Serial Cultivation of Single Keratinocytes from the Outer Root Sheath of Human Scalp Hair Follicles

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A method for the isolation of outer root sheath keratinocytes from plucked human hair follicles and for their subsequent cultivation has been developed. The selective trypsinization of outer root sheath keratinocytes provided a single cell suspension of defined origin within the hair follicle. The 3T3 feeder layer technique supports sustained growth of these cells in that as little as one single plucked hair follicle (yielding approximately 1.5×10^4 cells) consistently gave rise to a confluent 35-mm culture dish (with approximately 1.5×10^6 cells) within about 2 weeks. The outer root sheath keratinocytes can be serially passaged for up to 3 times and also cryopreserved. *J Invest Dermatol 87:485–488, 1986*

Plucked hair follicles were first used as a convenient biopsy material for the study of genetic disorders and for diagnostic purposes. Thereafter, it became evident that hair follicles provide an interesting model system of epithelial cells for more fundamental biomedical research [1].

In recent years several methods have been developed for establishing primary cultures of human hair follicle cells. Weterings et al [2] were first able to grow human hair follicle keratinocytes by explanting plucked scalp hair follicles on bovine eye lens capsules. Similarly, Wells succeeded in explanting plucked human hair follicles directly on tissue culture plastic [3]. Recently, we became interested in the study of epithelial–mesenchymal interactions, especially concerning the hair follicle biology. For this purpose, we aimed to initiate primary cultures of disaggregated hair follicle cells in order to increase the proliferative capacity of the primary hair keratinocytes and to get a more defined and standardized culture system.

In this report, we describe a method for the growth of primary cultures starting from dissociated human hair follicles and for their serial cultivation. The method consists essentially in disaggregating the outer root sheath keratinocytes with trypsin/EDTA and by plating them on a preformed 3T3 feeder layer. Cells from only one single hair follicle give rise to a confluent 35-mm Petri dish within about 2 weeks with a multiplication factor of 10^3 and 12 estimated cell generations.

MATERIALS AND METHODS

Cell Isolation and Cultivation Hair follicles were plucked from the scalp and the bulk of the hair shafts cut off. The follicles were immersed in Dulbecco's modified Eagle's medium (DMEM) buffered with 25 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and supplemented with 400 U/ml penicillin and 400 μg/ml streptomycin. Those in anagen phase, indicated by the visible bulb and intact outer root sheath, were carefully selected under the dissecting microscope. After 2 additional rinses, the follicles were transferred into a 35-mm Petri dish. The remaining liquid was sucked off and 10 follicles covered with 200 μl 0.1% trypsin (1:250) and 0.02% EDTA in phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} (pH 7.2) and incubated for 10 min at 37°C. Thereafter, a single cell suspension was obtained by vigorously pipetting the follicles in DMEM supplemented with 10% newborn calf serum. For some experiments, the remaining keratinocytes still adhering to the follicles were sequentially removed by 2 additional trypsinization steps of 10 min and 30 min, respectively. The dissociated keratinocytes were centrifuged for 10 min at 200 *g* and cells from one follicle resuspended in 2 ml of complete culture medium each. The culture medium consisted of 3 parts of DMEM (Seromed), containing sodium pyruvate and 1 g/liter glucose, and 1 part of Ham's F-12 (Seromed), supplemented with 10% fetal calf serum (Gibco), 5 μg/ml insulin (Sigma), 0.4 μg/ml hydrocortisone (Sigma), 0.135 mM adenine, 2 nM triiodothyronine, 0.1 nM choleratoxin (Sigma), 10 ng/ml epidermal growth factor (Seragen), and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) modified after Gilfix and Green [4]. The keratinocytes were seeded at a density of 2–3 $\times 10^3$ cells/cm^2 on a 3T3 feeder layer (2×10^4 cells/cm^2) (ATCC CCL 92) prepared according to Rheinwald and Green [5]. The 3T3 cell proliferation had been halted by a treatment with 6 μg/ml mitomycin C (Sigma) for 8 h.

The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and the medium changed twice a week. As a rule, the feeder layer was renewed once a week after selective removal of the 3T3 cells using 0.02% EDTA [5].

Subculture After 2–3 weeks in primary culture, the residual 3T3 cells were removed with 0.02% EDTA and the keratinocytes detached and disaggregated to single cells by incubating with 0.1% trypsin (1:250) and 0.02% EDTA in PBS (pH 7.2) at 37°C for 15–30 min. The dispersed cells were replated in culture medium at a density of 50 cells to 10^4 cells/cm^2 on a fresh 3T3 feeder layer.

