Serial Cultivation of Human Scalp Hair Follicle Keratinocytes

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A method is described for the serial cultivation of adult human hair follicle keratinocytes. Plucked scalp hair follices, placed on bovine eye lens capsules as a growth substrate, give rise to quickly expanding colonies within a few days. After trypsinization, the cells are replated with irradiated 3T3 cells as 'feeders'. Using this combination of techniques the keratinocytes can be subcultured up to four times. In this way about 10^7 keratinocytes can be generated from one single hair follicle. Moreover, the technique enables cryogenic storage of the cells, allowing for instance, convenient transportation. Subcultured hair follicle keratinocytes can be plated on glass coverslips. This allows immunofluorescence studies. The keratin cytoskeletons visualized using an antiserum against human keratin.
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Over the last 15 years, several methods for establishing cultures of normal skin keratinocytes have been developed. In early attempts, disaggregated epidermal keratinocytes were grown successfully on a plastic substrate (1, 3, 4, 9, 20). However, large inocula of the cells were necessary and serial cultivation was not satisfactory. In 1975, Rheinwald & Green developed a technique for the serial cultivation of human skin keratinocytes, using lethally irradiated Swiss 3T3 cells as a feeder layer (11). Concomitantly, much smaller inocula of keratinocytes were found sufficient to obtain growth. In recent years, we have employed human scalp hair follicles, being an extremely convenient biopsy material, for the detection of a number of genetic disorders (16).

In order to render this biopsy material more generally applicable, we tried to cultivate keratinocytes, obtained from follicles by trypsinization, in the 'feeder-cell' technique. However, this was successful only when a large number of hair follicles (more than 100) were used, which is obviously unsuitable for screening studies. Therefore, a different method was developed for the cultivation of keratinocytes from one single scalp hair follicle: a freshly plucked follicle is simply placed on the culture substrate, a bovine eye lens capsule, and within a few days a quickly expanding colony of epithelial cells is formed (17). This technique probably represents the easiest way of establishing primary cultures of purely epithelial cells from human skin, since fibroblasts have never been observed in cultures of anagen follicles.

Recently, we succeeded in the subcultivation of human hair follicle keratinocytes growing on lens capsules by transplanting small fragments of these cultures onto new capsules (18). However, the presence of the lens capsule impeded immunofluorescence studies on the cultured hair follicle keratinocytes. Moreover, further cultivation of the cells proved impossible after cryogenic storage.

In the present report we describe a method for the generation of large numbers of keratinocytes originating from single hair follicles. It requires a combination of our culture technique with bovine eye lens capsules for the primary culture, with the 'feeder-cell' technique for subcultivation. Cryogenic storage appeared possible, and immunofluorescence studies could be performed, as is shown by staining of the keratin cytoskeleton.

MATERIALS AND METHODS

Serial cultivation

Primary cultures of keratinocytes, originating from the outer root sheath of human scalp hair follicles, were established as described earlier (17). In brief, freshly plucked hair follicles were placed on bovine eye lens capsules mounted in culture dishes (Epicult, especially developed for this purpose and now commercially available from Sanbio, Nistelrode, The Netherlands). MEM was supplemented with 15% fetal bovine serum, 0.4 μg/ml hydrocortisone and 4 μg/ml insulin. The cultures were placed in an incubator with humidified air for an initial period of 3 days and subsequently transferred to an atmosphere of 5% $C●_2$ in air. After 15 days in culture, stratified colonies of keratinocytes are formed around each hair follicle. At that time the culture dishes were dismounted and the capsules with the adhering colonies were transferred to 0.2% trypsin and 0.5% EDTA in phosphate-buffered saline. Excess solution was carefully removed after 1 min and incubation was continued for 5 more min at 37°C. After the addition of culture medium the cells were gently suspended using a syringe and sedimented at 900 g. The cell pellet was resuspended in culture medium, and the quantity of the cells was determined using a hemocytometer. The contents of one Epicult dish (about 4×10^4 cells) were inoculated into a 90-mm culture dish containing 10^6 Swiss 3T3 cells, according to the technique of Rheinwald & Green (11). The Swiss 3T3 cells (CCL-92) were obtained from the American Type Culture Collection. The cells were irradiated with a dose of 3000 r using a cobalt source, as described by Kondo et al. (10). The medium used for subcultivation of hair follicle keratinocytes was Dulbecco's Modified Eagle Medium supplemented with 20% fetal bovine serum (Boehringer Mannheim), 0.4 μg/ml hydrocortisone (Sigma), 4 μg/ml bovine insulin (Organon, The Netherlands), 10^{-9} M cholera toxin (Schwarz/Mann), 10 ng/ml epidermal growth factor (Collaborative Research) and 50 μg/ml gentamycin. The cultures were refed every 3–4 days and were further subcultured every 2–3 weeks using the 'feeder-cell' technique.

