Expression of Specific Keratin Markers by Rabbit Corneal, Conjunctival, and Esophageal Epithelia during Vitamin A Deficiency

SCHEFFER C. G. TSENG, DIANE HATCHELL, NANCY TIERNEY, ANDREW J.-W. HUANG, and TUNG-TIEN SUN

Departments of Ophthalmology, Dermatology, and Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; Departments of Ophthalmology and Physiology, The Medical College of Wisconsin, Milwaukee 53226; Department of Ophthalmology, Duke University School of Medicine and Durham VAMC, Durham, North Carolina 27710; and Departments of Dermatology and Pharmacology, New York University School of Medicine, New York 10016

ABSTRACT Using an in vivo rabbit model system, we have studied the morphological and biochemical changes in corneal, conjunctival, and esophageal epithelia during vitamin A deficiency. Light and electron microscopy showed that the three epithelia undergo different degrees of morphological keratinization. Corneal and conjunctival epithelia became heavily keratinized, forming multiple layers of superficial, anucleated cornified cells. In contrast, esophageal epithelium underwent only minor morphological changes. To correlate morphological alterations with the expression of specific keratin molecules, we have analyzed the keratins from these epithelia by the immunoblot technique using the subfamily-specific AE1 and AE3 monoclonal antikeratin antibodies. The results indicate that during vitamin A deficiency, all three epithelia express an AE1-reactive, acidic 56.5-kd keratin and an AE3-reactive, basic 65–67-kd keratin. Furthermore, the expression of these two keratins correlates roughly with the degree of morphological keratinization. AE2 antibody (specific for the 56.5- and 65–67-kd keratins) stained keratinized corneal epithelial sections suprabasally, as in the epidermis, suggesting that these two keratins are expressed mainly during advanced stages of keratinization. These two keratins have previously been suggested to represent markers for epidermal keratinization. Our present data indicate that they can also be expressed by other stratified epithelia during vitamin A deficiency-induced keratinization, and suggest the possibility that they may play a role in the formation of the densely packed tonofilament bundles in cornified cells of keratinized tissues.

It has been well established that vitamin A plays an important role in regulating epithelial growth and differentiation (for reviews, see 8, 12, 32, 63, 67, 69). Fell and co-workers (14) have shown that vitamin A excess can induce mucous metaplasia in organ-cultured embryonic chick epidermis. Conversely, Mori (37), and Wolbach and Howe (66) have shown that vitamin A deficiency can cause squamous metaplasia and keratinization in a wide variety of nonkeratinized and secretory epithelia. Since metaplastic changes similar to those seen in vitamin A deficiency also occur in certain epithelia as an intermediate stage of carcinogenesis, and since vitamin A possesses anti-carcinogenic effects in some experimental animals (3, 25, 45), there has been a surge of interest in studying the mechanisms of vitamin A deficiency-induced squamous metaplasia and abnormal epithelial keratinization.

Normal epidermis is a keratinized tissue consisting of basal, spinous, granular and (anucleated) cornified layers (21, 27, 33, 36, 41). Recent studies have shown that the expression of keratins—the protein subunits of tonofilaments (16, 17, 53, 54)—changes during epidermal keratinization (19). The basal cells express an acidic 50- and a basic 58-kd keratin, whereas the suprabasal cells express in addition an acidic 56.5- and a

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basic 65–67-kd keratin (4, 47, 48, 56, 68). Interestingly, the expression of these latter two keratins is suppressed in cultured human epidermal cells forming nonkeratinized colonies (18, 52), although their expression is resumed when the cultured cells are induced to keratinize, either in a vitamin A-deficient culture medium (10, 20) or when the cells are provided with a proper in vivo permissive environment (4, 9). Such results, plus the fact that these two keratins are rarely detected in nonkeratinized normal tissues (34, 59; see below), suggest that the 56.5- and 65–67-kd keratins may be regarded as markers for epidermal keratinization[1] (55, 56, 59, 68).

In this paper, we describe the expression of these two keratin markers in rabbit corneal, conjunctival, and esophageal epithelia undergoing in vivo keratinization as a result of vitamin A deficiency. Keratins were analyzed by the immunoblot technique using the recently described AE1 and AE3 monoclonal antikeratin antibodies that are specific for the acidic (A) and basic (B) subfamilies of keratins, respectively (6, 10, 55, 57, 59). To correlate keratin changes with specific morphological alterations, we examined these epithelia by both light and electron microscopy. Our results indicate that in vitamin A-deficient rabbits, the three epithelia undergo different degrees of morphological keratinization, as indicated by the formation of various quantities of anucleated cornified cells. Immunoblot analysis illustrates that all three keratinizing nonepidermal epithelia have acquired the 56.5- and 65–67-kd keratins, at a level roughly proportional to the degree of morphological keratinization. The results thus suggest that these keratins are not epidermis-specific and may be regarded as markers for both normal and vitamin A deficiency-induced keratinization. The possibility that they may play an important role in the formation of densely packed tonofilament bundles in cornified cells will be discussed.

