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Change of Paracellular Permeability of Ocular Surface Epithelium by Vitamin A Deficiency

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Dietary vitamin A deficiency in young rabbits caused advanced squamous metaplasia with keratinization of conjunctival epithelium and concomitant reduced paracellular permeability to 3H-mannitol. Both morphologic and permeability changes were reversed with systemic administration of vitamin A. In adult rabbits, vitamin A deficiency caused milder changes of goblet cell loss and increased cellular stratification in conjunction with reduced permeability in the conjunctiva-like epithelium that covers the vascularized cornea after chemical injury with n-heptanol. Topically applied retinoid (tretinoin 0.1%) did not affect the morphology and permeability of the normal corneal or conjunctival epithelium of rabbits that were not vitamin A deficient. These studies showed that altered permeability is associated with the epithelial abnormality during vitamin A deficiency and helped clarify the physiologic function of retinoids in the ocular surface epithelia in the nondeficient state. Invest Ophthalmol Vis Sci 32:633-639, 1991

Earlier studies1-4 show that the ocular surface epithelium acts as a paracellular permeability barrier and maintains the homeostasis of fluid and solutes between the intraocular milieu and precorneal tear film. We recently confirmed that the ocular surface epithelium is the major paracellular permeability barrier to nonionic solute transport and reported that normal corneal paracellular permeability is 55 times less than that of conjunctiva.5 Furthermore, corneal permeability to mannitol is affected by the overlying epithelial phenotype, which is determined by the status of epithelial regeneration and differentiation during the process of corneal epithelial wound healing.6 Thus, we hypothesize that altering epithelial cellular differentiation may also affect paracellular permeability and that the degree of paracellular permeability may be an index for epithelial differentiation.

Vitamin A and its derivatives (retinoids) are known to alter epithelial cellular proliferation and differentiation both in vivo and in vitro.7-10 An excess of vitamin A induces mucinous metaplasia in embryonic chick epidermis.8 In the rabbit model of large corneal epithelial defects extending beyond the limbus, topical applications of retinoids cause retention of conjunctival characteristics on the cornea, inhibiting9 or reversing10 "conjunctival transdifferentiation." Conjunctival transdifferentiation is the process by which conjunctival epithelium migrating onto the corneal surface gradually transforms into a cornea-like morphology during wound healing in unvascularized corneas.11-14 In contrast, systemic vitamin A depletion can lead to squamous metaplasia in various nonkeratinized, secretory epithelia.15,16 We also showed that systemic vitamin A deficiency can induce the conjunctiva-like epithelium on vascularized corneas to transform into a cornea-like epithelium.17 Recently, we found that interrupting the local blood supply of vitamin A by occluding the corneal blood vessels, using photothrombosis, can induce conjunctival transdifferentiation of the epithelium to a non-mucinous character on vascularized corneas.18 These results indicate that when conjunctival epithelium migrates ectopically onto the corneal surface, its epithelial phenotype is influenced by the local supply of vitamin A. Morphologic transformation into a cornea-like epithelium during the process of conjunctival transdifferentiation might result from a deficiency of vitamin A.

Since vitamin A plays such an important role in modulating ocular surface epithelial differentiation, we examined the effect of systemic vitamin A deficiency on paracellular permeability to mannitol of the corneal and conjunctival epithelia and on para-
cellular permeability of the conjunctiva-like epithelium on the vascularized corneas in a model of conjunctival transdifferentiation. To see if there was a different susceptibility to the vitamin A supplementation between vitamin A-deficient and nondeficient states, the paracellular permeability of the normal nondeficient ocular surface epithelia was also examined after a short course of topical tretinoin treatment and compared with systemic administration of vitamin A to the vitamin A-deficient state.

Materials and Methods

The use of rabbits in this study conformed to the ARVO Resolution on the Use of Animals in Research.

