Effect of Vitamin A Deficiency on the Adhesion of Rat Corneal Epithelium and the Basement Membrane Complex


Purpose. To understand the underlying mechanisms responsible for the easy removal and sloughing of corneal epithelium in vitamin A deficiency.

Methods. An animal model of vitamin A deficiency, the vitamin A-deficient rat (A− rat), transmission electron microscopy, computer-assisted morphometric analysis and indirect immunofluorescence were used to study the adhesion of rat corneal epithelium to its basement membrane with emphasis on structure and molecular composition of the anchoring structures such as the hemidesmosome and bullous pemphigoid antigen.

Results. Transmission electron microscopy resolved numerous micro separations of the basal epithelial cell membrane from the basement membrane with intervening segmental basement membrane duplications and electron dense deposits. Morphometric analysis disclosed a statistically significant reduction in the frequency and size of hemidesmosomes. Four weeks after supplementing the diet with retinyl acetate (700 μg/week), significant reversal of these same structural abnormalities could be detected. Immunofluorescence staining for bullous pemphigoid antigen, a component of the adhesion complex, showed intense staining of the basal epithelial cytoplasm but weak and discontinuous staining of the basement membrane. Weak staining for laminin was also evident in A− corneas. In contrast, normal corneas displayed no cytoplasmic staining for bullous pemphigoid antigen and intense staining of the basement membrane for bullous pemphigoid antigen and laminin.

Conclusions. The authors propose that structural abnormalities of the epithelial basement membrane complex are responsible for the observed loose epithelial adhesion and sloughing, as well as other known abnormalities of healing in the vitamin A-deficient rat cornea. Invest Ophthalmol Vis Sci 1993;34:2646-2654.

Vitamin A is required for the normal growth and differentiation of epithelial tissues. Dietary deficiency results in keratinization of epithelial cells in the gastrointestinal tract, respiratory tract, and the ocular surface. Severe vitamin A deficiency results in xerophthalmia and keratomalacia. In the vitamin A-deficient human cornea, superficial keratinized layers have been seen to slough, carrying the deeper layers with them. In the course of studying the effect of vitamin A deficiency on inflammation and infection in the rat cornea, we noticed that the corneal epithelium of the A− rat could be easily dislodged. Thus it was hypothesized that vitamin A deficiency probably affected hemidesmosomes that are involved in anchoring basal cells to the underlying connective tissue and components of the basement membrane complexes (such as bullous pemphigoid antigen [BPA] and laminin).
The attachment of basal cells of stratified epithelia, such as corneal epithelium, to the subjacent connective tissue is mediated by specialized structures, comprising hemidesmosomes, basement membrane, and anchoring fibrils. The structures constitute the so-called basement membrane adhesion complex and are essential for the tight epithelial-stromal adhesion. Hemidesmosomes ultrastructurally appear as focal electron-dense plaques along the basal cell membrane into which cytoplasmic intermediate filaments insert. The best-characterized component of the hemidesmosome is the BPA initially identified in pemphigoid patients. Bullous pemphigoid antigen 1 (BPAG1) is a 230-kD non-collagenous protein and is the main autoantigen in bullous pemphigoid (BP). On the other hand, BPAG2, a 180-kD collagenous protein, is recognized by antibodies from some patients with BP and herpes gestationis. In the cornea, one cannot only localize BPA but can also find \( \alpha_6 \beta_4 \) and \( \alpha_6 \beta_1 \) integrin heterodimers. \( \alpha_6 \beta_4 \) and type VII collagen have been localized to the hemidesmosomes of the mouse cornea and to the basement membrane-anchoring fibril zone of both human and rabbit corneas, respectively. Type VII collagen has been detected in anchoring fibrils of other tissues, while laminin, a large glycoprotein found in the basement membrane, is also a key mediator of cell-substrate interactions.

In this communication, the effect of vitamin A deficiency on the ultrastructural morphology of the basement membrane adhesion complex and on the distribution of BPA and laminin is described.