MATERIALS AND METHODS

Rabbits: 20 New Zealand white rabbits, weighing 0.5 kg at the beginning of the experiments, were maintained on a vitamin A-deficient diet (test diet no. 77227; Teklad Test Diets, Madison, WI) as described previously (60, 61). Control rabbits were maintained either on the same diet supplemented with vitamin A palmitate (500,000 IU/kg; test diet no. 78403), or on regular Purina Rabbit Chow Checkers (Ralston Purina Co., St. Louis, MO). Both controls yielded identical results. To monitor the clinical progression of vitamin A deficiency, we examined weekly the eyes of all rabbits by slit-lamp biomicroscopy to detect any ocular surface abnormalities. In general, all the rabbits used in the present study were maintained on vitamin A-deficient diet for 3–6 mo and showed advanced keratinization of corneal epithelium (60).

Morphological Studies: Rabbits were sacrificed with an intracardiac overdose of sodium pentobarbital. Tissues were excised, fixed with 2.7% glutaraldehyde in phosphate buffer, embedded in a low-viscosity epoxy resin, and processed for both light and transmission electron microscopy (60).

Monoclonal Antikeratin Antibodies: The preparation and characterization of AE1, AE2, and AE3 mouse monoclonal antikeratin antibodies have been described elsewhere (59, 68). Tissue culture media conditioned by the hybridoma cells were used as the source of monoclonal antibodies.

Immunoblot Analysis of Keratins: Keratins were isolated by serial extraction as described (10, 52). They were then separated by SDS PAGE (12.5% acrylamide; [28]), transferred electrophoretically to a sheet of nitrocellulose paper (Millipore), stained with Fast green (10, 68), and then stained with monoclonal antibodies by the peroxidase-antiperoxidase technique (58, 68).

Immunofluorescent Staining: Unfixed, frozen tissue sections (8-μm thick) were stained with monoclonal antibodies by the indirect immunofluorescent staining technique (53).

RESULTS

Morphological Studies

The epidermis of the control and vitamin A-deficient rabbits showed similar morphology. Both are keratinized and consist of typical basal, spinous, granular, and cornified cell layers (Figs. 1, *a* and *b* and Fig. 2).

The morphology of normal corneal epithelium has been described as "nonkeratinized", with basal, wing (intermediate), and nucleated (nuclei-containing) superficial cells (Figs. 1 *c* and 3 *a*). However, the corresponding epithelium in vitamin A-deficient rabbit is drastically different and becomes morphologically similar, although not identical, to the epidermis, with the formation of keratohyalin granules and anucleated cornified cells possessing crosslinked (cornified) envelope (Figs. 1 *d* and 3 *b*; also see reference 60).

Rabbit bulbar conjunctival epithelium is normally a stratified epithelium composed of irregularly piled polygonal cells and is enriched in goblet cells (Figs. 1 *e* and 4 *a*). However, in vitamin A-deficient animals the epithelium is devoid of goblet cells, and becomes thickened, with multiple layers of superficial cornified cells (Figs. 1 *f* and 4 *b*). Keratohyalin granules can also be observed occasionally (arrows in Fig. 1 *f*).

Of the three rabbit epithelia studied, the esophageal epithelium undergoes the least morphological change. Normal rabbit esophageal epithelium is "parakeratinized," as defined by the lack of a granular layer and the formation of eosinophilic, nucleated cornified cells (Figs. 1 *g* and 5, *a–c*; 9, 31, 49). In vitamin A deficiency, this epithelium becomes thinner, exhibits thick keratin filament bundles (fibril-formation; see reference 24), and is covered with several layers of cornified cells that are more darkly stained and densely packed than normal (Figs. 1 *h* and 5, *d* and *e*). Since most of these cornified cells do retain their nuclei (Fig. 1 *h*), the tissue is still predominantly parakeratinized or at best only partially keratinized.

Keratin Analysis

Water-insoluble cytoskeletal proteins were isolated from skin, corneal, conjunctival, and esophageal epithelia of both normal and vitamin A-deficient rabbits, and were analyzed by the immunoblot technique using AE1 and AE3 monoclonal antikeratin antibodies. As we have shown earlier, these two antibodies are highly specific for the acidic (A) and basic (B) subfamilies of keratins, respectively (references cited). Consistent with morphological data, we found that normal and vitamin A-deficient epidermis express an identical set of keratins consisting of the 50- and 56.5-kd keratins of the AE1 (A) subfamily, and 58- and 65–67-kd keratins of the AE3 (B) subfamily (Fig. 6). In contrast, keratins of the corneal, conjunctival, and esophageal epithelia show distinct changes during vitamin A deficiency. Although these epithelia do not normally possess any significant amounts of the 56.5- and 65–67-kd keratins (59), they all express these two keratins during vitamin A deficiency-induced keratinization (arrows in Fig. 6). Correlation with morphological data show that the expression of these keratins is roughly proportional to the degree of morphological keratinization.

