Cell shape controls terminal differentiation of human epidermal keratinocytes

(cell adhesion/proliferation/involucrin expression)

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ABSTRACT   Cultures of human epidermal keratinocytes provide a useful experimental model with which to study the factors that regulate cell proliferation and terminal differentiation. One situation that is known to trigger premature terminal differentiation is suspension culture, when keratinocytes are deprived of substratum and intercellular contact. We have now investigated whether area of substratum contact, and hence cell shape, can regulate terminal differentiation. Keratinocytes were grown on circular adhesive islands that prevented cell–cell contact. By varying island area we could vary cell shape from fully spread to almost spherical. We found that when substratum contact was restricted, DNA synthesis was inhibited and expression of involucrin, a marker of terminal differentiation, was stimulated. Inhibition of proliferation was not a sufficient stimulus for involucrin synthesis in fully spread cells. When DNA synthesis and involucrin expression were plotted against contact area, classic dose–response curves were obtained. Thus cell shape acts as a signal for the terminal differentiation of keratinocytes in culture.

The epidermis is one of the tissues in the body that is continually renewed throughout adult life. Mitosis occurs in the basal layer, and cells that leave it stop dividing and undergo terminal differentiation as they move through the upper layers. As part of the program of terminal differentiation, synthesis of a number of different proteins is induced, including involucrin, a precursor of the cornified envelope (1, 2). Assembly of the cornified envelope in the outermost epidermal layers marks the final stage of keratinocyte terminal differentiation.

Human epidermal keratinocytes can be grown in culture under conditions in which they form stratified sheets with many of the properties of the tissue from which they were derived (3, 4). Mitosis is restricted to the basal layer, attached to the culture substratum; the cells undergo terminal differentiation in the suprabasal layers and finally detach into the culture medium. Such cultures therefore provide a useful experimental model with which to investigate the factors that regulate initiation of terminal differentiation.

Although terminal differentiation is normally linked to stratification, keratinocytes in culture can still initiate terminal differentiation when forced to grow as a monolayer (5, 6), and the commitment to terminally differentiate occurs in the basal layer *in vivo* (7). This commitment must be influenced by cell geometry or interrelationships, because when keratinocytes are placed in suspension culture they undergo premature terminal differentiation: they lose the ability to divide and they assemble cornified envelopes (8, 9). Cells in suspension are denied contact with other cells and with an adhesive substratum and either of these factors might act as a signal for terminal differentiation (10–13).

To investigate the role of cell–substratum contact, and hence cell shape, in regulating terminal differentiation, we have made use of a recently described technique that allows precise control of cell–substratum contact in the absence of cell–cell contact (14). We have looked at the effect of changes in cell shape on DNA synthesis and involucrin expression and conclude that a rounded morphology acts as a signal to keratinocytes to stop dividing and to undergo terminal differentiation; inhibition of proliferation in spread cells is not a sufficient signal for terminal differentiation.

MATERIALS AND METHODS

Cell Culture. Stock cultures of newborn human foreskin keratinocytes (2nd to 17th passage) were grown with a feeder layer of mitomycin C-treated Swiss 3T3 cells as described (3, 15). The culture medium consisted of one part Ham's F12 medium, and three parts Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 0.18 mM adenine, 5 μg of insulin per ml, 0.5 μg of hydrocortisone per ml, 0.1 nM cholera toxin, and 10 ng of epidermal growth factor (EGF) per ml (16). The EGF was prepared by C. George-Nascimento and generously donated by Chiron (Emeryville, CA).

To set up experimental cultures on island-patterned dishes or in methylcellulose, the 3T3 feeder layer was removed by aspiration with 0.02% EDTA (17) and keratinocytes were harvested with 0.05% trypsin/0.01% EDTA. Keratinocytes were seeded in suspension at 5×10^4 per ml in 2.0 ml of medium containing 1.3% methylcellulose (18). For island cultures, cells were seeded at 10^5 per 35-mm (diameter) culture dish and allowed to attach for 2–3 hr; then nonadherent cells were resuspended and the medium was immediately gelled with agarose (0.3%). Low-gelling-temperature agarose was purchased from Marine Colloids (Rockland, ME).