**MATERIALS AND METHODS**

**Vitamin A-Deficient Rats**

Nineteen-day-old, inbred, male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in screen-bottomed cages, in light- and temperature-controlled facilities. All animals were fed a casein-based vitamin A-deficient diet (Teklad, Madison, WI). Normal controls received 700 Mg of retinyl acetate (Sigma, St. Louis, MO or Teklad, Madison, WI) dissolved in Mazola brand corn oil and delivered orally once a week. After 4 weeks on the diet animals were placed in individual cages and monitored for signs of vitamin A deficiency. As determined by ocular changes, skin changes, and weight loss, early stages of xerophthalmia were reached by approximately 45 days of age, and severe ocular manifestations (enophthalmos, loss of corneal clarity, conjunctivitis, keratitis, blepharitis, and corneal vascularization), appeared within 90 days. In a representative group of animals (12 A- rats and 5 normal controls), mean serum retinol levels were determined by high performance liquid chromatography to be 0.54 \( \mu \)g/ml for A- animals as compared to 42.59 \( \mu \)g/ml for normal control animals. Experiments were conducted with severely A- rats that had been on the A- diet approximately 12 ± 1.3 weeks. To determine the effect of A supplementation, severely A- animals were placed on oral retinyl acetate, 700 \( \mu \)g per week for 4 weeks. All animal care conformed to the ARVO resolution on the use of animals and PHS Policy on Humane Care and Use of Laboratory Animals.

**Phase Contrast and Electron Microscopy**

After death by sodium pentobarbital overdose, whole corneas were immersed in half-strength Karnovsky's glutaraldehyde-formaldehyde fixative, postfixed in 2% osmium tetroxide, stained en bloc with uranyl acetate, dehydrated, and embedded according to standard technique. Semi-thin sections were cut and stained with para-phenylenediamine for phase contrast microscopy. Ultra-thin sectioned and uranyl acetate/lead citrate stained tissues were viewed with a Philips 410 transmission electron microscope (Eindhoven, The Netherlands).

**Morphometric Analysis**

Severely A- (n = 13), retinyl-acetate-supplemented (n = 7) and normal, nondeficient (n = 7) rat corneas were examined by transmission electron microscopy such that a randomly selected central area of the basal epithelium was photographed and printed at a uniform magnification of 31,200. Ten consecutive and adjacent fields representing 80 \( \mu \)m of the epithelial-stromal interface were analyzed. A Zeiss Videoplan Image Analysis System (Rainin Instruments, Woburn, MA) was used to count the number and measure the length of hemidesmosomes, as well as calculate the distance between the basal cell membrane and the lamina densa of the basement membrane. The average hemidesmosome length was calculated by dividing the combined length of all hemidesmosomes in the 80 \( \mu \)m standard field by the number of hemidesmosomes found in this same area. To calculate the mean distance between basal membrane and basement membrane, the cross-sectional area of the separation was divided by the 80-\( \mu \)m length of the basement membrane analyzed.

**Immunofluorescence**

Normal and A- corneas (two each) were frozen in Tissue Tek II O.C.T. compound (Lab Tek Products, Naperville, IL). Cryostat sections (5-6-\( \mu \)m thick) were placed on gelatin-coated slides and air dried overnight at 37°C, then rehydrated in phosphate-buffered saline, pH 7.2 for 10 minutes at room temperature. Non-specific binding sites were blocked by incubating the tissues in phosphate-buffered saline containing 1%...
bovine serum albumin for 10 minutes at room temperature. The primary antibody, either human anti-human BPA or rabbit anti-human laminin (Collaborative Research, Bedford, MA) were used at a dilution of 1:500 and 1:900, respectively, and were prepared in phosphate-buffered saline/bovine serum albumin. The tissues were incubated with the primary antibody for 30 minutes at 37°C, washed three times by gentle shaking in phosphate-buffered saline for 10 minutes at room temperature and treated with either donkey anti-human (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) or goat anti-rabbit (Sigma, St Louis, MO) immunoglobulin G conjugated with fluorescein isothiocyanate (FITC) for 30 minutes at 37°C. The tissues were washed again and mounted with coverslips using Gel/Mount (Biomeda Corp., Foster City, CA), and photographed using a Zeiss Axiophot microscope equipped for epi-illumination. Negative controls (primary antibody omitted and/or nonspecific antiserum from rabbit, mouse, or hybridoma fluid) were routinely included in every antibody binding study.