[1] The differentiation of hair and nail ("hard" tissues), although also known as keratinization, is morphologically and biochemically distinct from that of ("soft") epithelial tissues such as epidermis (22). Our results suggest that the 56.5- and 65–67-kd keratins may be regarded as markers for (soft) epithelial keratinization, but not for hair- or nail-keratinization (Lynch, M., L. Mak, and T.-T. Sun, unpublished).

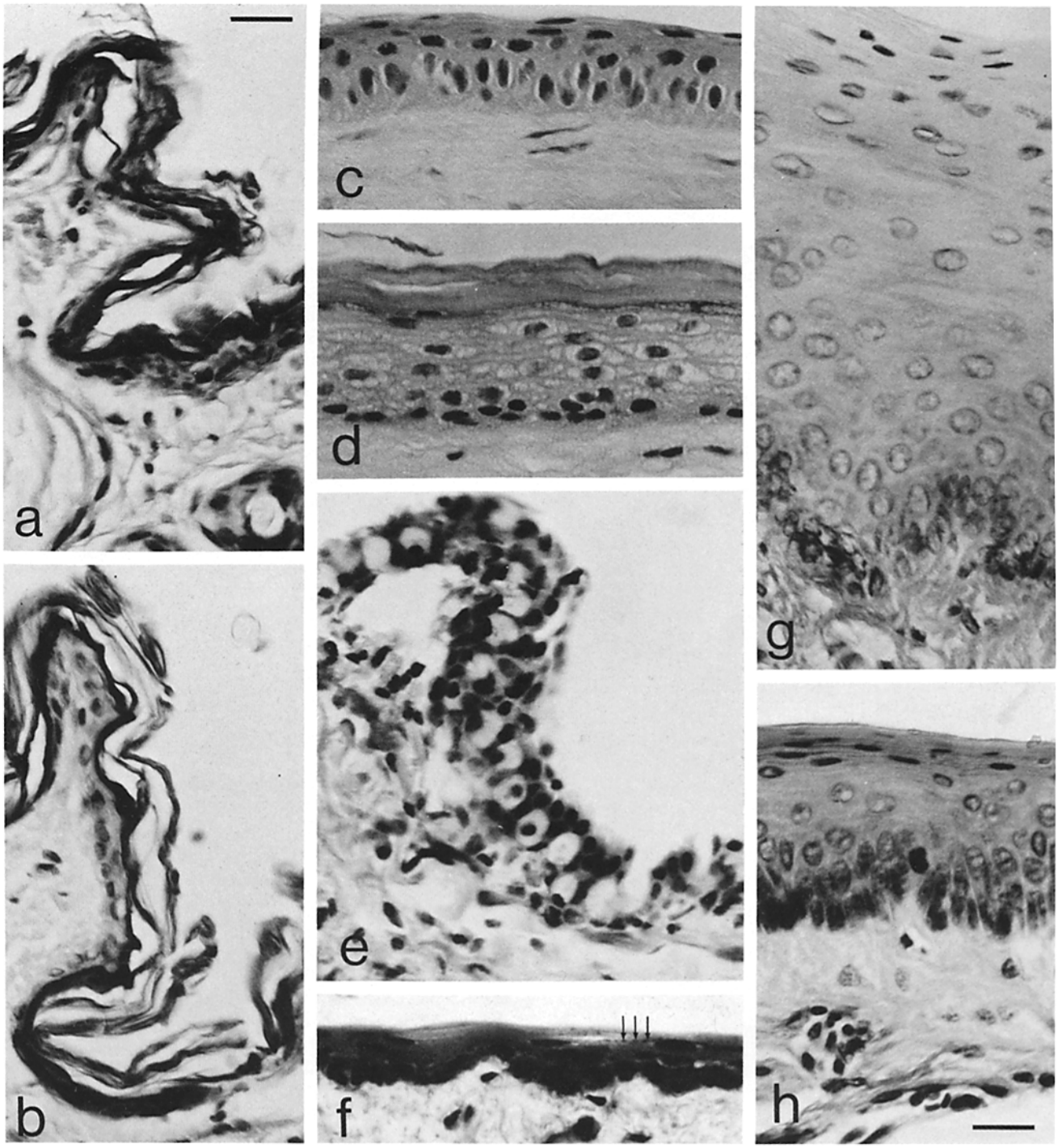


FIGURE 1 Light microscopy of various rabbit epithelial tissues from normal and vitamin A-deficient animals. (*a*) Normal skin; (*b*) skin from a vitamin A-deficient rabbit (A^-); (*c*) normal cornea; (*d*) cornea, A^-; (*e*) normal conjunctiva; (*f*) conjunctiva, A^-; (*g*) normal esophagus; (*h*) esophagus, A^-. Hematoxylin and eosin staining. Arrows in *f* indicate keratohyalin granules. Bar, 20 μm. × 500.

