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Human keratinocytes cultured without a feeder layer undergo progressive loss of differentiation markers

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Summary. Culture of keratinocytes in conventional medium without a mesenchyme-derived feeder layer leads to poor growth and impaired differentiation; however, the exact pathway and degree of differentiation achieved in such conditions is unclear. We have cultured normal human keratinocytes in Rheinwald and Green's medium, on plastic without a feeder layer, in order to investigate the degree of differentiation that they achieve in these conditions. Intermediate filament proteins, tonofibrils and desmosomes were assumed as markers of differentiation and their expression was analyzed by immunohistochemistry and electron microsçopy. Before reaching confluence, keratinocytes expressed keratin molecules, as well as vimentin, and formed tonofibrils and desmosomes. The expression of these markers was progressively reduced until confluence and was totally lost thereafter, while cultures could be propagated for at least six passages. On the contrary, reseeding on a feeder layer after the first passage led to rapid cell death. It could be concluded that signals from a feeder layer are relevant to support continuous synthesis of intermediate filaments proteins and formation of tonofibils and desmosomes, and that the derangement of the cytoskeleton in these conditions leads to altered, not simply defective, response to delayed stimulation by a feeder layer.

Key words: Desmosomes, Intermediate filaments, Keratins, Vimentin

Introduction

Human epidermal cells are able to proliferate and differentiate in vitro. The expression of intermediate filament bundles ("tonofibrils") in the cytoplasm and of desmosomes at the cell surface are relevant parts of the differentiation program of keratinocytes (Tseng et al., 1982; Lewis et al., 1987) and can be used to monitor the differentiation of these cells in vitro (Pillai et al., 1988; Fuchs, 1990; Wille et al., 1990; Zamansky et al., 1991; Eriksson et al., 1992). Fetal calf serum and a balanced cocktail of stimulating factors must be added to keratinocytes to achieve successful culture (Faure et al., 1987). Besides, dermal components, such as fibroblasts and matrix, promote keratinocyte growth and differentiation (Bell et al., 1981; Sengel, 1983); proper differentiation of keratinocytes in culture can be achieved in the presence of a feeder layer of lethally irradiated or mitomycin-treated fibroblasts (Rheinwald and Green, 1975), which is the most widely used technique to culture keratinocytes. Valuable alternatives to mitosis-blocked fibroblasts include whole dermis (Regnier et al., 1990), Matrigel, i.e. an equivalent of basement membrane, and type I collagen gel (Asselineau et al., 1986). The importance of dermal components is also shown by the fact that a feeder layer of whole dermis or fibroblasts from psoriatic patients, who have an enhanced epidermal growth and turnover in vivo, supports much more intense growth in vitro of keratinocytes than usual (Saiag et al., 1985; Krueger and Jorgensen, 1990). Culture methods have been devised also for the growth of keratinocytes in the absence of a feeder-layer; these methods take advantage of serum-free media, for a better understanding of the growth factors required by keratinocytes to grow in vitro (Boyce and Ham, 1983; Pillai et al., 1988; Wille et al., 1990). By high voltage electron microscopy of cells grown directly on grids, fixed and critical-point dried, Boyce and Ham (1983) found tonofilament bundles and keratohyalin granules at both low (less than 0.3 mmol/L) and high (more than 1.0 mmol/L) Ca^{2+} concentration, although the number of these organelles increased with Ca^{2+} concentration itself. However, further observations by conventional electron microscopy of routinely prepared specimens showed that cells grown without a feeder layer in serum-free medium at low Ca^{2+} concentration did not exhibit features of epidermal differentiation, such as tonofilament bundles, intercellular junctions, lamellar bodies, keratohyalin granules and cornified envelopes; these features, except keratohyalin granules, were, on the contrary present in cells cultured at high Ca^{2+} concentration (Pillai et al., 1993).