Statistical Analysis

To assess the significance of the data, means and standard error of the means were calculated and two-tailed, unpaired Student's t tests were done to obtain P values.

RESULTS

Clinicopathologic Observations of Corneal Epithelial Adhesion

In the A— cornea, the corneal epithelium is only loosely adherent to the underlying stroma; it can be dislodged as an intact sheet with gentle mechanical traction with jewelers forceps (Fig. 1A). In contrast, epithelium of normal corneas could only be removed in fragments. Histologic examination of the A— cornea revealed clear separation of the entire epithelial sheet from the stroma (Fig. 1B). Furthermore, transmission electron microscopy disclosed that mechanical separation of the epithelium did not disrupt the basement membrane (Fig. 1C), which remained adherent to the stromal surface, thereby suggesting that the adhesion defect involves the hemidesmosomes, which mediate adhesion between the basal cell membrane and the basement membrane.

Effects of Vitamin A Deficiency on Adhesion Complexes

Electron microscopic examination of the basal epithelium and basement membrane zone revealed that severely A— corneas exhibited fewer and shorter hemidesmosomes as compared to normal controls. As specified in Figure 2, A— corneas possessed 81.46 ± 6.99 hemidesmosomes per standard field compared to 210 ± 18.5 (P < 0.0001) in normal control corneas. Moreover, the average length of the hemidesmosome measured 0.10 ± 0.002 µm in the A— cornea, as compared to 0.13 ± 0.007 µm (P = 0.01) in normal controls. Approximately 11.9% (9.52 ± 0.98 µm) of the basement membrane in the A— cornea was occupied by hemidesmosomes, as compared to 35.2% (28.23 ±
Adhesion of Corneal Epithelium

Frequency of hemidesmosomes in vitamin A deficiency

<table>
<thead>
<tr>
<th>Condition</th>
<th>Number of Corneas</th>
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<tr>
<td>Normal Corneas (N=7)</td>
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<tr>
<td>A- Corneas (N=13)</td>
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<tr>
<td>Retinyl acetate (N=7)</td>
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FIGURE 2. Effect of vitamin A deficiency (A-) and supplementation (retinyl acetate) on the frequency of hemidesmosomes in the corneal epithelium. The results of morphometric analysis of transmission electron micrographs are presented as the mean number of hemidesmosomes standard error of the mean (SEM) per standard field of epithelium-basement membrane zone. All differences are statistically significant (P < 0.0001).

1.35 μm, P < 0.0001) in the normal cornea (Fig. 3). After four weeks of dietary supplementation with retinyl acetate, the frequency and size of hemidesmosomes had returned significantly toward normal with concomitant increase in the basement membrane area subtended by hemidesmosomes (Figs. 2, 3). However, microscopic defects in adhesion complexes were still evident at this time.

Transmission electron microscopy consistently revealed microseparations of the corneal epithelium from the basement membrane only in A- corneas (Fig. 4A), whereas no such areas were evident in the normal cornea (Fig. 4C). In the A- cornea, as hemidesmosomes disappeared and epithelium became separated from the basement membrane, intermediate filament organization in the vicinity was also lost (Figs. 4A, 4B). Thus the width of the lamina lucida (the distance between the basal cell membrane and the lamina densa of the basement membrane) was visibly increased in A- corneas (0.126 ± 0.01 μm) relative to controls (0.066 ± 0.01 μm; P = 0.01) (Fig. 5). Furthermore, within these areas of microseparation in the A- cornea, segmental duplications of the basement membrane and apparent generation of new hemidesmosomes were evident (Fig. 6). Even in corneas displaying severe corneal edema with thinning and disruption of the basement membrane, the segmental duplication of basement membrane and emergence of rudimentary hemidesmosomes could be observed (Fig. 7). After 4 weeks of retinyl acetate supplementation, these separations were reduced although not altogether to normal values. Similarly, in the retinyl-acetate-supplemented corneas, increases in the size and frequency of hemidesmosomes were also evident (Figs. 2, 3, and 4B). Corrugated, multilayered electron dense deposits (presumably cellular debris) between the basal cells and basement membrane were also detected (Fig. 7).

