Differentiation-related Expression of a Major 64K Corneal Keratin In Vivo and In Culture Suggests Limbal Location of Corneal Epithelial Stem Cells

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Abstract. In this paper we present keratin expression data that lend strong support to a model of corneal epithelial maturation in which the stem cells are located in the limbus, the transitional zone between cornea and conjunctiva. Using a new monoclonal antibody, AE5, which is highly specific for a 64,000-mol-wt corneal keratin, designated RK3, we demonstrate that this keratin is localized in all cell layers of rabbit corneal epithelium, but only in the suprabasal layers of the limbal epithelium. Analysis of cultured corneal keratinocytes showed that they express sequentially three major keratin pairs. Early cultures consisting of a monolayer of "basal" cells express mainly the 50/58K keratins, exponentially growing cells synthesize additional 48/56K keratins, and postconfluent, heavily stratified cultures begin to express the 55/64K corneal keratins. Cell separation experiments showed that basal cells isolated from postconfluent cultures contain predominantly the 50/58K pair, whereas suprabasal cells contain additional 55/64K and 48/56K pairs. Basal cells of the older, postconfluent cultures, however, can become AE5 positive, indicating that suprabasal location is not a prerequisite for the expression of the 64K keratin. Taken together, these results suggest that the acidic 55K and basic 64K keratins represent markers for an advanced stage of corneal epithelial differentiation. The fact that epithelial basal cells of central cornea but not those of the limbus possess the 64K keratin therefore indicates that corneal basal cells are in a more differentiated state than limbal basal cells. These findings, coupled with the known centripetal migration of corneal epithelial cells, strongly suggest that corneal epithelial stem cells are located in the limbus, and that corneal basal cells correspond to "transient amplifying cells" in the scheme of "stem cells → transient amplifying cells → terminally differentiated cells."

CORNEAL epithelium is a four- to five-layered, stratified squamous epithelium that has several unusual features. (*a*) To maintain its transparency, corneal epithelium is extremely flat with no papillary structures. Hence, the ratio between the basal and superficial surface areas approaches one; this corresponds to an exceptionally high demand on the regenerative capacity of the basal cells (Davanger and Evensen, 1971). (*b*) Because of the avascular nature of the underlying stroma, the bulk of corneal epithelium is remote from a capillary network, the nearest being located in the limbus which is the transitional zone between the cornea and conjunctiva (Hogan et al., 1971; Cogan and Kuwabara, 1973). (*c*) Corneal epithelium can be regenerated efficiently from the limbal epithelium and to a lesser extent from the conjunctival epithelium (Maumenee, 1964; Kinoshita et al., 1982). (*d*) The mitotic index of corneal epithelium tends to be higher toward the edge of the cornea (Friedenwald and Buschke, 1944). (*e*) Corneal epithelial neoplasms are in general quite rare, with most of the reported cases involving the limbus (Pizzarello and Jakobiec, 1978).

Recent studies indicate that specific keratin proteins can serve as excellent markers for different types or pathways of epithelial differentiation. Forming 10-nm filaments in almost all epithelia (Franke et al., 1978, 1979; Sun and Green, 1978 *b*; Sun et al., 1979), keratins are extremely complex. Immunological, biochemical, and cDNA analyses have established the existence of about 20 human keratins (Moll et al., 1982) that can be divided into an acidic (type I) and a basic (type II) subfamily (for recent reviews see Cooper et al., 1985; Fuchs et al., 1985; Quinlan et al., 1985). More important from an epithelial marker point of view, tissue distribution data have shown that acidic and basic keratins with similar "size ranks" within their respective subfamilies form specific "keratin pairs" as defined by frequent co-expression (Sun et al., 1984). According to this concept, the 56.5K acidic (Moll's catalogue no. 10 or HK10)[1] and 65–67K basic (HK1 and 2) keratins may be regarded as markers for "skin-

1. To facilitate a comparison of the rabbit and human data, we have identified keratins of both species not only by molecular weight, but also by catalogue numbers (Moll et al., 1982), prefixed when necessary by RK (rabbit keratin) or HK (human keratin) (see Table I and Fig. 1).

type" differentiation (keratinization), the 55K acidic (HK12) and 64K basic (HK3) keratins for "corneal-type" differentiation, the 51K acidic (HK13) and 59K basic (HK4) keratins for "esophageal-type" differentiation, the 50K acidic (HK14/15) and 58K basic (HK5) keratins for "keratinocytes and related cells," and the 48K acidic (HK16) and 56K basic (HK6) keratins for "hyperproliferative keratinocytes." Finally, the smaller 46K (HK17)/54K (HK7), 45K (HK18)/52K (HK8), and 40K (HK19) keratins are found as major components mainly in simple epithelia and thus may be viewed as markers for such cells (Sun et al., 1984, 1985; Cooper et al., 1985; also see Franke et al., 1981; Moll et al., 1982; Quinlan et al., 1985; Steinert et al., 1985).

Like many other keratinocytes, rabbit and human corneal epithelial cells undergo luxurious in vitro growth when plated in the presence of a lethally irradiated 3T3 feeder layer (Sun and Green, 1977; also see Rheinwald and Green, 1975). The differentiation of such cultured cells, however, has been shown to deviate significantly from the in vivo tissues (Doran et al., 1980). Thus cultured corneal epithelial cells express, in addition to the 55/64K (major) and 50/58K (minor) keratin pairs that are found in normal in vivo epithelium, the 48/56K and several other small keratins (Sun and Green, 1977; Doran et al., 1980; Gipson and Anderson, 1980; Franke et al., 1981; Kinoshita et al., 1983). These results, in conjunction with some recent data on cultured human epidermal cells and epidermal diseases (Eichner et al., 1984; Weiss et al., 1984), suggest that cultured corneal epithelial cells may serve as a model for studying hyperproliferative corneal epithelium.

Using a new monoclonal antibody, AE5, that is highly specific for the 64K corneal keratin, coupled with cell separation studies, we show here that the 55/64K corneal marker keratins are suprabasally located in corneal epithelial colonies (<10 d old) as well as in the limbal (edge) region of corneal epithelium. In contrast, basal cells of central corneal epithelium were found to be 64K keratin positive, suggesting that these corneal "basal" cells are in a more advanced state of differentiation than is currently appreciated. These results lend strong support to a model in which the stem cells of corneal epithelium are strategically located in the limbal area. This model provides explanations for many of the unusual properties of corneal epithelium, and has implications on the mechanisms of normal and abnormal corneal epithelial differentiation.

Materials and Methods

Monoclonal Antibody Production

Monoclonal antibodies highly specific for the corneal keratins were prepared as follows. BALB/c mice were repeatedly immunized by subcutaneous injection of 120 μg of SDS-denatured rabbit corneal epithelial keratins mixed with complete (primary) or incomplete (booster) Freund's adjuvants. Spleen cells from immunized mice were fused with P3X63Ag8.653 myeloma cells (kindly provided by Dr. M. Scharff of Albert Einstein College of Medicine) using 50% polyethylene glycol 4000 (Merck), grown in the presence of macrophage feeder cells, and selected with hypoxanthine/aminopterin/thymidine (HAT) medium (Kohler and Milstein; 1975). Anti-keratin antibody activities were differentially screened by the enzyme-linked immunosorbent assay (Woodcock-Mitchell et al., 1982) against both rabbit corneal and human epidermal keratins. Cells secreting corneal-specific antibodies were cloned by serial dilution.

The preparation and characterization of AE1 and AE3 antibodies have been described in detail elsewhere (Woodcock-Mitchell et al., 1982; Tseng et al., 1982; reviewed in Sun et al., 1984, 1985).

Culture of Rabbit Corneal Epithelial Cells

Corneal epithelia were isolated from young (2–3 kg), female albino rabbits by incubating the cornea (limbus excluded) in 0.1% trypsin (Gibco, Grand Island, NY), 0.01% EDTA in phosphate-buffered saline (PBS) for 30 min at 37°C, followed by gentle scraping of the corneal surface. The detached epithelial sheets were dispersed into a single cell suspension by pipetting. Epithelial cells from each cornea were plated (in Dulbecco's modified Eagle's medium containing 17% fetal calf serum and 5μg/ml hydrocortisone) into ten 60-mm dishes in the presence of mitomycin-treated 3T3 feeder cells (Rheinwald and Green, 1975; Sun and Green, 1977; Doran et al., 1980). These dishes usually reached their maximal cell number in 10–11 d; purity of such confluent cultures exceeds 95% according to keratin staining (Sun and Green, 1978*b*).

Keratin Extraction

Corneal epithelial cultures were treated briefly with 0.02% EDTA in PBS to remove the remaining 3T3 feeder cells and any contaminating corneal stromal cells (usually <3% of cells). The firmly attached epithelial cells were then extracted with 25 mM Tris-HCl (pH 7.4), 0.6 M KCl, 1% Triton X-100 plus a mixture of five protease inhibitors to remove the water-soluble proteins (Woodcock-Mitchell et al., 1982). The remaining cytoskeletal preparation containing mainly keratins was finally solubilized with either 1% SDS or 9 M urea in 25 mM Tris-HCl (pH 7.4).

Gel Electrophoresis and Immunoblotting

One- and two-dimensional SDS gel electrophoreses were performed according to Laemmli (1970) and O'Farrell et al., (1977), respectively. Immunoblotting was done according to Towbin et al., (1979), with some modifications so that the staining patterns generated by both fast green and the peroxidase–anti-peroxidase reaction can be recorded photographically from the same blot (Woodcock-Mitchell et al., 1982; Eichner et al., 1984).