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These references indicate that the differentiation program followed by human keratinocytes depends on culture conditions; however the influence of a mesenchyme-derived feeder-layer on the pathway and degree of differentiation of keratinocytes in vitro is poorly known (Fusenig, 1994). This point is relevant inasmuch as keratinocytes in vivo actually grow on dermis. To address this issue, we have used a medium designed for culture on a feeder-layer (Rheinwald and Green, 1975, 1977) and have simply omitted such a layer. Our results show that differentiation of keratinocytes in these conditions is impaired from early on, with loss of intermediate filament proteins (both keratins and vimentin), of tonofibrils and of desmosomes, which decrease progressively during the first passage, disappear completely from the second passage and cannot be recovered by late passage on a feeder layer.

Materials and methods

Reagents and disposables

Eagle's minimum essential medium, Dulbecco's modification (D-MEM), ethylenediamine-tetraacetic acid (EDTA), fetal calf serum (FCS), Ham's F-12, Hank's balanced salt solution (calcium- and magnesium-free), HEPES, $NaHCO_3$ and trypan blue were purchased from Seromed (Berlin, Germany); 3,3',5-triiodothyronine, adenine, cholera toxin, type I collagen, epidermal growth factor (EGF), glutamine, insulin, mitomycin-C, penicillin-streptomycin, transferrin, trypsin, TRIS (Tris(hidroxymethyl)aminomethane), Triton X-100, NaCl and $MgCl_2$ were from Sigma (Milan, Italy); dispase (grade II) was from Boehringer (Mannheim, Germany), and hydrocortisone from Calbiochem (Milan, Italy). Matrigel and disposable cell strainers were from Becton Dickinson (Franklin Lakes, NJ), centrifuge tubes and plastic culture flasks and Petri dishes from Costar (Concorezzo, Italy), sterile filters, with 0.22 μm pores, from Schleicher & Schuell (Dassel, Germany), and Lab-tek chamber slides from Nunc (Naperville, IL).

Culture of normal human keratinocytes

Human keratinocytes were obtained from plastic surgery keratomes of sun unexposed skin. The epidermis, after enzymatic separation from the dermis with dispase (1.2 U/ml in D-MEM, at 37 °C for 30 minutes), was cut into tiny pieces and incubated for 15 minutes at 37 °C with 0.25% trypsin and 0.02% EDTA in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4. After filtration through a sterile gauze and then a cell-strainer, the cells were centrifuged at x200g for 10 minutes in a Universal 30 RF centrifuge (Hettich, Tuttlingen, Germany), resuspended in minimum essential medium (60%) and Ham's-F12 (30%) and washed twice; $2x10^6/cm^2$ cells were injected into plastic Petri dishes and into chambers of Lab-tek slides and were incubated in air with 5% CO_2. The culture medium was a mixture of D-MEM (60%) and Ham's F12 (30%), plus FCS (10%), glutamine (4 mmol/L), penicillin (100 U/mL), streptomycin (100 μg/mL), adenine (0.18 mmol/L), insulin (5 μg/mL), hydrocortisone (0.4 μg/mL), 3,3',5-triiodothyronine (2 nmol/L) and EGF (10 ng/mL). In preliminary experiments, cholera toxin (0.1 nmol/L) was added to the culture medium, but this process was abandoned because cells could not survive beyond 48 h. In some preliminary experiments, EGF was added after 48 h, as in the method of Rheinwald and Green (1977), whereas in other experiments it was added since the beginning of culture. Since this latter protocol resulted in a slight improvement in the speed of adhesion and starting of proliferation, it was adopted in all further experiments. At confluence, the cells of each flask were detached with trypsin and EDTA for harvesting and reseeded into new flasks. This procedure could be repeated until the seventh passage. At the end of the first passage, some cells were seeded on mytomicin-C treated 3T3 fibroblasts, on Matrigel and on collagen type I.