Induction of Systemic Vitamin A Deficiency and Its Reversal by Systemic Vitamin A Administration

To study the effect of systemic vitamin A deficiency on the paracellular permeability of the cornea and conjunctiva, young New Zealand and albino rabbits, weighing less than 0.5 kg, were fed with a casein-based vitamin A-deficient diet (Test diet No. 7722; Teklad Test Diet, Madison, WI) for 4 months. Impression cytology specimens were taken from the bulbar conjunctiva and peripheral cornea to monitor the epithelial changes of the ocular surface. The rabbits that developed clinical xerophthalmia were killed, and their ocular surface tissues, without previously being touched by nitrocellulose acetate paper, were excised for perfusion studies.

To study the effect of vitamin A on the reversal of xerophthalmia, 4 months after systemic vitamin A depletion the rabbits were injected intramuscularly with a single dose of 200,000 IU of retinyl palmitate. Impression cytology was used to monitor the reversal of squamous metaplasia. Two weeks after the injection, the rabbits were killed, and their ocular surface tissues were removed for perfusion studies.

Induction of Conjunctival Transdifferentiation by Systemic Vitamin A Deficiency

The rabbit model of conjunctival transdifferentiation was induced by systemic vitamin A deficiency in the manner previously described. The entire corneal epithelium with a rim of conjunctival epithelium 2–3 mm beyond the limbus was denuded by n-heptanol and surgical scraping in a group of adult New Zealand albino rabbits with body weights of 2–3 kg. The extent of epithelial defect was confirmed by 1% methylene blue staining. The denuded corneas were healed by the surrounding conjunctival epithelium, and 65% of the corneas became extensively vascularized 2–3 weeks after healing. Four weeks after complete reepithelialization, four rabbits with extensive corneal vascularization were fed with a vitamin A-depleted diet for 5 months, and four control rabbits were fed regular chow. Impression cytology specimens were taken from the central corneas of either the control or vitamin A-deficient groups after 3 and 5 months of dietary depletion. The animals were killed 5 months after systemic vitamin A depletion, and the ocular surface tissues were obtained for perfusion studies.

Topical Tretinoin Treatment of Normal Ocular Surface Epithelia

To study the topical retinoid effect on the paracellular permeability of the normal cornea and conjunctiva, all-trans retinoic acid (tretinoin 0.1% w/w) ointment was prepared in mineral oil and petrolatum base. The ointment was applied to the lower conjunctival sac of one eye of the normal rabbits four times a day for 5 days with each application approximately 40 mg in weight. The contralateral eye received plain ointment base as a control. After treatment, the animals were killed, and whole corneal buttons and conjunctival tissues were excised carefully for perfusion studies.

In Vitro Perfusion Studies

To study the paracellular permeability, 3H-mannitol (27 Ci/mmol; New England Nuclear, Boston, MA) was used as a nonionic tracer in a lucite perfusion apparatus. The ocular surface tissues were mounted between two perfusion chambers. The tracer was placed in the chamber which bathed either the endothelial surface of the corneal buttons or the subconjunctival tissues of the conjunctival specimens. Permeabilities, measured by perfusion constants, were calculated from the linear regression of the kinetic curves derived from serial sampling of the tracer that permeated through the tissue into the other chamber. The initial concentration of tracer was 0.25 µM in Medium 199 (Bioproducts, Boston, MA), and all experiments were conducted at room temperature for 3 hr. Radioactivity was measured with a liquid scintillation counter with a counting efficiency of 40% for tritium.

