

Retinoids as Important Regulators of Terminal Differentiation: Examining Keratin Expression in Individual Epidermal Cells at Various Stages of Keratinization

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Abstract. When human epidermal cells were seeded on floating rafts of collagen and fibroblasts, they stratified at the air-liquid interface. The suprabasal cells synthesized the large type II (K1) and type I (K10/K11) keratins characteristic of terminal differentiation in skin. At earlier times in culture, expression of the large type II keratins appeared to precede the expression of their type I partners. At later times, all suprabasal cells expressed both types, suggesting that the accumulation of a critical level of K1 keratin may be a necessary stimulus for K10 and K11 expression. Expression of the terminal differentiation-specific keratins was completely suppressed by adding retinoic acid to the culture medium, or by submerging the cultures in normal medium. In submerged cultures, removal of vitamin A by delipidization of the serum restored the keratinization process. In contrast, calcium and transforming growth factor-beta did not influence the ex-

pression of the large keratins in keratinocytes grown in the presence of retinoids, even though they are known to induce certain morphological features of terminal differentiation. Retinoic acid in the raft medium not only suppressed the expression of the large keratins, but, in addition, induced the synthesis of two new keratins not normally expressed in epidermis *in vivo*. Immunofluorescence localized one of these keratins, K19, to a few isolated cells of the stratifying culture. In contrast, the other keratin, K13, appeared uniformly in a few outer layers of the culture. Interestingly, K13 expression correlated well with the gradient of retinoid-mediated disruptions of intercellular interactions in the culture. These data suggest that K13 induction may in some way relate to the reduction in either the number or the strength of desmosomal contacts between suprabasal cells of stratified squamous epithelial tissues.

THE epidermis consists of 10–20 layers whose cells possess distinct structure and properties. Only cells in the basal layer proliferate; as cells leave this layer and begin to migrate outward towards the skin surface, they cease to divide and start to terminally differentiate. The cells enter the spinous layer and increase steadily in size as they continue to be metabolically active (Yardley and Goldstein, 1976). During this phase, much of the cell's translational machinery is devoted to making keratins, the major product of the differentiated epidermis (Matoltsy, 1975). These proteins assemble into 8-nm filaments that increase in number and aggregate into bundles in the cytoplasm of the differentiating cell (Brody, 1960). As cells reach the granular layer, they enter a destructive phase and begin to lose their cytoplasmic organelles (Lavker and Matoltsy, 1970). At this stage, keratin filaments organize into even larger bundles, called macrofibrils (Dale et al., 1978). Shortly thereafter, the permeability of the cells increases, activating the enzyme transglutaminase. This enzyme catalyzes cross-linking of nonkeratin proteins into an envelope located just beneath the plasma membrane (Sun and Green, 1976; Rice and Green, 1977). Dead cells (squames) that reach the skin surface are merely cellu-

lar skeletons that are packed with macrofibrillar bundles of keratin. As they are released from the skin, the squames are continually being replenished by differentiating cells migrating outward.

A number of changes in protein synthesis take place during terminal differentiation. The major change is the induction of unusually large keratins (Fuchs and Green, 1980), which occurs as a cell leaves the basal layer (Viac et al., 1980). While basal cells express only two keratins, K5 (58 kD) and K14 (50 kD) (Nelson and Sun, 1983), suprabasal cells express keratins K1 (67 kD), K2 (65 kD), K10 (56.5 kD), and K11 (56 kD) in addition to basal keratins (Fuchs and Green, 1980; Eichner et al., 1984). With the exception of K2, which seems to be a proteolytic breakdown product of K1 (Fuchs and Green, 1980; Tyner and Fuchs, 1986), the shift to the expression of these large keratins is due to changes in specific keratin mRNAs (Fuchs and Green, 1980). The appearance of large keratin mRNAs is one of the earliest biochemical indications that a cell has undergone a commitment towards terminal differentiation. In humans, expression of these keratins is highly restricted to keratinizing cells of epidermis, exocervix, and anus (Moll et al., 1982a).

Another protein that seems to show a correlation with differentiation is involucrin, a constituent of the cross-linked envelope (Rice and Green, 1977; Green, 1977). In culture, its synthesis is exclusive to enlarged keratinocytes (Watt and Green, 1981). However, in vivo, only the outer spinous and stratum corneum layers of the epidermis stain with anti-involucrin antiserum (Rice and Green, 1979). Thus, in relation to the expression of large keratins, the expression of involucrin seems to take place at a later stage of differentiation. In addition, involucrin is more generally expressed in all types of stratified squamous epithelia, and does not appear to be as exclusive a marker of keratinization as the large keratins.

When human epidermal cells are cultured in medium containing vitamin A-depleted serum, they undergo stratification and extensive terminal differentiation (Fuchs and Green, 1981). Biochemically, the changes associated with the process of terminal differentiation take place: the large keratins are synthesized (Fuchs and Green, 1981; Eichner et al., 1984), and the transglutaminase-catalyzed cross-linking of involucrin generates the submembranous cornified envelope of the terminally differentiated cell (Green and Watt, 1982). The only other relatively defined cell culture method where these morphological and biochemical properties of terminal differentiation are retained is the seeding of keratinocytes on a floating raft of collagen and fibroblasts such that cells are fed at the air-liquid interface with vitamin A-containing medium (Karasek and Charlton, 1971; Lillie et al., 1980; Yang and Nandi, 1983; Asselineau et al., 1986).

In the submerged cultures, when retinoids are added back to medium containing delipidized serum, the level of stratification is reduced, and the biochemical properties of differentiation are suppressed (Fuchs and Green, 1981; Green and Watt, 1982; Yuspa et al., 1982). If the retinoid concentration exceeds the normal serum level, synthesis of two new keratins, a 40- and a 52-kD keratin, is enhanced (Fuchs and Green, 1981; Gilfix and Green, 1984; Eckert and Green, 1984; Kim et al., 1984). The 40-kD keratin, K19, is unusual in that in vivo it seems to be expressed in epidermis only after malignant transformation (Wu and Rheinwald, 1981). In other tissues, notably certain mucus-secreting simple epithelia, K19 is expressed under normal conditions (Moll et al., 1982a; Wu et al., 1982; Lane et al., 1985).

The 52-kD keratin, K13, is unusual in that its expression seems to be restricted to suprabasal cells of wet stratified squamous epithelia (van Muijen et al., 1986; Wild and Mischke, 1986). Like K19, this keratin is also seen in squamous cell carcinomas of the skin, but not in normal epidermis (Wu and Rheinwald, 1981). It has been suggested that since the expression of these two keratins is dependent on retinoids, squamous cell carcinomas might have an increased sensitivity to retinoids acquired through their tumorigenesis (Kim et al., 1984).

A number of questions concerning the influence of extracellular regulators on epidermal differentiation remain to be answered. (a) Can the expression of large keratins in cells grown on an air-liquid interface be suppressed by raising the retinoid levels in the nutrient medium? Does this alter the morphology of the stratified culture? (b) When epidermal cells undergo terminal differentiation, do they simultaneously express all of the large keratins, or is there a sequence to their expression? (c) When the level of retinoids is in-

creased and expression of K19 and K13 is induced, which cells of the population begin to express these keratins? (d) Can other putative regulators of terminal differentiation, e.g., calcium and transforming growth factor-beta (TGF- β),¹ compensate for the presence of retinoids and promote the expression of the differentiation-specific keratins in submerged cultures?

The experiments described in this manuscript were designed in an attempt to seek answers to these questions.

Materials and Methods

Preparation of Floating Epidermal Colonies on Fibroblast/Collagen Lattices

The procedure for making and seeding the collagen lattices was essentially the same as that described by Asselineau et al. (1985). Type I collagen was combined with culture medium at 4°C as described by the manufacturer (Seikagaku America, Inc., St. Petersburg, FL). Mouse 3T3 fibroblasts (Rheinwald and Green, 1975) were added to this solution at a concentration of 1.5×10^5 /ml, and the mixture was pipetted into 35-mm culture dishes (2 ml/dish). To gel, lattices were placed in the incubator at 37°C. After 2–3 h, medium was added at 2 ml/dish, and lattices were maintained in the incubator until ready for use (usually 12–72 h postgellation). Dulbecco's modified Eagle's medium was supplemented with hydrocortisone (0.4 μ g/ml) and serum as described previously (Asselineau et al., 1985).

To prepare epidermal cultures, each dermal lattice was seeded with 3×10^5 human keratinocytes derived from foreskin and kept as 3-d passage-cloned stocks under liquid nitrogen. Cells were grown submerged in culture medium for 7 d. At this point, lattices were removed from the dishes and placed on stainless steel grids (Asselineau et al., 1985, 1986). Grids were floated for 7–24 d on medium supplemented with a defined concentration of retinoic acid (Sigma Chemical Co., St. Louis, MO). Retinoic acid was stored at -70°C in the dark as 400 \times stocks in dimethyl sulfoxide, and diluted into medium at each feeding. Control plates with no retinoic acid still received a 1:400 dilution of dimethyl sulfoxide into the medium.