Immunofluorescence

For immunofluorescence, samples were collected at seeding, before subconfluence during the first passage and at confluence at the end of the first and third passages. Cells collected at seeding were cytospinned on glass slides; cells grown on Lab-tek were used for the other time points. The cells were fixed in methanol/acetone (1:1) and frozen until used. For immunostaining, they were kept at room temperature for 1 h, rehydrated, blocked with 1% bovine serum albumine, rinsed three times in phosphate-buffered saline and incubated with the primary monoclonal or polyclonal antibodies, for 1.5 h at 37 °C, followed by fluorescein isothiocyanate-conjugated goat anti-mouse or tetramethyl-rhodamine isothiocyanate-conjugated goat anti-rabbit antibodies, respectively (Sigma, Milan, Italy), for 1 h at 37 °C. The following monoclonal antibodies and dilutions were used: anti-HMB-45, 1:50 (Ortho, Milan, Italy); anti-HLA-Dr, undiluted (Ancell, Bayport, MN); anti-α-smooth muscle actin, 1:30 (Dako, Milan, Italy); anti-vimentin, 1:100 (Dako); anti-CD34, 1:20 (Immunotech, Marseille, France); anti-CD36, 1:20 (Immunotech); anti-CD14, 1:10 (Ortho); and a polyclonal antibody against all keratins, 1:50 (EuroDiagnostica B.V., Arnhem, NL) was used to label any such molecule, if present, independent of the type. The slides were mounted with Gel/Mount (Biomeda, Foster City, CA) and photographed in a Zeiss Axioskop microscope (Oberkochen, Germany) equipped for epifluorescence.

Some slides were immunostained with non-relevant, isotype-matched antibodies as negative controls. As a further negative control for keratin antibodies, these antibodies were preabsorbed with Triton-insoluble, isolated keratinocyte cytoskeletons before staining slides. Triton-insoluble keratinocyte cytoskeletons were

prepared as follows (Edds, 1979). Keratinocytes were isolated from skin biopsies as indicated above and lysed with 0.5% Triton X-100 in 0.12 mol/L NaCl, 2 mmol/L $MgCl_2$ and 5 mmol/L Tris, pH 7.5, for 15 minutes at 4 °C. The unlysed nuclei were pelleted by centrifugation at x 1,500g for 10 minutes. The resulting supernant, that was highly enriched in cytoskeletons, was concentrated by centrifuging twice at x17,000g for 15 minutes, without resuspending the pellet after the first centrifugation. Slides of skin biopsies and buffy coats were run in parallel as positive controls, as appropriate.

Electron microscopy

For electron microscopy, samples were collected at seeding, at subconfluence after seeding and at confluence at the end of each passage. At each time point after seeding, keratinocytes grown in plastic Petri dishes were washed in PBS and then fixed directly in the dish with a mixture of 2% formaldehyde and 2.5% glutaraldehyde in 0.1 mmol/L cacodylate buffer, pH 7.4, at room temperature for 10 minutes; the cells were then removed with a scraper, osmicated and embedded in Epon. Ultrathin sections were stained with uranyl acetate followed by bismuth tartrate or lead citrate and observed in a Siemens (Munich, Germany) 102 electron microscope, at 80 kV.

Results

Cell culture

In all experiments, adhesion to plastic was complete within 24 h; confluence at the first passage required about 14 days and about 10 days at all further passages (Fig. 1). After confluence, attempts to reseed cells on 3T3 mytomicin-C-treated fibroblasts, on Matrigel or on type I collagen were unsuccessful; the cells first attached to the substrate, but then vacuolated, detached and eventually died within 24 h.

Immunofluorescence

Semiconfluent and confluent cells at the first and third passages were challenged with all the above mentioned antibodies, in order to explore the expression of intermediate filament proteins and to exclude markers of hematopoietic, monocyte-macrophage and melanocyte lineages. All cells before confluence, at the first passage, stained intensely for keratins and weakly for vimentin (Fig. 2); confluent cells at the first passage stained weakly for keratins alone (Fig. 3); no labeling with any antibody was obtained on cells at the third passage. In particular, cells were always unreactive for anti-HMB-45, anti-HLA-DR, anti-α-smooth-muscle actin, anti-vimentin, anti-CD14, anti-CD34, anti-CD36. All negative controls were unstained by the antibodies; positive controls were stained as expected.

