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In vitro Growth and Differentiation of Epithelial Cells derived from Post-embryonic Hair Follicles

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Abstract

Cell aggregates formed during the first 2 days of culture of cells derived from hair follicle tissue of young rats. Aggregates occurred at an accelerated rate at higher cell densities, and contained a high proportion of epithelial cells although a variable proportion of mesenchymal (fibroblast) cells were present. Citrulline was detected in the cultured cell proteins. Electrophoretic analysis of the proteins showed the presence of hair cortical keratin in the cultures, but these proteins were not synthesized during the culture period, in conflict with previous findings. Protein synthesized by the cultured cells was not of inner root sheath or epidermal origin and the only protein which could be identified was actin. It is concluded that the present culture system is unsuitable for the study of keratinization as occurs in the intact hair follicle.

Introduction

Hair follicles, which are derived from epithelial-type cells, possess a complex cellular organization consisting of concentric layers of cells with completely distinct differentiation patterns to produce a distinct complement of keratins. These patterns are different from those of the epidermal cells. In most mammals the cells of the hair follicle undergo periodic cycles of growth (anagen phase) and degeneration (catagen phase) associated with the regular shedding of hair, a phenomenon which is unique to the hair follicle.

The complexity of the cellular organization makes the function of the hair follicle extremely difficult to study *in vivo* and necessitates the use of *in vitro* cultures from follicle tissue. Cultured cells derived from keratinizing tissue are potentially valuable for the study of the control of the specialized processes which are involved in keratin production. To date, few culture systems have been developed for cells derived from hair follicles (Frater 1979). Frater (1975, 1976, 1979) has developed methods for the isolation and culture of cells from post-embryonic hair follicles, and some properties of the cultured cells have been described (Frater 1975, 1976, 1979; Frater and Hewish 1980). In a preliminary report it was suggested that the cultured cells synthesized keratin proteins (Frater and Hewish 1980).

The cell culture system has been further investigated in order to evaluate it as a potential model for hair growth. This paper describes some more properties and characteristics of the cells in culture including a more detailed examination of the proteins formed *in vitro*.

Materials and Methods

Cell Culture

Cells were isolated from the lower dermis of Hooded Wistar rats 12–14 days old, and cultured by the methods of Frater (1976, 1979).

Identification of Cell Types

Epithelial and mesenchymal cells were distinguished by two staining procedures. The presence of aminopeptidase (cytosol-1) in mesenchymal cells was detected by the procedure of Jacquemont and Pruniéras (1969). The second method was with dichrome stain (Everett and Miller 1978), which differentially stains epithelial and mesenchymal cells in primary culture by an unknown mechanism.

Labelling of Cultured Cells

Cells were grown for 4 days in culture and labelled with 20 μCi [^{35}S]cysteine in 1 ml of culture medium plus 10% (v/v) foetal calf serum for 16 h. The medium was removed and the cells (adhering to the dish) washed with 0·14 M sodium chloride–0·01 M sodium phosphate, pH 7·4. The cells were scraped from the surface of the culture dish with a glass rod and washed twice with saline.

Preparation of Soluble Proteins

Epidermal keratin was obtained from new-born rats. The skin was removed and the epidermis stripped by the procedure of Walker *et al.* (1977). Inner root sheaths of adult rat vibrissae were obtained by the method of Rogers (1958).

Approximately 0·5 mg of hair, 1 mg of inner root sheath, 1 mg epidermis or 2 mg of cell culture were extracted at room temperature for 18 h with 50 μl of a solution containing 8 M urea, 0·05 M dithiothreitol and 0·05 M Tris (pH 9·3). Radiolabelling by *S*-carboxymethylation was carried out by two slightly different procedures by adding (1) 5 μl of an aqueous solution containing 60 μCi of [2-^{3}H]iodoacetic acid and 5 μl of 3 M Tris or (2) 5 μl of an aqueous solution containing 6 μCi of [2-^{14}C]iodoacetic acid, and 0·5 μl of 3 M Tris. After 10 min, 25 μl of a solution containing 1 M iodoacetate and 2·3 M Tris (pH 8·5) was added, followed 10 min later by 5 μl of 2-mercaptoethanol (Marshall 1981).

For the cell culture radiolabelled *in vitro* with [^{35}S]cysteine, the step involving the addition of radiolabelled iodoacetic acid was eliminated.