AE2 Antibody Staining

To determine in what cell layers the 56.5- and 65–67-kd keratins were expressed, we stained frozen sections of various epithelia from both control and vitamin A-deficient rabbits using another monoclonal antikeratin antibody, AE2. As we have shown previously, in normal epidermis this antibody recognizes specifically the 56.5- and 65–67-kd keratins (59, 68). Immunofluorescent staining shows that although normal corneal epithelium is AE2-negative (Fig. 7*a*), the keratinized corneal epithelium from vitamin A-deficient rabbits exhibits positive AE2-staining of suprabasal cells (Fig. 7, *c* and *d*). This staining pattern is similar to that of normal epidermis, and suggests that the 56.5- and 65–67-kd keratins are expressed mainly during advanced stages of epithelial keratinization (cf. 68).

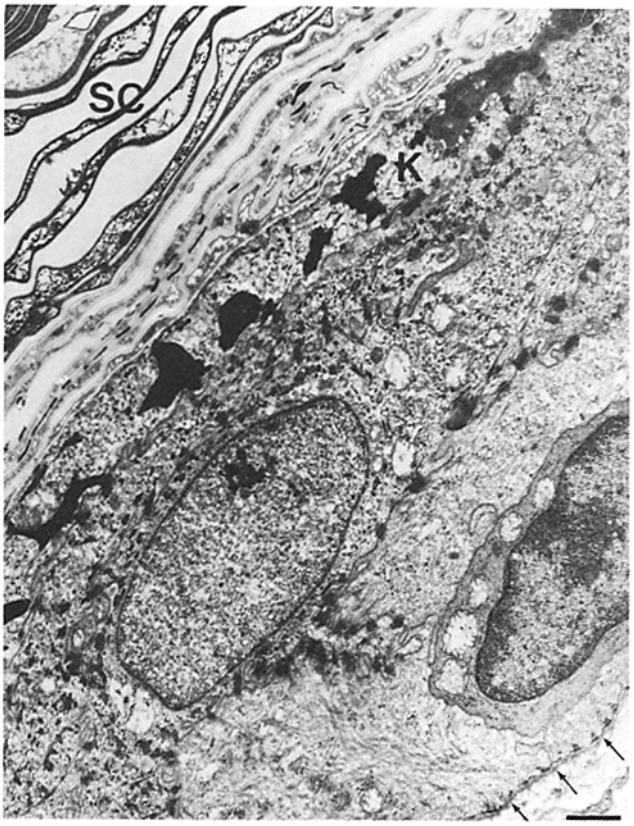


FIGURE 2 Electron microscopy of the epidermis from a vitamin A-deficient rabbit. SC: stratum corneum (with cornified cells); K: keratohyalin granules; Arrows: epidermo-dermal junction. Bar, 1 μm.

DISCUSSION

Morphological Keratinization

The morphological hallmarks of a fully keratinized epithelium (e.g., epidermis) include lamellar granules, keratohyalin granules, and eosinophilic, anucleated cornified cells (stratum corneum; 11, 33, 36, 40, 41). These cornified cells are characterized by a lack of synthetic activity, a specialized cellular envelope (cornified or crosslinked envelope; 21, 33), and a cytoplasm that is largely devoid of any cellular organelles except densely packed tonofilaments. The cornified cells contribute to the permeability barrier and provide physical protection to the underlying living layers, and are therefore functionally important (27).

Although it is customary to use the term "keratinization"[2] to describe the morphological changes that occur in various nonepidermal epithelia during vitamin A deficiency, our results indicate that different epithelia, even in the same rabbit, undergo different degrees of morphological keratinization. Of the three nonepidermal epithelia studied, corneal epithelium usually achieves the highest degree of morphological keratinization, forming abundant keratohyalin granules and anucleated cornified cells (Figs. 1*d* and 3, *b–d*; 60). Bulbar conjunctival epithelium also undergoes a significant degree of morphological keratinization (Fig. 4*b*; cf. references 1, 5). In contrast, esophageal epithelium shows only relatively minor morphological alterations (Figs. 1*h* and 5, *d* and *e*). These cell-type-specific responses to vitamin A deficiency are most likely due to the intrinsic divergence of their differentiation programs (9), and/or different sensitivities to vitamin A (23).

Biochemical Keratinization

The fact that all stratified epithelia can undergo various degrees of morphological keratinization (65) in a cell-type-specific fashion (Figs. 1–5) makes it sometimes difficult to determine, based on morphology alone, to what extent a particular nonepidermal tissue may be keratinized, and accordingly whether this tissue should be called, perhaps dogmatically, "keratinized" or "nonkeratinized."[3] It would therefore be desirable to be able to define the keratinization process in biochemical terms.

Using an in vivo rabbit model system, we have shown in this paper that an acidic 56.5- (AE1-reactive; acidic subfamily; PI 5.3; equivalent to the no. 10 human keratin of Moll et al. [34]) and a basic 65–67-kd keratin (AE3-reactive; basic subfamily; PI 6–8; nos. 1 and 2) are expressed by several rabbit nonepidermal epithelia during vitamin A deficiency-induced keratinization. The 65–67-kd keratin is also present in some other partially keratinized normal human epithelia (34, 35)[3] and is synthesized by human conjunctival epithelial cells cultured with delipidized serum (20). These results suggest that the 56.5- and 65–67-kd keratins are not epidermis-specific, but can also be expressed by other keratinocytes[4] during normal or vitamin A deficiency-induced keratinization.

