Conjunctival Transdifferentiation Induced by Systemic Vitamin A Deficiency in Vascularized Rabbit Corneas

Scheffer C. G. Tseng,* Mahmood Farazdaghi,† and Agatha A. Rider‡

Conjunctival transdifferentiation, the process in which conjunctival epithelium transforms into a cornea-like epithelium with the loss of goblet cells during the healing of a total corneal epithelial defect, can be retarded or reversed by corneal neovascularization. We have previously shown that this process normally occurring on non-vascularized corneas can be retarded or reversed by topical retinoids, suggesting that vitamin A may be one of the factors from blood circulation which is responsible for modulating transdifferentiation. Herein, we have examined the effect of systemic vitamin A deficiency on vascularized corneas starting 4 months after epithelial denudation, and compared this deficient group with their vascularized and non-vascularized controls. Mean serum retinol level (μg/dl) (n = 4) measured by HPLC was gradually reduced from 83 of the controls to 20 in a 10 month follow-up. Topographical analysis disclosed a centrifugal loss of goblet cell density with time. Histology showed complete transdifferentiation in vascularized areas at 9 months, initiated by the loss of mucin contents from receding zones first noted at 2 months. Using impression cytology, all corneas were not keratinized and all conjunctivas maintained a normal goblet cell density at 10 months. These results indicate that conjunctival epithelium on corneal surface is more sensitive to the decrease of serum vitamin A levels than that on conjunctiva, and support the hypothesis that the relative vitamin A deficiency on vascularized corneas can also result in the conjunctival transdifferentiation. Invest Ophthalmol Vis Sci 28:1497-1504, 1987

When a total corneal epithelial defect is created extending 2 to 3 mm beyond limbus, the epithelial source for wound healing is derived from the surrounding conjunctiva. During the healing process, the fate of the migrating conjunctival epithelium on the denuded corneal surface will be determined by the presence or absence of corneal neovascularization. In its absence, the conjunctiva-like epithelium will undergo serial stages of morphological transformation into a cornea-like epithelium with the loss of goblet cells,1-3 a process now termed conjunctival transdifferentiation. However, if corneal vascularization is present during the healing period1,3,4 or introduced afterwards,5,6 conjunctival transdifferentiation is either inhibited1,3-5 or reversed,6 as evidenced by the persistence of goblet cells. These investigations indicate that corneal vascularization may be a causative factor in modulating conjunctival transdifferentiation.

We speculated that the persistence of goblet cells on the corneal surface in this model may be supported by some modulating factor(s) from the blood circulation.7 We further hypothesized that vitamin A or retinoids may be among the factors that can inhibit conjunctival transdifferentiation by maintaining goblet cell differentiation, based on the following information about vitamin A. First, it has been shown that under normal conditions, the conjunctival and scleral blood vessels at the limbal region are the major source of vitamin A for the cornea when the distribution of retinol-binding protein is analyzed,8 or when the blood-borne horseradish peroxidase tracer is studied.9 Second, retinoids are essential for epithelial growth and differentiation.10,11 Numerous studies, both in vivo and in vitro, have demonstrated that deficiency of this vitamin can convert a secretory epithelium to a squamous epithelium (squamous metaplasia), and a vitamin excess can convert a stratified squamous epithelium to a secretory epithelium (mucous metaplasia).10,11 Because of the relative deficiency of vitamin A in the normal avascular cornea compared to that of vascularized conjunctiva, conjunctival transdifferentiation occurs.

To test the above hypothesis, we have previously...
demonstrated that topical retinoids, ie etretinate and 13-cis retinoic acid, can inhibit conjunctival transdifferentiation in non-vascularized corneas by maintaining goblet cell differentiation.12 Recently, we also demonstrated that topical application of all-trans retinoic acid or increased all-trans retinol levels in tears and aqueous by a model of immunogenic uveitis can reverse the transdifferentiated cornea-like epithelium in the non-vascularized corneas to a conjunctiva-like epithelium with the reappearance of goblet cells.13 In the present study, we further tested this hypothesis in vascularized corneas by inducing conjunctival transdifferentiation, which would otherwise not occur in vascularized conditions, using the model of systemic vitamin A deficiency.