Isolation of Basal and Suprabasal Cells

Post-confluent cultures of rabbit corneal epithelial cells were treated at 37°C for 5 h with 1.4 mM EGTA (the precise concentration varies slightly with different batches of serum) in calcium-free Dulbecco's modified Eagle's medium (M. A. Bioproducts, Walkersville, MD) containing 17% fetal calf serum. Vigorous pipetting with the medium resulted in the clean detachment of suprabasal layers, leaving a monolayer of basal cells adhering to the dish (see Fig. 10). This procedure also worked well for isolating the basal cells from cultured rabbit esophageal epithelial cells (Schermer, A., J. Yu, C. Hardy, and T.-T. Sun, manuscript in preparation).

Immunofluorescent Staining Techniques

Cells were grown on 12-mm glass coverslips, fixed and permeabilized with methanol at 4°C for 30 min, and stained with antibodies (singularly or doubly) as described (Sun and Green, 1978*b*, 1979; O'Guin et al., 1985). Intact sheets of post-confluent corneal epithelial cells were detached from the dish by scraping, mounted in OCT medium (Miles Scientific, Naperville, IL), cryostat sectioned at 7 μm, and stained by standard procedures.

Results

Rabbit Epithelial Keratins Are Homologous to Human Keratins

Although most of our previous work on keratin expression has been carried out on human epithelia (for reviews see Sun et al., 1984, 1985), we have chosen to use the rabbit as our experimental animal for studying corneal epithelium not only because of the feasibility of performing in vivo experiments, but also because a detailed analysis of rabbit keratins showed that they were strikingly similar to human keratins

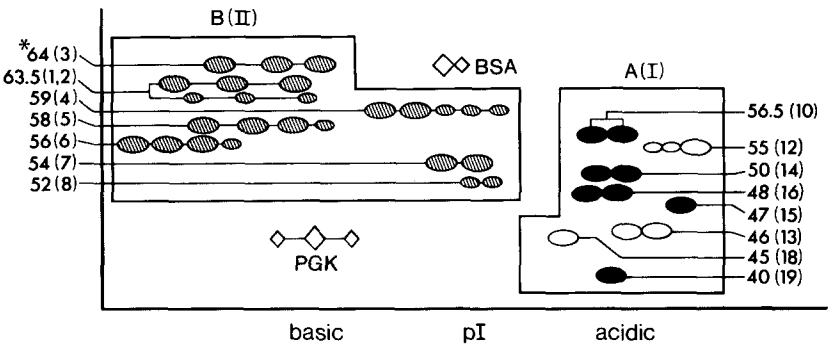

Figure 1. A schematic diagram of the two-dimensional electrophoretic pattern of all known rabbit epithelial keratins. Keratins are identified by molecular weights (*K*) (that is, *64* is the 64,000-mol-wt keratin, etc.) and are divided into the acidic (*A*; type I) and basic (*B*; type II) subfamilies according to their immunoreactivities. Note that all basic keratins are recognized by AE3 (hatched ovals); many but not all acidic keratins are recognized by AE1 (black ovals). Numbers in parentheses denote catalogue numbers of Moll et al. (1982) for the corresponding human keratins, as determined by their expression patterns (see Table I). *pI*, isoelectric point; *BSA*, bovine serum albumin; *PGK*, 3-phosphoglycerate kinase. Asterisk (*) denotes the AE5-reactive, 64K corneal keratin.

(Fig. 1; Table I), thus allowing us to compare our rabbit data with the available information on human keratins.

AE5 Antibody Is Highly Specific for a 64K Rabbit Corneal Keratin

To generate probes for studying corneal epithelial differentiation, we set out to make monoclonal antibodies specific for the 55K (RK12)/64K (RK3) corneal marker keratins. After fusing mouse myeloma cells with splenocytes from BALB/c mice that were immunized with rabbit corneal keratins, we assayed the hybridoma supernatants by the enzyme-linked immunosorbent assay against both rabbit corneal and human epidermal keratins. Most of the antibodies were found to cross-react with both antigens; cells secreting such broadly reactive antibodies were discarded. Three hybridoma lines (AE5, AE6, and AE7) showing preferential binding to corneal keratins were cloned and used for subsequent studies.

One- and two-dimensional immunoblotting showed that AE5 antibody was highly specific for a basic 64K rabbit keratin (RK3) that was present in both normal rabbit corneal epithelium (Fig. 2, *a* and *b*, lane *1*; Fig. 3) and cultured rabbit corneal epithelial cells (Fig. 2, *a* and *b*, lane *2*). In cultured corneal cells, this AE5 antigen exhibited a typical filamentous configuration (Fig. 4 *a*) which can be converted into a net-like structure in cells that had been treated with cytochalasin B (Fig. 4 *b*; Knapp et al., 1983); these results confirmed the keratin nature of the 64K antigen. This keratin was undetectable, however, in all the non-corneal tissues tested (Fig. 2 *b*, lanes *3–12*). Similar results were obtained

Table I. Classification and Expression of Rabbit Epithelial Keratins

Keratin Subfamily							
Acidic (type I)			Basic (type II)				
Molecular weight ($\times 10^{-3}$)	No.	Ab	Molecular weight ($\times 10^{-3}$)	No.	Ab	dMW $\times 10^{-3}$ (B–A)	Markers for
56.5	RK10	AE1 AE2	63.5 (65–67)	RK1,2	AE3 AE2	7	Skin-type differentiation
55	RK12	–	64	RK3	AE3 AE5	9	Cornea-type differentiation
46 (51)	RK13	– AE8	59	RK4	AE3	13	Esophageal-type differentiation
50/47 (50/50′)	RK14/15	AE1	58	RK5	AE3	8	Keratinocyte and related cells
48	RK16	AE1	56	RK6	AE3	8	Hyperproliferative keratinocytes
45	RK18	–	52	RK8	AE3	7	"Simple" epithelia
40	RK19	AE1					

This table is based on an analysis of keratins from rabbit skin, cornea, esophagus, bladder, trachea, mesothelium, intestine, and cultured skin, corneal and esophageal keratinocytes (Fig. 2; also see Tseng et al., 1982, 1984; Cooper et al., 1984; O'Guin et al., 1986). The catalogue numbers (*No.*) are those of the corresponding human keratins (Moll et al., 1982). The molecular weights of most of the rabbit keratins are nearly identical to the corresponding human keratins except the 46K, 47K, and 63.5K keratins (in these cases the human molecular weights are shown in parentheses). Although not listed, all rabbit keratins react with the anti-intermediate filament antibody (Pruss et al., 1981; Cooper et al., 1984); – denotes a lack of AE1 reactivity; and AE8 data are from Schermer, A., J. Yu, C. Hardy, and T.-T. Sun (manuscript in preparation). Note the extremely small size of the RK13 (46K) as compared with the corresponding HK13 (51K); an unusually small esophageal, acidic keratin marker has also been found in cow (BK13, 43K, AE8 positive; Cooper and Sun, 1986). *AB*, antibody reactivity; *dMW* $\times$ 10^{-3} *(B-A)*, molecular weight difference between the basic and acidic members of a keratin pair.

