Selective Migration of Terminally Differentiating Cells from the Basal Layer of Cultured Human Epidermis

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ABSTRACT How terminally differentiating cells are selectively expelled from the basal layer of epidermis has been a source of interest and speculation for many years. The problem can now be studied in culture, using involucrin synthesis as an early marker of terminal differentiation in human keratinocytes. When keratinocytes are forced to grow as a monolayer by reducing the calcium ion concentration of the culture medium, they still begin to synthesize involucrin. Raising the level of calcium ions induces stratification, and cells that are synthesizing involucrin are selectively expelled from the basal layer. I have found that during calcium-induced stratification no new proteins or glycoproteins are synthesized, and the rate of cell division does not change. Movement of involucrin-positive cells out of the basal layer was found to be unaffected by cycloheximide, tunicamycin, or cytosine arabinoside. These results suggest that keratinocytes growing as a monolayer already have the necessary properties to determine their position when stratification is induced. Addition of calcium simply allows formation of desmosomes and other intimate cell contacts required for stratification. The properties of involucrin-positive cells that determine their suprabasal position include a reduced affinity for the culture substrate and preferential adhesion to other cells at the same stage of terminal differentiation. The molecular basis of these adhesive changes is discussed.

Two of the greatest challenges in cell biology are to understand what factors direct cells towards terminal differentiation and how the properties of individual differentiated cells in turn influence tissue architecture. The cultivation of human epidermal cells provides a useful model for studying these problems, because, as in intact epidermis, the cells grow as stratified colonies in which mitosis is restricted to the basal layer, and cells terminally differentiate as they migrate through the outer layers (11).

In stratified cultures of human keratinocytes, synthesis of involucrin, a precursor of the cross-linked envelope, begins immediately above the basal layer and therefore provides a useful marker for an early stage in terminal differentiation (1, 27, 34). When keratinocytes are prevented from stratifying by reducing the calcium ion concentration of the medium, they are still able to initiate involucrin synthesis. This demonstrates that, although stratification and terminal differentiation are normally linked, stratification is not a prerequisite for terminal differentiation. Raising the level of calcium ions induces keratinocyte monolayers to stratify, and results in the selective migration of involucrin-positive cells from the basal layer. Thus, attainment of the suprabasal position appears to be a consequence, not a cause, of terminal differentiation (35).

In the past, two problems have hampered a detailed analysis of how terminally differentiating keratinocytes are selectively expelled from the basal layer. It is impossible to identify cells that are committed to leaving the basal layer and it is hard to regulate cell migration experimentally, since the rate of migration reflects a steady state between basal cell proliferation and superficial cell shedding. These problems can be circumvented by studying calcium-induced stratification. In low-calcium monolayers, the cells that will leave the basal layer are those already synthesizing involucrin; and all the involucrin-positive cells enter the suprabasal layers within 24 h of raising the level of calcium ions.

Since involucrin-positive cells are hardly ever found in the basal layer of stratified cultures (1), it is likely that by the time keratinocytes have terminally differentiated as far as the initiation of involucrin synthesis, they have all the properties needed to determine their position in the suprabasal layers. In support of this conclusion, I have found that calcium-induced stratification can proceed normally in the absence of new protein synthesis and glycosylation, and when mitosis is inhibited. The properties of involucrin-positive cells that determine their suprabasal position include a reduced affinity for the culture substrate and preferential adhesion to other

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MATERIALS AND METHODS

Cell Culture

Human keratinocytes (strain a, second to sixth passage), isolated from newborn foreskin, were grown in the presence of 3T3 cells pretreated with 4 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, MO) for 2 h (24, 25). The culture medium was Dulbecco's modified Eagle's medium (DME)[1] supplemented with 20% fetal calf serum, 0.5 μg/ml hydrocortisone (25), 10^{-10} M cholera toxin (10), and 10 ng/ml epidermal growth factor (Bethesda Research Laboratories, Inc., Gaithersburg, MD) (26).