For radiolabeling of proteins, medium was supplemented with 100 μ Ci/ml [³⁵S]methionine (1 Ci/mmol) for 8 h, and keratins were extracted as described previously (Fuchs and Green, 1980).

Fixation and Preparation of Tissues

For hematoxylin-eosin staining, portions of rafts were gently removed from grids with tweezers and placed in Carnoy's solution for fixation. Fixed cultures were embedded in paraffin, sectioned (5–8 μ m), and stained. Wherever possible, Carnoy's fixed sections were used for immunofluorescence staining, since the histology was far superior to that of frozen sections.

Sometimes, antibodies showed specific binding only when frozen sections were used. In these situations, rafts were placed in isopentane cooled to -140 to -160°C with liquid nitrogen. Frozen tissue was embedded in Tissue-Tek (Polysciences, Inc.), and sectioned (4 μ m) using a cryostat.

Antibodies and Immunofluorescence Microscopy

A polyclonal antiserum against gel-purified human K1 was raised in a New Zealand White rabbit as described previously (Kim et al., 1984). As judged by immunoblot analysis, the anti-K1 antiserum showed some cross-reactivity with the basal epidermal keratin K5 (Kim et al., 1984). However, as judged by immunofluorescence of frozen epidermal tissue sections, the antibodies seemed to be specific, indicating that the immunoreactive antigenic site for K5 is masked in the tissue. Recently, we raised a more specific rabbit antiserum against a synthetic peptide to a portion of the human K1 sequence, and verified its specificity by immunoblot analysis. The immunofluorescence studies involving our original anti-K1 antibody were repeated with this antiserum, and identical results were obtained.

A mouse monoclonal antibody (K_k 8.60), monospecific for K10/K11 (O. Gigi-Leitner et al., 1986), and a mouse monoclonal antibody (K_m 4.62), monospecific for K19 (O. Gigi-Leitner et al., 1986) were obtained from Dr. Benjamin Geiger (Weizmann Institute of Science, Rehovot, Israel). A rabbit

1. *Abbreviations used in this paper:* EGF, epidermal growth factor; TGF- β , transforming growth factor-beta.

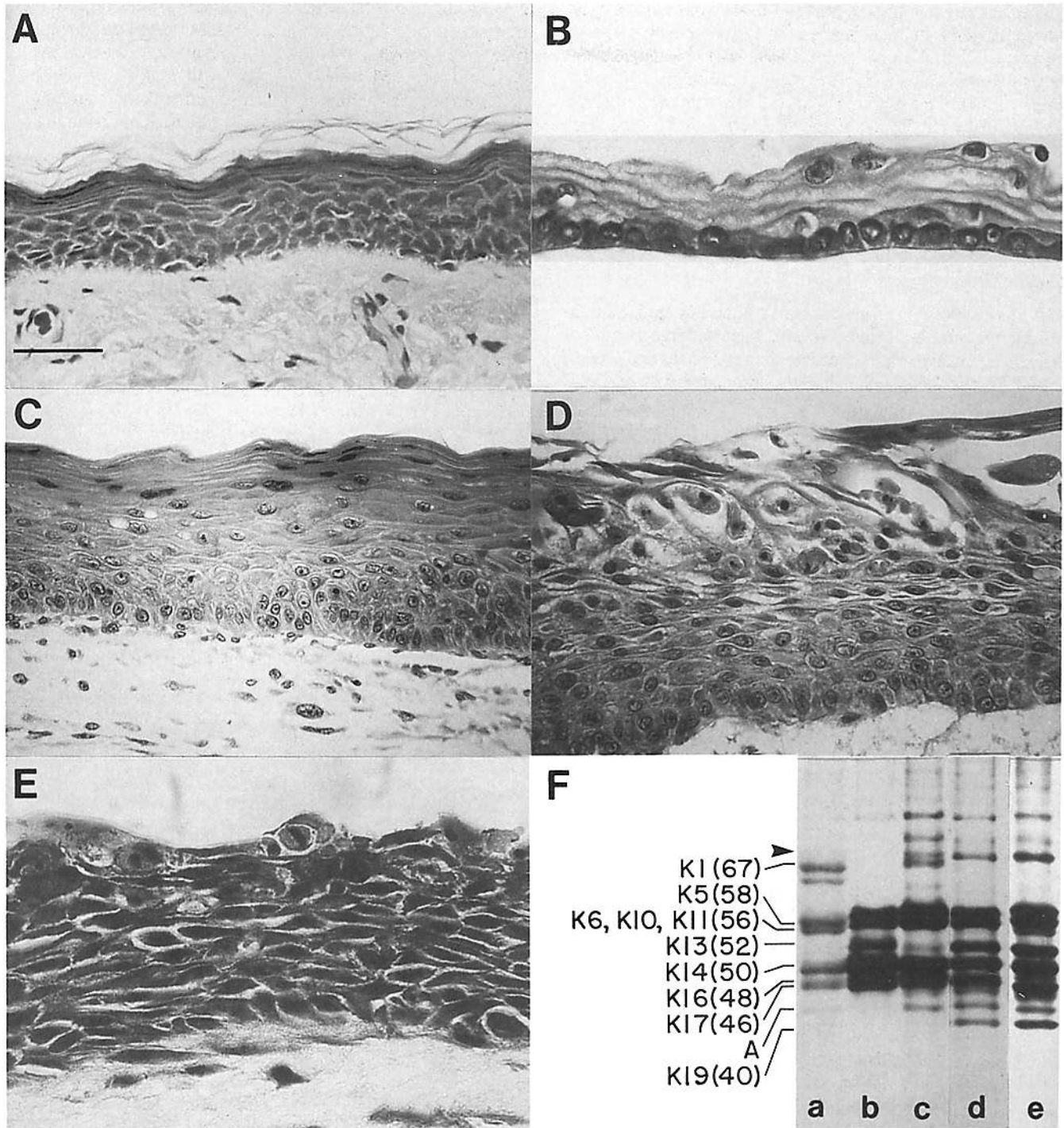


Figure 1. Effects of retinoic acid on morphology and keratin expression in human epidermal cells cultured on collagen rafts. Foreskin epidermal cells were seeded on lattices of collagen and fibroblasts. The rafts were then floated on serum-containing medium supplemented with varying levels of retinoic acid (RA). 10 d after seeding, a portion of the rafts were fixed in Carnoy's solution, embedded in paraffin, and sectioned (5 μ m). Tissue sections were stained with hematoxylin and eosin and examined by light microscopy. (A) Keratinizing trunk skin epithelium; (B) submerged epidermal culture, no RA supplement; (C) epidermal raft, no RA supplement, 1 wk after floating; (D) epidermal raft, 1.0×10^{-6} M RA, 1 wk after floating; (E) epidermal raft, 1.0×10^{-5} M RA, 1 wk after floating. Bar, 20 μ m. (F) Tissues and cultures described above were incubated in [35 S]methionine containing medium (100 μ Ci/ml; 1.0 Ci/mmol), and the keratins were extracted 8 h later (Fuchs and Green, 1980). Equal amounts of radioactively labeled proteins were subjected to SDS-PAGE followed by fluorography and autoradiography. The gel was overexposed for keratins K5, K6, K14, K16, and K17 so that keratins K1, K13, and K19 could be readily detected (if present). Note that three bands (molecular mass 70–90K, possibly lamins) were often seen as contaminants in the keratin extracts. Lanes a–e of the gel correspond to the samples shown in panels A–E, respectively.