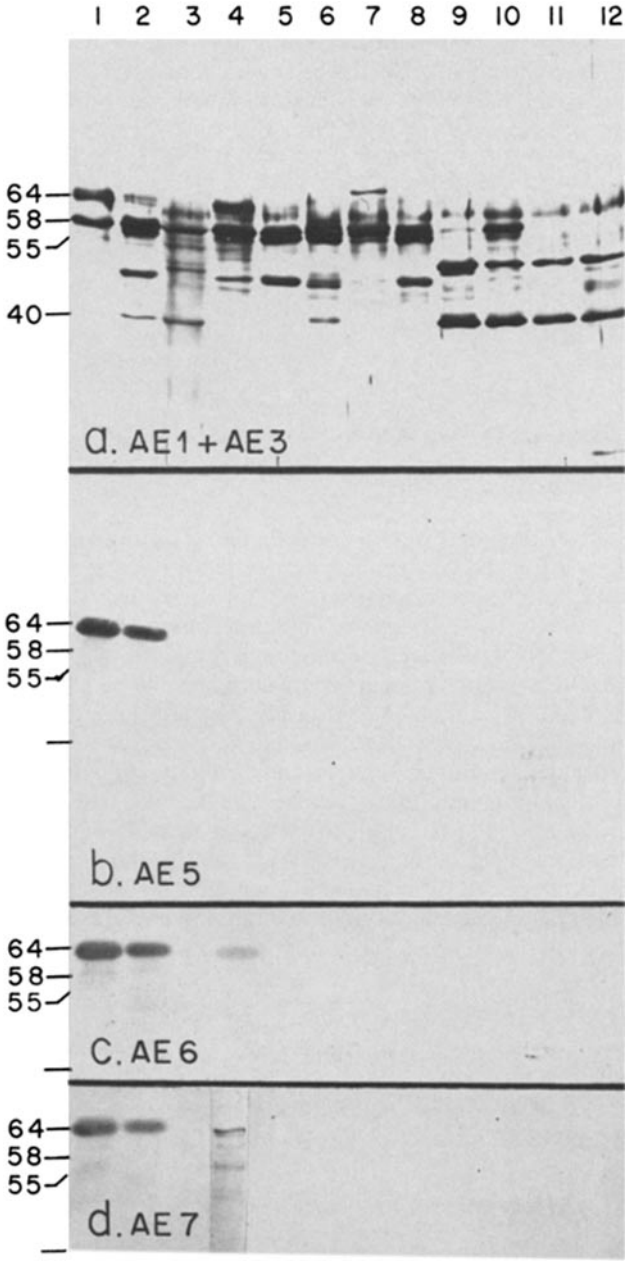

Figure 2. One-dimensional immunoblot analyses of rabbit epithelial keratins. (*a*) Blotted with a mixture of AE1 and AE3 antibodies; these two antibodies, in combination, allow the detection of most mammalian epithelial keratins (Tseng et al., 1982; Sun et al., 1984). (*b*) AE5. (*c*) AE6. (*d*) AE7. Rabbit keratins from (lanes *1*) corneal epithelium in vivo; (lanes *2*) cultured corneal epithelial cells; (lanes *3*) conjunctival epithelium; (lanes *4*) epidermis; (lanes *5*) cultured epidermal cells; (lanes *6*) cultured human epidermal cells included as a reference; (lanes *7*) esophageal epithelium; (lanes *8*) cultured esophageal epithelial cells; (lanes *9*) bladder epithelium; (lanes *10*) tracheal epithelium; (lanes *11*) mesothelium; and (lanes *12*) intestinal epithelium. Note the strong reaction of AE5, AE6, and AE7 with a 64K (RK3) corneal keratin and the weak cross-reaction of AE6 and AE7 with a 63.5K (RK1 or 2) epidermal keratin. The 55K corneal keratin reacts with neither AE1 nor AE3 (see Table I), and is therefore not detected in *a*.

with AE6 and AE7 antibodies, although these two antibodies were less specific because they cross-reacted significantly with a 63.5K (RK1/RK2) epidermal keratin (Fig. 2, *c* and *d*; lane *4*).

AE5 Antibody Stains Limbal Epithelium Suprabasally but Corneal Epithelium Uniformly

To study the expression of the 64K keratin in normal epithelium, we used AE5 antibody to stain frozen sections of rabbit cornea and neighboring tissues. Strong staining was observed in corneal epithelium, with no staining in the adjacent conjunctival epithelium (Fig. 5). Interestingly, the detailed AE5 staining pattern in corneal epithelium varied depending on anatomical location. While central corneal epithelium stained uniformly (basal cells included; Fig. 5 *a*), the limbal portion of the epithelium stained suprabasally (Fig. 5 *b*; for similar results obtained with AE6 and AE7 antibodies, see Fig. 5, *d* and *e*). These results suggest that the mode of 64K keratin expression may vary (suprabasally versus uniformly) in different parts of the cornea. Since we know from our

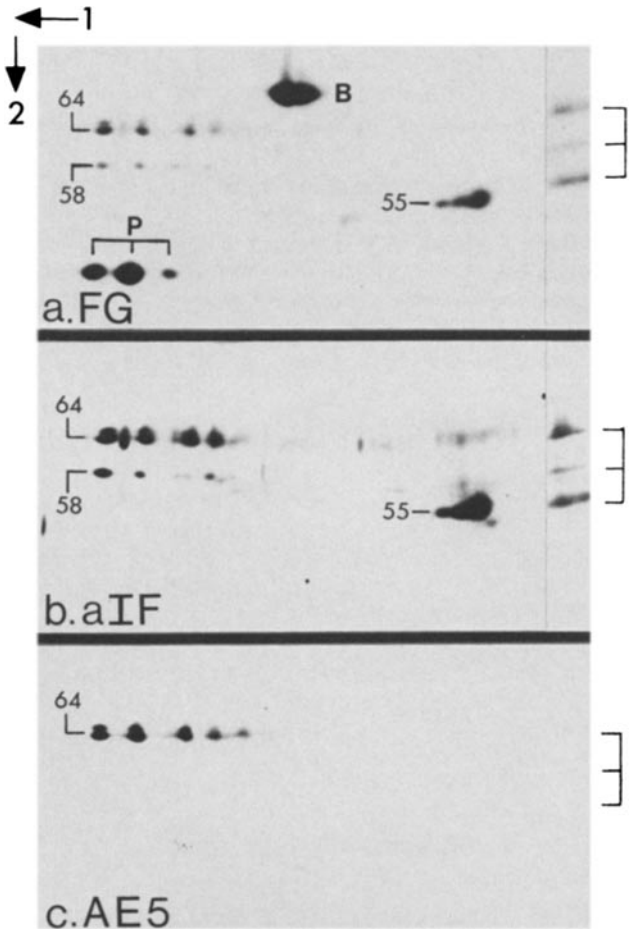

Figure 3. Two-dimensional immunoblot analyses of rabbit corneal keratins. (*a*) Fast green staining showing the major 55/64K keratins and the minor 58K keratin (50K keratin is barely detectable; Tseng et al., 1982). *B* and *P* denote bovine serum albumin and 3-phosphoglycerate kinase, respectively, which were added as references. (*b*) Anti-intermediate filament antibody. Note the identification of all corneal keratins (Pruss et al., 1981; Cooper et al., 1984). (*c*) AE5 staining. Note strong staining of all the (presumably phosphorylated; Vidrich et al., 1985) isomers of the 64K keratin. Top left arrows denote the directions of (*1*) first dimensional nonequilibrium pH gradient gel electrophoresis from acidic to basic, and (*2*) second dimensional SDS PAGE (O'Farrell et al., 1977). Marks on right indicate positions of the 64K, 58K, and 55K keratins. Compare with Fig. 1.

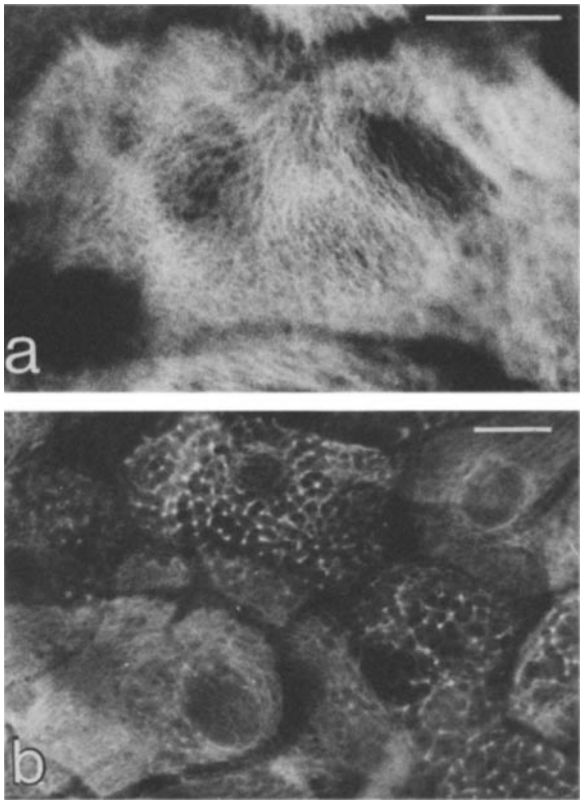

Figure 4. Immunofluorescent staining of cultured rabbit corneal epithelial cells with AE5. (*a*) Some upper cells located in the center of a large colony demonstrating filamentous staining of keratin fibers. (*b*) Cells that have been treated with cytochalasin B (10 μg/ml for 2 h) before fixation, many showing a net-like staining pattern that is typical of keratins in such drug-treated cells (Knapp et al., 1983). Bars, 20 μm.

previous study (Woodcock-Mitchell et al., 1982) that the expression of another high molecular weight keratin pair (the 56.5/65–67K markers for "skin-type" differentiation) is primarily suprabasal, these results regarding cornea were unexpected. To better understand the biological meaning of this finding, we studied keratin expression in cultured rabbit corneal epithelial cells at different stages of growth and differentiation.

Sequential Expression of 50/58K, 48/56K, and 55/64K Keratin Pairs in Cultured Rabbit Corneal Epithelial Cells

Rabbit corneal keratinocytes form stratified colonies in the presence of lethally irradiated or mitomycin-treated 3T3 feeder cells (Sun and Green, 1977). One- and two-dimensional immunoblot analyses showed that in early cultures (<6 d), which consisted of a monolayer of "basal" cells, there existed predominantly a 50K (RK14)/58K (RK5) keratin pair (Fig. 6, lanes *1–2*). This pair was later supplemented by a 48K (RK16)/56K (RK6) pair when the cells entered a period of exponential growth (days 7–8; Fig. 6, lanes *3–4*; for two-dimensional gel pattern, see Fig. 7, *a* and *a'*), and finally by a 55K (RK12)/64K (RK3) keratin pair when the colonies became heavily stratified (>9 d; Fig. 6, lanes *5–6*; for two-dimensional gel pattern of an 11-d culture, see Fig. 7, *b* and *b'*).

Immunofluorescent staining provided independent support for the differentiation-dependent expression of the 64K keratin. Rabbit corneal epithelial cells were fixed after 5, 8, and 11 d in culture, and double stained with both a broadly reactive rabbit antiserum to keratin (Fig. 8, *a*, *c*, and *e*) and mouse AE5 antibody (Fig. 8, *b*, *d*, and *f*). The results indicated that AE5-positive cells were mainly located in the suprabasal layers of post-confluent, stratified cultures (Figs. 8 and 9).