Polyacrylamide Gel Electrophoresis

Protein mixtures were characterized by two-dimensional polyacrylamide gel electrophoresis (Marshall 1981). The proteins were initially separated at pH 8·9 in 8 M urea in 6% (w/v) acrylamide followed by SDS electrophoresis in a gel slab containing a step-wise acrylamide concentration (7·5 cm of 10%, then 2 cm of 15%). Following electrophoresis the proteins were located by fluorography (Bonner and Laskey 1974).

Amino Acid Analysis

Cell cultures (grown for 24 h and 4 days, harvested by scraping) and inner root sheath were hydrolysed for 24 h *in vacuo* at 108°C with constant-boiling HCl containing a trace of phenol. Acid was removed by freeze-drying before the samples were subjected to amino acid analysis. Citrulline values were corrected for the decomposition to ornithine which occurred during the acid hydrolysis (Rogers 1962).

Reagents

Culture media and foetal calf serum were obtained from the Commonwealth Serum Laboratories, Parkville, Vic. Collagenase A (EC 3.4.24.3) and Type I actin from rabbit muscle were obtained from the Sigma Chemical Company, St. Louis, U.S.A. Trypsin (1/250 grade) was obtained from Difco Laboratories, Detroit, U.S.A. Radioisotopes were from The Radiochemical Centre, Amersham, U.K.

Results

Cell cultures showed patterns of development similar to that previously described (Frater 1979). The cells were originally rounded and highly refractile but gradually spread onto the surface of the culture dish. After several days, many of the cells formed round aggregates, the size and rate of formation of the aggregates being influenced by the initial cell density (Fig. 1). With continued time, the aggregates reached a limiting size of approximately 0·1 cm, after which little further growth occurred.

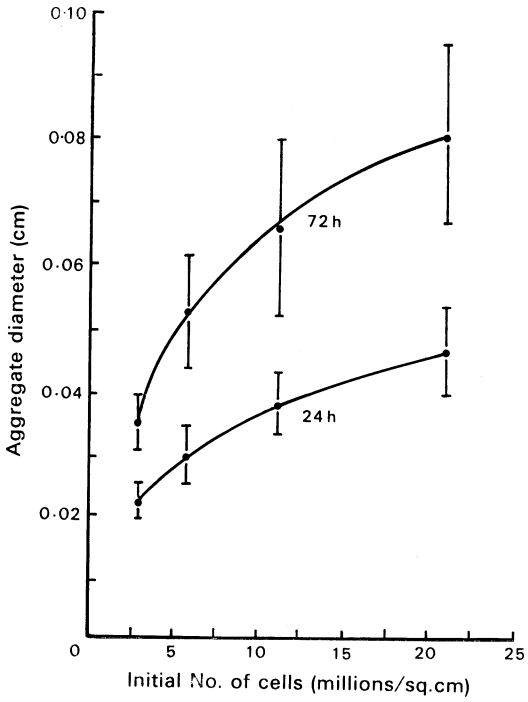

Fig. 1. Effect of cell density on the density of cell aggregates. Dermal cultures were plated at the indicated densities. The cell densities were estimated using a Wild M40 microscope and eyepiece with micrometer grid at 100× magnification and phase-contrast. Aggregate diameters were measured with the same apparatus after the indicated times.

The origins of the cells participating in the formation of the aggregates was investigated by the use of differential staining techniques. Both the aminopeptidase stain and the differential dichrome stain gave similar results, although the aminopeptidase stain gave lower estimates of the numbers of mesenchymal cells than the dichrome stain, probably because of the difficulty of detection of the stained granules within very flattened cells. The two stains indicated the presence of epithelial-derived and mesenchyme-derived cells both within the aggregates and among the non-aggregated cells. The epithelial type strongly predominated within the aggregates. Free cells were largely mesenchymal (fibroblasts) and their numbers increased with time in culture relative to the epithelial population.