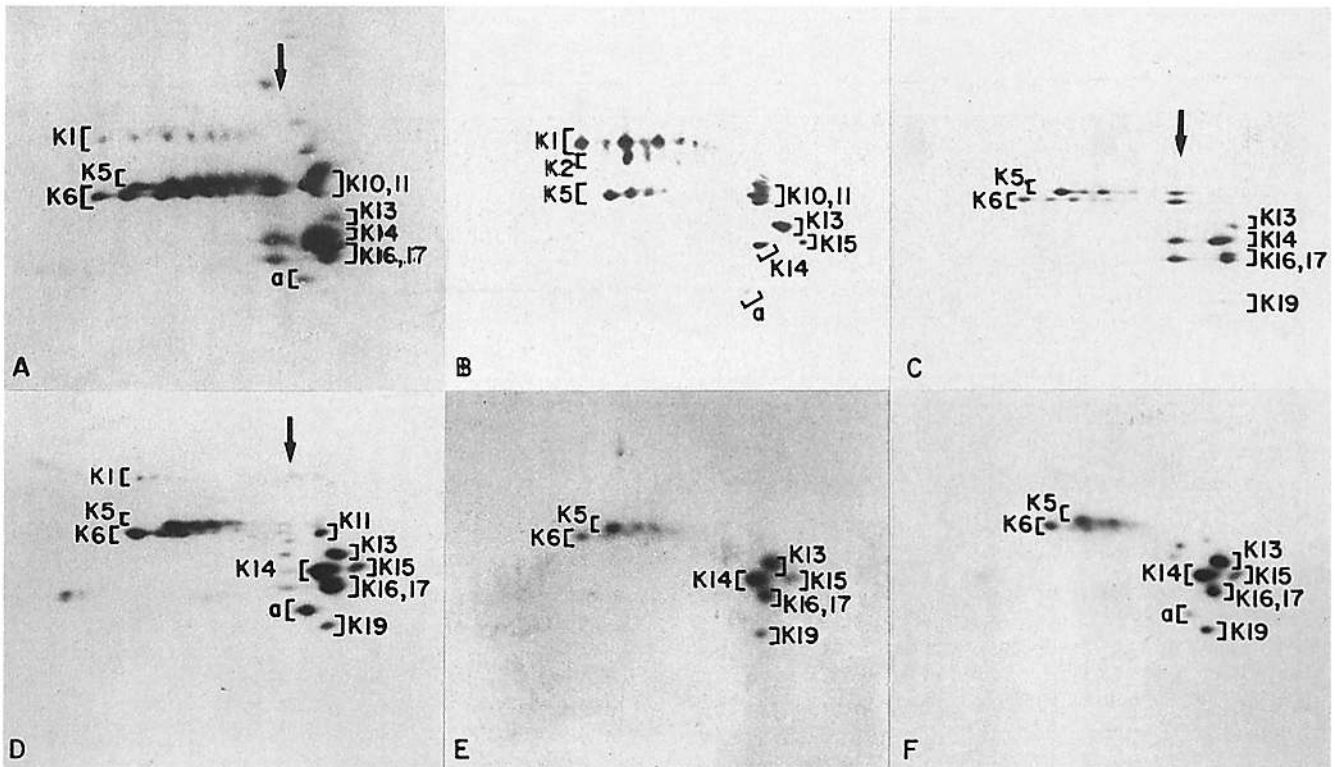


Figure 2. Changes in keratin biosynthesis in response to increased levels of retinoic acid. Epidermal cultures were prepared and radiolabeled as described in the legend to Fig. 1. Radiolabeled keratins were resolved by two-dimensional gel electrophoresis. Nonequilibrium pH gradient electrophoresis was used in the first dimension and SDS-PAGE in the second dimension (O'Farrell, 1977). To visualize the keratins, gels were either silver stained (*B*) or subjected to fluorography and autoradiography (*A* and *C-F*). Keratin extracts were from: (*A*) floating epidermal culture, no RA supplement; (*B*) human foreskin; (*C*) submerged epidermal culture, no RA supplement; (*D*) floating epidermal culture, 1.0×10^{-7} M RA; (*E*) floating culture, 1.0×10^{-6} M RA; (*F*) floating culture, 1.0×10^{-5} M RA. Overhead arrows in *A*, *C*, and *D* indicate complexes formed between type I and type II keratins.

polyclonal antiserum that is monospecific for K19 was obtained from Dr. James Rheinwald (Wu and Rheinwald, 1981). A mouse monoclonal antibody (RKSE 60) that is likely to be specific for K10/K11 was obtained commercially from Clark Laboratories (Ramaekers et al., 1983). A mouse monoclonal antibody (1C7) monospecific for K13 was obtained from Dr. Goos van Muijen (van Muijen et al., 1986).

As second antibodies, the following conjugates were used: fluorescein-labeled goat antibodies against rabbit IgG (Cooper Biomedical, Malvern, PA); Texas red goat antibodies against mouse IgG (Tago, Inc., Burlingame, CA).

Immunofluorescence was performed as described previously (Franke et al., 1979).

Two-dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis was performed with nonequilibrium pH gradient in the first dimension (O'Farrell et al., 1977) and SDS 8.5% polyacrylamide gel electrophoresis in the second dimension.

Results

Retinoids Disrupt the Intercellular Interactions in Cultures Grown on Floating Rafts

In human epidermis, the cells stratify extensively and show a gradual series of morphological changes characteristic of the process of terminal differentiation (Fig. 1 *A*). When cells were cultured in the presence of a 3T3 fibroblast feeder layer submerged in serum-containing medium, only a few layers formed, and a sharp morphological distinction was seen be-

tween the nucleated basal cells and the largely enucleated, flat suprabasal cells (Fig. 1 *B*). An *in vitro* system which more closely paralleled the *in vivo* morphology was to culture epidermal cells on a floating lattice matrix of collagen and 3T3 fibroblasts (Asselineau et al., 1985). After 1 wk flattened enucleated cells formed in the upper layers, and numerous desmosome-like intercellular attachments could be seen (Fig. 1 *C*). Although in some regions of the raft (e.g., the one shown) the number of cell layers was somewhat greater than that typically seen on the trunk and limbs of the body (compare with the cross section of thigh skin seen in Fig. 1 *A*), the thickness was within the range of variability observed *in vivo*. More importantly, the gradient of morphological changes in the cultures was much more gradual and characteristic of epidermis than that observed in epidermal cultures grown on a plastic substratum submerged in serum-containing medium (*B*).

Since submerged cultures grown in medium containing delipidized serum undergo extensive terminal differentiation which can subsequently be suppressed by retinoid supplements (Fuchs and Green, 1981), we reasoned that keratinization in the floating epidermal cultures might be successful primarily because the stratifying cells are able to migrate away from the vitamin A ($\sim 5.2 \times 10^{-7}$ M; Kim et al., 1984) in the serum-containing medium. To examine this possibility, we tested whether the addition of retinoic acid to the culture medium is sufficient to inhibit the differentiative pro-

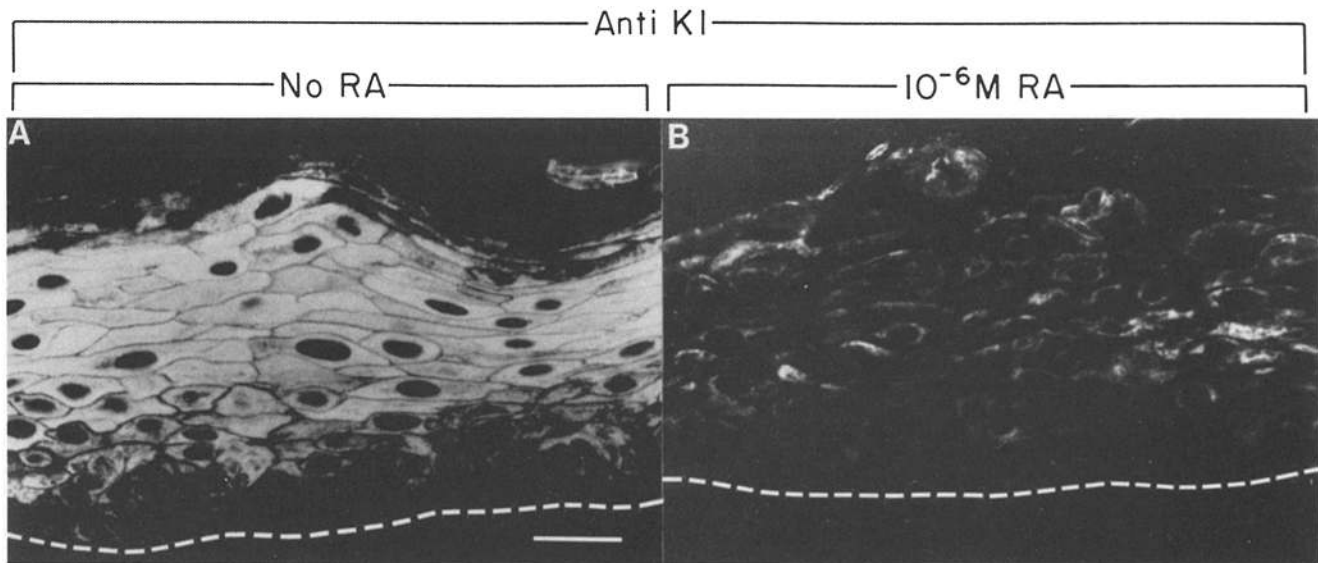


Figure 3. The location of K1 in floating epidermal cultures treated with increasing levels of retinoids. Epidermal rafts were seeded and floated on medium containing serum and supplements of retinoic acid (RA) as indicated. After 10 d, rafts were fixed in Carnoy's solution and embedded in paraffin. Samples were sectioned (8 μm) and subjected to indirect immunofluorescence using an antiserum against K1 (Kim et al., 1984). Sections were from (A) floating epidermal raft, no added RA; (B) floating epidermal raft, 1.0×10^{-6} M RA. Dotted lines indicate the borders between the basal epidermal layer and the collagen/fibroblast lattice. Bar, 20 μm .

cess. Floating epidermal cultures were fed medium supplemented with retinoic acid at concentrations of 1×10^{-8} – 1.0×10^{-5} M, a range previously shown to be effective for submerged cultures (Gilfix and Green, 1984).