Electron microscopy

Electron microscopy showed that the vast majority of cells at seeding were keratinocytes with well developed tonofibrils, formed by the bundling of

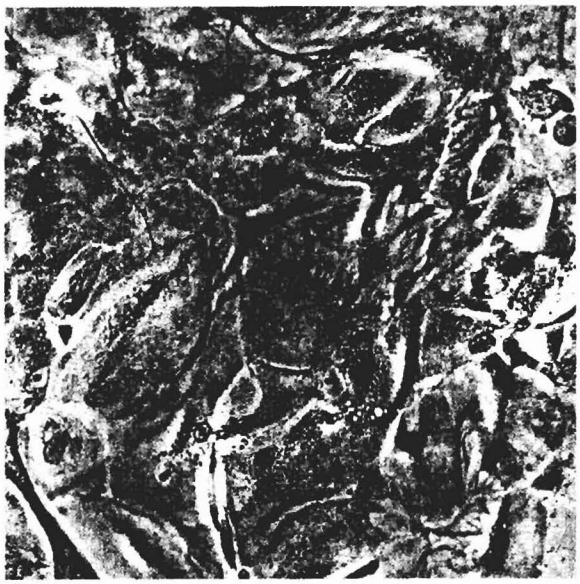

Fig. 1. Confluent cells after the third passage. Epidermal cells grown without a feeder layer appear wide and flat. Phase contrast microscopy, x 320.

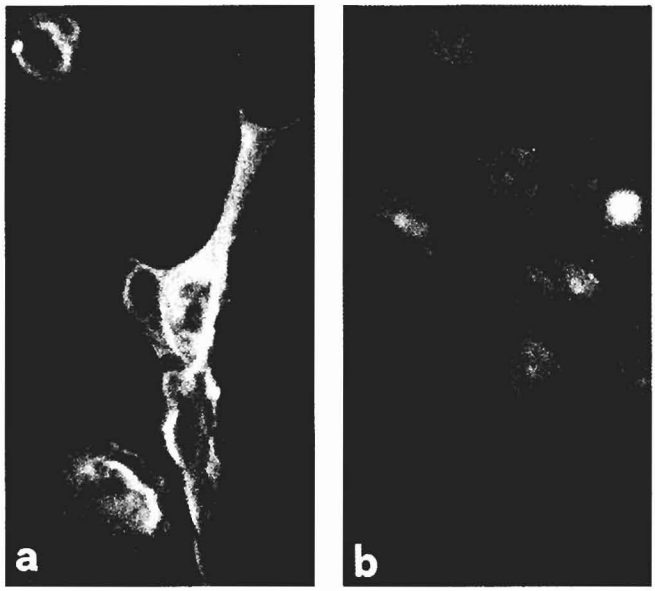

Fig. 2. Non confluent cells during the first passage. Cells grown without a feeder layer stain intensely with an anti-pan keratin polyclonal antibody **(a)** and weakly with an anti-vimentin monoclonal antibody **(b)**. Note unspecific positivity of nucleoli with anti-vimentin antibody. Indirect immunofluorescence, x 100

intermediate filaments, and with half desmosomes at the cell surface, which resulted from trypsinization (Fig. 4). Few cells were non-keratinocytes, including some melanocytes.

At subconfluence, cells were flat, contained few intermediate filaments gathered into thin fibrils and were connected with each other by small desmosomes, where tonofibrils inserted to the plasma membrane (Fig. 5). Cells contained many ribosomes not attached to membranes and only a few cisternae of endoplasmic reticulum; the Golgi apparatus was small. A mesh of thin filaments was adjacent to the plasma membrane in the areas where cells were not in contact with each other (Fig. 5).