Cryogenic storage

For storage in liquid nitrogen the cells were released from the lens capsule as described above. After one wash with culture medium the cell pellet was resuspended in 1 ml of fetal bovine serum (inactivated; 30 min at 56°C) containing 5% dimethyl sulphoxide (DMSO). The freeze medium was at 4°C. The cell suspension was transferred into freeze vials with a silicon gasket between cap and tube (Nunc, Denmark) and immediately moved to a −80°C freezer. Under these conditions the cooling rate was about 1°C per min. After 24 h the vials were transferred to a liquid nitrogen tank. To recover the cells from frozen storage the vial was warmed to 37°C. The suspension was diluted in culture medium to reduce the concentrationof DMSO and centrifuged at 900 *g*. The cell pellet was inoculated into a 90-mm dish in the presence of irradiated 3T3 cells.

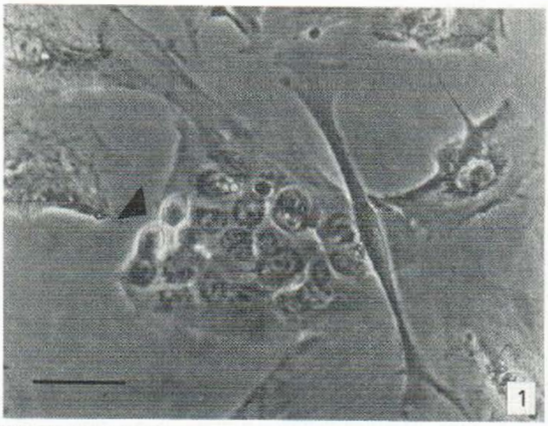
Determination of the colony-forming efficiency

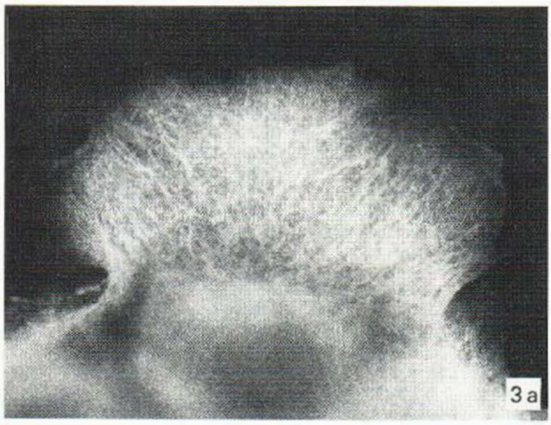
In order to determine the colony-forming efficiency (CFE) after freezing of the cells, two primary cultures of keratinocytes growing on lens capsules were trypsinized as described. Half of the cells were immediately plated together with 10^6 irradiated 3T3 cells in a 90-mm dish. The other half of the cells were quickly frozen in freeze medium at −80°C. After 3 h the freeze vial was transferred to a liquid nitrogen container and after 3 more hours the vial was thawed. The cells were recovered and plated as described above. At day 5 the colonies with more than 10 cells were counted using a phase-contrast microscope.