Unlike submerged cultures, which showed little stratification but substantial desquamation with increasing levels of retinoids (Fuchs and Green, 1980), floating epidermal colonies showed greater stratification and less desquamation. As the concentration of retinoic acid was increased from 1.0×10^{-7} to 1.0×10^{-5} M, the morphology of the epidermal layers was progressively disturbed (Fig. 1, D and E). Cells within the outermost layers seemed to be most sensitive; even at low levels of retinoic acid, these cells had a "looser" almost hyperplastic appearance, with fewer cell-cell contacts (Fig. 1 D). At higher retinoid concentrations, the number of cell layers was often diminished, and the intercellular contacts seemed perturbed throughout the culture (Fig. 1 E). These results suggest that retinoids disturb normal intercellular interactions in cultured epidermal cells, and that these more delicate contacts are completely lost during cycles of feeding in submerged cultures.

The Differentiation-specific Keratins Are Differentially Repressed by Retinoids

To examine the expression of keratins in epidermal cultures that had been floating for 7 d, [^{35}S]methionine was added to the medium 8 h before keratin extraction. Radiolabeled keratins were initially separated by one-dimensional gel electrophoresis. An autoradiogram of the gel (Fig. 1 F) shows clearly that the one of the differentiation-specific keratins of trunk epidermis (K1, lane a) was also synthesized by the floating epidermal culture (lane c), but not by the submerged cultures (lane b). Although the level of synthesis of K1 in the floating cultures was substantial, it was expressed in seven isoelectric variants, as can be seen by two-dimensional gel analysis (Fig. 2 A). In foreskin epidermis, the more basic K1

variants were seen preferentially (Fig. 2 B). K2 was detected only in skin and not in floating cultures (Fig. 1 F, cf. lanes a and c; Fig. 2, cf. A and B) and may be a proteolytic processing product of K1 (Fuchs and Green, 1980; Tyner et al., 1986).

Since the electrophoretic mobilities of K10 and K11 were similar to those of K5 and K6, they could only be seen when resolved by two-dimensional gel electrophoresis (Fig. 2). These acidic differentiation-specific keratins did not show much heterogeneity in their isoelectric points and hence were easily visible in the autoradiograms. Both K10 and K11 were expressed in abundance in floating epidermal cultures (Fig. 2 A) and in epidermis (B), but not in submerged cultures (C).

When the concentration of retinoic acid in the medium was raised to 1.0×10^{-7} M, expression of all differentiation-specific keratins was markedly reduced (Fig. 1 F, lane d; Fig. 2 D). At 1.0×10^{-6} – 1.0×10^{-5} M, expression of K1, K10, and K11 was blocked (Fig. 1 F, lane e; Fig. 2, E and F). These data were later confirmed by immunofluorescence studies (see below).

To determine (a) which cells in floating epidermis express differentiation-specific keratins, and (b) whether all of these cells respond in the same fashion to increasing levels of retinoids, we used a polyclonal antibody largely specific for K1. Immunofluorescence of fixed sections of floating cultures showed marked staining by this antiserum in nearly all suprabasal layers (Fig. 3 A). While some areas showed only a single K1-negative cell layer, other regions had as many as three such layers. In retinoid-treated cultures, all K1-positive cells displayed a uniform response with reduced K1 staining. At 1.0×10^{-6} M retinoic acid, K1 was suppressed in all cells (Fig. 3 B).

As shown in Fig. 2, the only raft which expressed appreciable levels of differentiation-specific keratins was the one that had not been cultured in medium containing retinoic

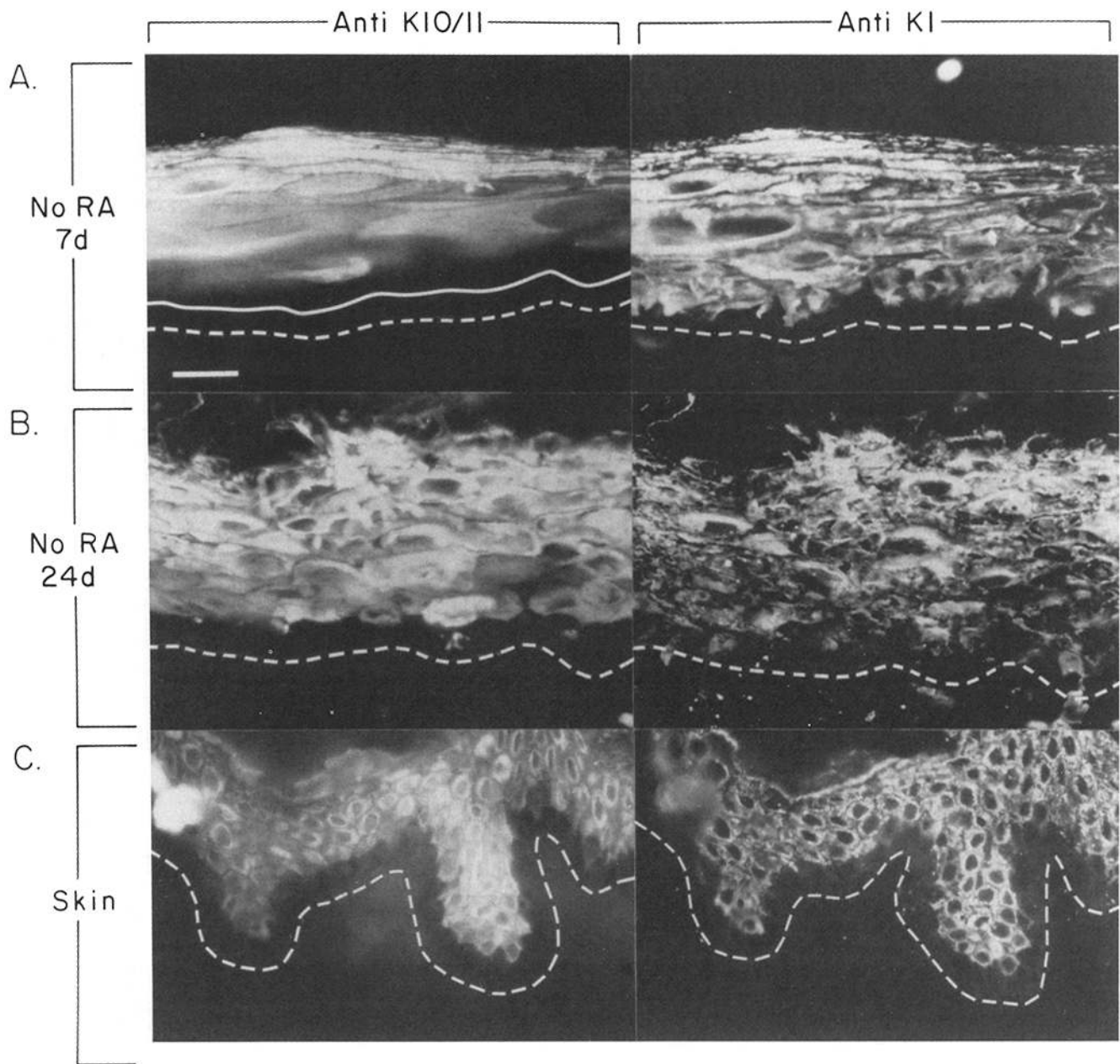


Figure 4. K10/K11 show a change in distribution with increasing time in culture. Epidermal rafts were seeded and floated on normal serum-containing medium. After the times indicated below, rafts were frozen in isopentane at -160°C . Samples were then sectioned ($4\ \mu\text{m}$) and subjected to indirect double immunofluorescence microscopy, as described in Materials and Methods. Antibodies were against: (*left*) K10/K11; (*right*) K1 oligopeptide (see Materials and Methods). Frozen sections were from: (*A*) epidermal raft, 7 d after floating; (*B*) epidermal raft, 24 d after floating; and (*C*) the keratinizing side of human foreskin. Both monoclonal antibodies RKSE60 (Ramaekers et al., 1983) and K_k 8.60 (Gigi-Leitner et al., 1986) showed identical staining patterns. Solid line in *A*, *left* frame, designates the border of anti-K1 staining observed in the *right* frame. Dotted lines indicate the border between the basal epidermal layer and either the collagen lattice (*A* and *B*) or the dermis (*C*). Note that in a few places the raft separated between the basal and the suprabasal epidermal layers during sectioning. In these cases, the results of the immunofluorescence could still be assessed, but the lower part of the raft does not appear in the figure. Bar, $20\ \mu\text{m}$.

acid supplements. As judged by double immunofluorescence, the K10/K11 in these cells appeared to be restricted to the more superficial outer epidermal layers, whereas K1 was detected in all suprabasal layers (Fig. 4 *A*, left and right). Interestingly, the difference in anti-K10/K11 and anti K1 staining disappeared when rafts were floated for an additional 17 d before harvesting (Fig. 4 *B*, left and right). Correspond-

ingly, at these later times the synthesis of all the differentiation-specific keratins was higher than in the 7-d cultures (data not shown). With respect to the pattern and levels of K10/K11 and K1 expression, these long-term cultures more closely resembled human skin, where K10/K11 and K1 were coexpressed in abundance in all suprabasal layers (cf. Fig. 4 *C*, left and right).