Basal Cells Contain Primarily 50/58K Keratins, Whereas Suprabasal Cells Contain Additional 55/64K and 48/56K Keratins

The suprabasal staining of cultured cells by AE5 did not prove unequivocally that the 64K antigen was absent from the basal layer because of the possibility of antigenic masking (Woodcock-Mitchell et al., 1982; Franke et al., 1983). To circumvent this problem, we wanted to isolate the basal cells from a well-stratified culture (11 d) in order to perform direct keratin analysis. This was accomplished by treating the culture for 5 h with a medium containing 1.4 mM EGTA (see Materials and Methods). The suprabasal cells could then be dislodged by pipetting, leaving behind a well-defined basal layer (Fig. 10). Consistent with the results on 5-d cultures (Fig. 6, lane *1*), SDS PAGE and immunoblotting analyses showed that the basal cells from 11-d cultures also contained primarily the acidic 50K and basic 58K keratins (Fig. 11, lane *1*). In contrast, suprabasal cells contained, in addition to the 50/58K keratins, the 55/64K "corneal" keratins and the 48/56K "hyperproliferation" keratins (Fig. 11, lane 2). Moreover, these suprabasal cells also contained an increased amount of the 45/52K and 40K "simple epithelial" keratins (Fig. 11, lane 2).

Can Basal Cells Ever Express the 64K Keratin?

The suprabasal location of the 55/64K keratins in cultured cells suggests that they may be associated with an advanced stage of corneal epithelial differentiation. Since it is known from earlier studies of cultured human epidermal keratinocytes that basal cells can, under certain experimental conditions, acquire differentiated properties (Watt and Green, 1981), we prepared basal cells by the EGTA technique from cultures that had been maintained post-confluently for various periods of time, and stained them with AE5. In older cultures (>15 d), an increasing proportion of basal cells were found to become AE5-positive (Fig. 12), proving that suprabasal location is not an absolute requirement for the cells to express the 64K keratin marker. This finding (that AE5 stained basal cells of only older cultures) raised the possibility that the limbal and central corneal epithelia may correspond to the "young" and "old" corneal epithelial colonies, respectively (see Discussion).

Discussion

The 50/58K Keratin Pair Is First Expressed by Basal Cells

We have demonstrated here that the 50/58K keratins are the predominant cytoskeletal proteins not only in early corneal epithelial cultures before they undergo stratification (Fig. 6, lanes *1* and *2*), but also in basal cells isolated from a highly

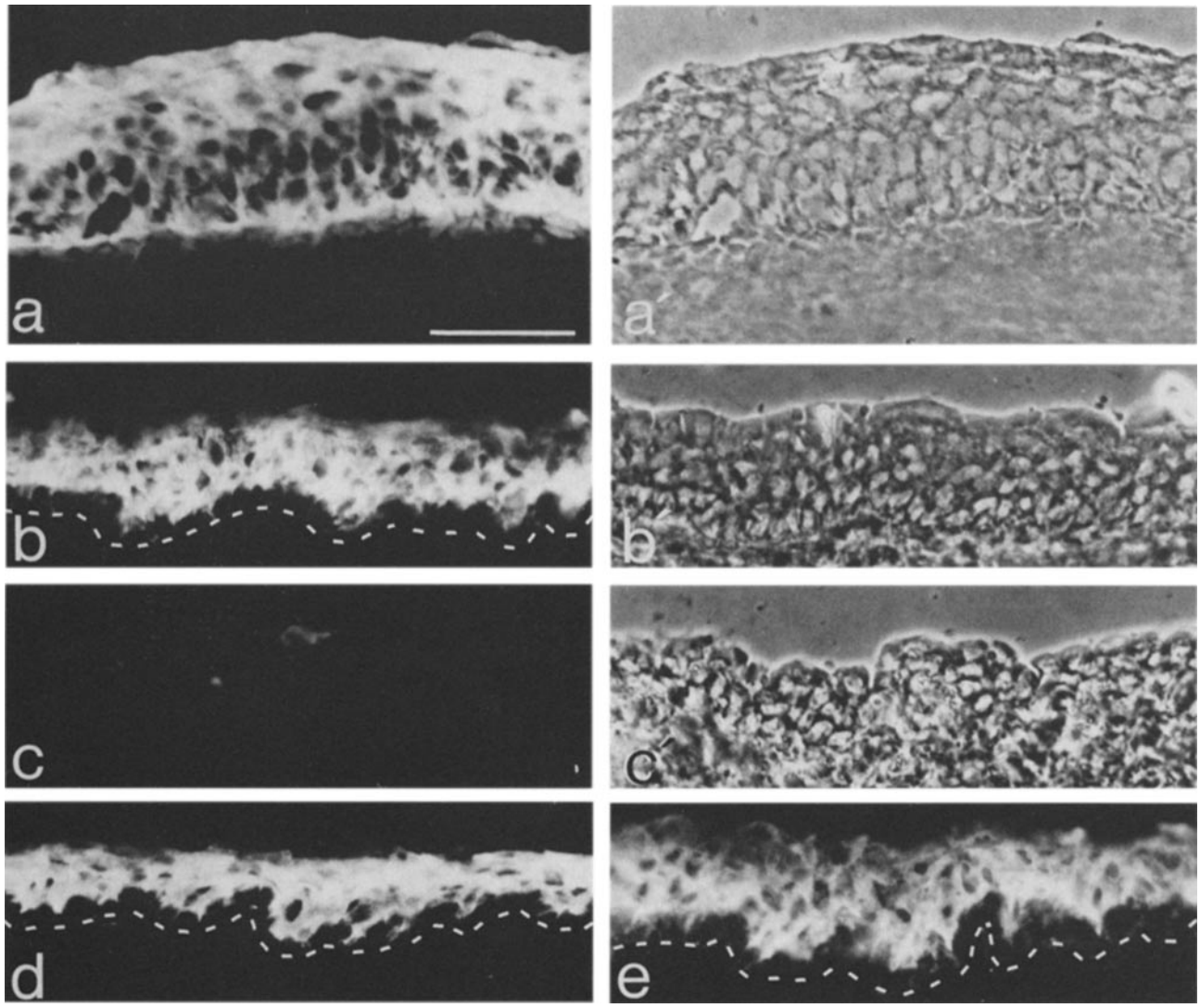

Figure 5. Immunofluorescent staining of frozen rabbit corneal sections. *a* to *e* are immunofluorescent micrographs; *a'–c'* are corresponding phase-contrast images of *a–c*, respectively. (*a*) AE5 staining of rabbit corneal epithelium. Note the staining of the entire epithelium, basal cells included. (*b*) AE5, limbal epithelium. Note suprabasal staining. (*c*) AE5, conjunctival epithelium showing no significant staining. (*d*) AE6, limbal epithelium. (*e*) AE7, limbal epithelium. Although AE6 and AE7 cross-react with some epidermal keratins, in cornea these two antibodies are monospecific for the 64K keratin (Fig. 2 and data not shown). Dashed lines in *b*, *d*, and *e* correspond to the location of epithelial–stromal junction. Bar, 50 μm.

stratified culture (Fig. 11, lane *1*). Interestingly, these two keratins have also been found to represent the major intermediate filament subunits in epidermal basal cells both in man (Woodcock-Mitchell et al., 1982; Skerrow and Skerrow, 1983) and in mouse (Breitkreutz et al., 1984; Schweizer et al., 1984). Since an acidic and a basic keratin are known to be required for 10-nm filament formation, and since the 50K and 58K keratins can form a highly stable complex (Franke et al., 1983; Hatzfeld and Franke, 1985; Eichner et al., 1986), our results support the idea that these two keratins may interact with each other to form tonofilaments in the basal cells of all 50/58K-positive epithelia (Woodcock-Mitchell et al., 1982).

Our keratin localization data from the present work and from a previous study (Woodcock-Mitchell et al., 1982) are in disagreement with a recent claim that basal cells of normal human epidermis contain 50/58K plus the 48/56K and 46K keratins (Fuchs, 1983; Kim et al., 1984; Fuchs et al., 1985). This claim was based on the assumption that long-term cultured epidermal cells represent a pure population of basal cells ("cultured basal cells"; Kim et al., 1984) and therefore all keratins made by such cultured cells must be present in the basal layer of in vivo epidermis. However, this does not take into account the fact that some of the major keratins synthesized by cultured keratinocytes (i.e., the 46K, 48K, and 56K components) are not present at all in normal trunk epidermis (Fuchs and Green, 1978; Eichner et al., 1984). Moreover, cultured keratinocytes do stratify (Rheinwald and Green, 1975; Sun and Green, 1976) and can in some cases undergo their own "basal-to-suprabasal" transition in keratin synthesis (Figs. 9 and 11).