Cryopreservation For storage in liquid nitrogen the cells were released from culture dishes as described above. After 1 wash with culture medium, 10^6 cells were resuspended in 1 ml culture medium containing 10% glycerol, transferred into a cryotube (Nunc), and placed within a styrofoam container in a −80°C freezer. After 24 h the tubes were transferred to a liquid nitrogen tank. To recover the cells from frozen storage, the tubes were warmed to 37°C and the suspension diluted in culture medium.

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Abbreviations:
DMEM: Dulbecco's modified Eagle's medium
PBS: phosphate-buffered saline

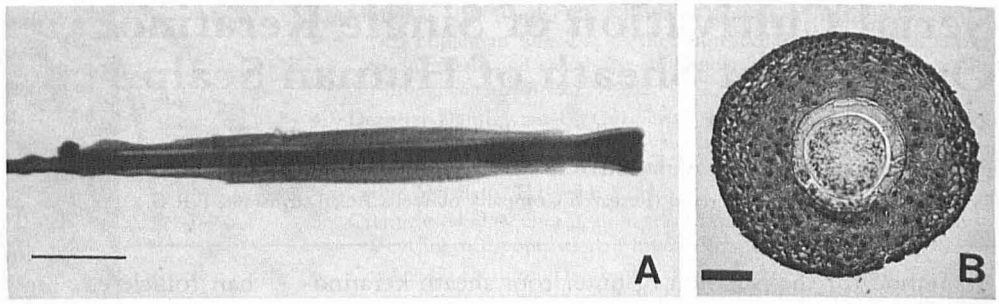

Figure 1. Plucked adult human scalp hair follicle. *A*, Whole hair follicle with its usually almost intact outer root sheath in the portion between the upper part of the hair bulb and the presumptive level of the sebaceous gland which is normally absent. Unstained, *bar* = 0.5 mm. *B*, Transversal section of the middle third of a hair follicle. The peripheral layer of the columnar basal cells is often preserved over an extensive portion of the circumference. H&E, *bar* = 50 μm.

The cells were washed and then inoculated at a density of 2×10^4 cells/cm^2 onto a feeder layer.

Histologic Examination Confluent cultures of hair follicle keratinocytes grown in culture dishes were detached as epithelial sheets according to Green et al. [6] and Watt [7]. Briefly, the cultures were incubated with Dispase II (Boehringer Mannheim) at 1.2 units/ml in PBS. After about 15 min at 37°C, the epithelial sheet started to separate from the plastic surface. The detachment could be accelerated by carefully lifting the sheet with tweezers. Pieces of the sheet were punched out, stretched over a Whatman No. 1 filter paper, and washed with PBS. They were then fixed in Bouin's solution for 24 h and processed for wax embedding. Serial sections were prepared at 7 μm and stained with hematoxylin and eosin.

In order to facilitate their handling the plucked hair follicles were embedded in a solution of 3% agar in PBS prewarmed at 40°C prior to the fixation. The subsequent processing of the hair follicles was carried out as described above.

RESULTS

Cell Isolation The plucked anagen hair follicles from adult human scalp exhibited an almost intact epithelial outer root sheath (Fig 1*A,B*). Only the outer root sheath portion at the level of the infundibulum and around the upper part of the hair bulb was usually lacking. In most cases the lower part of the hair bulb and the sebaceous gland remained in the tissue.

The disaggregation of the outer sheath keratinocytes with 0.1% trypsin and 0.02% EDTA for 10 min yielded a mean cell number of $1.5 \pm 0.4 \times 10^4$ (n = 7) per follicle. Comparative studies of the untreated and disaggregated follicles indicated that cells from the external layers of the outer root sheath had been removed (Figs 1*A,B*, 2*A,B*).

When the remaining follicle was trypsinized for an additional 10 min, $0.5 \pm 0.25 \times 10^4$ (n = 11) cells were additionally obtained per follicle. The thickness of the residual outer root sheath depended on individual differences in the diameter of the plucked hair follicles and ranged from roughly half of the outer sheath cells still present (Fig 3*A,B*) to their almost complete removal. A final enzymatic digestion of 30 min removed the remainder of the outer root sheath cells, leaving behind the denuded inner root sheath (Fig 4).