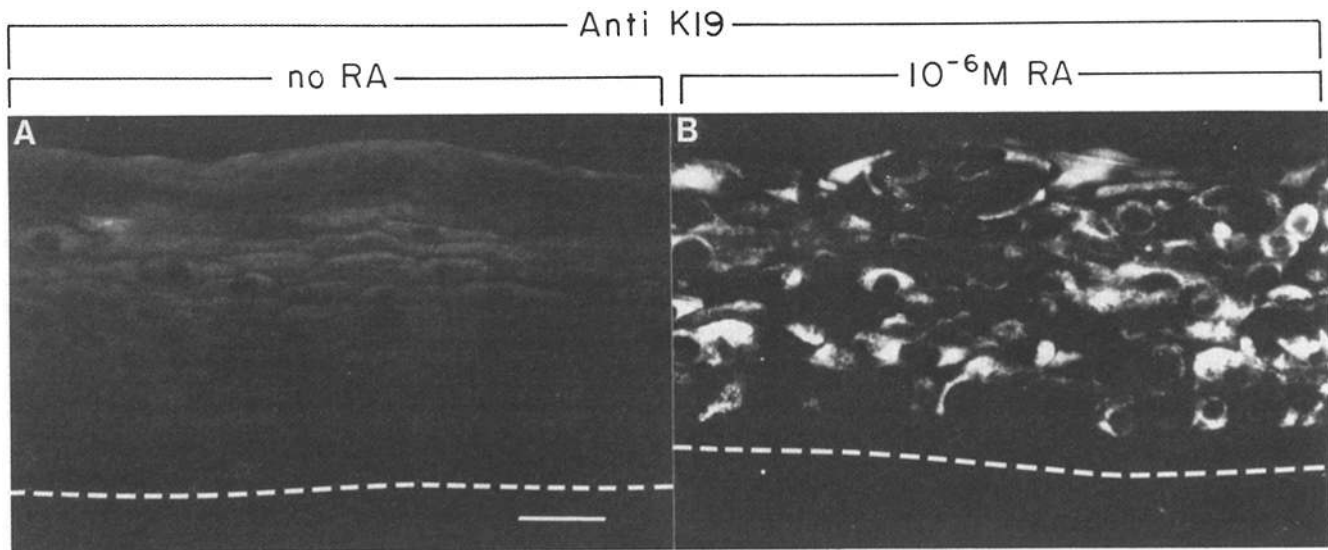


Figure 5. The localization of K19 in retinoid-treated cultures. Epidermal rafts were seeded and floated on medium containing serum and supplements of retinoic acid (RA). After 10 d in culture, rafts were fixed in Carnoy's solution and embedded in paraffin. Samples were sectioned (8 μm) and subjected to indirect immunofluorescence microscopy using a polyclonal antiserum specific for K19. Sections were from: (A) epidermal raft, no RA supplement; (B) epidermal raft, 1.0×10^{-6} M RA. The dotted lines indicate the border between the basal epidermal layer and the collagen lattice. Bar, 20 μm .

Expression of Basal and Hyperproliferation-associated Keratins In Floating Cultures Is Not Mediated by Retinoids

Some keratins were expressed irrespective of the levels of retinoids in the medium. This group included K5 and K14, the only keratins expressed in basal epidermal cells *in vivo* (Fig. 2). In addition, K6, K16, and K17, keratins expressed transiently in skin under conditions of hyperproliferation (E. Birgitte Lane, personal communication; Weiss et al., 1984; Tyner et al., 1986), were synthesized constitutively in the floating cultures, as they are in submerged cultures (Fuchs and Green, 1981).

Retinoic Acid Seems to Be Playing Different Roles in Mediating the Induction of Keratins K19 and K13

Two keratins, K19 and K13, are not normally present in epidermis (Moll et al., 1982a), in floating epidermal cells grown *in vitro* (Asselineau et al., 1986), or in submerged epidermal cells grown at low density (Sun and Green, 1978). However, in response to elevated levels of retinoids in the medium, both of these keratins are induced in submerged keratinocytes (Fuchs and Green, 1981; Kim et al., 1984). The results shown in Figs. 1 F and 2 illustrate that retinoic acid also induces the expression of K19 and K13 in floating cultures. In addition, a spot corresponding to K15 was also detected in all cultures treated with retinoic acid (Fig. 2, D–F).

K19, a keratin typically seen in certain simple epithelial cells (Moll et al., 1982a) and in other epithelial cells undergoing transition (Lane et al., 1985), was only detected when floating cultures were grown in media supplemented with 1.0×10^{-7} M retinoic acid or higher (Fig. 1 F, lanes d and e; Fig. 2, D–F). K13, whose expression has been identified in suprabasal cells of several nonkeratinizing stratified squamous epithelia (van Muijen et al., 1986), was expressed at very low levels in floating epidermal cultures grown in nor-

mal medium (Fig. 2 A), but its synthesis increased greatly in response to very low levels of retinoic acid (Fig. 1 F, lanes d and e; Fig. 2 D). That K13 is expressed at all in floating cultures fed with normal serum-containing medium is seemingly contrary to the findings of Asselineau et al. (1986). However, since K13 appears to be extremely sensitive to retinoid concentration, and since retinoid concentrations can vary as much as 10-fold in different serum lots (cf. Fuchs and Green, 1981 with Kim et al., 1984), this variation could easily account for the difference in results.

The antithetical behavior of K1 and K13/K19 led us to wonder whether there might be a direct correlation between suppression of K1 and induction of K13/K19. To examine this question, we stained frozen sections of floating cultures with antibodies specific for K19 and K13. Unexpectedly, the distributions of staining for K19 and K13 were distinctly different.

About 10% of the stratifying cells showed strong staining for K19 when retinoic acid was added at 1.0×10^{-6} M (Fig. 5). The rest of the suprabasal cells showed only a very low level of K19 staining. The number of brightly stained cells did not increase appreciably as the level of retinoic acid was elevated. Thus, as judged by immunofluorescence, the inverse relation between K1 and K19 did not seem to be constant throughout the epidermal population.

In contrast to K19, K13 showed a uniform gradient of expression depending upon the level of retinoic acid in the medium (Fig. 6). In untreated cultures, K13 was seen only in all the most superficial stratifying cells (Fig. 6 A, left). Double immunofluorescence of these cultures indicated that the outermost layers expressed both K13 and K1, whereas the innermost stratified layers stained only with anti-K1 and not with anti-K13 (Fig. 6 A, right). When the medium was supplemented with 1.0×10^{-7} M retinoic acid, K13 expression was induced in most suprabasal layers, and correspondingly, K1 expression was repressed (Fig. 6 B, left and right).