Confluent cells at the end of the first passage contained very few intermediate filaments that gathered into thin bundles in a strict minority of cells (Fig. 6); desmosomes were seen but exceptionally. Thin filaments appeared similar to those in subconfluent cells. On the contrary, both rough and smooth endoplasmic reticulum and the Golgi apparatus were well developed; most commonly, separate cell regions showed either one or the other type of reticulum, with the rough one closer to the nucleus.

Confluent cells from the second passage on were always apparently deprived of intermediate filaments and poor in microfilaments; they had well developed rough endoplasmic reticulum and Golgi apparatus but only few elements of smooth endoplasmic reticulum. Intercellular junctions were never recognized (Fig. 7).

Discussion

In this study, we found that epidermal cells undergo loss of morphological and immunophenotypical differentiation markers when grown in a Rheinwald and Green (1975, 1977) medium without a mesenchyme-derived feeder layer; cells could be propagated for several passages but differentiation markers were never regained in these conditions, nor could these markers evoked by reseeding on a mesenchyme-derived feeder layer at later passages.

Immunohistochemical and electron microscopic analyses showed that proliferating cells adhering to plastic were keratinocytes, since they depended on EGF for growth, did not express markers of other possible epidermis-derived cell types and, most important, expressed typical differentiation markers of this cell type during the first passage in culture. These markers include the expression of keratin molecules, besides vimentin, and the formation of tonofibrils and desmosomes. However, the intensity of expression of these markers decreased progressively during the first culture passage and was totally lost thereafter. Tonofibril aggregation and desmosome formation seemed to be more markedly affected than the synthesis of intermediate filament proteins, since the former structures were recognized in only a few confluent cells at the first passage, whereas almost all these cells expressed keratin

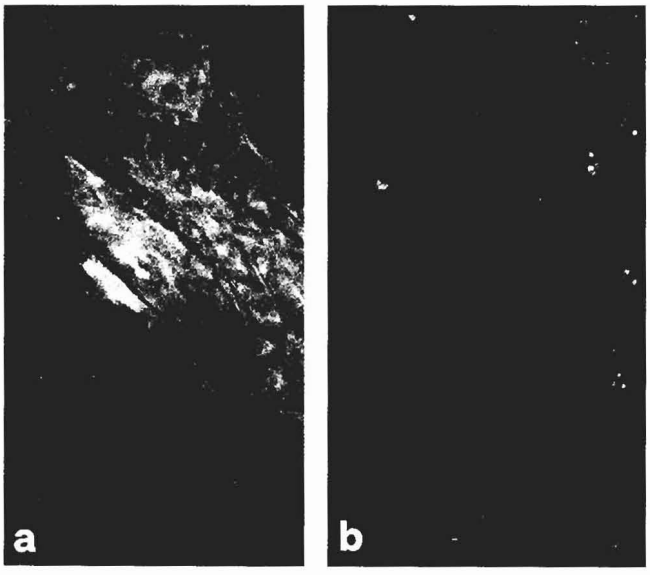

Fig. 3. Confluent cells at the first passage. Epidermal cells grown without a feeder layer stain weakly with an anti-pan keratin polyclonal antibody **(a)** and are unstained by an anti-vimentin monoclonal antibody **(b**, except for the unspecific labeling of nucleoli. Indirect immunofluorescence, x 200