Results

Effect of Systemic Vitamin A Deficiency and Subsequent Supplementation on Ocular Surface Epithelia of Young Rabbits

After 4 months of systemic vitamin A depletion, the rabbits developed conjunctival xerosis and corneal surface keratinization (Fig. 1A). Impression cy-
Fig. 1. Four months after systemic vitamin A deficiency in young rabbits, advanced xerophthalmia with corneal surface keratinization was noted (a). Impression cytology showed total loss of goblet cells and numerous keratinized cells on the conjunctival surface (b). One week after intramuscular injection of vitamin A, the corneal keratinization was significantly reduced (c), and conjunctival squamous metaplasia was partially reversed with the disappearance of keratinized cells (d). Two weeks after the treatment, the corneal keratinization resolved and the corneal surface became lustrous (e), with reappearance of normal conjunctival epithelium and regeneration of goblet cells (f). Arrows indicate goblet cells.

tology from the bulbar conjunctiva revealed marked squamous metaplasia characterized by total loss of goblet cells, increased ratio of nucleus:cytoplasm (N:C) to 1:4–1:8, and the presence of keratinized cells (Fig. 1B). One week after systemic administration of 200,000 IU of retinyl palmitate, the keratinized plaques on the cornea were partially resolved (Fig. 1C). Impression cytology showed partial regeneration of goblet cells (indicated by arrows) and return of the conjunctival epithelial cells with N:C ratio of 1:2–1:4 (Fig. 1D). Two weeks after treatment, the cornea resumed its smooth and lustrous surface (Fig. 1E). Impression cytology revealed a normal pattern of conjunctival epithelium with a N:C ratio of 1:1–1:2 and numerous goblet cells (Fig. 1F). This rapid reversal of the corneal keratinization and conjunctival squamous metaplasia after systemic vitamin A supplementation confirms that these surface changes are specific to vitamin A deficiency or xerophthalmia.

The permeability to mannitol of the keratinized cornea of the vitamin A-deficient rabbits was similar to that reported in normal corneas (Table 1); however, the conjunctival permeability of the vitamin A-deficient rabbits was significantly reduced from that of the normal conjunctiva (P < 0.005, paired t-test). Two weeks after systemic vitamin A supplementation, corneal permeability was unchanged, but the permeability of conjunctival tissue including Tenon’s capsule increased significantly compared with that of the vitamin A-deficient conjunctiva (P < 0.005, paired t-test). It reached a level similar to that of normal conjunctiva with Tenon’s capsule (Table 1). This

Table 1. Reversal of epithelial permeability by systemic vitamin A treatment in young rabbits

<table>
<thead>
<tr>
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<th>Permeability (×10⁻⁸ cm/sec)</th>
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<tr>
<td></td>
<td>Cornea</td>
</tr>
<tr>
<td>Systemic vitamin A deficiency (Fig. 1a, b)</td>
<td>0.14 ± 0.02 (n = 6)</td>
</tr>
<tr>
<td>Two weeks after systemic supplementation (Fig. 1e, f)</td>
<td>0.13 ± 0.01 (n = 4)</td>
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From ref. 5: normal conjunctiva with Tenon’s capsule 2.55 ± 0.30 (n = 4); normal cornea 0.12 ± 0.02 (n = 9).
result indicates that further keratinization of the stratified corneal epithelium would not decrease the permeability, but progression of the squamous metaplasia of the conjunctival epithelium could significantly reduce the permeability in the more permeable conjunctival tissue.

Effects of Systemic Vitamin A Deficiency in Adult Rabbits With Vascularized Corneas

The process of conjunctival transdifferentiation was induced on vascularized corneas of adult rabbits with systemic vitamin A deficiency. No evident squamous metaplasia of the ocular surface epithelium was noted after 5 months of vitamin A depletion (compare Figs. 2A–B with Figs. 1A, 2B); however, regression of the corneal vessels was apparent (Fig. 2B). Before dietary depletion, impression cytology of the corneal surface showed a conjunctival epithelial phenotype with many goblet cells interspersed among small conjunctival epithelial cells (Fig. 2C). After vitamin A depletion, goblet cells disappeared, and the superficial cells of the resultant epithelium adopted the morphology of cornea-like superficial squamous epithelial cells that had a N:C ratio of 1:3–1:4 (Fig. 2D).