Characterization of Proteins Synthesized by the Cultured Cells

Amino acid analyses

Amino acid analysis was used as an aid to the identification of the proteins of the cultured cells. The amino acid citrulline has been reported to be present specifically

in protein derived from the inner root sheath and medulla of hair follicle (Rogers and Simmonds 1958; Rogers 1962) although it may occur as well in low amounts in epidermal protein (Kubilus *et al.* 1979). Total proteins from cell cultures which had been incubated for 24 h and 4 days respectively were subjected to acid hydrolysis and amino acid analysis (Table 1). The presence of citrulline was confirmed by amino acid analysis but although the citrulline content decreased from 1·7 residues per 100 residues after 24 h to 1·3 residues per 100 residues after 4 days, the total amount of citrulline in the culture increased by 41% during this period, indicating that citrulline was present in the proteins synthesized by the cultured cells. The remainder of the amino acids were found to occur in similar proportions to those of inner root sheath protein but the overall variation was too great to allow conclusive identification.

Table 1. Amino acid compositions (as residues per 100 residues) of inner root sheath and culture protein

Amino acid	Inner root sheath	Culture protein 24 h	Culture protein 4 days
Lysine	5·9	6·4	2·6
Histidine	1·7	1·9	0·8
Arginine	6·3	5·2	3·1
Aspartate	9·2	9·7	9·2
Threonine	4·3	5·0	5·2
Serine	8·0	6·9	8·9
Glutamate	17·7	14·4	14·9
Proline	4·6	5·3	4·9
Glycine	9·0	6·8	10·6
Alanine	6·2	7·9	8·0
½Cystine	1·0	1·1	1·2
Valine	4·6	5·4	9·5
Methionine	1·6	—	2·0
Isoleucine	3·4	4·2	4·5
Leucine	8·0	9·0	9·2
Tyrosine	2·3	2·7	3·0
Phenylalanine	2·8	3·4	3·5
Citrulline[A]	3·4	1·7	1·3

[A]Includes ornithine.

Gel Electrophoresis

The proteins of the follicle cells after culture for 4 days were examined by two-dimensional polyacrylamide gel electrophoresis (Fig. 2) and compared with those from rat hair, inner root sheath and epidermis. Identity of protein species in different samples (as indicated in Fig. 2) was confirmed by co-electrophoresis of ^{3}H-labelled culture proteins and ^{14}C-labelled keratin standards, as described by McConkey (1979). Keratin proteins of both the low-sulfur (labelled 1–5 in Fig. 2) and high-sulfur (6–9 in Fig. 2) types were present in the culture material, and some culture proteins (1–3, 5, 13 in Figs 2*a*–2*c*) possessed the same mobilities as proteins in the inner root sheath sample. The origin and type of components 10–12 are unknown. Possibly components 1–3 and 5 in the inner root sheath pattern result from hair contamination. The protein indicated in Figs 2*a*, 2*c* as *A*, which was present in the culture and inner

root sheath, was identified as actin as it exhibited co-electrophoresis with commercially purified rabbit muscle actin. However, the proteins synthesized by the cultured cells, as demonstrated by those proteins labelled after incubation of the cultures with [^{35}S]cysteine, comprised a small subset of the total present (Fig. 2*e*) and, with the exception of actin, contained no species which showed co-electrophoresis with either inner root sheath or hair keratin proteins.