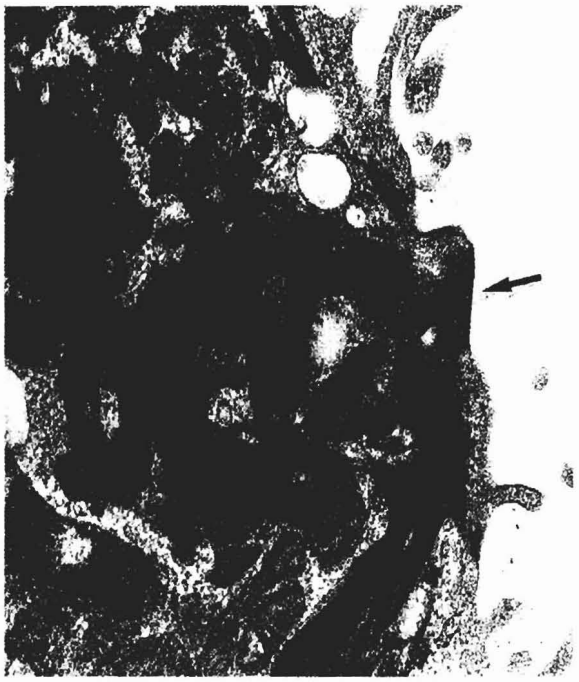

Fig. 4. Keratinocytes at seeding. The cells show a well developed cytoskeleton, with thick tonofibrils formed by aggregated tonofilaments. Half desmosomes can be recognised at the cell surface, where tonofibrils insert to the plasma membrane (arrow). Electron microscopy, x 40,000

proteins, although at a low level. At further passages, all differentiation markers searched for were lost. Electron microscopy showed an absence of differentiation markers at an ultrastructural level. Besides, the lack of any keratin molecule, which would otherwise have been tagged by the antibody used in this study, is of special interest, since keratin synthesis preceeds that of all other differentiation markers of keratinocytes, such as those connected with cornified cytoplasm and cornified envelope formation (Byrne, 1997).

Therefore, we should conclude that keratinocytes actually de-differentiate during the first passage in culture without a feeder layer, and remain de-differentiated therafter.

Several groups (Boyce and Ham, 1983; Wille et al., 1990; Pillai et al., 1993; Varani, 1998) described the presence of tonofibrils and desmosomes in cells grown in a feeder layer-free, serum-free medium at high calcium concentration. They also reported a decrease in the expression of differentiation markers by lowering Ca^{2+} concentration, although these markers did not disappear completely, except in the experiments of Pillai et al. (1993). These data and ours together suggest that the presence of a feeder layer is indeed necessary for the formation of tonofibrils in keratinocytes. We used a Rheinwald and Green (1975, 1977) medium, with fetal calf serum and high Ca^{2+} concentration, so that the only experimental variable under study was the presence or absence of a feeder layer. We have not tested the effects of lowering Ca^{2+} concentration since we could not expect even lesser differentiation than that achieved and documented in this study. The results stress the role of a mesenchyme-derived feeder layer on the differentiation of keratinocytes in culture. Experience from many laboratories indicates that culture in Rheinwald and Green (1975, 1977) medium with a feeder layer represents a reliable model for keratinocytes growing in vivo on the dermis (Sengel, 1983). Therefore, it can be reasonably speculated that also in vivo, as in our study, signals from a mesenchyme-derived layer are necessary to maintain continued synthesis of intermediate filament proteins and hence to preserve or reconstitute the network of tonofibrils and desmosomes, at least in the basal cell layer. The signals from a mesenchyme-derived