The 65–67- and 56.5-kd keratins are expressed by cells in the suprabasal layers (Fig. 7; references 56, 68) which are conventionally thought to be terminally differentiated. Tritium-thymidine incorporation experiments provided clear evidence, however, that at least some of these suprabasally located cells are still capable of replicating (13, 29, 30, 42) and probably represent "transient amplifying cells" in the scheme of "stem cell → transient amplifying cell → terminally differentiated cell" (30, 43). Recently, Van Neste et al. (62) studied the expression of the 67-kd keratin by doing [^{3}H]-thymidine autoradiography and anti-67-kd keratin staining on the same epidermal sections (62). Their results illustrate that all suprabasal cells, including some replicating (transient amplifying) cells, are 67-kd keratin-positive, suggesting that the expression of at least this keratinization marker is cell

[2] The formation of eosinophilic, cornified cells (nucleated or anucleated) has been described as "cornification." This term may be distinguished from "keratinization," which is more restrictive and emphasizes the formation of granular cells and anucleated cornified cells. Since the 56.5 and 65–67 kd keratins are absent from psoriatic epidermis (see, e.g., 65) or rodent esophageal epithelium (Figs. 1, *g* and 6) that are clearly cornified but not keratinized, these two keratins may be related to keratinization, but not to cornification in general.

[3] The 65–67-kd keratin has been found in human thymic Hassall's corpuscles, which are morphologically keratinized (2, 35). This keratin has also been detected in normal exocervical epithelium and 14-wk old embryonic epidermis which, like rabbit esophageal epithelium undergoing vitamin A-deficiency-induced keratinization (Fig. 1*h*), show little or no sign of morphological keratinization (15, 35, B. Dale and K. Holbrook, in preparation). Taken together, these data suggest that biochemical keratinization (expression of 56.5- and 65–67-kd keratins) precedes morphological keratinization.

[4] "Keratinocyte" is the major cell type of all stratified squamous epithelia (21, 31, 49, 54). Although keratinocytes of various tissues can be specialized, they share the properties of (a) having a high keratin content (>30% of total cellular protein; 54); (b) synthesizing specific keratin molecules (e.g., the 50- and 58-kd keratins; 34, 38, 39, 57, 59); and (*c*) making involucrin and other precursors of the cornified envelope (21, 44, 51).