Construction of Island Dishes. Dishes were imprinted with island patterns as described (14). The dishes were first coated with a nonadhesive substratum of polyHEMA (Hydron Laboratories, New Brunswick, NJ) and the islands were deposited by vacuum evaporation of palladium through a mask of copper film (Allan Agar, Stansted, UK). The island patterns imprinted with these masks were of two types, graduated and uniform. Both were based on a rectangular grid with 150 μm between the centers of the islands. The graduated pattern consisted of a regular series of 10 circular island sizes, ranging from 400 to 5000 μm², with rectangular islands of 300 × 750 μm distributed at intervals over the pattern. Two uniform patterns, consisting of circular islands of a single size, were also constructed: in one the island area was 500 μm² (25 μm in diameter) and in the other the area was 2000 μm² (50 μm in diameter). All island patterns covered 50% of the surface of the dish, amounting to a total of >20,000 individual islands.

Electron Microscopy. Cells were rinsed briefly in warm phosphate-buffered saline (PBS: 0.15 M NaCl/0.003 M KCl/0.01 M Na$_2$HPO$_4$/0.015 M KH$_2$PO$_4$) and then fixed in 6% glutaraldehyde buffered to pH 7.4 with phosphate and rendered isotonic with sucrose. After 5 min the fixed cells were washed in water and then progressively dehydrated in a series of alcohol concentrations during a total time of no more than 10 min (longer exposure to alcohol caused the poly-HEMA coat underlying the islands to dissolve). After critical point drying, the dish was mounted on a stub and examined at an incident angle of 60° in a Jeol JSM 35 scanning electron microscope.

Light Microscopy. All cells for light microscopy were stained during the last hour of culture with 50 μg of Hoechst DNA-specific stain 33258 per ml in the growth medium. Staining allowed islands with more than one cell to be detected and also helped to identify cells with cornified envelopes. Cells with envelopes were distinguished by their large size, collapsed shape, and dense outline as well as by the absence of a nucleus.

Autoradiography. Standard methods of autoradiography were modified because of two special problems presented by island cultures: the rounded shape of the cells significantly increases the distance between the nucleus and the overlying film, and the metallic palladium of the islands catalyzes the reduction of silver halide to silver metal in the presence of photographic developer. Chemical interaction between the palladium and the silver halide was prevented by covering the dishes with a 50-nm film of polyvinyl chloride as described by Rodgers (19). The relative attenuation of the silver image caused by the long pathlength was avoided by using 2 μCi (1 Ci = 37 GBq) of carrier-free thymidine per ml (25 Ci/mmol) and exposing the cells to stripping film for 7 days at a temperature of −50°C and a relative humidity of 40%. Under these conditions the distinction between positive and negative nuclei was as clear as in control, unconfined, cultures.

Polyacrylamide Gel Electrophoresis (PAGE). Keratinocytes were labeled for 24 hr with 15 μCi of [^{35}S]methionine per ml (specific activity, 8000 Ci/mmol; Amersham), extracted in PAGE sample buffer containing 2-mercaptoethanol, and electrophoresed on 7.5% acrylamide gels, using the buffer system of Laemmli (20). Gels were stained with 0.1% Coomassie brilliant blue R, fluorographed (21), dried down, and exposed on prefogged Fuji RX film (22).

Immunofluorescence. Cells were fixed in 3.7% formaldehyde in PBS for 10 min. They were then permeabilized in 0.5% Triton X-100 in PBS for 2 min at room temperature and incubated for 45 min with a rabbit antiserum to involucrin (6). After thorough washing in PBS, the cells were incubated with fluoresceinated goat anti-rabbit IgG (Bio-Yeda, Rehovot, Israel) as before and then washed and mounted in Gelvatol (Monsanto).

RESULTS

Induction of Involucrin Synthesis in Suspension. The effect on involucrin expression of growing keratinocytes in suspension culture was measured by PAGE and immunofluorescence microscopy. Fig. 1 shows the total [^{35}S]methionine-labeled proteins of keratinocytes grown in suspension or on tissue culture plastic for 24 hr. A band that comigrated with purified involucrin was prominent in extracts of suspended cells (lanes 3 and 4) but barely detectable in extracts of attached cells (lanes 1 and 2). The level of expression of several other proteins also differed between suspended and attached cells (Fig. 1, small arrows).