Cell Culture Primary cultures of hair follicle keratinocytes could be routinely established by plating cells disaggregated from one single hair follicle on a preformed 3T3 feeder layer in a 35-mm culture dish or from 10 follicles on a 3T3 feeder layer in a 100-mm culture dish. The seeding efficiency of the keratinocytes at the initiation of the primary cultures was $2.6 \pm 0.5\%$ (n = 4). After 2–3 days in culture, groups of 2–4 keratinocytes surrounded by 3T3 cells were visible. These cells formed quickly expanding colonies pushing away the 3T3 cells. The keratinocytes were of similar small size and closely packed in a polygonal epitheloid arrangement (Fig 5). As long as the colonies were of limited size, the weekly renewal of the feeder layer proved to be beneficial. Otherwise, the cells at the margin of the colonies enlarged and eventually ceased to divide in the absence of a feeder layer, as it has been noted for interfollicular epidermal keratinocytes [8]. When reaching a larger size the colonies started to pile up and to stratify in their center, leading occasionally to the formation of domes (Fig 6). These cultures were confluent after 2–3 weeks of incubation. As approximately 10^7 cells could be generated from 10 follicles in a 100-mm dish, the number of actively growing cells increased about 1000-fold during the primary culture lifetime. Due to the virtual absence of dermal components in the plucked hair follicle and to the renewal of the feeder layer, an overgrowth by fibroblasts has never been encountered. Outer root sheath keratinocytes originating from more internal layers removed by the second trypsinization step also consistently gave rise to cultures displaying similar morphology but with a seeding efficiency of $1.1 \pm 0.5\%$ (n = 3). This result indicates that more axially located outer sheath cells still retain their proliferative capacity in vitro. Furthermore, even cells disaggregated by the third trypsinization step were still able to attach and proliferate in culture.

A cross-section through a confluent culture detached from the plastic by means of dispase treatment reveals the multilayered structure with the polygonal basal cells underneath and the more flattened cells of the upper layers (Fig 7).

As for the epidermal keratinocytes [8], the hair follicle kerat-

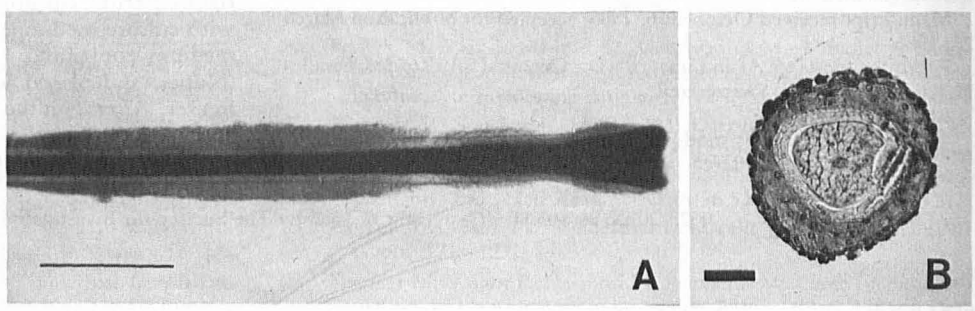

Figure 2. Plucked adult human scalp hair follicle following a treatment with 0.1% trypsin and 0.02% EDTA for 10 min. *A*, Whole treated hair follicle. The extent of the cell removal can vary over the follicle length leading sometimes to an uneven thinning of the hair follicle. Unstained, *bar* = 0.5 mm. *B*, Transversal section of the middle third of a treated hair follicle. At this level, usually about half of the outer root sheath cells have been removed. H&E, *bar* = 50 μm.

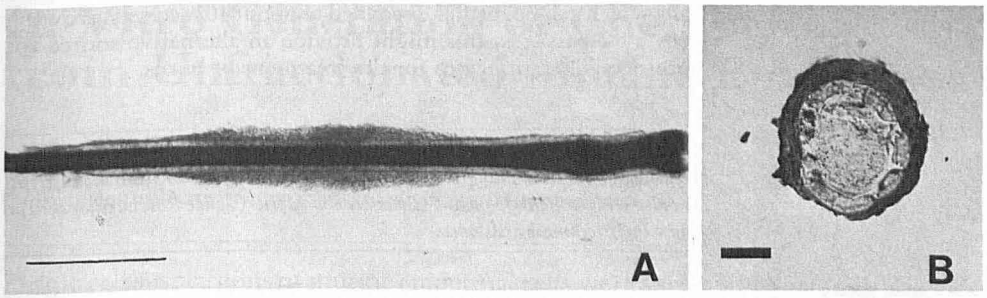

Figure 3. Plucked adult human scalp hair follicle following a treatment with 0.1% trypsin and 0.02% EDTA for 20 min. The corresponding pictures (*A*, unstained, *bar* = 0.5 mm; and *B*, H&E, *bar* = 50 μm) show that the outer root sheath cells have been removed to a great extent.