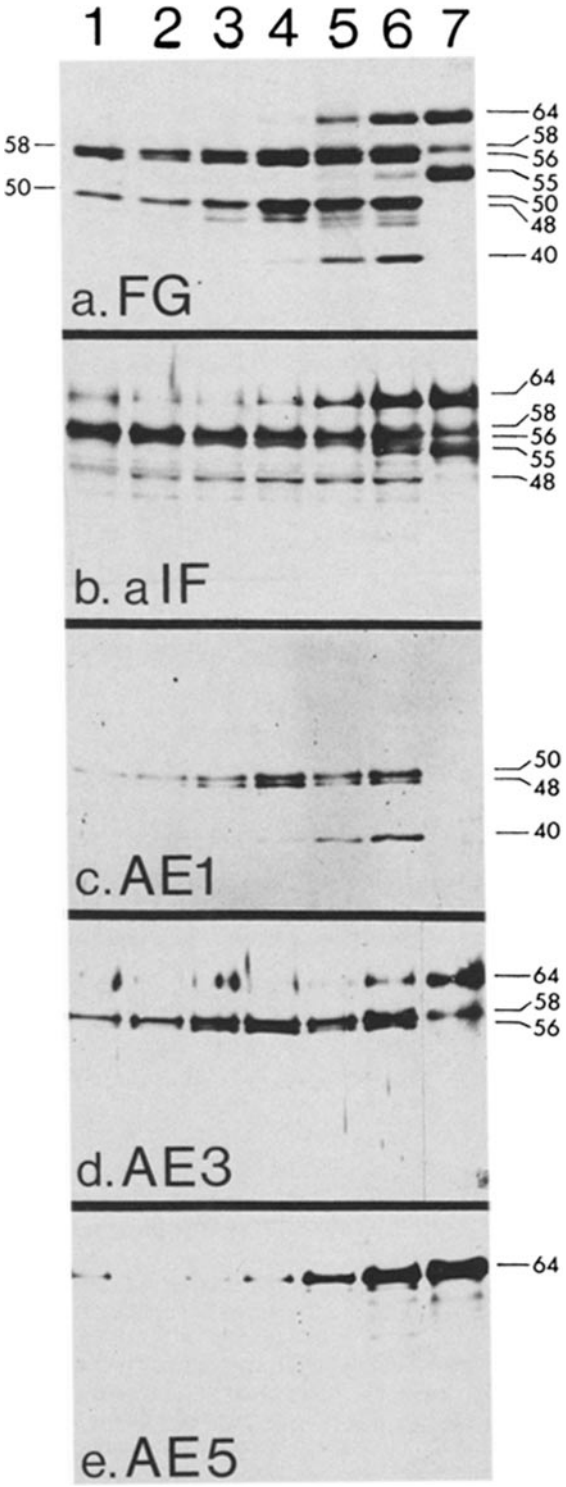

Figure 6. Immunoblot analyses of keratins from cultured rabbit corneal epithelial cells during successive stages of growth. (*a*) Fast green. (*b*) Anti-intermediate filament antibody (known to recognize all keratins, albeit with heterogeneous affinity) (Pruss et al., 1981; Cooper et al., 1984). (*c*) AE1. (*d*) AE3. (*e*) AE5. Lanes *1* to *6* represent samples of days 5, 6, 7, 8, 9, and 11, respectively; lane *7* is in vivo rabbit corneal epithelial keratin. Note that in *a*, 5- and 6-d samples contain, in addition to the AE1-positive 50K and AE3-positive 58K keratins, a "56K" fast green–stained band. This band is identified as vimentin (from 3T3 feeder cells which are difficult to remove completely from early cultures) because it is anti-intermediate filament antibody positive (*b*) but AE3 negative (*d*), and because of its position in 2-D gels (Fig. 7).

The 48/56K Keratin Pair Is Localized Primarily in Suprabasal Cells

When the cells enter a phase of exponential growth they begin to express the 48/56K keratin pair (Fig. 6, lanes *3* and *4*), which, as we and others have shown previously, is characteristic of cultured keratinocytes (Sun and Green, 1977; Nelson and Sun, 1983) as well as various hyperproliferative keratinocyte diseases (McGuire et al., 1984; Moll et al., 1984; Nelson et al., 1984; Weiss et al., 1984). Our cell separation data indicate that in an 11-d confluent culture, these two keratins are mainly located in the suprabasal layers (Fig. 11, lane *2*). This finding is consistent with our earlier observation that AE1, which shows a relatively strong reaction with the human 48K keratin, stains human psoriatic epidermis suprabasally (Weiss et al., 1983).

The 55/64K Keratin Pair Is Present Mainly in Suprabasal Cells

In more advanced cultures (>9 d old), corneal epithelial cells begin to express suprabasally the 55/64K keratin pair which, as we have suggested recently, may be regarded as a marker for "corneal-type" differentiation (Figs. 6, 9, and 11; Cooper et al., 1985; Cooper and Sun, 1986).[2]

The fact that basal cells of normal corneal epithelium contain the 64K keratin (Figs. 5 *a* and 13 *a*) strongly suggests that the expression of this keratin does not signal that such cells are in a "terminally" differentiated state, because they can still undergo a significant degree of in vivo and in vitro proliferation (Maumenee, 1964; Sun and Green, 1977). Once plated in culture, these AE5-positive basal cells can become AE5 negative (Figs. 6–9, 11), apparently as a consequence of antigen dilution through cell proliferation. Our results from both time course studies (Fig. 6) and some preliminary [^{35}S]methionine incorporation experiments (data not shown) suggest that, on a tissue level, the expression of the 55/64K pair (markers for corneal type differentiation) and that of the 48/56K pair (markers for "hyperproliferative" keratinocytes) are roughly reciprocal. This is analogous to human epidermis where the expression of the 56.5/65–67K keratins (markers for skin-type differentiation) was found to be approximately reciprocal to that of the 48/56K markers (Weiss et al., 1984). Taken together, these observations support our earlier suggestion that, on a cellular level, the expression of the "hyperproliferation" markers and that of the "differentiation-related" markers may be mutually exclusive (Weiss et al., 1984). This possibility will have to be tested eventually by in situ hybridization once all the appropriate cDNA probes become available.

Simple Epithelial Keratins

Like many other keratinocytes, cultured rabbit corneal epithelial cells express a significant amount of simple epithelial (small molecular weight) keratins including the 45/52K and 40K components (Moll et al., 1982; Wu et al., 1982; Eichner et al., 1984). While our data clearly establish that suprabasal cells possess large quantities of these proteins, we are not

2. We have recently shown that cow snout epithelium, which is a highly specialized, non-epidermal tissue, contains the AE5-positive, 64K "corneal" keratin (Cooper and Sun, 1986). This finding suggests that the 64K keratin is not strictly corneal specific, and that snout cells also undergo, at least in part, "corneal-type" differentiation (Cooper et al., 1985).

