Studies of Developing Human Hair Shaft Cells In Vitro

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Methods for studying aspects of hair formation in vitro have been devised on the basis of isolating developing hair shaft cells. These cells were obtained using a sterile microdissection technique. Plucked anagen follicles were dissected free of surrounding tissues (inner and outer root sheaths), and presumptive hair shaft cells (including germinal epithelia) were cultured directly on mammalian fibroblasts or in media preconditioned by fibroblasts. Specimens were cultured either as dispersions or in whole tissue pieces. Tryptsinized whole tissue specimens in culture were sometimes observed to form increased bulk, while dispersed cells appeared to elongate and form larger colonies. In sections of these colonies examined by transmission electron microscopy, intracellular hard keratin intermediate filaments (IFs) together with IF-matrix hard keratin complexes were observed. Radiolabelled cysteine [35S] was added to cultures (3–20 days), showing a continuing but reduced synthesis of hard keratin IF proteins (low-sulfur) over the period of study. Matrix protein (high-sulfur) production was drastically reduced after 3 days. Monoclonal antibodies directed against hair keratin IF components were used in Western transfers and immunofluorescent studies to help assess the specificity of proteins synthesized in culture. Our observations indicate that, with some refinement, the presently described methods enable preparation of hair shaft precursor cells suitable for observing certain hair-forming processes in vitro. J Invest Dermatol 90:58–64, 1988

Advances in understanding mechanisms of most body functions normally arise from the use of in vitro techniques, but as yet no suitable model system has been described for monitoring mammalian hair shaft growth. While detailed chemical and structural studies have advanced our knowledge of keratin fiber/follicle characteristics, methods for studying mechanisms of hair growth (kinetics of formation, growth requirements, and differentiating factors) are still required.

Culturing presumptive hair shaft (PHS) or other follicle cells has been considered appropriate to solving such problems. In early studies, Frater [1] cultured hair follicle cells (enzyme-prepared mixtures) from mouse skin and found that proportions of cells differentiated morphologically to resemble mature cuticle and cortical cells. After using similar cell preparative methods, Hewish and Marshall [2] observed formed aggregates containing both epithelial and mesenchymal cells after a 2-day culture period. In harvested cell hydrolysates, citrulline was detected, but the only identifiable protein was actin. Wool follicle cell suspensions (trypsinized) prepared by the araldite method were also observed to contain a heterogeneous population of cells after culture [3]. These cells were metabolically active and incorporated labeled leucine, uridine, and thymidine.

In other experiments, alternative approaches used plucked anagen follicles in culture [4,5]. Weterings et al [5–7] showed that culture of human follicle cells could be achieved on bovine eye lens capsules. The cells demonstrating prolific mitotic activity were derived from the outer root sheath, and specific cellular and/or biochemical activity of developing hair shaft cells was not achieved. Hair germs (follicles) isolated (from newborn mice) and cultured by Martinet et al [8] were used to study cell morphology and biochemical activity. Observed hair-like differentiation by some cells was inconsistent with the electrophoretic patterns of extracts. These did not indicate hard keratin protein synthesis.

Cell-free systems have been developed as viable alternatives for studying hair keratin protein synthesis in vitro [9–15]. In early developments [9–11], cell-free systems were found to have low activities of amino acid incorporation into hard keratin proteins. Wilkinson [12] obtained good yields of polysomes from wool roots, and found that wool follicle mRNA was relatively stable.

Subsequent studies led to improved activity for incorporating amino acids in proteins using very young guinea pigs [13–15]. Steinhert and Rogers [14,15] demonstrated both de novo synthesis of low- and high-sulfur keratin proteins and that the mechanism of protein synthesis was ribosomal-dependent. They found evidence indicating that initiation and translation rates of low- and high-sulfur protein synthesis were different.

An underlying problem associated with most of the methods used in preparing cells for culture often involved the formation of complex mixtures of cell types. Hair follicles possess concentric layers of specialized cells arising from germinal epithelia arranged around a dermal papilla located at the base of the follicle [16,17]. The dermal

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Abbreviations:
DMEM: Dulbecco’s modified Eagle’s medium
PBS: fetal bovine serum
HABM: high-antibiotic medium
HSP: high-sulfur proteins
IAA: iodoacetatic acid
IFs: intermediate filaments
IRS: inner root sheath
LSP: low-sulfur proteins
ORS: outer root sheath
PBS: phosphate-buffered saline
PHS: presumptive hair shaft
SCM: S-carboxymethyl
MATERIALS AND METHODS