Materials and Methods

Rabbit Model of Conjunctival Transdifferentiation

A rabbit model of conjunctival transdifferentiation was created in New Zealand albino rabbits, body weight 2-3 kg, in a manner similar to a previous method3 using n-heptanol originally described by Cintron et al.14 Corneas that healed within 6 days were excluded because the resultant epithelium may have originated from corneal remnants. On the 10th day after wounding, the remaining corneas were screened for epithelial defects and corneal vascularization. Those corneas healed without epithelial defects could then be classified into the following two major groups: the non-vascularized group with vessels extending less than 2 mm into the cornea, and the vascularized group with vessels extending more than 2 mm into the cornea and involving more than three quadrants. The occurrence of these two corneal types was haphazard, unpredictable, and possibly related to the severity of the chemical debridement. Corneas that had not healed completely by day 10 were re-screened on day 12. A total of 38 rabbits were used in this experiment, which gave 43 non-vascularized corneas, 24 vascularized corneas, and 9 corneas which were excluded due to vascularization occurring only in part of the cornea. Our study conformed to the ARVO Resolution on the Use of Animals in Research.

Induction of Vitamin A Deficiency

Four months after re-epithelialization, 18 rabbits with 24 vascularized corneas were subjected to the vitamin A-depleted diet (Test diet No. 7722, Teklad Test Diets, Madison, WI), originally described by Van Horn et al in the rabbit xerophthalmia model.15 Three to four corneas were obtained from the experimental group at 2, 4, 6, 7, 9, and 10 months after the special diet by sacrificing the rabbits with an overdose of intravenous phenobarbital.

Tissue Preparation For Morphological Study and Goblet Cell Analysis

After sacrifice, we removed the cornea with a 1 to 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, and counterstained with hematoxylin and eosin (H-E). Goblet cell analysis was performed as previously described.3 In brief, flat-mount preparations were made on each corneal half and then subjected to fixation and sequential stainings with 0.5% Alcian blue (pH 2.5) and PAS reagents. The topographical density of goblet cells was counted at ×400 magnification at each 1 mm zone from the center to the limbus. The mean value of the three meridians of the three pies of each corneal half was designated as the value of that corneal specimen. The mean value and its standard error of three or four of such specimens was reported here for comparison at any given time of sacrifice, in a way similar to that of our previous report.13

Measurement of Serum Retinol Levels by HPLC

In order to monitor the progress of vitamin A depletion, serum level of all-trans retinol (retinol) was measured by a HPLC method. Aliquots of 0.2 ml of serum were mixed with an equal volume of 125 retinol equivalent (RE)/dl of retinyl acetate in absolute ethanol which was used as an internal standard. After addition of 1.0 ml hexane, the mixture was agitated on a Vortex Mixer for 60 sec. It was then centrifuged at 2000 rpm for 15 min. The supernatant was removed and evaporated at room temperature in a stream of nitrogen. The residue was then dissolved in 0.2 ml absolute ethanol, of which an aliquot of 30 μl was injected into a Varian High Performance Liquid Chromatography (HPLC) apparatus (Sunnyvale, CA), which was a Model 5000 equipped with a Varichrom U-Vis detector set at 328 nm. The reverse phase chromatography was run using a Waters C-18, 10 μ, 3.9 × 30 cm column at a flow rate of 2.5 ml/min of methanol:water (96:4). This method was similar to that reported previously by Driskell et al.16 The concentration of serum retinol was calculated using the internal standard method by comparing the peak heights. Duplicate runs were made on each sample. Before use, the samples were covered by aluminum foil and stored at −20°C. The sample preparation
was proceeded under yellow fluorescent light. All reagents used here were of HPLC grade.

Impression Cytology

Ten months after vitamin A depletion, impression cytology was performed on both corneal and conjunctival surface using our previous method to determine if there was any change of squamous metaplasia on the ocular surface as a result of vitamin A deficiency.

Results

Four months after total removal of corneal and limbal epithelia, conjunctival epithelium on the non-vascularized corneas transdifferentiated into a cornea-like epithelium with total disappearance of goblet cells. In contrast, vascularized corneas still maintained a conjunctiva-like epithelium containing goblet cells (results not shown). These findings were consistent with those of our previous reports. It should be noted that the conjunctiva-like epithelium would persist indefinitely without spontaneous transdifferentiation on the vascularized corneas, since vascularized controls maintained these conjunctival features for more than 2 yr after wounding in a separate survey (unpublished results). Figure 1 shows the finding of numerous goblet cells on such a vascularized control cornea 20 months after debridement.