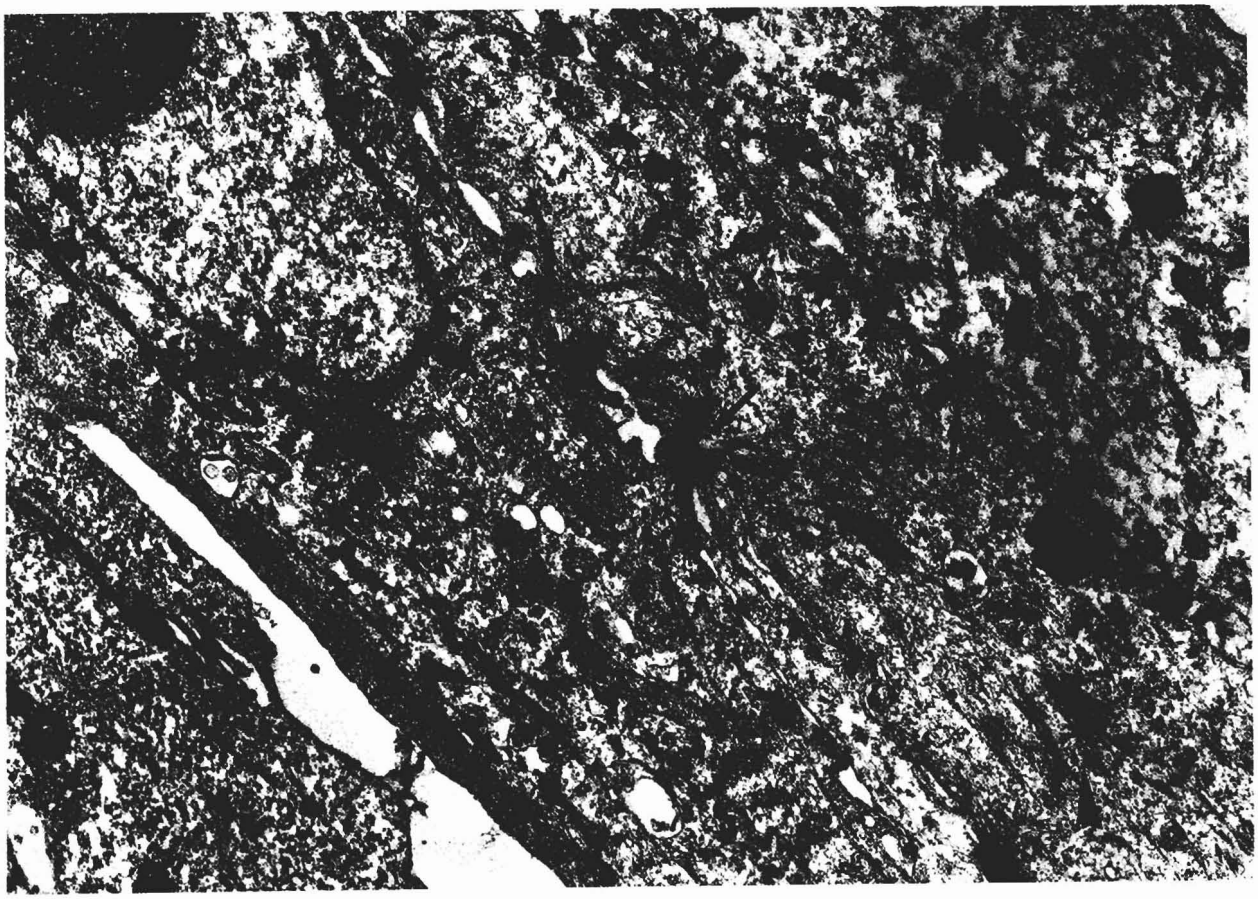

Fig. 5. Keratinocytes at the first passage before reaching confluence. The cytoplasm contains many free polyribosomes and some tonofilaments aggregated into thin fibrils (arrowheads), which insert to the plasma membrane at desmosomes (arrow). Note the layer of thin filaments beneath the plasma membrane of a cell. Electron microscopy, x 25,000

layer are probably transduced by adhesion receptors (Gumbiner et al., 1988; Carter et al., 1991; Sjaastad and Nelson, 1997) and this transduction would obviously not occur in the absence of the ligands. The derangement of the cytoskeleton could in turn lead to further disturbances in the localization and function of cell surface receptors (Luna, 1991; Troyanovsky et al., 1993) and this might be responsible for the altered (not simply defective) response that we observed to reseeding on a feeder layer at later passages, which led to cell death. This behaviour was observed not only when mitomycin-C-treated 3T3 fibroblasts were used as a feeder layer, but also when Matrigel and collagen were used for this purpose. Since both the components of Matrigel and collagen interact with cells through adhesion receptors, these receptors, rather than others interacting with soluble molecules, appear to be relevant in determining cell death upon reseeding on any feeder layer.