After 24 hr in suspension the proportion of cells expressing involucrin, as assessed by immunofluorescence microscopy, increased 3-fold: from 25% to 69% in a typical experiment (Fig. 2; see also Table 1). Involucrin expression is normally
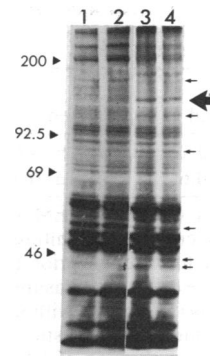

FIG. 1. Polyacrylamide gel of [^{35}S]-methionine-labeled cell extracts. Cells were grown on tissue culture plastic (lanes 1 and 2) or in suspension (lanes 3 and 4) for 24 hr. The large arrow indicates the involucrin band. Small arrows mark the positions of other proteins that differ in abundance between the two culture conditions. Molecular weights are shown as $M_r \times 10^{-3}$.

correlated with an increase in cell size (23), and the modal diameter of cells in suspension culture was greater than in the starting population (compare Fig. 2 *a* and *b*). In contrast, when cells were seeded on plastic for 24 hr, the proportion of involucrin-positive cells fell slightly, because some of the involucrin-positive cells in the starting population did not attach (Table 1).

These experiments show that involucrin expression was induced by 24 hr in suspension culture. We therefore went on to investigate the effect on involucrin expression of varying the substratum contact area of isolated cells.

Behavior of Cells on Island Arrays. Petri dishes with patterns of circular palladium islands superimposed on a nonadhesive polyHEMA coating were seeded with keratinocytes. After 2–3 hr nonadherent cells were resuspended and captured in agarose above the island layer to prevent further cell attachment while maintaining the original plating density. Involucrin-negative keratinocytes adhere and spread more rapidly than involucrin-positive cells, and therefore a seeding time of 2 hr ensured that about 95% of the attached cells were involucrin-negative (24). Typically, >50% of the islands were occupied, and >90% of the occupied islands bore a single cell.

Coulter Counter measurements of the volumes of cells that attached to islands indicated a modal diameter of 13.5 μm and
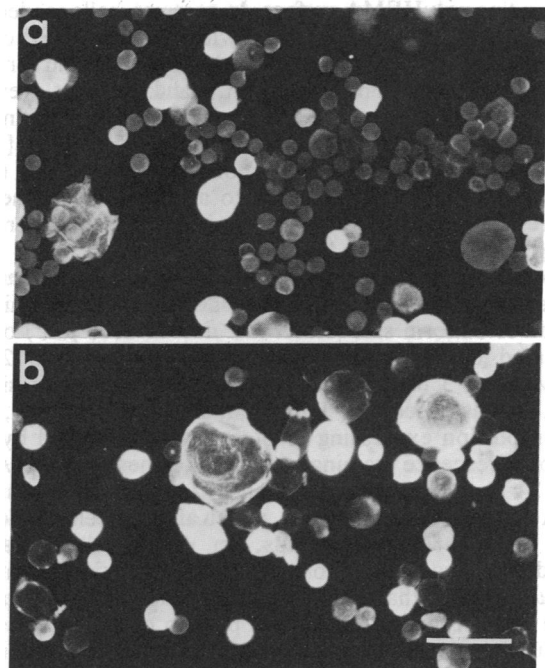

FIG. 2. Keratinocyte suspensions stained with involucrin antiserum. (*a*) Cells grown on tissue culture plastic. (*b*) Cells from the same starting population as in *a* but after 24 hr in suspension culture. (Bar = 50 μm.)