Rabbits with vascularized cornea(s) were then placed on a vitamin A-depleted diet. The dietary effect on the serum retinol concentration was monitored monthly by HPLC in a total of 10 month follow-ups except at the 3rd and 8th month after vitamin A deficiency. As shown in Figure 2, mean serum retinol levels gradually decreased from 83 μg retinol equivalent (RE)/dl of the pre-dietary control to 20 μg RE/dl at 7 months and then remained stationary thereafter until the end of 10 months of vitamin A deficiency.

The density and distribution of goblet cells were then analyzed on the vascularized corneas during this 10 month period of study. The results are summarized in the Figures 3 and 4. The goblet cell density, measured as number of cells per mm² at 2 months of vitamin A deficiency, was high and distributed throughout the entire vascularized corneas from the center to the mid-periphery and the periphery of the cornea (Fig. 4, upper panel). As shown in Figure 3, at up to 4 months goblet cells were still found from the limbus (L) to the center (C) of the cornea. Thereafter, one began to see the gradual centrifugal receding of goblet cells from the central part of the cornea to the periphery (Figs. 3, 4, middle panel), and finally at the end of 10 months, goblet cells could only be found in the peripheral 1 to 2 mm from limbus (Figs. 3, 4, lower panel). Accompanied with the centrifugal loss of goblet cells was the morphological transformation of the conjunctiva-like epithelium containing goblet cells to a cornea-like one with increased cellular stratification which was first noted at 9 months (Fig. 4, upper through lower panels).

The changes of goblet cells were then deliberately examined at the receding zone. It became clear that goblet cells followed sequential steps of cellular degeneration before they disappeared. These degenerating goblet cells were quantitated in the same manner as mentioned above from C to L of the cornea, the results of which are presented in Figure 5. The detailed features of the sequential degenerative changes are illustrated in Figure 6. The first degenerative change was noted at 2 months after vitamin A deficiency using flat-mount preparations, on which one
could see sporadic emptying of central mucin content in some goblet cells (Fig. 6A). Following this, at 4 months, the remaining mucin further disintegrated, and some goblet cells became smaller in size, containing bluish mucin, and some formed halos as a result of the loss of goblet cells in the center of a group of goblet cells (Fig. 6B). Since the bluish coloration signifies the acidic nature of the mucin, acidic mucin was used here. These degenerating goblet cells occurred sporadically and were distributed throughout the entire vascularized cornea at 2 months and increased in density at 4 months; the density at the latter time is displayed in Figure 5. The distribution curve indicated that the peak density was at the 2 mm zone from limbus, or 4 mm from the central cornea where the goblet cells could still be found. As the vitamin A depletion further progressed to 6 months, the degenerating goblet cells at the receding zone exhibited coalescence of the halo formation as well as acidic mucin rings, resulting in a total loss of goblet cells (Fig. 6C). At this time, the density of the degenerating goblet cells with halo formation receded centrifugally, and the peak density moved to 1 mm from limbus, or, interestingly, 4 mm from the goblet cell leading edge, which was 5 mm from the limbus (Fig. 5). At 7 months or later, this density curve of degenerating goblet cells further receded centrifugally and the peak density could only be found at the limbus, and the goblet cell leading edge was at 3–4 mm from the limbus (Fig. 5). The centrifugal receding of the degenerating goblet cells was accompanied by the disappearance of goblet cells from the central cornea to the leading edge, which was characterized by the coalescence of residual acidic coloration into a cluster or acinus pattern without any staining distributed along the remaining underlining vasculatures (Fig. 6D). The results of the changes of the density curves of degenerating goblet cells from 4 to 9 months along with the centrifugal shift of the peak density zone, which was always at 3–4 mm from the receding edge, strongly indicated that the conjunctival transdifferentiation was under the influence of a centrifugal gradient difference from the limbus to the center of the cornea.

It should be noted that we did not note any keratinization of the corneal surface (Fig. 4), nor any squamous metaplasia of the conjunctival surface using impression cytology (results not shown) even at the end of the 10 month period of study.

Discussion

The rabbit model of conjunctival transdifferentiation is an intriguing and ideal system to study the cellular differentiation of conjunctival epithelium. This process was first described by Friedenwald and subsequently thoroughly studied by several other investigators, including Thoft, Friend et al, Liu et al, and us. These pathophysiological studies of this experimental model have given us insights into the ocular surface changes caused by a total loss of corneal and limbal epithelia. This information is helpful in understanding such clinical problems as chemical burns, in which the first histopathological description of a similar finding of conjunctival transdifferentiation was given by Maumenee and Scholz.