Experimental Manipulation of Cells

LOW-CALCIUM MONOLAYERS: Calcium salts were omitted from the DME formulation and fetal calf serum was depleted of divalent cations by treatment with Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA) as described by Brennan et al. (3). The calcium concentration, determined by flame photometry, was ~2.0 mM in normal keratinocyte medium, and 0.1 mM in calcium-free DME containing 20% Chelex-treated fetal calf serum (low-calcium medium). Keratinocytes in low-calcium medium were grown in the presence of a feeder layer of 3T3 cells, and the medium was supplemented with hydrocortisone, cholera toxin, and epidermal growth factor.

HIGH DENSITY PLATING: At confluence, the normal yield of keratinocytes from a 35-mm-diam petri dish (Falcon Plastics, Oxnard, CA) is ~1–1.5 × 10^6. For high density plating experiments, a mixture of involucrin-positive and -negative keratinocytes was plated out in the absence of 3T3 cells, at a density of 3–5 × 10^6 per 35-mm dish, that is, three times the normal cell number per dish at confluence.

KERATINOCYTE AGGREGATION: 2.5% agarose was prepared by autoclaving 5% low melting point agarose (Bethesda Research Laboratories, Inc.) in distilled water, cooling it to 37°C, and adding an equal volume of 2 × DME. The agarose was set in the shape of a hollow capsule. 1–2 × 10^6 keratinocytes were pelleted to the base of the capsule by low speed centrifugation. The capsule was then sealed with agarose and incubated in medium for 24 h. Keratinocytes were also plated out at high density in an agarose-coated petri dish: the cells did not adhere to this but formed large aggregates in suspension within 24 h.

Metabolic Inhibitors

The effect of different concentrations of various inhibitors on incorporation of radioactivity into trichloroacetic acid-precipitable material was measured. 2 μg/ml cycloheximide (Sigma Chemical Co.) inhibited [^{35}S]methionine incorporation by >90%; 5 μg/ml caused 97% inhibition. 5 μg/ml tunicamycin (Sigma Chemical Co.) inhibited 92% of [^{3}H]mannose incorporation. 15 μg/ml cytosine arabinoside (Sigma Chemical Co.) inhibited [^{3}H]thymidine incorporation by 98%. Radiochemicals were supplied by Amersham International, Amersham, England. Higher concentrations of the drugs did not cause further inhibition of incorporation and were toxic to the cells.

Preparation of Sections through Keratinocyte Cultures and Examination by Immunofluorescence

Stratified sheets of keratinocytes were detached intact from the culture dish by incubation with 2.5 mg/ml dispase (grade II, Boehringer Mannheim, Federal Republic of Germany) in serum-free DME (1). Each sheet was rinsed with isotonic PBS and draped over a piece of Whatman No. 1 filter paper (Whatman Ltd., Maidstone, England) to provide support and to establish the orientation of the sheet. Detached sheets were fixed in 3.7% formaldehyde in PBS at room temperature for 15–30 min, then dehydrated and embedded in paraffin wax. 8-μm sections were cut at right angles to the surface of the filter paper.

For immunofluorescence studies, sections were rehydrated, washed in PBS, and incubated with rabbit antiserum to involucrin, diluted 1:20 with PBS (27), for 30 min at room temperature. After the sections were further washed in PBS, a 1:32 dilution of fluoresceinated goat anti-rabbit IgG (Miles Scientific, Naperville, IL) was added and the preparation was incubated as before. Finally, the sections were washed thoroughly in PBS, mounted in Gelvatol (Monsanto Petrochemicals Ltd., St. Louis, MO), and examined with a Zeiss photomicroscope III.

Labeled Cell Extracts

Keratinocytes in 35-mm-diam petri dishes were incubated in 2 ml of medium containing 10 μCi/ml [^{35}S]methionine (>800 Ci/mmol), 5 μCi/ml [^{14}C]mannose (200–300 mCi/mmol), or 5 μCi/ml [^{14}C]glucosamine hydrochloride (50–60 mCi/mmol) (Amersham International). After being labeled, cultures were rinsed in PBS and solubilized in a modification of the sample buffer for PAGE: it consisted of 2% SDS, 0.125 M Tris-HCl, pH 6.8.