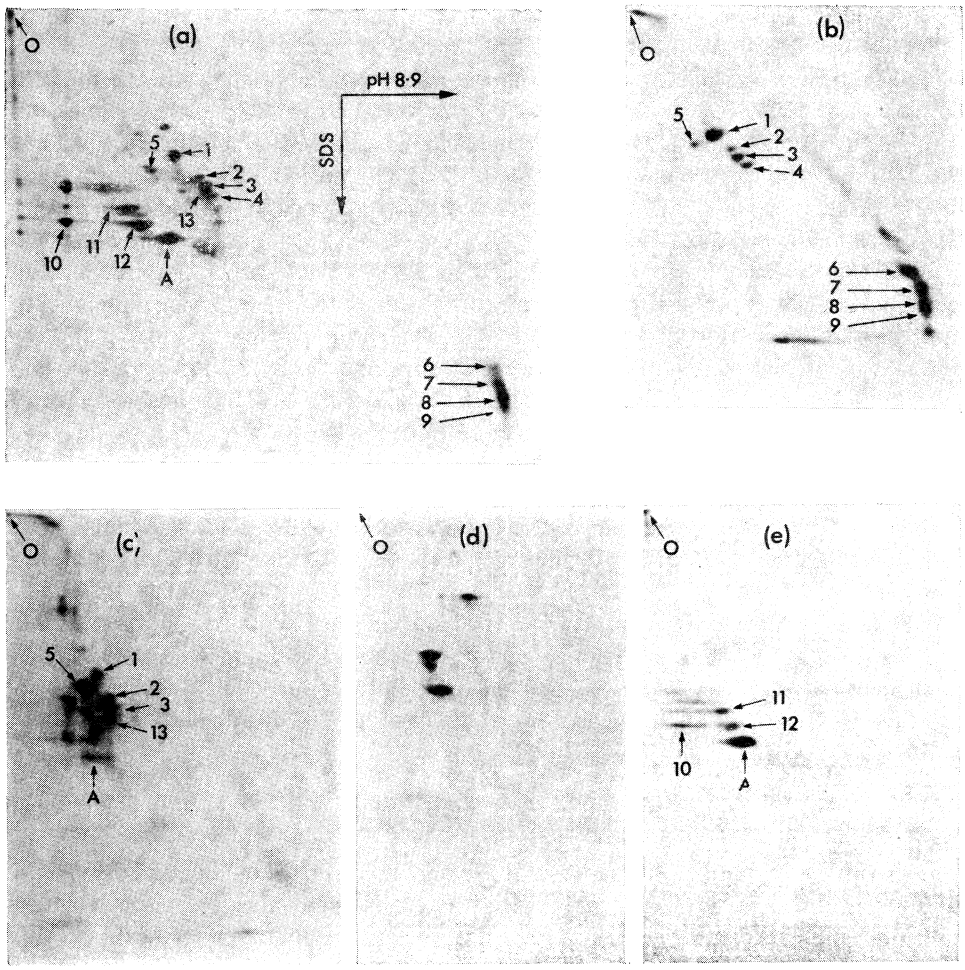

Fig. 2. (*a*)–(*d*) Two-dimensional gel electrophoresis of [^{3}H]iodoacetate-labelled proteins from (*a*) culture; (*b*) rat hair; (*c*) inner root sheath; (*d*) rat epidermis. The proteins in (*a*) numbered 1–13 correspond with proteins from the other sources. The protein labelled *A* has been found to exhibit co-electrophoresis with purified actin. (*e*) Two-dimensional gel electrophoresis of proteins from a cell culture labelled by incubation with [^{35}S]cysteine. Electrophoresis and the labelling of proteins is as in (*a*)–(*d*). o Origin of electrophoresis.

Discussion

Frater (1976, 1979) showed that cells isolated from whole dermal tissue containing hair follicles formed aggregates on the surface of the culture dishes. These aggregates,

which contained a high proportion of epithelial cells, are assumed to be the sites of differentiation, on the basis of histological studies (Frater 1979), although direct evidence was not obtained. The work described here further characterizes the cell culture system. The cells in culture which remained unassociated with the aggregates were found to consist of a mixture of epithelial and fibroblast cells, with fibroblasts predominating with increasing time in culture. It was found that cellular morphology was insufficient as a guide to the nature of the cells, and the two cell types could only be distinguished by differential staining. This observation has also been made by other workers (Jacquemont and Pruniéras 1969).

The cultures were found to contain hair cortical proteins but, despite labelling the cultures with [^{35}S]cysteine, which would be expected to emphasize the synthesis of sulfur-rich proteins such as keratins, no evidence for the synthesis of hair proteins by the cultured cells was obtained. Presumably the hair proteins were carried through into the cultures as components of fully differentiated cortical cells, and their relative concentrations were found to vary between preparations. A major protein which was synthesized by the cultures was identified as actin. Some other culture proteins exhibited co-electrophoresis with inner root sheath proteins, but were not labelled after incubation with [^{35}S]cysteine. No epidermal proteins were present in the cell culture. Thus, the increase in the amount of citrulline occurring during culture was not due to the synthesis of inner root sheath or epidermal proteins, and the protein species containing citrulline have not been identified.

The observation that no hair proteins are synthesized by the cell culture system is in conflict with a previous report (Frater and Hewish 1980). However, the use of a higher resolution electrophoretic procedure and the co-electrophoresis double-label technique in the present study lead us to infer that the previous conclusion was incorrect.

The lack of hair cortical keratin synthesis and atypical synthesis of glycosaminoglycans (Frater and Hewish 1981) by cultures of this type eliminates the possibility of their use as a model system for hair growth. Further work must, therefore, concentrate on modifications to the system which encourage the survival and differentiation of cortical keratinocytes.

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