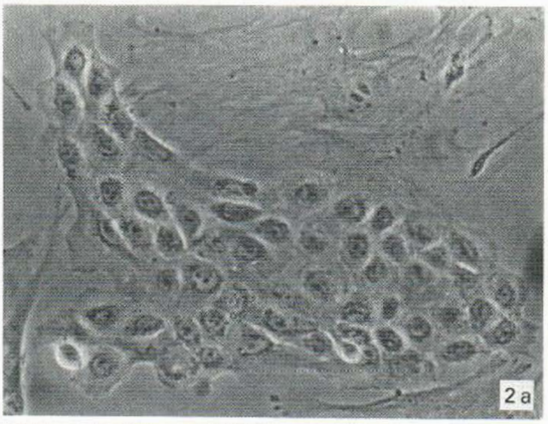
Indirect immunofluorescence microscopy

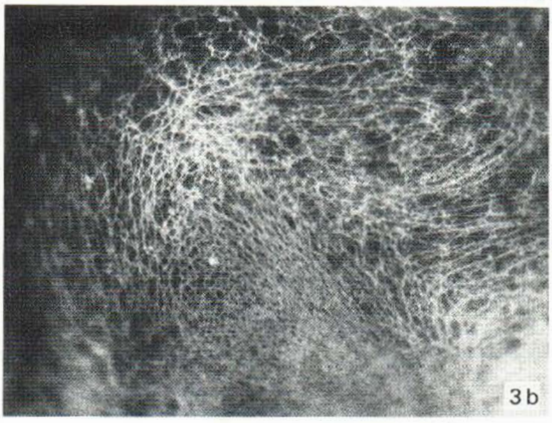
An antiserum specific for keratins of human stratum corneum was prepared as described by Sun & Green (13), and tested by double diffusion in agar *ad modum* Yen et al. (19). The antiserum was partially purified by 50% ammonium sulphate precipitation of the immunoglobulins.

For indirect immunofluorescence microscopy, hair follicle keratinocytes originating from one single primary culture were seeded together with 2×10^5 irradiated 3T3 cells onto sterilized glass coverslips in a 50-mm culture dish. After 5 days of cultivation, the coverslips were briefly rinsed with phosphate-buffered saline (PBS) and fixed in a 4% formaldehyde solution in PBS for 15 min at room tempera-









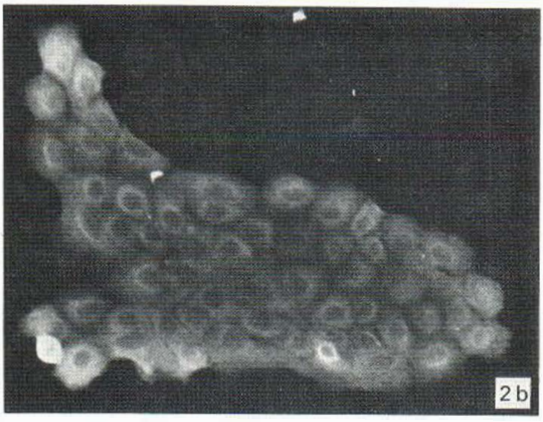


Fig. 1. Picture of a colony of hair follicle keratinocytes surrounded by irradiated 3T3 cells. Note the cell division that is taking place (arrow). The photograph was taken using a Leitz phase-contrast inverted microscope (bar: 50 μm).

Fig. 2. Phase-contrast microscopy (a) and prekeratin immunofluorescence microscopy (b) of the same colony of hair follicle keratinocytes surrounded by 3T3 cells. Note the brightly fluorescing cell in the corner of the colony, indicating mitosis.

Fig. 3. (*a*) Keratinocyte at the border of a colony, showing fluorescence around the nucleus and more peripherally radiating into the cytoplasm. (*b*) Detail of the flattened part of a well-spread hair follicle keratinocyte, revealing the filamentous nature of prekeratin in the cell.

ture. The fixed cells were treated for 5 min with prechilled acetone (−20°C), and air-dried. 100 µl of the partially purified keratin antiserum (diluted 1 : 20 in PBS) was placed on the coverslips and incubated for 45 min at 37°C in a humidified atmosphere. The coverslips were thoroughly rinsed using an incubator–shaker (New Brunswick Scientific Co., USA) for 20 min at room temperature, while the buffer was changed three times. Fluorescein-conjugated swine anti-rabbit IgG (Dako, Denmark) was applied at a dilution of 1 : 40. After incubation for a further 45 min at 37°C, the coverslips were rinsed in PBS for 20 min as above and mounted cell-side down in glycerol/PBS (1 : 1) on slides. Nail polish was used to prevent evaporation. The preparations were viewed in a Zeiss microscope using epifluorescence ilumination and 10× and 95× oil-immersion objectives. Photographs were taken with Kodak tri-X film.