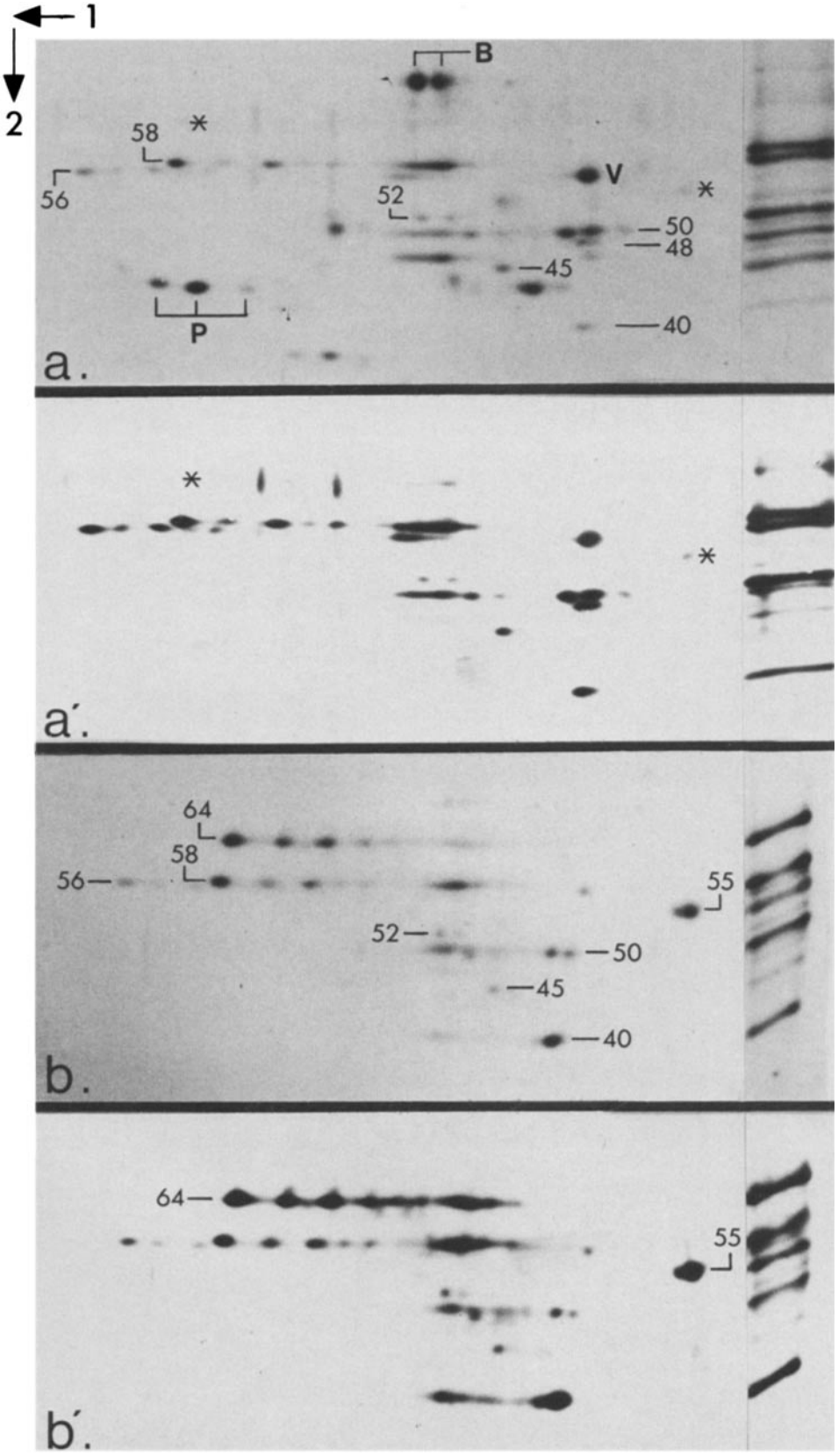

Figure 7. Two-dimensional immunoblot analyses of keratins from cultured rabbit corneal epithelial cells. (*a* and *a'*) 7-d culture; (*b* and *b'*) 11-d culture. (*a* and *b*) Fast green staining; (*a'* and *b'*) immunoblot performed on the same blots as shown in *a* and *b* using a mixture of the anti-intermediate filament antibody (see Fig. 6 *b*) and AE1 monoclonal anti-keratin antibody. Note the large quantities of the 55K and 64K corneal marker keratins in *b* and *b'*, and their absence from *a* and *a'*. The horizontal smears under the bovine serum albumin (*B*) marker are keratins involved in complex formation (Franke et al., 1983). *P*, 3-phosphoglycerate kinase also added as a marker. *V*, vimentin as detected by the anti-intermediate filament antibody in the 7-d sample most likely due to the incomplete removal of feeder cells. Asterisks in *a* and *a'* denote the position of the 55K and 64K keratins. For the relative position of various keratins, compare with Fig. 1.

sure whether the small amounts of such keratins found in the basal cell fraction are truly synthesized by these cells or due to incomplete cell separation. Additional immunolocalization data (cf. Figs. 8 and 9) using highly specific antibodies will be required to clarify this point.

Within a Keratin Pair the Synthesis of the Basic Member Can Precede That of the Acidic Member

Although the 55K and 64K keratins are present in roughly equimolar quantities in normal corneal epithelium (Fig. 6,

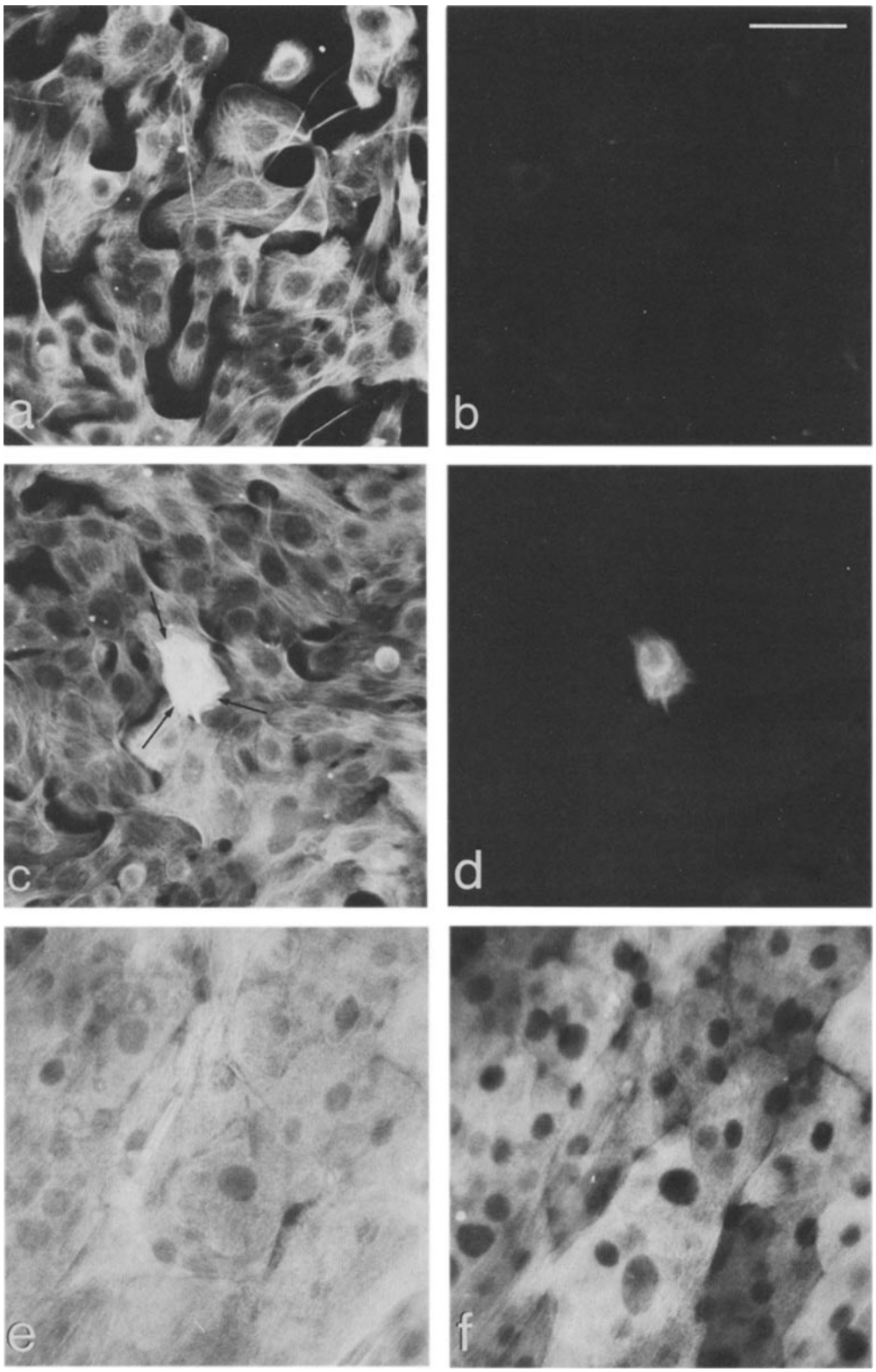

Figure 8. Double immunofluorescent staining of cultured rabbit corneal epithelial cells during successive stages of growth with a broadly reactive rabbit anti-keratin antiserum (*a*, *c*, and *e*) and the 64K keratin-specific mouse AE5 antibody (*b*, *d*, and *f*). Cell cultures were fixed on days 5 (*a* and *b*), 8 (*c* and *d*), and 11 (*e* and *f*) and processed for double immunofluorescent staining. Note that the rabbit anti-keratin antiserum stains almost 100% of the cells at all stages of growth (*a*, *c*, and *e*), whereas AE5 stains only suprabasal cells (*d* and *f*). The arrows in *c* denote an AE5-positive, suprabasal cell (cf. panel *d*). *a*, *c*, and *e* are identical fields as *b*, *d*, and *f*, respectively. Bar, 50 μm.

lane 7) and have been classified as a "pair" (Sun et al., 1984), detailed analyses of cultured corneal epithelial cells have revealed that the synthesis of the basic 64K keratin precedes by 1–2 d that of the acidic 55K keratin (Fig. 6, lanes *5* and *6*). A similar situation exists in normal esophageal, corneal, and bladder epithelia which possess small amounts of the basic 58K and sometimes 56K keratins, without their corresponding acidic members (i.e., the 50K and 48/46K keratins, respectively; Moll et al., 1982). This raises the question as to whether these basic keratins may form complexes with some other acidic members such as the 40K keratin (Fig. 7 *b*) which happens to be present in significant quantities not only in cultured corneal keratinocytes (Fig. 6, lanes *5* and *6*), but also in many other epithelia (Wu and Rheinwald, 1981;

