,14} Except for xerophthalmia, which is known to be caused by systemic vitamin A deficiency, the pathogenesis of squamous metaplasia underlying all other disorders remains obscure.

By studying human conjunctival flaps and histopathological characteristics of these surface disorders, we previously identified intense inflammation and the loss of vascularization in the subepithelial stroma secondary to scar formation as two common pathologic processes for squamous metaplasia.\textsuperscript{14} The intense inflammation may account for the development of squamous metaplasia in the acute inflammatory stage in various ocular surface disorders. Loss of vascularization simulates the process of experimental transdifferentiation described earlier. Our present study demonstrates that vitamin A or retinoids may be the factor in the blood circulation that exerts an important function in maintaining goblet cell differentiation on the surface epithelium. The absence of this factor resulting from loss of vascularization may account for the loss of goblet cells and subsequent keratinization in these ocular surface disorders, particularly those characterized by the cicatricial change in the chronic stage.

Furthermore, all conventional nonsurgical therapies for squamous metaplasia are palliative and incapable of reversing the process of this disorder, although they may alleviate the symptoms. Our findings that topical retinoid supplements can maintain goblet cell density in avascular corneas may lead to an effective treatment for squamous metaplasia disorders. Recently, we reported the efficacious use of topical tretinoin or all-trans retinoic acid in treating some of these ocular surface disorders.\textsuperscript{15,16} Further exploration of the modulating mechanism of conjunctival transdifferentiation may enhance our understanding of the pathogenesis of these disorders and help us treat them more effectively.

Key words: conjunctival transdifferentiation, Etretinate, goblet cells, Isotretinoin, retinoids

References