Table 1.   Effect of [³H]thymidine on involucrin expression

	Involucrin-positive cells, %		
Culture	Starting population	Attached	Suspended
Control	32.3 ± 3.9	10.5 ± 3.1	66.7 ± 4.6
[³H]Thymidine	26.3 ± 3.9	9.7 ± 0.6	67.9 ± 0.9

The percentage of involucrin-positive cells (mean ± SEM of duplicate dishes) was determined in control cultures or cultures treated with 2 μCi of [³H]thymidine per ml for 3 days prior to harvesting. The proportion of involucrin-positive cells was measured in the starting population (i.e., after 3 days with or without [³H]thymidine) and after 24 hr attached to tissue culture plastic or suspended in methylcellulose in the presence or absence of [³H]-thymidine.

a mean of 14.5 μm, in good agreement with previous measurements of the size of involucrin-negative keratinocytes (23). Assuming that the cells adopted a regular lenticular shape, the height of cells on the smallest islands (24 μm in diameter) would be between 3 and 8 μm. Although it was not possible to measure directly cell size 1 or more days after seeding, microscopical observation indicated that terminal differentiation on islands was accompanied by an increase in cell size.

Fig. 3 illustrates the shapes adopted by keratinocytes on islands of different areas, ranging from fully spread to almost spherical. In some cases, cornified envelopes assembled within the cells: such cells were enlarged and often collapsed. Collapse could result in bizarre doughnut shapes (Fig. 3c); it seems possible that this collapse is part of a physiological process that normally contributes to squame formation, since cornified cells are highly flattened *in vivo*. However, envelope formation was never very common. Even after 3 days in culture the proportion of cells with envelopes did not exceed 10% on the smallest islands and 5% on the largest.

Cultures seeded onto island dishes were observed by time-lapse cinematography. No cell ever moved from one island to another. Movement on islands was limited to the extension of pseudopods not more than 5 μm over the surrounding polyHEMA surface. In contrast, cells seeded on ordinary untreated plastic moved with an initial speed of 30 μm/hr, until they made contact with other cells and formed immobile groups. Confinement on islands did not prevent mitosis or cytokinesis. However, both processes were inhibited by continuous exposure to high specific activity [³H]-thymidine in the medium, as reported for fibroblasts (14). Since [³H]thymidine was added to all of the experimental cultures, the number of cells per island did not increase during the course of the experiments.

Effect of Cell Shape on DNA Synthesis and Involucrin Expression. Keratinocytes were seeded on dishes containing islands of uniform size (either 500 μm² or 2000 μm²) or on untreated tissue culture plastic and were fixed at 24-hr intervals for 3 days. The proportion of cells synthesizing DNA was assessed by [³H]thymidine autoradiography, and the proportion expressing involucrin was measured by immunofluorescence staining of parallel dishes (Fig. 4). After only 24 hr, a greater proportion of cells was synthesizing DNA on the 2000-μm² islands, which allowed cell spreading, than on the 500-μm² islands, on which cells remained rounded. Conversely, more cells expressed involucrin on 500-μm² islands than on 2000-μm² islands. On days 2 and 3, there was an increase in the proportion of cells expressing involucrin or synthesizing DNA, an increase that was more marked for DNA.

The proportion of cells seeded on tissue culture plastic that synthesized DNA, or expressed involucrin, was intermediate between the values on the two island sizes on all 3 days (also shown in Fig. 4). Morphometric analysis showed that the
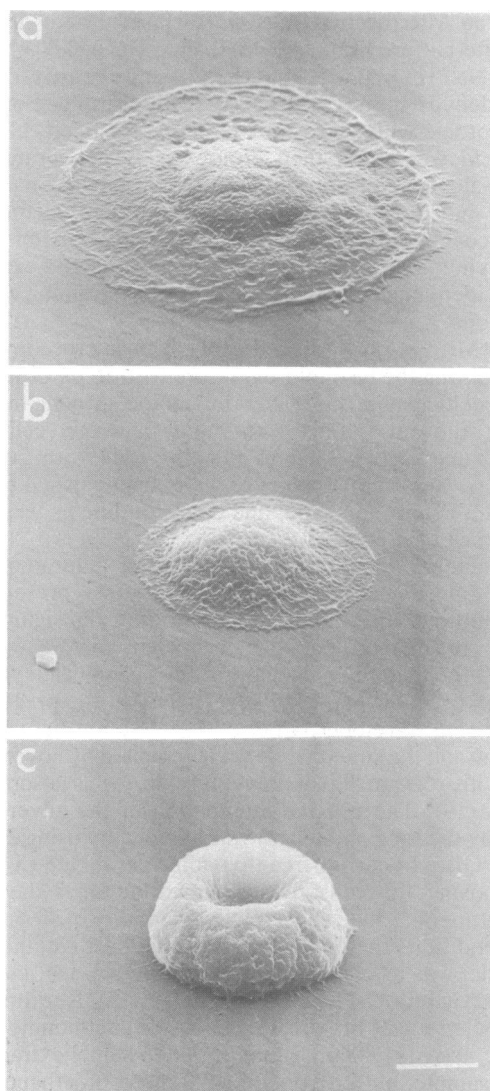