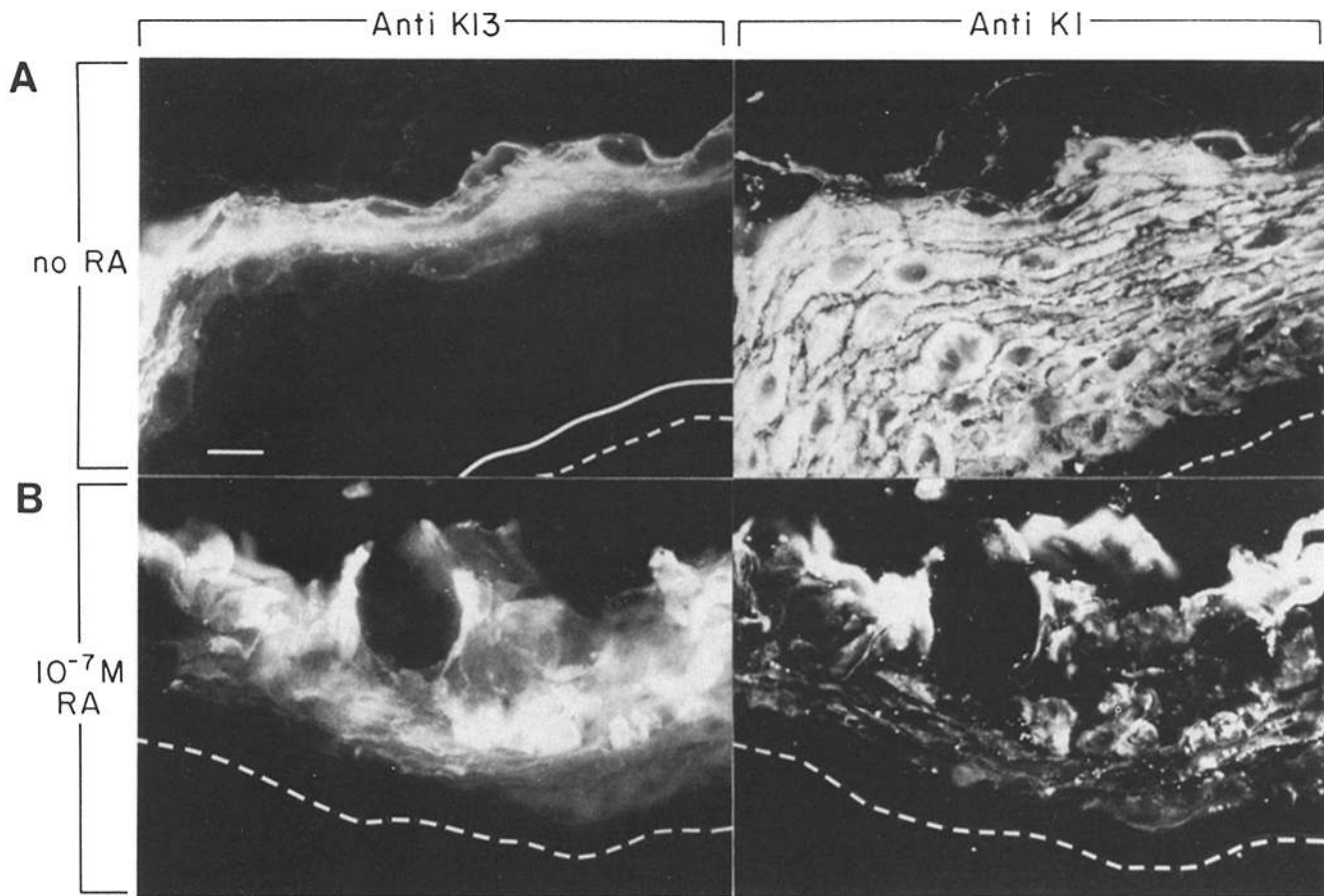


Figure 6. The localization of K13 in retinoid-treated cultures. Epidermal rafts were seeded and floated on medium containing serum and supplements of retinoic acid (RA). After 10 d in culture, rafts were frozen at -160°C in isopentane and sectioned ($4\ \mu\text{m}$). Sections were subjected to indirect double immunofluorescence using a monoclonal antibody against K13 (*left*) and a polyclonal antiserum against K1 (*right*). Sections were from: (A) epidermal raft, no RA supplement; (B) epidermal raft, $1.0 \times 10^{-7}\ \text{M}$ RA supplement. Results similar to those shown in B were obtained for all epidermal rafts floated on medium containing RA in the range of 1.0×10^{-7} – $1.0 \times 10^{-5}\ \text{M}$. Solid line in A (*left*) indicates border of anti-K1 staining observed in A (*right*). Dotted lines indicate borders between basal epidermal layers and collagen lattices. Bar, $20\ \mu\text{m}$.

Thus, while a correlation was found between increased expression of K13 and decreased expression of K1, the gradient of K13 induction occurred progressively from the outer to the inner stratifying layers.

Expression of the Terminal Differentiation-specific Keratins Is Not Induced by Calcium or by TGF- β

The experiments described above and previously (Fuchs and Green, 1981; Kim et al., 1984; Eckert and Green, 1984; Gilfix and Green, 1984) suggest that the process of inducing the terminal differentiation-specific keratins and of suppressing K13 and K19 in both floating and submerged cultures may rely upon reducing the exposure of stratifying epidermal cells to retinoids. Since the expression of K1, K10, and K11 seems to be one of the earliest biochemical markers of terminal differentiation (Fuchs and Green, 1980, 1981), we wondered whether other extracellular factors known to influence various features of the keratinizing process might also have an effect on keratin expression.

Previous reports have indicated that although calcium induces many morphological features of differentiation, it does not trigger the expression of large keratins (Roop et al.,

1983), nor does it influence synthesis of the cornified envelope protein involucrin (Watt and Green, 1982; Watt, 1984) or desmosomal proteins (Watt et al., 1984). In the experiments reported here, the medium contained $2.0\ \text{mM}$ calcium, a level shown to induce certain characteristics of terminal differentiation *in vitro* (Hennings et al., 1980). Since rafts grown on this medium did not express the large keratins when retinoic acid was present, calcium cannot override the effects of retinoids in controlling differentiation. However, we cannot exclude the possibility that calcium may be required for the retinoid-mediated synthesis of the terminal differentiation-specific keratins.

Recently, a report by Masui et al. (1986) suggested that TGF- β may influence terminal differentiation in bronchial epithelial cells, as evidenced by a TGF- β -mediated increase in stratification and cornified envelope formation. To determine whether TGF- β can elicit biochemical changes similar to those seen at low retinoid levels, we grew submerged epidermal cultures in normal serum-containing medium in the presence of varying levels of epidermal growth factor (EGF) and TGF- β . The level of TGF- β in medium containing 10% serum has been estimated at $0.02\ \text{nM}$ (Childs et al.,

1982), although the amount of active TGF- β may be less than this (Masui et al., 1986). For the experiments described here, added TGF- β was tested at a range of 0.01–1.0 nM, in the presence or absence of 0.8 nM EGF.

At three quarters confluence and at 2 wk postconfluence, cells were radiolabeled with [35 S]methionine, and keratins were subsequently extracted. Over the concentration range examined, neither TGF- β nor EGF- β induced the expression of large keratins (Fig. 7 A), even though the extent of stratification may have been slightly increased at a concentration of 1.0 nM TGF- β (Fig. 7 B).

Although these results failed to demonstrate any positive biochemical effect of TGF- β on terminal differentiation of epidermal cells, the gel profiles did indicate that several marked changes in protein synthesis took place when the cells reached confluence. As shown in Fig. 7 A, actin synthesis was substantially repressed, a finding which correlates with a decrease in cell movement and reduced mitotic index. More importantly, synthesis of K13 was markedly elevated in confluent cultures maintained at a constant level of retinoids. This phenomenon was only observed when retinoids were present in the culture medium. K13 was not induced in confluent cultures grown in medium containing delipidized serum (Fuchs and Green, 1981).

In Normal Human Epidermis, K13 is Only Expressed in the Inner Epithelium of the Foreskin

Interestingly, while K13 is not typically expressed in human skin (Moll et al., 1982a; Tyner et al., 1986; Fig. 1 F, lane a), it could be seen in keratins extracted from foreskin (see Fig. 2 B). Since K13 expression in epidermis seems to be unique to foreskin, we anticipated that it might be present only in the wet inner epithelium of this tissue. Except for the most superficial layers of the inner foreskin, its morphology is very similar to retinoid-treated floating cultures (Fig. 8 A). Stratifying cells are nucleated, and they are larger and looser in appearance than the keratinizing epithelium of the outer surface of foreskin (Fig. 8 E).

To examine the expression of keratins in the inner and outer epithelia of foreskin, we stained frozen sections with antibodies against K13 and K1. As expected, K13 stained all suprabasal layers of the wet (inner) epithelium (Fig. 8 B), but none of the layers of the dry (outer) epithelium (Fig. 8 F). This observation is consistent with an earlier observation made by van Muijen et al. (1986).

All suprabasal cells of both the inner and outer epithelia of foreskin stained with our antibody against K1 (Figs. 8 C and 4 C). We were surprised to discover that the pattern of anti-K10/K11 staining in the inner part of the foreskin differed from the pattern of anti-K1 staining. Anti-K10/K11 showed no staining in the densely packed, nucleated outermost layer of the wet epithelium (Fig. 8 D). In the dry epithelium, K10/K11 and K1 showed identical distributions (Fig. 4 C, left and right).

Discussion

Retinoid Deficiency, Terminal Differentiation, and the Expression of Large Keratins

Vitamin A has been known to affect epithelial differentiation since the early studies of vitamin deficiency (Mori, 1922;

Wolbach and Howe, 1925) and vitamin excess (Fell and Melanby, 1953). Early cell culture studies reported morphological changes in keratinocytes treated with varying amounts of retinoids (Yuspa and Harris, 1974; DeLuca and Yuspa, 1974; Sporn et al., 1975; Milstone and McGuire, 1979; Elias et al., 1981). More recently, retinoid-mediated changes in keratin synthesis (Fuchs and Green, 1981; Kim et al., 1984; Eckert and Green, 1984; Gilfix and Green, 1984) and in involucrin synthesis (Green and Watt, 1982) have been reported. In these cases, growth of keratinocytes in medium containing delipidized serum induced both morphological and biochemical changes associated with terminal differentiation. Although it was not determined whether retinoids were the only fat-soluble serum components that suppressed keratinization, the addition of physiological concentrations of retinoids back to the medium counteracted this response.

In the experiments reported here we have shown that retinoic acid can even suppress the extensive terminal differentiation achieved by epidermal cell cultures floating on rafts of collagen and fibroblasts. In this system vitamin A-depleted serum was not necessary to induce the keratinizing process, and somewhat higher concentrations of retinoic acid were necessary to suppress the response. However, at relatively low (10^{-6} M) retinoic acid, this suppression was similar to that attained by keeping the rafts submerged in serum-containing medium.