Before vitamin A depletion, corneal permeability of the vascularized corneas was elevated to 0.75 ± 0.04 (×10^-8 cm/sec) (n = 4), similar to our previous report. After the induction of conjunctival transdifferentiation by systemic vitamin A deficiency, the corneal permeability decreased to 0.15 ± 0.03 (n = 4), a level similar to that of normal and nonvascularized corneas as previously reported. This result indicates that even when the conjunctival epithelium moved ectopically onto the corneal surface, its phenotype and permeability remained susceptible to modulation by vitamin A deficiency.
Effect of Topical Tretinoin on Normal Ocular Surface Epithelia

Tretinoin ointment (0.1%) was applied to one eye of normal adult rabbits four times a day for 5 days, and the fellow eye received a control ointment base. The ocular surface of both the experimental and control eyes remained uninflamed and wet. Mild lid swelling with tarsal hyperemia was noted in the tretinoin-treated group; however, neither damage to the ocular surface epithelium nor injection of bulbar conjunctiva was noted in either group. In vitro perfusion studies revealed that there was no difference in corneal permeability between the experimental and control eyes (Table 2). When compared with normal corneal permeability, these data indicate that neither topical tretinoin nor control ointment affected corneal permeability. Although tretinoin-treated eyes had a slightly higher conjunctival permeability than control eyes, no significant difference was noted (P > 0.1, paired t-test; Table 2). This result indicates that epithelial phenotype and paracellular permeability are not altered by topically applied retinoid if the ocular surface epithelia are not in a vitamin A-deficient state.

Discussion

We previously showed that ocular surface epithelium is the principal paracellular permeability barrier to nonionic solutes diffusing from the intraocular milieu and that this paracellular permeability can be modulated by the differentiative status (phenotype) of ocular surface epithelia. In this study, we further demonstrated that vitamin A deficiency can modulate epithelial differentiation and thereby affect paracellular transportation of nonionic solutes, such as mannitol.

Systemic depletion of vitamin A in young rabbits caused advanced squamous metaplasia with keratinization of the conjunctival epithelium, as previously reported by Hatchell et al. In the adult rabbits, vitamin A deficiency induced the transformation of the conjunctival epithelium on the vascularized corneas to a cornea-like epithelium with the loss of goblet cells and increased cellular stratification. This finding was consistent with our previous report. These results indicate that the nonkeratinized conjunctival epithelium, whether situated in situ or ectopically migrated onto the corneal surface, is susceptible to the depletion of vitamin A. In the former case, conjunctival squamous metaplasia could be readily reversed after systemic supplementation of vitamin A. In either case, morphologic alteration is accompanied by a decrease of paracellular permeability to mannitol.

Table 2. Topical tretinoin treatment on normal ocular tissue

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cornea (X10^-6 cm/sec)</th>
<th>Conjunctiva (X10^-6 cm/sec)</th>
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<tr>
<td>Control ointment</td>
<td>0.13 ± 0.02 (n = 4)</td>
<td>6.75 ± 0.12 (n = 4)</td>
</tr>
<tr>
<td>0.1% Tretinoin ointment</td>
<td>0.12 ± 0.02 (n = 4)</td>
<td>7.04 ± 0.32 (n = 4)</td>
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From ref. 5: normal cornea—0.12 ± 0.02 (n = 9); normal conjunctiva—6.78 ± 0.21 (n = 5).

On the corneal surface, epithelial changes induced by systemic vitamin A deficiency were different between the normal corneal epithelium-covered avascular corneas of the young rabbits (Fig. 1) and the conjunctival epithelium-covered vascularized corneas of the adult rabbits (Fig. 2). Four months after dietary deprivation, advanced keratinization was observed in the former but not in the latter group. This difference can be explained as follows. First, the age of the rabbits with dietary deprivation was different between the two groups. In the rabbits with the vascularized corneas, vitamin A depletion was initiated 4–6 months after the creation of ocular surface trauma. By that time, the rabbits had reached an adult size, weighing 2–3 kg, and liver storage of vitamin A had increased. As reported previously, dietary deprivation in these adult rabbits for 4–5 months only results in a mild decrease of serum vitamin A levels to 20–60 μg/dl. In contrast, serum vitamin A level was undetectable after 3–4 months of dietary deprivation if the young rabbits with initial body weights of 0.5 kg. Second, the vascular supply of vitamin A to the corneal surface will be deprived more in the avascular normal cornea than in vascularized corneas. These reasons explain why normal corneal epithelium became keratinized when the serum vitamin A level was lowered markedly, and the overlying conjunctival epithelium of the corneal surface transformed into a cornea-like morphology when the serum level of vitamin A was mildly decreased.