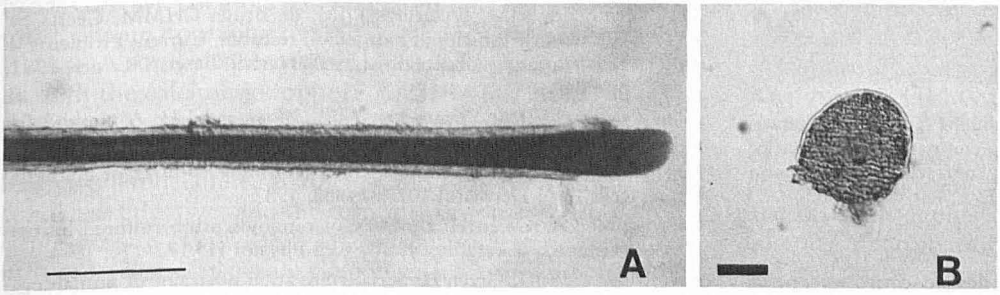

Figure 4. Plucked adult human scalp hair follicle following an ultimate treatment with 0.1% trypsin and 0.02% EDTA for 50 min. The corresponding pictures (*A*, unstained, *bar* = 0.5 mm; and *B*, H&E, *bar* = 50 μm) show that all the outer root sheath cells have been removed leaving behind the denuded inner root sheath on (*A*) and the denuded hair shaft on (*B*).

inocytes were best subcultured within 2 weeks after plating, before individual colonies had enlarged too much. The subcultivation on a fresh feeder layer enabled clonal seeding densities (50 cells/cm^2) with a cloning efficiency in the range of 0.5–5%. Until now cells could be successfully passaged 3 times.

Cells recovered from liquid nitrogen storage and plated in the presence of a 3T3 feeder layer showed no morphologic differences compared with the nonfrozen cells. The seeding efficiency of the keratinocytes recovered from cryogenic storage was in the range of 1–5%.

DISCUSSION

Previously, growth of human hair keratinocytes in primary cultures has been reported using explant cultures of plucked hair follicles on bovine eye lens capsules [2] or on plastic [3]. Using this approach, major drawbacks were rather limited cell numbers and cultures of low reproductivity. Preliminary attempts to grow dispersed hair keratinocytes on different biologic substrates (rat tail collagen, fibronectin, extracellular matrix secreted by bovine corneal endothelial cells [9] failed (unpublished results). The cells neither attached nor spread out under these conditions. However, this impairment could be circumvented by adopting the 3T3 coculture conditions established by Rheinwald and Green [5]. Cells from one single hair follicle routinely gave rise to a confluent culture in a 35-mm dish after about 2 weeks. The combined effect of the medium composition and of the feeder layer apparently promoted rapid cell growth and, concomitantly, delayed the terminal differentiation. Most cells isolated by the method reported here originated from the outer root sheath portion located between the opening of the sebaceous gland and the upper hair bulb. Thus, it is very unlikely that any of the keratinocytes growing in culture originated from the infundibulum where the outer root sheath is indistinguishable from interfollicular epidermis. In addition, the results obtained by sequential trypsinization of the outer root sheath suggest that cells from all layers retain a high capacity for proliferation in culture. This behavior contrasts to the situation in the outer root sheath which is believed to be a rather static structure as judged from morphologic observations. However, both necrotic cells and mitotic figures are occasionally found in the outer sheath, indicating that cell division and cell death occur at a well balanced, but low level within this tissue [10].