PAGE

Samples were electrophoresed in 7.5% acrylamide slab gels (16). Gels were stained with 0.1% Coomassie Brilliant Blue R (Sigma Chemical Co.), destained, and prepared for fluorography by the procedure of Bonner and Laskey (2). Prefogged Kodak X-Omat AR film was used (17).

RESULTS

Protein Synthesis and Stratification

Three different aspects of calcium-induced stratification were examined to see whether keratinocytes growing as a monolayer already had the properties that would determine their position after stratification, or whether addition of calcium triggered new biosynthetic events.

Protein synthesis was examined first. The proteins synthesized by monolayer, stratified, or stratifying keratinocytes were compared by PAGE of cells extracted after labeling for 24 h with [^{35}S]methionine. As shown in Fig. 1 (lanes *1–3*),

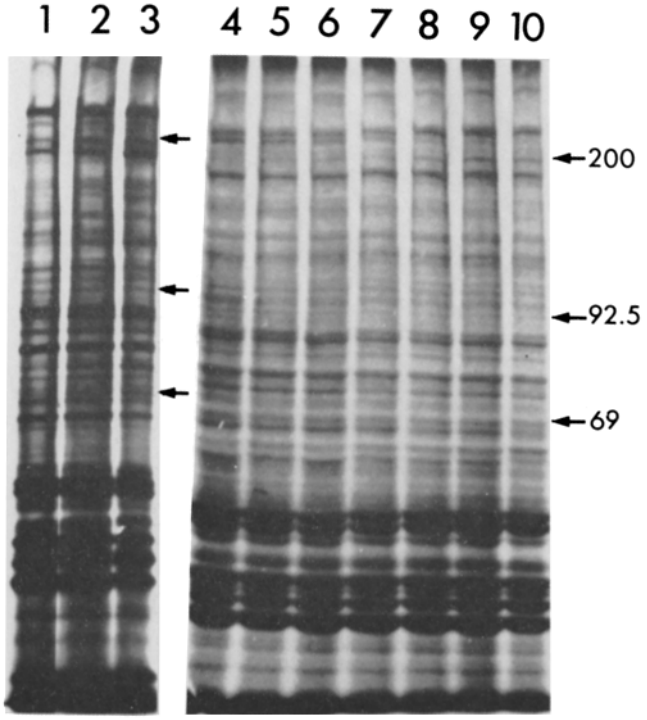

FIGURE 1 PAGE of keratinocyte proteins labeled with [^{35}S]methionine. Lanes *1–3*: 24-h label. Lane *1*, monolayer keratinocytes in low-calcium medium; lane *2*, monolayer keratinocytes, induced to stratify by addition of normal medium at the start of the labeling period; lane *3*, control, stratified keratinocytes grown in normal medium. Lanes *4–10*: 1-h label. Lane 4, monolayer keratinocytes in low-calcium medium. Lanes *5–10*, addition of normal medium to monolayer keratinocytes to induce stratification. Lane *5*, 1 h after addition of normal medium; lane 6, 2 h; lane *7*, 3 h; lane *8*, 6 h; lane *9*, 9 h; lane *10*, 12 h. Arrows indicate position of protein standards (molecular weights × 10^{-3}).

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium.

there was no difference either in the number of proteins or in their relative abundance. To determine whether calcium induced a transient change in the type of proteins synthesized, stratifying cultures were labeled for 1 h in [^{35}S]methionine at intervals after addition of calcium to the monolayers. The proteins synthesized during the first 12 h, by which time stratification is well advanced, were the same as in nonstratifying cultures (Fig. 1, lanes *4–10*). The rate of incorporation of label into trichloroacetic acid-precipitable material was stimulated slightly after the first 6 h (data not shown).

The effect of cycloheximide on calcium-induced stratification was also examined. Keratinocytes were grown to confluence in low-calcium medium (0.1 mM), and then normal medium (2.0 mM calcium) containing 2 or 5 μg/ml cycloheximide was added. Protein synthesis was inhibited within minutes (32). After 24 h, the cultures were harvested with dispase, fixed, embedded, and sectioned. Cycloheximide treatment caused some cells to detach from the culture surface, particularly at the higher concentration tested; this was not observed in control cultures, and was probably due to the toxicity of prolonged exposure to the drug. Nevertheless, stratification did occur in the presence of cycloheximide and, as in control cultures, involucrin-positive cells were located almost exclusively in the suprabasal layers (Fig. 2, *a* and *b*).