We found an interference between the effect of cholera toxin and that of a feeder layer, in that the addiction of the former proved to be incompatible with culture without a feeder layer. Cholera toxin stimulates an increase in intracellular cAMP (Green, 1978; Wilkinson et al., 1987), which most commonly results in increased proliferation (Rahman and Tsuyama, 1993).

Fig. 6. Keratinocytes at confluence after the first passage. Tonofilaments aggregated into thin fibrils are contained in the cytoplasm of a cell, but do not insert to desmosomes. Electron microscopy, x 15,000

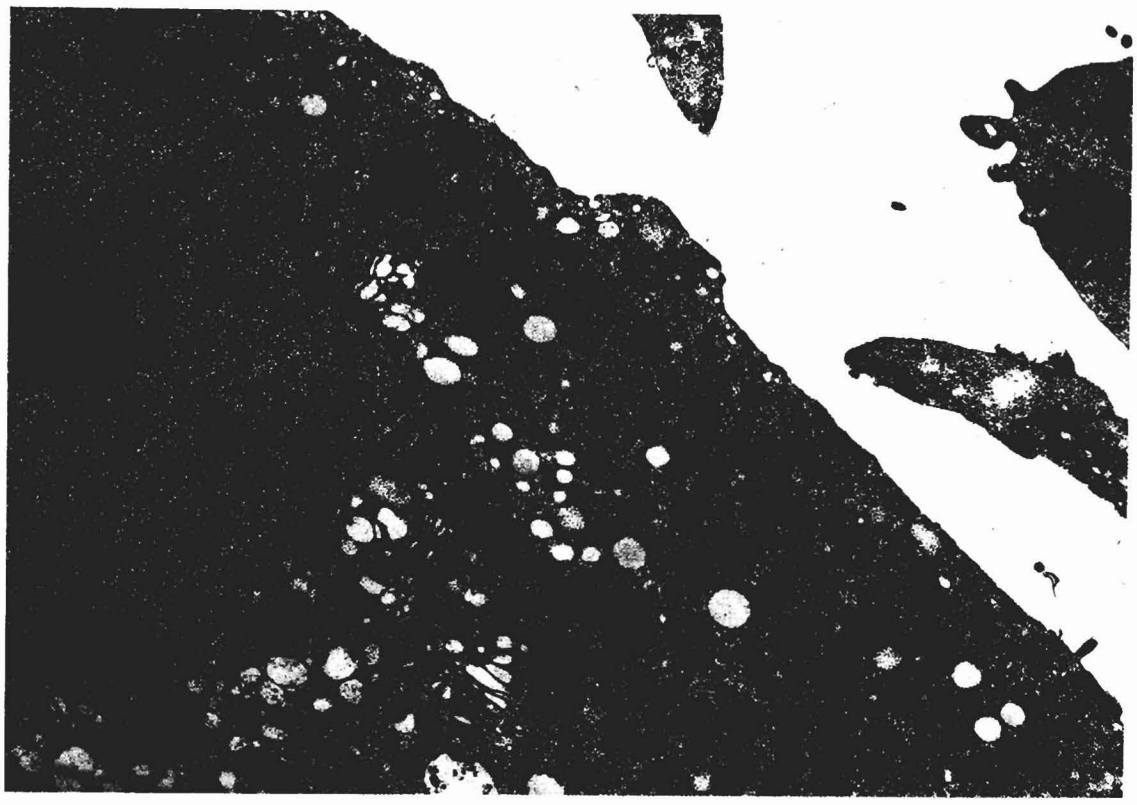

Fig. 7. Keratinocytes at confluence after the first passage. The cytoplasm contains a well developed Golgi apparatus, several mitochondria and many free polyribosomes, but no intermediate filaments. Electron microscopy, x 15,000

However, Okada et al. (1982) had shown that, for human keratinocytes grown directly on plastic, cholera toxin is beneficial at a low cell density, moderately beneficial at medium cell density and harmful at high cell density. In our study, cells were seeded at high density and this may explain why we had to withdraw cholera toxin from the beginning of the experiment, to achieve cell growth.

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