IF for the detection of BPA and Laminin

We observed redistribution of the BPA antigen in the A- basal corneal epithelial cells, as intense cytoplasmic staining was detected (Fig. 8A) in contrast to the linear staining of the basement membrane zone (Fig. 8C) in the normal cornea (Fig. 8B). In the A- cornea,
FIGURE 4. Effect of vitamin A deficiency and retinyl acetate supplementation on the width of the lamina lucida in the cornea epithelium. (A) Transmission electron micrograph of vitamin A-deficient (A−) cornea reveals microseparations (*) of plasma membrane (arrows) of basal epithelial cells (EC) from the basement membrane (arrowheads) at the stromal surface (Str). Discontinuous segments of the duplicated basement membrane are evident within the microseparations. (B) Transmission electron micrograph of the A− cornea after 4 weeks of retinyl acetate supplementation shows less pronounced microseparations (*) between plasma membrane with few well-developed hemidesmosomes (arrows) and continuous basement membrane (arrowheads). (C) Transmission electron microscopy of nondeficient normal cornea illustrates normal epithelium-basement membrane zone, featuring numerous well-developed hemidesmosomes (arrows) along the plasma membrane closely apposed to the basement membrane (arrowheads). (IF = intermediate filaments; all X31,200)

staining for laminin was extremely weak in the basement membrane zone as compared to bright and linear staining in normal controls (Fig. 8D). Normal rabbit serum used as a control did not stain the corneas (Fig. 8E).

DISCUSSION

These findings afford an explanation for the clinical observations of defective adhesion of the corneal epithelium in the A− rat. There are fewer and smaller
Separation of basal epithelium from basement membrane in vitamin A deficiency

**FIGURE 5.** Effect of vitamin A deficiency (A−) and supplementation (retinyl acetate) on the width of the lamina lucida in the corneal epithelium. The results of morphometric analysis of transmission electron micrographs are presented as the mean distance SEM between basal cell plasma membrane and the lamina densa of the basement membrane. (Also see Figure 4.)

hemidesmosomes in the A− cornea and as a result, the surface area of the basement membrane occupied by adhesion complexes is substantially reduced. It is suggested that this paucity of adhesive junctions is responsible for the microseparations of the epithelium from the basement membrane. The greatest degree of separation between the basal cell plasma membrane and basement membrane occurred in areas where the frequency and size of hemidesmosomes was the least. This is in contrast to diabetic corneal epithelium where the basement membrane separates from the stroma

**FIGURE 6.** Transmission electron microscopy of vitamin A-deficient cornea resolves microseparations (*) of basal plasma membrane (BC) from original basement membrane (arrowheads), and segmental duplication of basement membrane (large arrows) with attendant hemidesmosomes (small arrows). (Str = stroma; ×31,200).

**FIGURE 7.** Survey transmission electron micrograph of a vitamin A-deficient cornea demonstrates severe intercellular edema (*), with elevation of entire epithelial layer from the original basement membrane (arrowheads), accumulation of fibrocellular material (left and central) and formation of rudimentary and incomplete basement membrane (large arrows) plus hemidesmosomes (small arrows) (×6000).
FIGURE 8. Effect of vitamin A-deficiency on the basement membrane components of the corneal epithelium. (A) In the A− cornea, bullous pemphigoid antigen is retained intracellularly within the basal epithelial cells. (B) In the normal cornea, bullous pemphigoid antigen exhibits discrete extracellular staining in the basement membrane zone. (C) In the A− cornea, staining for laminin in the basement membrane zone is reduced and very diffuse. (D) In the normal cornea, laminin displays bright linear staining. (E) Normal rabbit serum was used as control to determine nonspecific binding (all indirect immunofluorescence, ×440).

Because of reduced penetration of the anchoring fibrils into the stroma.25 Taken together, our data suggest that vitamin A deficiency causes a reversible decrease in the frequency and size of hemidesmosomes of the corneal epithelium, leading to decreased basement membrane surface area covered by hemidesmosomes. Such reduction in hemidesmosome size and frequency might then account for loose adherence of the epithelium to the basement membrane and its easy mechanical removal in the A− cornea.