Thus, no new proteins were detected after addition of calcium to keratinocyte monolayers, and stratification and selective migration of involucrin-positive cells could occur in the virtual absence of protein synthesis. This suggests that the desmosomal junctions formed within 2 h of addition of calcium are assembled from proteins already present in the cell, and are not synthesized *de novo* (12; F. M. Watt and D. R. Garrod, manuscript in preparation).

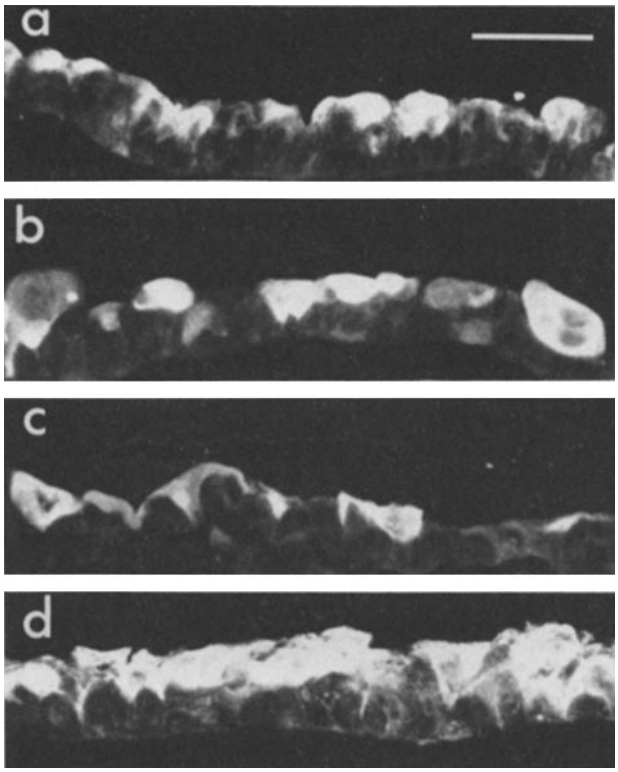

FIGURE 2 Effect of various inhibitors on calcium-induced stratification. (*a*) Control, (*b*) 2 μg/ml cycloheximide, (c) 5 μg/ml tunicamycin, (*d*) 15 μg/ml cytosine arabinoside. In each case, virtually all of the involucrin-positive cells are in the suprabasal layers. Bar, 50 μm. × 320.

Protein Glycosylation and Stratification

In both natural and cultured epidermis, changes in keratinocyte membrane glycosylation occur during terminal differentiation, and these could play a role in determining the orderly migration of keratinocytes through the different cell layers (33). To discover whether changes in protein glycosylation occur during calcium-induced stratification, monolayer, stratified, and stratifying keratinocytes were labeled for 24 h with [^{14}C]mannose or [^{14}C]glucosamine, and the labeled protein bands were analyzed by PAGE (Fig. 3). No differences between keratinocytes grown under the different conditions were detected.

Tunicamycin specifically inhibits addition of N-linked carbohydrate to protein (31), and its effect on calcium-induced stratification was examined. Normal medium was added to low calcium monolayers in the presence of 5 μg/ml tunicamycin. After 24 h the cultures were harvested with dispase, fixed, embedded, and sectioned. Areas of stratification were found in the treated cultures, and in these, as in control cultures, involucrin-positive cells were selectively expelled from the basal layer (Fig. 2*c*). Any glycoproteins that play a role in determining the position of keratinocytes within the different cell layers must therefore be synthesized in monolayers as well as in stratified cultures.

Mitosis and Stratification

The third aspect of calcium-induced stratification to be examined was whether mitosis of involucrin-negative cells is required. Some years ago, it was suggested that terminally differentiating keratinocytes might be forced from the basal layer by pressure from neighboring cells in mitosis (19). This is not the case in intact epidermis, because when mitosis is inhibited by ionizing radiation, keratinocytes still enter the suprabasal epidermal layers normally (7). Two experiments in culture also ruled out a role for mitosis in the selective migration of terminally differentiating cells.