We previously reported that the loss of goblet cells in the process of conjunctival transdifferentiation always follows a centrifugal pattern from central to peripheral cornea in conjunction with the regression of the corneal blood vessels (Fig. 2). We attributed the epithelial changes observed in this experiment to the change in blood supply of vitamin A, a concept which was also proposed by Rask et al. and Raviola. We recognize that lacrimal tear fluid could be another route of vitamin A supply to the ocular surface epithelium. Ubels et al. showed free retinol in rabbit tears and lacrimal gland fluid in concentra-
junctional complexes which can be observed ultra-
enough for the nonkeratinized epithelia to show
the treatment in this study might not have been long
application of retinoids. Furthermore, the duration of
the more advanced the squamous metaplasia, the
keratinized epithelia, eg, cornea and conjunctiva, to topi-
topical supplementation of vitamin A. In other words,
we believe that vitamin A, delivered from corneal
blood vessels, is one of the circulatory factors that is
responsible for maintaining conjunctival epithelial
differentiation on vascularized corneas.

Supplementation of vitamin A to ocular surface
epithelia that had previously been deprived of vitami
A could reverse the process of squamous meta-
plasia and thereby change paracellular permeability.
In contrast, topical tretinoin treatment did not
change the paracellular permeability of normal cor-
nea or conjunctiva, at least for 5 days of the treatment
course. Previously, Elias et al.25 indicated that topical
cutaneous application of tretinoin and other retinoids
increase transepidermal water evaporation. They
attributed this effect to an alteration of the epidermal
junctional complexes which can be observed ultra-
structurally within 24 hr after treatment.25 Because
this topical tretinoin effect was not apparent in our
experiment suggests that keratinized epithelium, eg, epidermis, may be more susceptible than nonkera-
tinized epithelia, eg, cornea and conjunctiva, to topi-
cal supplementation of vitamin A. In other words,
the more advanced the squamous metaplasia, the
more susceptible it is to modulation by the topical
application of retinoids. Furthermore, the duration of
the treatment in this study might not have been long
enough for the nonkeratinized epithelia to show
changes in their paracellular permeabilities. In a vitami
A-deficient state, additional supplementation of
vitamin A can reverse the morphologic change and
permeability. In contrast, in a nondeficient state, vi-
tamin A supplementation does not induce a notice-
able change in morphology and permeability. This
information might help clarify the physiologic func-
tion that retinoids serve in the ocular surface epithelia
in the nondeficient state.

The corneal vascular regression during systemic vi-
tamin A deficiency is important (Fig. 2). Previously,
we noted that when pellets containing retinoids were
placed in the corneal stroma, they were not angio-
genic.11,12 Persistent elevation of mitotic activity of
the vascular endothelium in vascularized corneas has
been reported.26 Thus, it is conceivable that depletion
of vitamin A in the blood circulation may depress the
mitosis of vascular endothelium and lead to vascular
regression. Since corneal vascularization is always ac-
companied by a conjunctival epithelial phenotype in
the healing of a large corneal epithelial defect extend-
ning beyond the limbus,27-29 further exploration of this
mechanism is important to understand the pathogen-
esis of corneal angiogenesis under the influence of
epithelial cell differentiation.

Key words: cornea, conjunctiva, epithelium, paracellular
permeability, retinoids, transdifferentiation, vitamin A de-
ciciency, xerophthalmia

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