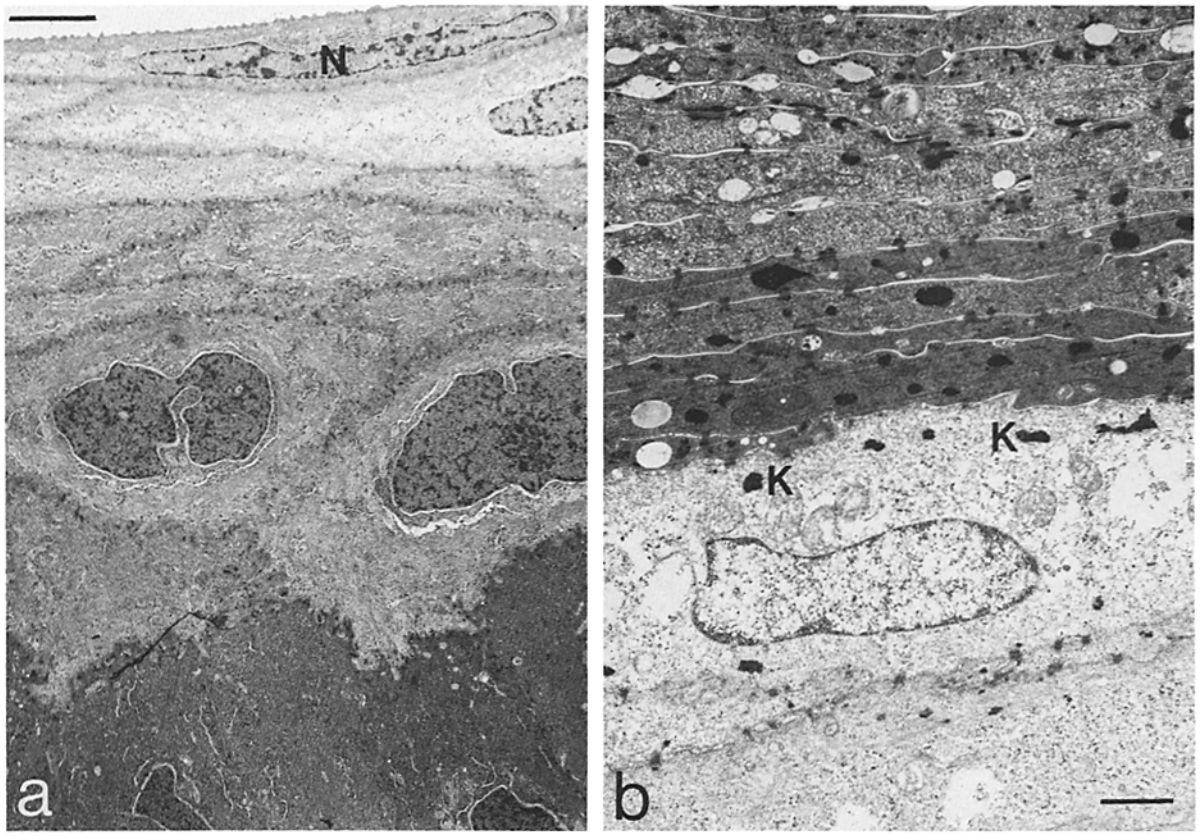


FIGURE 3 Electron microscopy of corneal epithelia from (a) control and (*b*) vitamin A-deficient rabbits. Note the presence of nuclei (*N*) in superficial cells of normal corneal epithelium, and the formation of keratohyalin granules (*K*) and anucleated stratum corneum during vitamin A-deficiency. Bars, 2 μm.

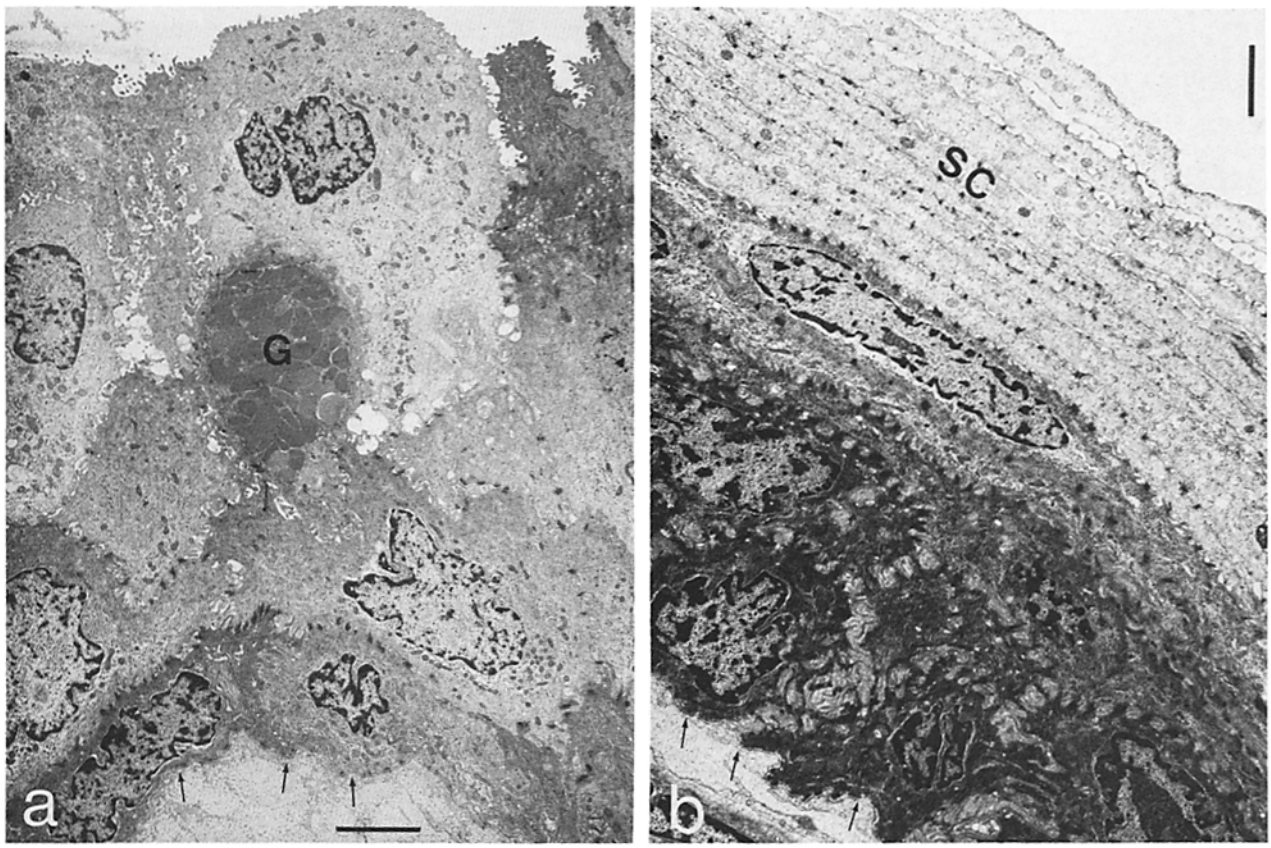


FIGURE 4 Electron microscopy of conjunctival epithelia from (*a*) normal and (*b*) vitamin A-deficient rabbits. Note in normal conjunctival epithelium the round, nonsquamous superficial cells, and mucous-filled goblet cells (*G*), and in vitamin A-deficient epithelium the lack of goblet cells and the appearance of the squamous, anucleated superficial stratum corneum (*SC*) cells. Arrows: epithelial-mesenchymal junction. Bars, 3 μm (*a*); 2 μm (*b*).