We do not yet know whether retinoids exert their action directly via the epidermal cells, or indirectly via the fibroblasts located in the collagen lattice. However, since the collagen lattice remains in contact with the medium whether it is floating or submerged, yet keratin expression in the epidermal cells undergoes marked changes, it seems most likely that retinoic acid is acting directly on the epidermal cells and that the barrier of retinoid diffusion is the basal epidermal layer. If this assessment is correct, then the enhancement of terminal differentiation caused by growing cells at an air-liquid interface may arise from the ability of stratifying cells to migrate away from the nutrient supply of vitamin A. Interestingly, since blood vessels supplying vitamin A to skin are located in the dermis, it might be that terminal differentiation *in vivo* could be programmed by progressive migration of differentiating epidermal cells away from the dermis and towards the skin surface. Further studies examining the specificity of various biologically active and inactive retinoids in suppressing the differentiative process will be necessary in order to study the mechanism underlying the events reported in this paper.

While other extracellular factors such as calcium and TGF- β influence stratification and cornified envelope formation *in vitro* (Hennings et al., 1980; Watt and Green, 1982; Watt, 1984; Masui et al., 1986), only retinoid-deficient cells seem to express the large molecular mass keratins characteristic of all suprabasal epidermal cells *in vivo*. These keratins account for ~85% of the total protein in fully differentiated squamiae, and as such are major biochemical markers of the differentiative process (Fuchs and Green, 1980). The functional significance of their differential expression has not yet been determined. Recently, however, *in vitro* filament assembly studies using different purified keratins revealed that while basal epidermal keratins (K14 and K5) form isolated, disperse filaments, differentiation-specific keratins assemble into filaments that aggregate (Eichner et al., 1986). Although

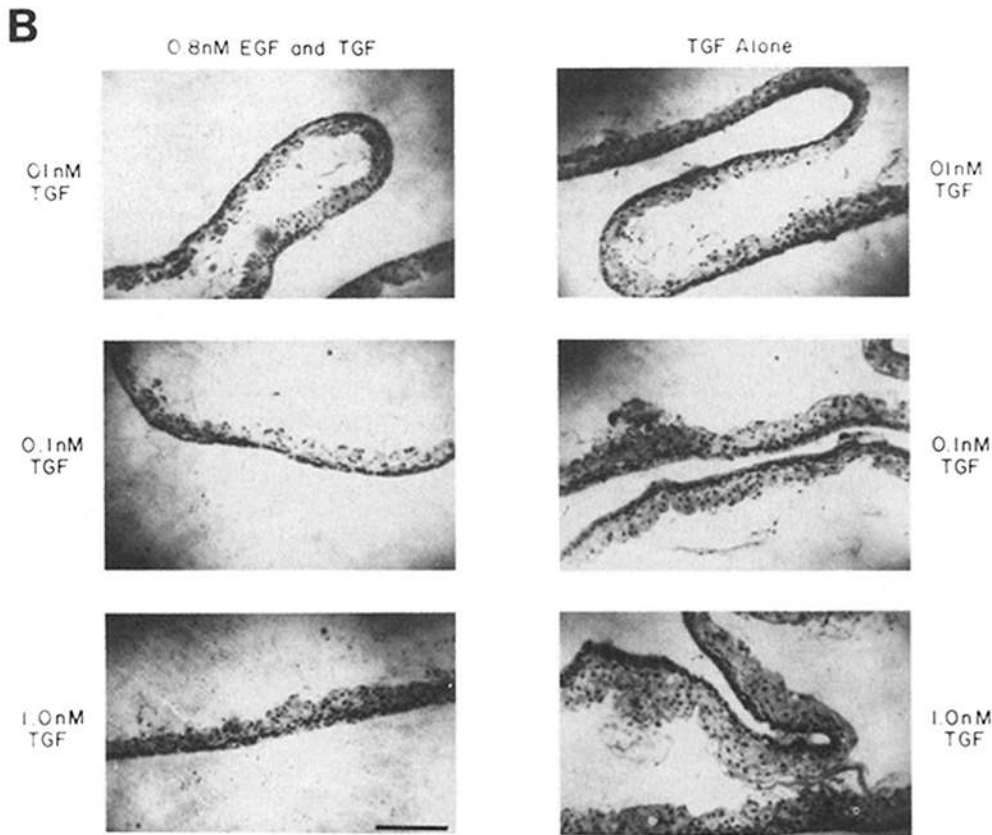
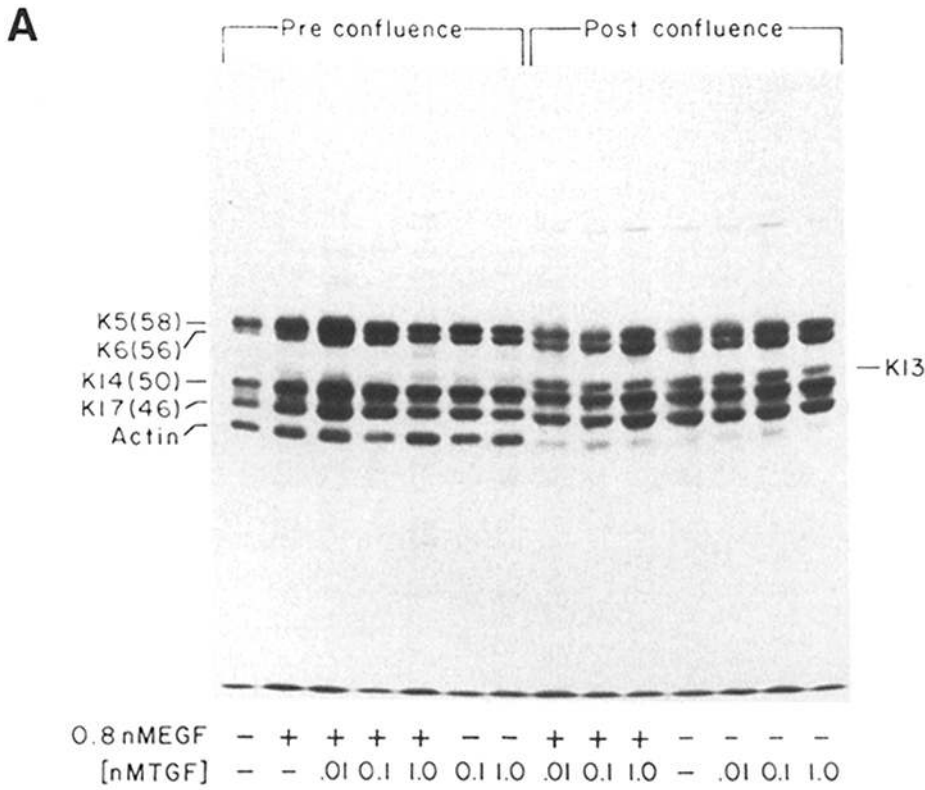


Figure 7. The effects of TGF- β and EGF on keratin synthesis in submerged epidermal cultures. Epidermal cells were seeded on lawns of mitomycin C-treated fibroblasts and cultured in the standard (submerged) fashion. Cells were grown in triplicate in serum-containing medium that was supplemented with EGF and/or TGF as indicated. (A) At three-fourths confluence and again at 2 wk postconfluence, one dish of each set was radiolabeled with [35 S]methionine (100 μ Ci/ml) overnight and the keratins were extracted and analyzed by SDS-PAGE. The gel was fluorographed and subjected to autoradiography. (B) At 2 wk postconfluence, the third dish of each set was treated with the enzyme dispase to remove the culture from the dish surface. Cultures were fixed in Carnoy's solution and embedded in paraffin. Cross sections (4 μ m) of each culture were stained in hematoxylin and eosin and visualized by light microscopy.