Our major interest in this model has been the modulating mechanism by which conjunctival transdifferentiation occurs on non-vascularized corneas. Based on previous observations that conjunctival transdifferentiation can be either inhibited or reversed by corneal neovascularization, we speculated that the modulating factor(s) might be coming from the blood circulation and should meet the following
two criteria: (1) be present in systemic vascular circulation in a high concentration, but relatively low or negligible in extravascular tissues; and (2) possess the ability of modulating goblet cell differentiation. Because vitamin A or retinoids have the above two properties, we hypothesized that vitamin A might be one of the candidate factors. Our previous studies together with the present report have supported this hypothesis, which is summarized in Figure 7.

Conjunctival epithelium growing on the denuded corneal surface can exhibit one of the following two morphologies: either conjunctiva-like containing goblet cells or cornea-like without goblet cells but with increased cellular stratification. The transformation of original conjunctiva-like epithelium to a cornea-like one is termed transdifferentiation, and occurs as a result of relative local vitamin A defi-
ciency. This relative deficiency state can in turn occur either on normally non-vascularized cornea in which blood-borne vitamin A is relatively lower than in normally vascularized conjunctiva, or on vascularized corneas in which systemic vitamin A deficiency is induced (this report). The notion of the relative local vitamin A deficiency is verified in this report by the finding that serum retinol levels were subnormal but not undetectable. As a matter of fact, the first change of goblet cell degeneration was noted at 2 months and became pronounced from 4 to 7 months, during which time serum retinol levels remained in the range of 20 to 60 μg RE/dl. Even when transdifferentiation was completed at 9 months, its level was still around 20 μg RE/dl. At that time, goblet cell density over conjunctiva remained unchanged and cornea showed no keratinization, indicating that the vitamin A-deficient state of the ocular surface was a relative one. In other words, under the above two conditions, the conjunctival epithelium growing on the denuded corneal surface will lose goblet cell differentiation earlier than that growing on the normal conjunctival area.

The ocular surface changes of this vitamin A deficiency model should not be confused with those originally described by Van Horn et al. and recently reproduced by us, since the vitamin A storage level was low in young animals. In this report, vitamin A depletion was created 4 months later on rabbits with a starting body weight of 2–3 kg, which had a high vitamin A storage level and thus made the clinical xerophthalmia inapparent.

According to this hypothesis, one can imagine that transdifferentiation can also be inhibited or reversed by increasing a local supply of vitamin A to the corneal surface. Indeed, we have shown that this was achievable by either making the corneas vascularized or by applying topical retinoids onto the non-vascularized corneas (Fig. 7). All the evidence points to a close relationship between local vitamin A supply and goblet cell differentiation, and the latter is very sensitive to the former.

Previous studies by Rask et al. and Raviola indicate that the major supply of vitamin A to the epithelium on the corneal surface is from blood circulation. This notion is supported by all of our previous experiments and the present study. They all show that the loss of goblet cells in conjunctival transdifferentiation is always centrifugal and that their regeneration in the reversal process is always centripetal. Recently, Uibel et al. demonstrated free retinol in rabbit tears and lacrimal gland fluid in the concentration of 6.9 μg/dl and 6.2 μg/dl respectively. The facts that these values are ten times less than that of serum and that the goblet cell changes actually follow a radial
As stated above, conjunctival transdifferentiation involves morphological transformation of the conjunctiva-like epithelium to a cornea-like one, which is indeed a process of cellular differentiation. In the present report, the findings that acidic mucin came to dominate neutral mucin together with the shrinkage of goblet cell size occurring in an early stage of vitamin A deficiency are similar to our earlier study of the transdifferentiation on non-vascularized corneas, and to our previous finding on mucin changes in xerophthalmic rats. These results indicate that biochemical changes of conjunctival mucin might occur far ahead of those of goblet cell density. We therefore speculate that one of the major action mechanisms for vitamin A modulation of conjunctival goblet cell differentiation is through the control of mucin expression. Recently, we have purified and partially characterized rabbit ocular mucin and developed monoclonal antibodies to it (see the companion papers in this issue). In the future, we hope to further explore the molecular controlling mechanism by which vitamin A modulates conjunctival transdifferentiation.

**Key words:** conjunctival transdifferentiation, goblet cells, retinoids, vascularization, vitamin A deficiency

**References**

12. Tseng SCG, Hirst LW, Farazdaghi M, and Green WR: Inhibi-