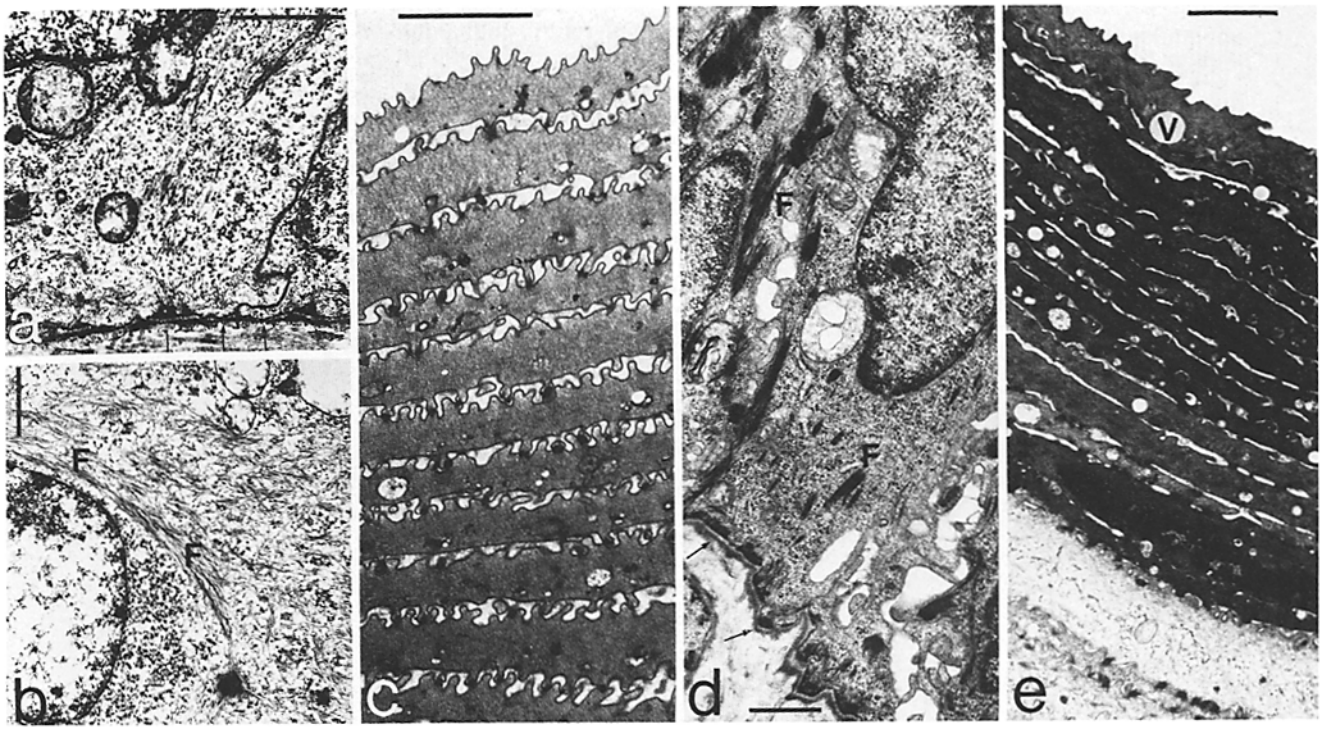
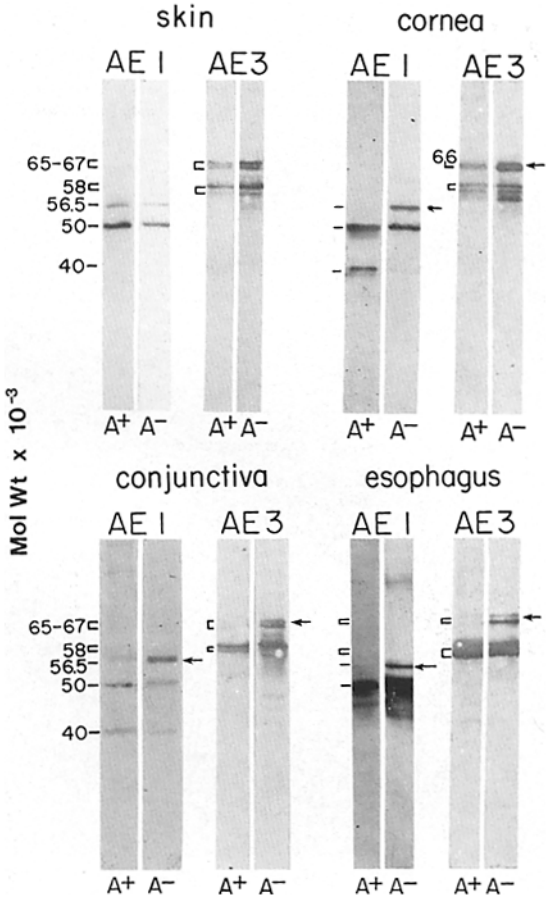


FIGURE 5 Electron microscopy of esophageal epithelia from (*a–c*) normal and (*d* and *e*) vitamin A-deficient rabbits. *a*, *b*, and *c* show the basal, intermediate, and superficial cells of normal esophageal epithelium, respectively. Note the sparse, loosely arranged keratin filaments (*F*) throughout the normal epithelium. *d* and *e* show the basal and superficial cells of vitamin A-deficient epithelia, respectively. Note the keratin filament bundles (*F*; 24) and the densely packed, darkly stained cornified cells. Also note in e the abrupt transition between the lower, living cells and the superficial cornified cells. Compared with the control, the cornified cells of A^- animals possess a much smoother cell surface and frequently contain vacuoles (*V*). Arrows in *a* and *d* denote epithelium-mesenchymal junction. Bars, 2 μm (*a*, *c*, and *e*); 1 μm (*b* and *d*).



position-dependent (basal vs. suprabasal; 68), and does not require the cells to be in the "terminally differentiated" compartment.