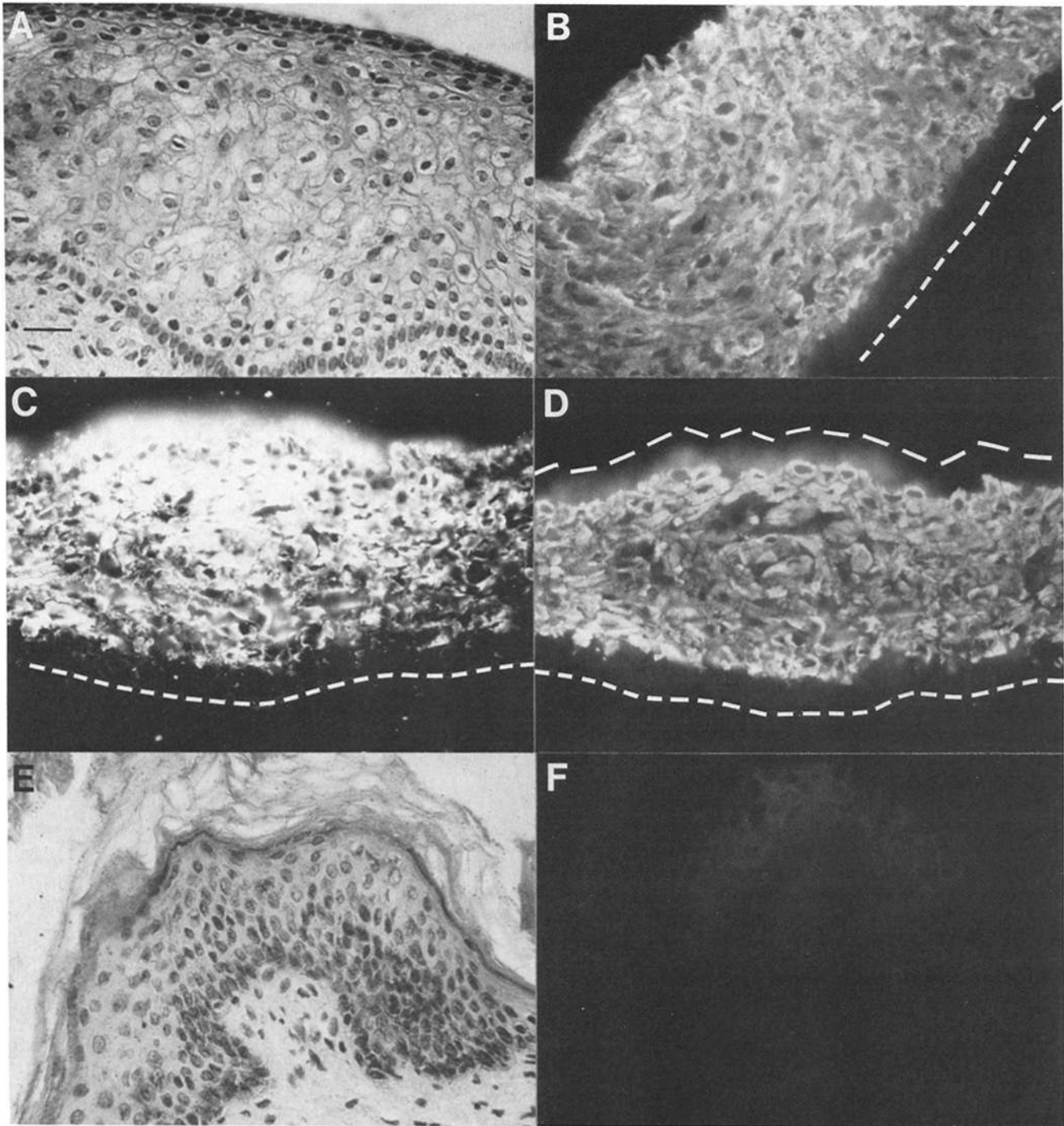


Figure 8. Differential expression of K13 in the inner and outer sides of the foreskin. Human foreskins were either fixed in Carnoy's solution and embedded in paraffin (*A* and *E*) or frozen in isopentane at -160°C (*B-D* and *F*). Samples were sectioned ($4\ \mu\text{m}$) and subjected to either hematoxylin and eosin staining (*A* and *E*) or indirect immunofluorescence (*B-D* and *F*). (*A*) Nonkeratinizing stratified squamous epithelium of the inner (wet) side of the foreskin; (*B*) inner side of foreskin, stained with anti-K13; (*C*) inner side of foreskin stained with anti-K1; (*D*) same section as *C*, stained with anti-K10/K11; (*E*) keratinizing stratified squamous epithelium of the outer (dry) side of the foreskin; (*F*) outer side of foreskin, stained with anti-K13. Dotted lines demarcate tissue boundaries. Bar, $20\ \mu\text{m}$.

the *in vivo* formation of macrofibrillar bundles of keratin filaments in the suprabasal cells may not arise solely from expression of these large keratins, the aggregation may assist keratin filaments in surviving the destructive phase of the differentiative process. It might also interfere with further cell division. As such, the synthesis of large keratins could

play an important role in the commitment of a cell to terminally differentiate.

A Critical Level of K1 Expression May Be Required for K10/K11 Induction

The keratins have been subdivided into two distinct types ac-

ording to their sequences (Fuchs et al., 1981). Type I and type II keratins seem to be expressed in specific pairs according to tissue and to relative stage of differentiation and development (Eichner et al., 1984). In vitro assembly experiments have shown that at least one member of each type is necessary for filament formation (Hatzfeld and Franke, 1985; Eichner et al., 1986).

Recently, gene transfection studies have suggested that the forced expression of a type II epidermal keratin in a fibroblast cell can trigger the expression of an endogenous type I epidermal keratin, suggesting the possibility that accumulated type II keratin may act as a signal to induce its partner, type I keratin (Giudice and Fuchs, 1987). In our in vitro studies, at times when the expression of terminal differentiation-specific keratins was significantly lower than in skin, the type II keratin K1 were seen in some layers where the type I keratins K10 and K11 were not detected. In our in vivo studies we discovered that the outermost layer of cells in the wet epithelium of the foreskin seemed to express K1, but not K10/K11. Recent examples of type II expression in the absence of type I expression have also been reported in other laboratories (Darmon, 1985; Schermer et al., 1986). Since, in many of these cases, type I keratin expression seems to subsequently follow type II expression, it is possible that a critical level of type II expression is required for type I induction.

Retinoid Excess, Part I: K19 Expression

We were surprised to discover that only a few suprabasal epidermal cells showed strong staining with anti-K19 antiserum in response to elevated levels of retinoids. If the level of antibody staining reflects the true level of K19 in epidermal cells, then K19 expression in some cells is at least an order of magnitude higher than we initially anticipated. Alternatively, if the antigenic sites for the antibody are masked in most cells, then a few cells must have a different organization of K19 in their cytoskeleton. In either case, no clear morphological distinction could be made between cells showing different levels of K19 staining.

In addition to its induction in retinoid-treated cultures, K19 has been found (a) in certain simple epithelial cells, e.g., mesothelium (Wu et al., 1982); (b) during early fetal development of epidermis (Moll et al., 1982b; Dale et al., 1985); (c) in basal and squamous cell carcinomas of skin (Wu and Rheinwald, 1981; Moll et al., 1984); and (d) in epithelial cells in the process of transition (Lane et al., 1985). Even though it is the smallest keratin known, K19 can still assemble into filaments both in vivo (Wu and Rheinwald, 1981) and in vitro (Hatzfeld and Franke, 1985). How this keratin might influence the resulting filaments and how this might be important for the biology of cells which express K19 is presently unknown.

Retinoid Excess, Part II: K13 Expression

When epidermal cells are grown submerged in medium containing 10^{-7} – 10^{-5} M retinyl acetate, they show an increase in cell movement, as indicated by rearrangement of groups of cells into ridges and eventually whorls (Fuchs and Green, 1981). Increasing concentrations of retinoids also seem to reduce adhesiveness in cultures, as evidenced by the increased detachment of the most mature cells from the upper layer of

the epithelium (Milstone and McGuire, 1979; Fuchs and Green, 1981). Electron microscopy studies have shown that desmosome and tonofilament formation are also repressed by retinoids (Yuspa and Harris, 1974).

Unlike submerged cultures, where weaker intercellular interactions can be disrupted with every feeding, retinoid-treated cultures on floating collagen/fibroblast rafts maintained delicate interactions between superficial cell layers. This enabled us to observe a retinoid-mediated gradient of disorganization in the cells, from the outer to the inner layers. Interestingly, this perturbation in cell-cell contacts closely paralleled the expression of K13 in the retinoid-treated cultures. The apparent correlation between weakening of intercellular interactions, stratification, and K13 expression may explain why submerged cultures in normal medium only expressed large amounts of K13 when they reached confluence, and stratified and desquamated extensively. Moreover, it offers a possible explanation for why cultures grown in retinoid-depleted medium did not synthesize K13 even though they stratified extensively. Under these conditions, the intercellular interactions were extremely strong and the epithelium showed no tendency to discharge cells from its upper surface (Fuchs and Green, 1981).

If vitamin A has a direct effect on K13 synthesis, it might be expected that the gradient of expression is from the inner to the outer layers, rather than the reverse. The discovery that there is a gradient of K13 expression which seems to be opposite to the expected concentration gradient of retinoids suggests that K13 regulation may be mediated by retinoids via weakening of intercellular interactions. We do not yet know whether disruption of cell-cell contacts in the absence of retinoids can induce K13 expression. However, it now seems likely that, in vivo, biochemical differences between intercellular interactions within different stratified squamous epithelia account for their variation in K13 expression.

Precisely how differences in intercellular interactions might lead to an alteration in keratin synthesis is not known. It is well known that keratin filaments interact specifically with desmosomes at the inner side of the plasma membrane (Skerrow and Matoltsy, 1974). In epidermis, the large keratins of terminally differentiating cells may be preferentially involved in these interactions (Drochmans et al., 1979). Possible associations of specific keratin filaments with desmosomes in other cell types have not yet been examined. However, if high levels of retinoids repress desmosome formation as initially suggested by Yuspa and Harris (1974), then intermediate levels of retinoids might be expected to influence the complex interactions between desmosomal proteins and keratin filaments.

In summary, our results indicate that retinoids influence keratin gene expression in a number of different ways. Their suppressive effect upon the expression of the large epidermal keratins and their inductive effect upon K13 suggest that retinoids might play an important role in development and differentiation in stratified squamous epithelia. Additional experiments will be necessary to define the molecular pathway(s) by which retinoids exert their action on these cells.

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