FIG. 3.   Scanning electron micrographs of keratinocytes seeded on different sizes of island: 2000-μm² island (a); 400-μm² islands (b and c). The doughnut-shaped cell in c has enlarged, collapsed, and developed a roughened surface, indicating the development of a cornified envelope and marking the end stage of terminal differentiation. (Bar = 10 μm.)

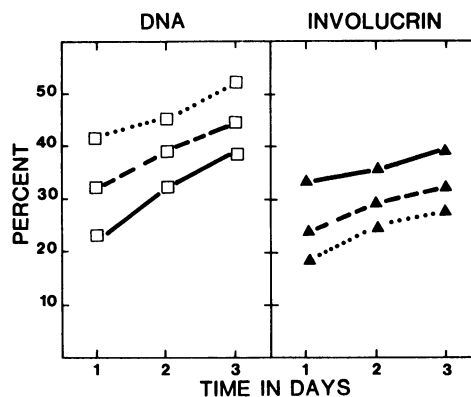mean area of substratum contact of these control cells was 1200 μm², also intermediate between the values for cells on

FIG. 4.   Percentages of cells synthesizing DNA (□) or expressing involucrin (▲) on 2000-μm² (dotted lines) or 500-μm² islands (continuous lines) or on tissue culture plastic (dashed lines) at different times after plating.

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the two island sizes. Hence, paradoxically, unconfined cells spread less than cells on the largest islands, perhaps as a mechanical consequence of the crowding that follows cell contact.

Keratinocytes seeded on uniform 500-μm^2 or 2000-μm^2 islands were labeled with [^{35}S]methionine and extracted in sample buffer 24 hr after plating. Densitometric analysis of the proteins separated by PAGE showed that there was twice as much involucrin in the rounded cell extracts, from small islands, as in the spread cell extracts, from large islands (not shown). Thus, the difference in the proportion of cells expressing involucrin on different-sized islands was reflected in the involucrin content of the cultures.

Titration of Cell Response to Island Area. To determine the detailed relationship between cell shape, DNA synthesis, and involucrin expression, keratinocytes were cultured in dishes containing a graded series of 10 different sizes of island. These sizes ranged between 400 μm^2 and 5000 μm^2 (25–80 μm in diameter). Graded dishes also contained larger rectangles of 0.2 mm^2, to allow unconfined cells to be studied under the same conditions (14). Cells were incubated for 3 days on the island array and at the end of this period the proportion of cells synthesizing DNA was assessed by autoradiography, whereas the proportion expressing involucrin was scored after immunofluorescence staining of parallel dishes (Fig. 5).

As shown in Fig. 6, the proportion of cells incorporating [^{3}H]thymidine was about 15% on the smallest islands (400 μm^2) and rose to a maximum of 45% on the largest islands. Thus, as observed for fibroblasts (14), there was a dose–response relationship between DNA synthesis and substrate contact area. A dose–response relationship was also observed between island area and involucrin expression, although in the opposite direction. Up to 50% of the cells on the smallest islands expressed involucrin compared with only 5% on the largest islands.

We have confirmed the immunofluorescence data by photometry. Rounded cells inevitably appear brighter to the eye than flattened cells, even when the amount of antibody bound is the same, because the fluorescence is concentrated over a smaller area. However, photometry provides a measure of the total light emission from single cells, irrespective of shape, and thus any significant increase above background provides an objective indication of the presence of involucrin. The proportion of cells scored as positive in this way began to rise at between 2000 μm^2 and 1000 μm^2 and approached a maximum at 400 μm^2 (Fig. 7). Plots of the average emission from the cells on each island size gave a similar curve (not shown). Our visual estimates (also shown in Fig. 7), made on the same plates, were in good agreement with these photometric measurements.