Segmental duplication of the basement membrane and infoldings of the basal cell plasma membrane with or without attendant hemidesmosomes resolved in these A− corneas were ultrastructurally reminiscent of adhesion complex alterations described in human cases of corneal edema and recurrent epithelial erosion.26,27 Membranous infoldings and short segments of basement membrane were also evident along the basal epithelial cell surface of rabbit corneas in which basement membrane regeneration had been induced by superficial keratectomy.28,29 It has been suggested that interruption of the basement membrane induces the corneal epithelium to attempt restoration of the integrity of this layer by synthesizing and depositing a new basement membrane. Moreover, basement membrane duplication has also been observed in the diabetic cornea.28 Our data therefore extends the observation of segmental duplication of basement membrane to the A− cornea and supports the notion that the epithelial cell is responsible for the synthesis and deposition of the basement membrane.

Vitamin A is widely known to affect the differen-
tion of corneal epithelial cells. In the eye, retinol deficiency is associated with both a reduction in the goblet cell population and an increase in conjunctival epithelial cell mitosis, but a paradoxical delay in conjunctival wound healing. We can now also extend the effect of vitamin A deficiency to include interference with the transcription and translation of the molecular components of the basement membrane and adhesion complexes. The abnormally thin and duplicated basement membrane in the A− cornea supports this interpretation, as does the deficiency of critical hemidesmosome components such as laminin.

Despite the observed paucity of hemidesmosomes, BPA, a key component of these structures, was produced by A− basal epithelial cells. However, the staining pattern strongly suggested redistribution of the antigen to the cytoplasm, perhaps due to the reduction of hemidesmosome and/or due to the lack of a signal from the basal cell plasma membrane or the basement membrane. This staining pattern was reminiscent of BPA produced by cultured epithelial cells, which also usually lack hemidesmosomes and hence present with cytoplasmic staining for this antigen.

Integrins have not only been shown to be present in the hemidesmosome but have also been shown to bind to discrete regions of laminin. Because integrins are transmembrane protein complexes and have been located to the hemidesmosome, it could be hypothesized that these proteins mediate the adhesion of basal epithelial cells by interacting with both laminin and perhaps other as yet unidentified extracellular matrix components on the stromal side of the adhesion junction and with BPA and other unknown molecules in the basal cell plasma membrane. We have demonstrated poor localization of anti-laminin antibodies to the A− basement membrane indicating either a reduction of the laminin content or modification of the its structure in these corneas. Vitamin A deficiency could also influence the production and quality of integrins and could therefore cause poor adherence of the epithelium via this mechanism. We therefore predict an important role for laminin in the adhesion of epithelium to the basement membrane. However, there is no evidence indicating that vitamin A deficiency affects the expression of any of the several integrins.

Can these morphologic and functional changes be reversed by replacing dietary vitamin A? In fact, even short-term supplementation with retinyl acetate had a major effect on A− corneas. Within 4 weeks, clinical ocular, and skin changes as well as the ultrastructural morphology of the cornea began to revert to normal. As these animals were observed for only 4 weeks, we were not surprised that residual effects of vitamin A deficiency remained ultrastructurally detectable. These normalizing changes were however substantial, suggesting that vitamin A indeed has a vital effect on the epithelial-stromal interactions in the cornea.

In summary, we demonstrated a statistically significant reduction in the frequency and size of hemidesmosomes in the vitamin A-deficient rat cornea. This was also accompanied by redistribution of BPA to the cell cytoplasm and changes in the staining pattern for laminin, leading us to propose that structural abnormalities of the epithelial basement membrane complex are responsible for the loose epithelial adhesion as well as other known abnormalities of healing in the vitamin A-deficient rat cornea.

Key Words
cornea, epithelium, vitamin A deficiency, basement membrane, hemidesmosome, adhesion complex, bullous pemphigoid antigen

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References
11. Jones JCR, Yoko KM, Goldman RD. Is the hemides-


25. Sonnenburg A, Linders CJT, Godderman PW, Damsky CH, Aumailley M, Timpl R. Integrin recognition of different cell binding fragments of laminin (P1, E3, E8) and evidence that $\alpha_6\beta_4$ but not $\alpha_2\beta_1$ functions as a major receptor for fragment E8. *J Cell Biol.* 1990;110:2145–2155.