The detailed functions of the 56.5- and 65–67-kd keratin remain to be elucidated. Our present finding that the expression of 56.5- and 65–67-kd keratins can be correlated with the formation of densely packed keratin filaments (in cornified cells) suggests the possibility that these keratins may enhance filament-filament interaction or filament-matrix affinity (see, e.g., 7, 50). Ongoing experiments utilizing tonofilaments reconstituted with purified keratin components should allow us to test this hypothesis.

FIGURE 6 Immunoblot analysis of epithelial keratins using AE1 and AE3 monoclonal antikeratin antibodies. Water-insoluble cytoskeletal proteins from rabbit skin, corneal, conjunctival, and esophageal epithelia were separated by SDS PAGE, transferred to nitrocellulose sheets, and stained with AE1 or AE3 antibodies by the peroxidase-antiperoxidase technique. A^+ and A^- denote specimens from control and vitamin A-deficient rabbits, respectively. Note the detection of a 56.5- and a 65–67-kd keratin (arrows) in all A^- specimens by AE1 and AE3 antibodies, respectively. Also note the relative decrease in the AE1-positive 40-kd keratin (equivalent to no. 19 human keratin; [34]) in A^- corneal and conjunctival epithelia (20). The AE3-positive 66-kd band in normal corneal epithelium represents a cornea-specific keratin that is distinguishable from the 65–67-kd keratin by two-dimensional gel electrophoresis and by immunoreactivity with AE5 and AE6 monoclonal antibodies (Schermer, A. and T.-T. Sun, unpublished; cf. 6, 26, 34, 46).

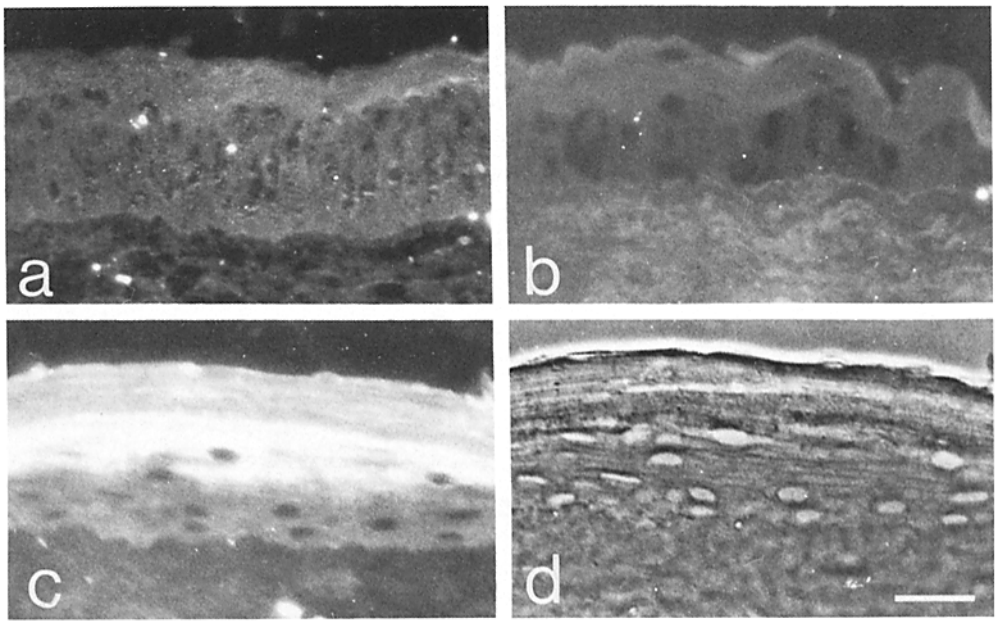


FIGURE 7 Indirect immunofluorescent staining of frozen sections of rabbit corneal epithelia with AE2 monoclonal antikeratin antibody. (a) Normal rabbit cornea stained with AE2 showing no reaction. (b) Vitamin A-deficiency specimen stained with P3 myeloma supernatant (as a control) showing no reaction. (c) Vitamin A-deficiency specimen stained with AE2 showing intense staining of cells above the basal layer. (d) Same field as c, phase contrast microscopy. Bar, 20 μm. × 500.

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Reprint requests should be sent to Dr. Tung-Tien Sun, Department of Dermatology, New York University School of Medicine, 550 First Avenue, New York, NY 10016.

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