First, addition of calcium to keratinocyte monolayers did not affect cell division. In a typical experiment, 30 plates of keratinocytes were seeded at the same density and grown to confluence in low-calcium medium. Normal medium was then added to 15 of the plates, and low-calcium medium to the other 15. After 24 h, the mean number of cells per dish was 8.0×10^5 (1.1×10^5 SD) in normal medium, and 7.5×10^5 (1.2×10^5 SD) in low-calcium medium. This difference is not significant.

Second, inhibition of mitosis did not affect stratification. When normal medium containing 15 μg/ml cytosine arabinoside was added to low-calcium monolayers, stratification and selective migration of involucrin-positive cells occurred normally, even though DNA synthesis was completely inhibited (4) (Fig. 2*d*). Therefore, mitosis was not required for selective expulsion of involucrin-positive cells from the basal layer. Inhibition of mitosis for 24 h did not stimulate involucrin synthesis (F. M. Watt, unpublished observation).

In conclusion, the selective migration of involucrin-positive keratinocytes from the basal layer during calcium-induced stratification did not require new protein synthesis or N-linked glycosylation, and did not depend on mitosis of involucrin-negative cells. Adding to these results the observation that

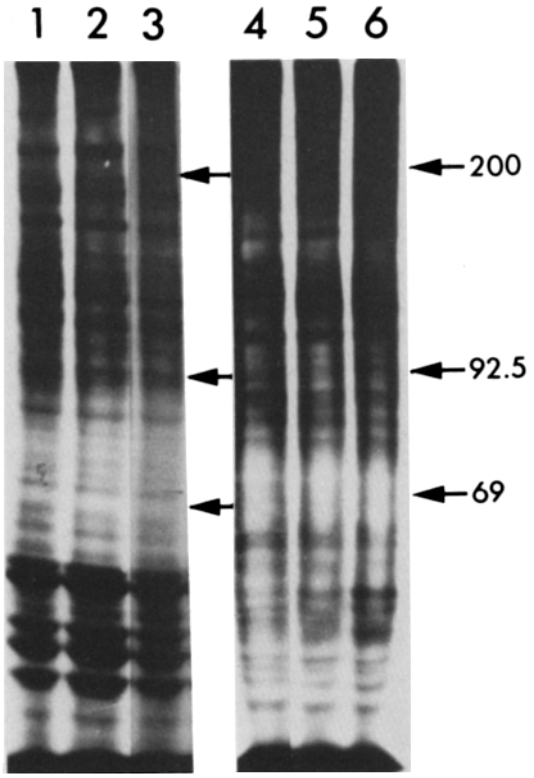

FIGURE 3 PAGE of keratinocyte glycoproteins labeled for 24 h. Lanes *1–3*: [^{14}C]mannose. Lanes *4–6*: [^{14}C]glucosamine. Lanes *1* and *4*, monolayer keratinocytes in low-calcium medium; lanes *2* and *5*, monolayer keratinocytes induced to stratify by addition of normal medium at start of labeling period; lanes *3* and *6*, control, stratified keratinocytes grown in normal medium. Arrows indicate position of protein standards (molecular weights × 10^{-3}).

involucrin-positive keratinocytes are very rarely seen in the basal layer of stratified cultures (1), it seems that by the time that keratinocytes have terminally differentiated as far as the initiation of involucrin synthesis, they have all the properties required to determine their position above the basal layer, even if migration from the basal layer is prevented. What these properties may be is considered in the next section.

Adhesive Properties of Terminally Differentiating Keratinocytes

When monolayers of keratinocytes are maintained in low-calcium medium for 1–2 wk after reaching confluence, increasing numbers of involucrin-positive cells round up and either remain loosely attached to the surface of the monolayer or detach into the medium (35). This suggests that terminal differentiation of keratinocytes is accompanied by a decrease in substrate adhesiveness, which, in turn, could result in migration from the basal layer. I have now looked for evidence of a link between terminal differentiation and altered cell adhesion under conditions in which the calcium concentration is normal, enabling keratinocytes to form desmosomes and extensive cell-to-cell contacts.