RESULTS

Serial cultivation

Primary cultures of hair follicle cells were used for subcultivation after 2 weeks of culture. Older cultures became progressively more stratified and contained an increasing number of keratinized cells. After 2–3 days in subculture, groups of 2–4 keratinocytes surrounded by 3T3 cells were visible. These cells formed quickly expanding colonies (Fig. 1), pushing away the 3T3 cells and finally covering a large area of the culture dish. The presence of epidermal growth factor and cholera toxin in the culture medium has been shown to prolong considerably the culture lifetime and to enhance the rate of cell proliferation in cultures of epidermal keratinocytes (6, 12). Since we also observed a beneficial effect on the cultures of hair follicle cells growing on the lens capsule (18), these substances are new routinely included in our culture medium.

The CFE of the keratinocytes at their first passage, determined as described in Materials and Methods, was 1.0±0.2% (n=4). A maximum of four subcultivation steps could be performed, with gradually decreasing CFE. After 3 days in culture, the outgrowth from a single hair follicle placed on a lens capsule contained 50 to 100 cells. In a total culture period of about 12 weeks, approx. 10^7 keratinocytes can be generated from one single scalp hair follicle. Compared with the external root sheath, which contains about 10000 cells (as judged from cell-counts after trypsinization), the increase is 1000-fold during the culture lifetime. Cells recovered from cryogenic storage and plated in the presence of irradiated 3T3 cells attached to the culture substrate just as quickly as non-frozen cells. There were no morphological differences between the colonies formed by these cells. However, the CFE of the cells after freezing was lower: 0.20±0.05% (n=4). The CFE did not decrease significantly upon storage of the keratinocytes for as long as 6 months (n=4).

Immunofluorescence microscopy

The anti-keratin serum, prepared as described in Materials and Methods, produced a strong precipitin band against stratum corneum keratins as well as against extracts from plucked hair follicles and cultured cells in a double diffusion assay, according to Yen et al. (19). Cultured hair follicle keratinocytes, when grown on glass coverslips in the presence of irradiated 3T3 feeder-cells, stained strongly with the antiserum, while the 3T3 cells surrounding the hair follicle cell colonies remained unstained (Fig. 2). At high magnification, a filamentous pattern was revealed in the flattened part of some of the hair follicle keratinocytes (Fig. 3). This pattern is comparable to that of epidermal keratinocytes, as described earlier by Sun & Green (14).

DISCUSSION

The use of hair follicles as a biopsy material for biomedical research and diagnosis started only a decade ago. In recent years the hair follicle has been increasingly used as an

enzyme source for molecular diagnosis of inborn errors of metabolism (for review, see 16). Since the development of a method for the cultivation of hair follicle keratinocytes (17), this approach has found further applications. For example, in some studies related to cancer, it is necessary to incubate for long periods of time with carcinogens (2, 5, 21). This can only be done when cultured cells are used (7, 8).

We recently described a method for subcultivation of human hair follicle keratinocytes by means of transplantation of colony fragments onto new bovine eye lens capsules (18). This was an improvement of the culture method described earlier (17), since it enabled us to cultivate more cells derived from a single hair follicle. The present technique of co-cultivation of irradiated 3T3 cells and hair follicle cells derived from primary cultures on bovine eye lens capsules allows serial cultivation of hair follicle keratinocytes. The advantage of this method is that the lens capsule is only essential for the primary culture and in consequence about 10 times more cells can be generated than by transplantation. Alternatively, for biochemical studies the transplantation technique is preferable, since the determination of biochemical parameters is not disturbed by the presence of other cell types, and since the cells have not been altered by proteolytic enzymes that have been shown to alter some of the biochemical parameters of cells in culture (15).

The technique presently described not only allows the generation of a large amount of cells, but also enables fluorescence studies and cryogenic storage. As a result, transportation of the cells between laboratories is now possible. This is especially important for the investigation of relatively uncommon genetic diseases that manifest themselves in epithelial cells.

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