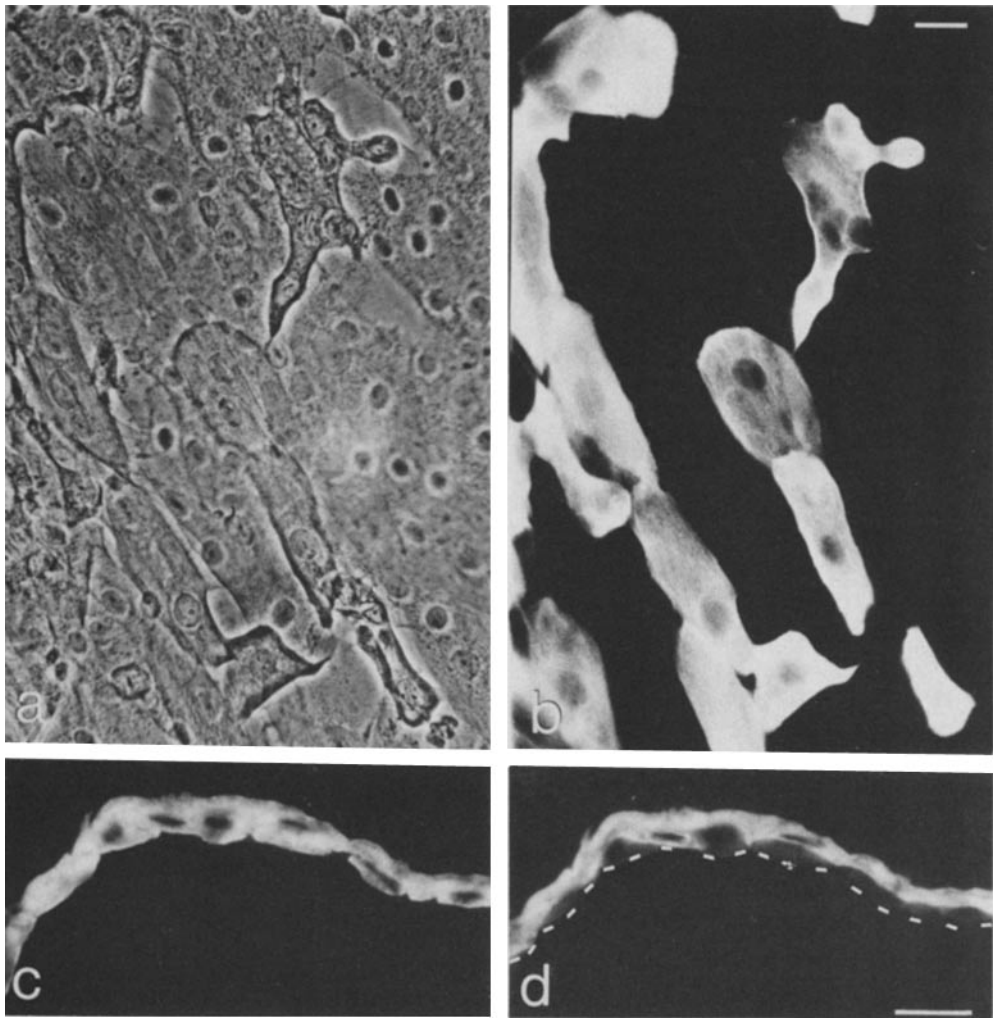

Figure 9. Suprabasal staining of cultured rabbit corneal epithelial cells with AE5 antibody. (*a*) Phase-contrast, center of a large colony. (*b*) AE5 staining of the same field as in *a* showing preferential decoration of suprabasal cells. (*c* and *d*) Double staining of a vertical (7 μm) frozen section of a rabbit corneal epithelial colony (day 11) with a rabbit antikeratin antiserum, and AE5, respectively. Note in *d*, the suprabasal staining by AE5. Dashed line in *d* indicates the bottom surface of basal cells. Bars, 20 μm.

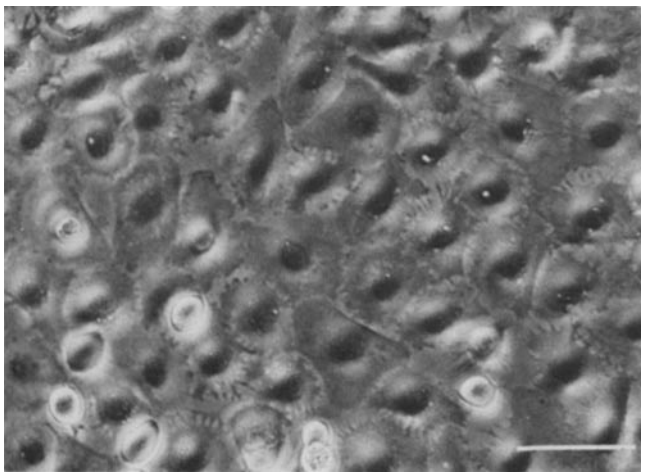

Figure 10. Morphology of basal cells prepared from rabbit corneal epithelial cultures by the EGTA technique. An 11-d culture was incubated at 37°C in a medium containing 1.4 mM EGTA for 5 h, then vigorously sprayed with the medium to dislodge suprabasal cells (see Materials and Methods). Note a monolayer of basal cells interconnected with numerous cellular processes. Phase-contrast microscopy of living cells. Bar, 20 μm.

Moll et al., 1982; Tseng et al., 1982). This 40K keratin is unique in that it is one of the few acidic keratins that does not seem to have a regular basic "partner" (Sun et al., 1984). Also, being the smallest intermediate filament subunit, this keratin probably possesses only very limited NH_2-terminal

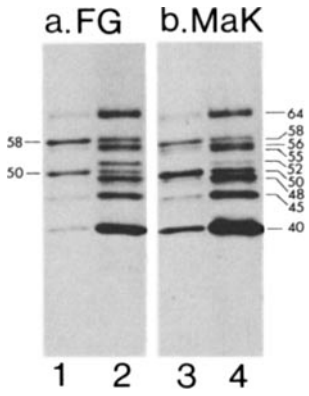

Figure 11. One-dimensional immunoblot analyses of the keratins of basal and suprabasal cells isolated from an 11-day-old, stratified culture of rabbit corneal epithelial cells. (*a*) Fast green. (*b*) Immunoblotting using a mixture of mouse anti-intermediate filament and AE1 antibodies (*MaK*). Lanes *1* and *3* are basal cell keratins; lanes *2* and *4* are suprabasal cell keratins. Note (in lane *1*) that basal cells contain almost exclusively the 50/58K keratins, while (in lane *2*) suprabasal cells contain additional 55/64K, 48/56K, 45/52K, and 40K keratins.

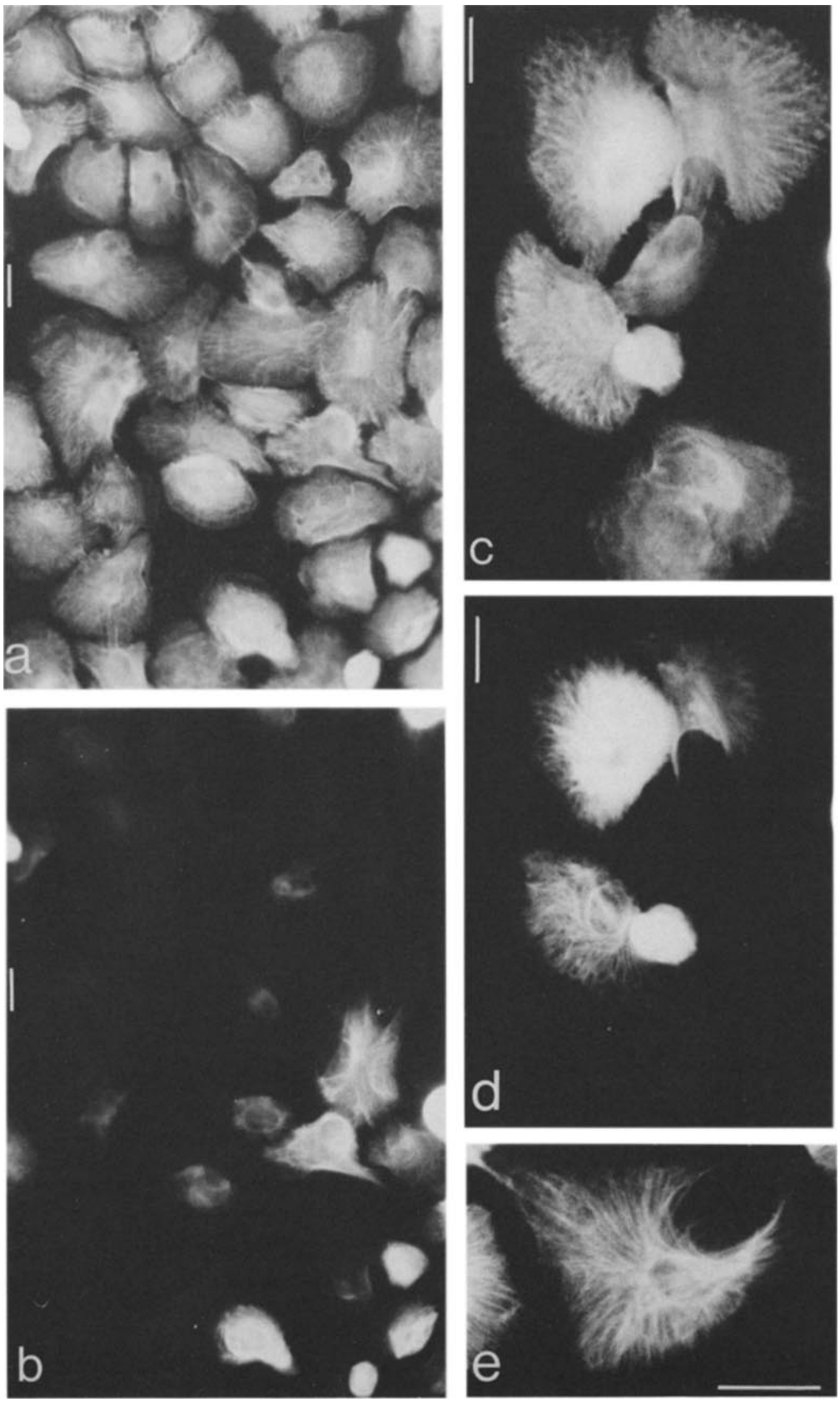

Figure 12. Immunofluorescent (AE5) staining of basal cells isolated by the EGTA technique from a 15-d-old, rabbit corneal epithelial culture. (*a*) Staining with a broadly reactive rabbit anti-keratin antiserum showing the staining of all basal cells. (*b*) AE5 staining of the same field as in *a* showing the selective staining of some basal cells. (*c*) Rabbit anti-keratin antiserum staining at higher magnification than *a* showing keratin filaments of basal cells. (*d*) AE5, same field as in *c* demonstrating filamentous and somewhat uneven distribution of the 64K keratin. (*e*) AE5 staining of another basal cell. Bars, 20 μm.