In the first experiment, stratified cultures containing a mixture of involucrin-negative and involucrin-positive keratinocytes were harvested as a single-cell suspension. The cells were plated out in normal medium, without 3T3 cells, at three times the usual confluent density. After 24 h, the cultures were fixed and sectioned, to determine whether sorting out of involucrin-negative and -positive cells had occurred, and, if so, which cells adhered preferentially to the culture dish.

Fig. 4*a* shows that sorting out of keratinocytes did occur. The basal layer contained almost exclusively involucrin-negative cells, and cells synthesizing involucrin were found only in the outermost layers. These results confirm that keratinocytes that have not yet begun to terminally differentiate adhere preferentially to the culture substrate.

The sorting out of involucrin-positive and -negative keratinocytes during calcium-induced stratification or after high density plating may result not only from a decreased affinity of involucrin-positive cells for the culture substrate, but also from preferential cohesion of cells at the same stage of terminal differentiation. This has been studied by allowing keratinocytes to aggregate either in a pellet or free in suspension.

Stratified cultures of keratinocytes, consisting of a mixture of involucrin-negative and -positive cells, were harvested close to confluence. The resulting single-cell suspension was pelleted into an agarose capsule. The capsule was sealed and incubated in normal medium for 24 h, then sectioned. Large aggregates had formed at the periphery of the pellet; these consisted of clusters of involucrin-negative cells surrounded by involucrin-positive cells (Fig. 4*b*). The same results were obtained when keratinocytes were allowed to aggregate in suspension for 24 h (Fig. 4, *c* and *d*). The average cell cycle time of dividing keratinocytes is ~22 h (10), and, therefore, the clusters of involucrin-negative cells seen in aggregates could not have arisen by mitosis alone.

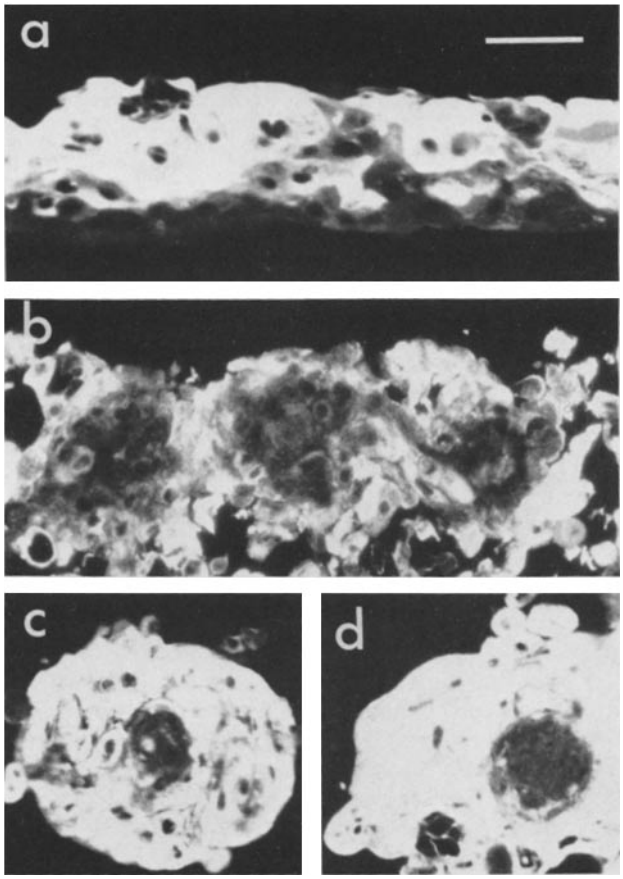

FIGURE 4 Sorting out of involucrin-positive and -negative keratinocytes. (*a*) High density plating, (*b*) aggregation in agarose pellet, (*c* and *d*) aggregation in suspension. Bar, 50 μm. × 260.