Inhibition of Proliferation Is Not a Sufficient Signal for Initiation of Involucrin Expression. In epidermis and in
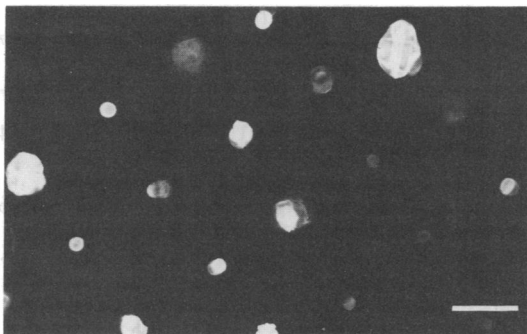

FIG. 6. Proportion of cells synthesizing DNA ($\square$) or expressing involucrin ($\blacktriangle$) as a function of island area (μm^2) in 3-day-old cultures.

keratinocyte cultures, the first event in terminal differentiation is normally withdrawal from the cell cycle. In the experiments involving islands, the presence of [^{3}H]thymidine in the medium inhibited proliferation, presumably because the cells arrested in G$_2$ stage to carry out DNA repair; nevertheless, the proportion of cells expressing involucrin varied as a function of island area. Thus growth arrest did not appear to be a sufficient signal for induction of involucrin expression. To confirm this, the following experiment was carried out.

Two microcuries of [^{3}H]thymidine per ml was added to stock cultures of keratinocytes in logarithmic-phase growth. After 3 days, the cells were harvested and the number of cells per dish was compared with control (untreated) cultures. In a typical experiment the number of cells rose 3- to 4-fold in control dishes but did not increase in the presence of [^{3}H]thymidine. [^{3}H]Thymidine-treated and control cells were replated in suspension in methylcellulose or on tissue culture plastic in the presence or absence of [^{3}H]thymidine and the proportion of involucrin-positive cells was measured 1 day later. As shown in Table 1, the proportion of involucrin-positive cells rose to >65% in suspension but was ≈10% in attached cultures. Thus, although attached and suspended [^{3}H]thymidine-treated cells were growth arrested, involucrin synthesis was only stimulated in suspension.

DISCUSSION

We have shown that the area of contact with the substratum, and hence cell shape, regulates proliferation and terminal

FIG. 5. Immunofluorescence staining of keratinocytes with antiserum to involucrin on the graduated pattern of islands. (Bar = 100 μm.)
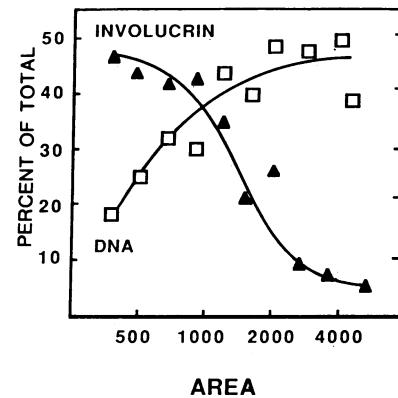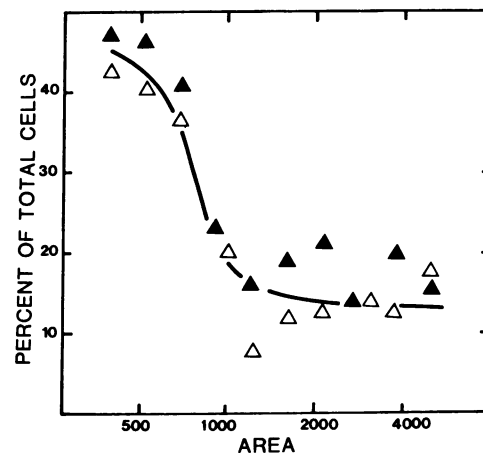

FIG. 7. Proportion of cells expressing involucrin as a function of island area (μm^2) in 3-day-old cultures. $\triangle$, Photometric measurement; $\blacktriangle$, visual estimate.