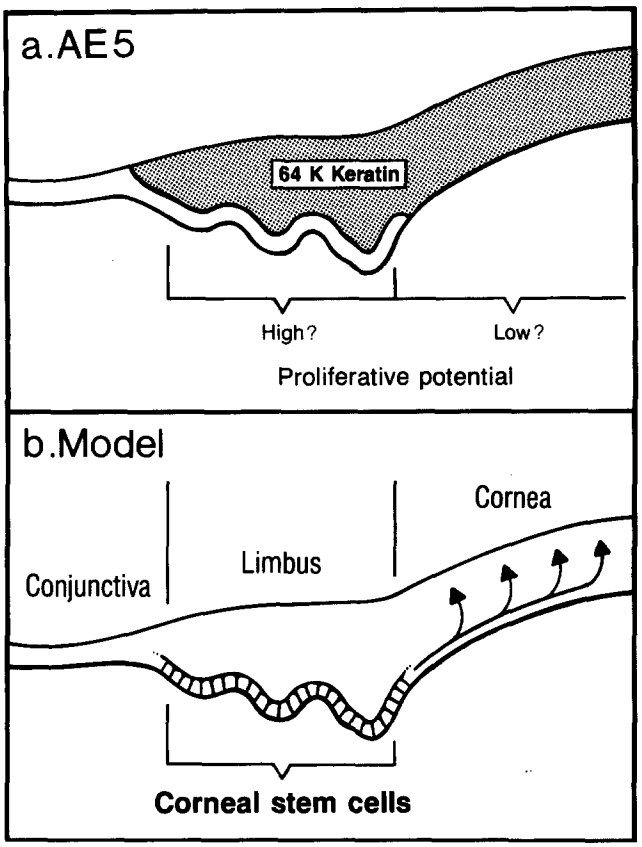

Figure 13. A model of corneal epithelial maturation. (*a*) Expression of the 64K keratin in corneal and limbal epithelia. Based on the earlier statements by Friedenwald and Buschke (1944), we presume that the proliferative capacity and/or rate of the 64K keratin-negative limbal basal cells is higher than the 64K keratin-positive corneal basal cells; more data will be required, however, to test this point (thus the question marks in the figure). (*b*) A model showing that corneal epithelial stem cells are located in the basal layer of the limbal region. Arrows indicate the directions of cell migration during normal corneal epithelial maturation.

and/or COOH-terminal sequences which are believed to protrude from the filaments and to be mainly responsible for any keratin type-specific functions (Steinert et al., 1985).

Possible Mechanisms of Basal to Suprabasal Transition in Keratinocyte Differentiation

Our data from cell separation experiments have clearly demonstrated the striking changes in keratin expression when cultured corneal keratinocytes leave the basal compartment (Fig. 11). The molecular mechanism of this transition is at present unclear. Although the physical contact between keratinocytes and basement membrane most likely plays a critical role in maintaining a "basal" state of differentiation, separating basal cells from their substratum is apparently not sufficient in triggering the expression of some suprabasal keratin markers (Sun and Green, 1978*a*). Moreover, suprabasal cells seem to be able to choose among multiple, alternate pathways of differentiation (i.e., "hyperproliferative" mode versus one of the several pathways of keratinocyte differentiation; Cooper et al., 1985), but we know very little about what controls such decision making. The mechanism by which basal corneal epithelial cells are able to synthesize large quantities of 55/64K keratins in situ is also obscure, although the possibility of subtle changes in the basement membrane composition in central cornea (versus limbus) cannot be ruled out. Further investigations aimed at elucidating the nature of keratinocyte stem cells as well as the interaction between keratinocytes and basement membrane will have to be conducted in order to answer these questions.

Limbal Location of Corneal Epithelial Stem Cells: A Model

Although the differentiation of corneal epithelium is classically envisaged as a "vertical" process in which the cells lost from the superficial surface are continuously replaced by cells originating from the basal layer, Davanger and Evensen (1971) observed that some pigmented limbal cells form streaks suggesting centripetal migration (i.e., cells moving toward the center of the cornea). Based primarily on this observation, these authors were the first to speculate that cells at the limbal area may be involved in normal corneal epithelial renewal. Fig. 13 *b* shows a schematic model which is a more refined version of this speculation, which has so far been supported mainly by circumstantial evidence and therefore has received relatively little attention. The three major elements of this model and their supporting evidence are summarized below.

(a) The Expression of the 64K Keratin. Our AE5 staining data showed clearly that while the 64K keratin is suprabasally located in the limbus, this protein is present throughout the central corneal epithelium, basal cells included (Figs. 5 and 13 *a*). Assuming that the 64K keratin is a marker for an advanced stage of corneal epithelial differentiation (Figs. 8, 9, and 11), these findings strongly suggest that corneal basal cells are in a differentiated state more advanced than we have previously recognized. Moreover, the fact that corneal basal cells can still undergo significant replication suggests that corneal basal cells must correspond to "transient amplifying cells" in the scheme of "stem cells → transient amplifying cells → terminally differentiated cells" (Potten et al., 1979; Lavker and Sun, 1982, 1983).

(b) Centripetal Migration of Corneal Epithelial Cells. In an elegant series of studies, Thoft and co-workers have shown that after corneal transplantation there is a gradual replacement of the donor epithelium by the host (pericorneal) cells (Kinoshita et al., 1981; Thoft and Friend, 1983).[3] Assuming there is no rejection, this observation suggests centripetal migration. That such a migration actually occurs even in normal corneal epithelium is supported not only by the migratory pattern of pigmented limbal cells as cited earlier, but also by some recent data from "cell-tagging" experiments (Buck, 1985; Dong, G., and R. Lavker, personal communication).

(c) Mitotic Activity of Limbal Epithelium. Although the mitotic activity of corneal epithelium has been studied extensively, relatively little attention has been paid to the limbal area. However, it has been reported that in corneal epithe-

3. Based on cell migration data, Thoft and Friend (1983) have proposed an "x,y,z-hypothesis" which states that corneal epithelial cell shedding equals the sum of vertical and centripetal cell movements. This hypothesis is different from our current model mainly in that the former does not specify the source of centripetally migrating cells (location of corneal epithelial stem cells) which can therefore originate from either limbus or conjunctiva.

lium mitotic figures are more numerous toward the edge of the cornea, i.e., in a region that is closest to the limbus (Friedenwald and Buschke, 1944). This finding is consistent with the idea that corneal epithelial stem cells are physically located in the limbus, presumably in the basal layer (Fig. 13 *b*; cf. Lavker and Sun, 1983). More extensive studies will be required, however, in order to confirm the enhanced limbal replication, to investigate the regulation of limbal mitotic activity as a function of circadian cycle (Scheving et al., 1978) and physiological perturbances, and to determine whether different parts of the limbus (i.e., temporal vs. nasal, upper vs. lower) may behave differently (Goldberg and Bron, 1982; Dong, G., A. Schermer, T.-T. Sun and R. Lavker, experiments in progress).

Implications of the Model

The localization of corneal epithelial stem cells in the limbus has implications in the following areas.

(a) Corneal Epithelial Neoplasms. Although corneal epithelial dysplasia and neoplasms are relatively rare, a great majority of them seem to involve the limbus (Pizzarello and Jakobiec, 1978; Waring et al., 1984). Mechanical debridement of abnormal corneal epithelium, without cryotherapy or excision of the neighboring limbal area, brings prompt relief from the symptoms, but recurrences are common (Roberson, 1984). Significantly, such recurrences usually happen near the originally involved limbal area (Waring et al., 1984), where the transformed stem cells presumably reside.

(b) Other Corneal Epithelial Diseases. Persistent corneal epithelial defect has a distinct predilection for the central cornea (Cavanagh et al., 1979). This could be readily explained if the peripheral stem cells have an abnormally low rate or capacity of proliferation. Our model also emphasizes that more attention should be paid to studying the possible role(s) of limbal cells in other corneal epithelial diseases.

(c) Corneal Epithelial Regeneration. Although it is generally thought that both limbal and conjunctival epithelia can serve as sources for corneal epithelial regeneration (Friedenwald and Buschke, 1944; Maumenee, 1964), the former seems to work better (Kinoshita et al., 1982). Concerning the question of whether the narrow limbus has enough reparative power, one should note that limbal epithelium contains many rete ridges and can therefore, at least in some areas (e.g., "Palisades of Vogt" in man), be quite thick (Duke-Edler and Wybar, 1958; Goldberg and Bron, 1982). Consequently, the limbus (defined as a band ~0.5–1 mm wide around the corneal periphery) has sufficient cells to cover a total corneal abrasion (Heydenreich, 1959; Kinoshita et al., 1982). In this regard, it would be interesting to determine whether a complete loss of limbal epithelium (including the presumptive stem cells in rete ridges) will explain the occasionally observed delay or even regression of corneal epithelial healing (Srinivasan et al., 1977).

(d) A Model System for Studying Epithelial Stem Cells. The "horizontal," physical separation of the limbal stem cells from their descendent corneal cells makes this system uniquely suitable for studying the properties of epithelial stem cells. The eventual isolation and characterization of these stem cells are essential steps toward a better understanding of their role(s) in epithelial differentiation, wound healing, and tumorigenesis.

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