The cell aggregation experiments demonstrate that keratinocytes at different stages of terminal differentiation can 'sort out', just as cells from different tissues sort out in culture (21). Involucrin-negative cells adhere preferentially to involucrin-negative cells, and involucrin-positive cells adhere preferentially to involucrin-positive cells. According to the differential adhesion hypothesis of Steinberg (30), during cell sorting the more strongly cohesive cells assume the internal position within the aggregate. If this is so, keratinocytes become less cohesive as they terminally differentiate. Whether the molecular basis of the altered cohesiveness is the same as, or different from, the reduced affinity for the substrate remains to be investigated.

DISCUSSION

The mechanism by which terminally differentiating cells are selectively expelled from the basal layer of epidermis has been a source of interest and speculation for many years (7, 14, 19). Two recent discoveries have allowed a detailed analysis of the process in cultures of human keratinocytes. The first is that the onset of involucrin synthesis, while occurring about half way through the living layers of intact epidermis, is immediately suprabasal in culture (1). The second is that keratinocytes can still initiate involucrin synthesis when forced to grow as a monolayer in low-calcium medium; however, these cells migrate to a suprabasal position within 24 h of raising the concentration of calcium ions (35). Human keratinocytes proliferate both in low-calcium (0.1 mM) and normal (2.0 mM calcium) medium and the proportion of involucrin-positive cells is the same under both conditions (35). Note that, in contrast, mouse keratinocytes only proliferate in low-calcium medium; the entire population is induced to terminally differentiate when the level of calcium ions is raised (13).

Several aspects of calcium-induced stratification were investigated. PAGE did not reveal any changes in the proteins synthesized after addition of calcium; and stratification was not inhibited by cycloheximide. No changes in keratinocyte glycoproteins were found, and tunicamycin did not inhibit stratification. Furthermore, raising the level of calcium ions did not affect cell division, and keratinocytes stratified normally in the presence of cytosine arabinoside. These results suggest that involucrin-positive cells growing as a monolayer already have the properties that will determine their suprabasal position. Calcium ions are required for the formation of desmosomes (28) and tight junctions (20) and it seems likely that the only effect of increasing the concentration of calcium ions in the medium is to allow these intimate cell contacts to form. The ability to stratify is an intrinsic property of a mixed population of keratinocytes at different stages of terminal differentiation.

The properties of keratinocytes that determine their position within the different cell layers appear to be adhesive. Involucrin-positive cells had a reduced affinity for adhesion to the culture substrate. Keratinocytes adhered preferentially to other keratinocytes at the same stage of terminal differentiation and involucrin-negative cells were probably more cohesive than involucrin-positive cells.

In the keratinocyte aggregates formed in culture, terminally differentiating cells surrounded basal cells. In contrast, when keratoinocytes are injected into animals, they form cysts in which basal cells surround and enclose terminally differentiating cells (5, 18, 23). The two situations are not analogous however. Aggregates were formed in the absence of an adhesive substrate, whereas cysts are usually adherent to host tissue. The presence of a suitable substrate for basal cell adhesion may be the major factor that determines the relative positions of basal and terminally differentiating keratinocytes; cellular cohesiveness may only become important when no such substrate exists, as in agarose capsules. If this idea is correct, a pellet of keratinocytes enclosed in a substance to which basal cells can adhere would form aggregates with basal cells at the periphery and terminally differentiating cells in the center, as seen in cysts.

At present, it is only possible to speculate on the molecular basis of the changes in keratinocyte adhesiveness during terminal differentiation. Although desmosomes are probably essential for stratification, it is unlikely that they confer selectivity of entry into the suprabasal layers. My experiments suggest that suprabasal cells are less cohesive than basal cells, yet, in human epidermis, the number of desmosomes per cell increases above the basal layer (15). Furthermore, the reduced substrate adhesiveness of involucrin-positive cells is observed in low-calcium medium (35), in which desmosome formation is inhibited (12, 13).

An alternative possibility is that changes in nonjunctional membrane glycoproteins alter cellular adhesion. In several differentiating systems, notably slime molds (29) and neural tissue (6), glycoproteins conferring specificity in cell recognition and adhesion have been identified. Changes in membrane glycosylation occur during terminal differentiation of keratinocytes, both in intact tissue (8, 9, 22, 36) and in culture (33). Whether any of these changes affect keratinocyte adhesion and cause terminally differentiating cells to leave the basal layer is currently under investigation.

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