In vivo, the columnar basal cells, instead of forming hemidesmosomal contacts, send out minute basal cytoplasmic processes through the surrounding basement membrane [11,12]. Moreover, the outer sheath cells of the lower two-thirds of the follicle have

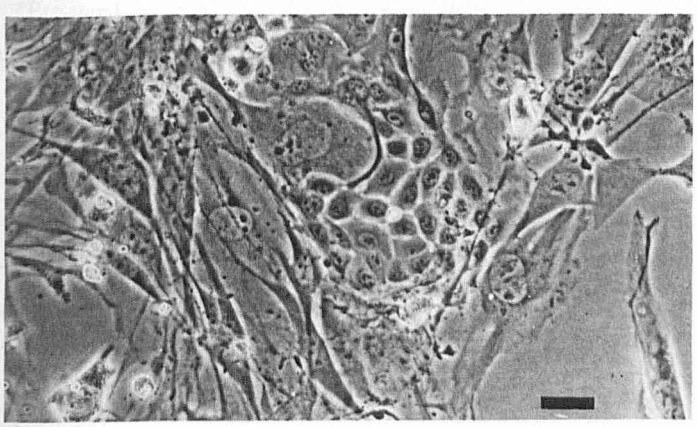

Figure 5. Primary hair follicle keratinocytes surrounded by mitomycin C-treated 3T3 cells, 5 days after plating. Phase contrast, *bar* = 40 μm.

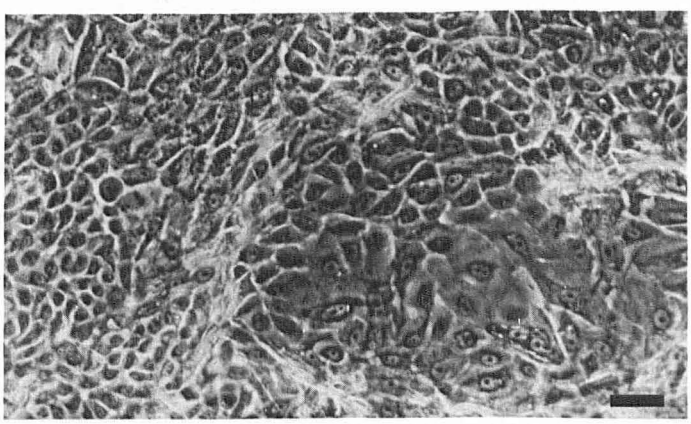

Figure 6. Primary hair follicle keratinocytes, 18 days after plating. Phase contrast, *bar* = 40 μm.

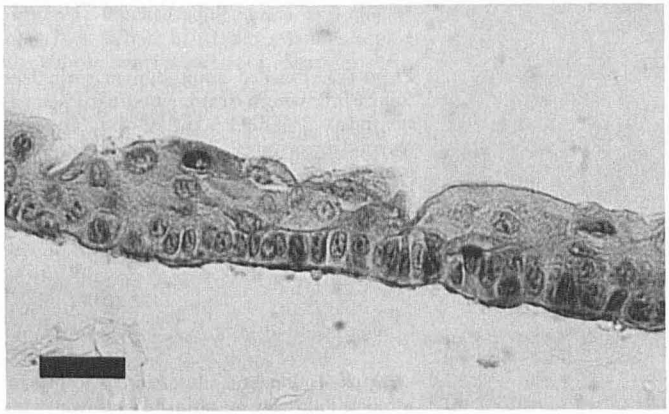

Figure 7. Transversal section through a confluent hair follicle keratinocyte culture detached with dispase. The very compact arrangement of the basal cells has probably been enhanced by a contraction phenomenon occurring during the detachment procedure. H&E, *bar* = 25 μm.

been described to be virtually devoid of desmosomes except at the contacts with cells of the Henle's layer where these structures are abundant [11]. Another characteristic of the outer sheath cells is their high content of glycogen granules [11,12]. In explant cultures, however, numerous desmosomes are found, as shown by Weterings et al [13]. In addition, glycogen accumulations have been demonstrated mainly in the basal cells, whereas the keratin polypeptide pattern resembled that of the outer root sheath in situ [13; manuscript in preparation]. We are currently investigating the role of different culture conditions for the modulation of phenotypic expression, such as the effect of dermis on the differentiation of outer root sheath cells cultured at the air–liquid interphase. These studies might help to elucidate to what extent these cells retain in vitro their orginal properties or shift toward a more epidermal-like phenotype. Such a finding would not be suprising since the regeneration of epidermis after wounding has been discussed to proceed from the outer root sheath of intact hair follicles.

In summary, the technique described here enables the generation of a large amount of hair follicle cells readily available for biomedical research and diagnosis, provided the initial presence of the foreign feeder cells is not prejudicial. In particular this might contribute to the elucidation of the biologic role of outer root sheath cells in induction and maintenance of hair growth and differentiation. Since one single follicle gives rise to 10^6 viable cells which can be further expanded to about 10^8 actively growing cells by 2 passages, this might provide an alternative source for autologous keratinocytes for the treatment of burns.

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