differentiation of human keratinocytes in culture. When spreading was restricted and the cells remained rounded, involucrin expression was stimulated and DNA synthesis was inhibited. Control experiments showed that inhibition of DNA synthesis was not, in itself, a sufficient stimulus for involucrin synthesis. Keratinocytes did not appear to have a shape threshold that had to be reached to trigger a response; when the proportion of cells incorporating [³H]thymidine or expressing involucrin was plotted against substratum contact area, a classic dose–response curve was obtained (Fig. 6). In the past, a number of workers have noted correlations between cell morphology and differentiated gene expression (see, for example, refs. 25, 26), but our experiments provide evidence for a direct role of cell shape in the absence of cell–cell interactions.

It is clear from Fig. 1 that involucrin is not the only protein whose expression is regulated by cell shape. We have preliminary evidence that in suspension culture and on small islands there are changes in the pattern of expression of keratins and components of the extracellular matrix (P. Newman and F.M.W., unpublished data). Thus, restricted substratum contact may result in coordinate regulation of a number of different genes in a way that may mimic the program of terminal differentiation *in vivo*.

The proportions of keratinocytes synthesizing DNA or involucrin on tissue culture plastic were intermediate between those on islands of different sizes (Fig. 4). This is most simply explained by their intermediate area of spreading, but other factors might also be involved. For example, cell–cell contact could modulate proliferation and terminal differentiation: the extent of keratinocyte communication by means of gap junctions is reduced during terminal differentiation and the equilibrium concentration of molecules transmitted by gap junctions might affect the proportion of undifferentiated keratinocytes that enter mitosis (13). A further possibility is that keratinocytes on the largest islands are subject to tensile stress when they conform to the circular contour of the substratum and that this acts as a positive stimulus for DNA synthesis (27).

We have shown here that reduced substratum contact can act as a signal for terminal differentiation. However, it is also known that the affinity of keratinocytes for the substratum is reduced as a consequence of terminal differentiation (15, 24). Similarly, the size of the cells increases as they terminally differentiate (23, 28), and if space on the substratum is limited an increase in size would also reduce the proportion of the cell surface in contact with it. Hence, reduction in substratum contact can be cause and consequence of differentiation. We speculate that these relationships might form the basis of a positive feedback system *in vivo*, in which crowded cells are at the same time induced to differentiate and to leave the basal layer.

In stratified cultures on plastic the basal keratinocytes are highly spread, but in the epidermis they are cuboidal. In consequence, substratum contact area can be very much greater *in vitro*. We have shown here that restricted substratum contact area is a signal for terminal differentiation, but it is possible that the critical parameter is in fact the strength of the adhesive interaction and that the basement membrane might offer greater adhesion than any of the substrata we have used.

In culture, involucrin synthesis begins immediately above the basal layer. *In vivo* it usually begins in the upper spinous layers and is sometimes, though not always, correlated with cell flattening (29). The reason for such an association may lie in changes in cell–cell adhesiveness and in cell volume. We have consistently noticed a correlation between envelope formation and cell collapse on islands (Fig. 3); *in vivo* collapse (and flattening) may precede envelope assembly.

The nature of the terminal differentiation signal to which rounded cells repond is unknown. One possibility is that the signal involves interactions between the plasma membrane and the cytoskeleton (reviewed in refs. 11, 12, 30). Cell–substratum adhesion is initially mediated by focal membrane specializations (31) and it is known that island culture can result in striking alterations in the number, arrangement, and turnover of focal contacts (32). Confinement on small islands might therefore stimulate terminal differentiation by means of its effects on focal contacts, which could in turn affect the state of assembly of the cytoskeleton. Evidently, this hypothesis is open to test, and the smooth nature of the dose–response curve relating shape and terminal differentiation makes specific predictions about the nature of the proposed control signal.

Finally, the results show that cell shape can act as a signal for terminal differentiation and inhibition of DNA synthesis. Irreversible inhibition of proliferation normally precedes terminal differentiation, but arrest of cells in G_2 stage by prolonged exposure to high specific activity [³H]thymidine was not a sufficient signal for induction of involucrin expression in our experiments. The extent of overlap between the two signaling pathways remains to be determined.

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