Isolation of Germinal and PHS Cells

The methods used for preparing precursor hair shaft cells were based on a previously described microdissection protocol developed for isolating hard keratin structural components [20-22]. Follicles were plucked from human volunteers and immediately immersed in a high-antibiotic tissue culture medium (HABM) containing gentamycin (50 μg/ml) (Schering Corp.), polymyxin B sulfate (12.5 μg/ml; Wellcome Australia, Ltd.), 8% vol/vol fetal bovine serum (FBS) (Flow Laboratories, McLean, VA), Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories). Before microdissection, follicles in the anagen phase were carefully screened for damage or presence of contaminating cells resulting from the plucking procedure (Fig 1). Dermal papilla cells were rarely observed in the plucked follicles. Only those follicles possessing intact germinal cells were used for subsequent dissection. About 40 follicles were collected in this manner, but these were usually prepared in groups of 5. Follicles were dissected aseptically at 25°C with a polarizing filter fitted to a binocular microscope as an aid for complete removal of IRS layers from PHS cells (Fig 1). The lower bulb regions containing germinal epithelial cells (tissue pieces) were dissected from their respective PHSs and transferred to a sterile (1 ml) centrifuge tube (Eppendorf) containing HABM at 0°C. After gentle pelleting they were washed 3 times with a sterile solution (washing solution) containing glucose (0.1 g), NaCl (0.88 g) and KCl (0.04 g; reagent weights are per 100 ml distilled H2O). After carefully removing the washing solution the tissue pieces were incubated in a 0.25% wt/vol trypsin (Flow Laboratories) solution containing 0.02% wt/vol EDTA (disodium) in phosphate-buffered saline (PBS) for 1-2 h, or for 15-20 h at 4°C. Some preparations had no trypsin treatment.

Culture Conditions

Trypsinized PHS tissues were placed in 2-ml wells (Linbro) containing previously irradiated ([60Co], 3,000-4,000 rad) [23] mouse dermal 3T3 cells or mitomycin C (Sigma Chemical Co., St. Louis, MO)-treated human dermal fibroblasts [24]. The tissue pieces were cultured in 20 mM Hepes-buffered DMEM containing 10% vol/vol FBS, sodium benzyl penicillin B.P. (60 μg/ml; Glaxo), streptomycin sulfate B.P. (100 μg/ml; Glaxo), D,L-isopropenol HCl (0.001 μM; Sigma Chemical Co.) and hydrocortisone (1.5 μg/ml; Sigma Chemical Co.). Alternatively, trypsinized tissue pieces were placed in 2-ml wells that had been precoated with 0.1% wt/vol swine skin gelatin type 1 (Sigma Chemical Co.). These PHS tissues were cultured in fresh medium containing an equal volume of conditioned medium that was obtained from 3-4-day-old human fibroblast cultures and filtered (0.2 μm) before use.

In plating tissue pieces (no trypsin or 1-2 h trypsinization) considerable care was required, since these pieces were barely detectable to the unaided eye. Usually 5 pieces were added to each well. When PHS tissue was dispersed into cells and cellular clumps (15-20 h trypsinization), aliquots were taken for plating.

All cultures were placed in a 37°C, 5% vol/vol CO2 humidified incubator and fed weekly.

Transport of Human Follicle Specimens

For clinical and aberrant hair studies, follicle specimens are not always readily accessible, hence in the present study transport feasibility of plucked specimens was conducted by holding tissue in HABM at room temperature or by carrying on persons for periods up to 48 h. Before labeling, extraction, and electrophoretic studies, PHS tissue was dissected from surrounding tissues as described above.

Radiolabelling and Harvesting Cultures

We assessed protein synthesis of cells in culture by incorporation of [35S] cysteine into cultures at 3, 8, 14, and 17 days. Wells containing PHS tissue pieces were rinsed with cysteine/cysteine-free medium plus dialyzed FBS (1 ml) before addition of cysteine/cysteine-free medium (0.4 ml) containing 200 μCi of l-[35S]cysteine (Amersham Corp., Amersham, United Kingdom). After incubation for 16 h at 37°C, cultures were harvested in Durham tubes and washed (3 or 4 times) with the washing solution described above. Excess liquid was carefully removed with a Pasteur pipette. The remaining fluid surrounding PHS tissue pieces was absorbed into finely pointed strips of filter paper.

Extraction of Labelled Cultures

Harvested PHS tissues were extracted with a solution (20 μl) containing 9 μM urea, 0.05 M dithiothreitol, 0.25 M Tris (pH 9.5), 0.01 M EDTA. From a 0.1 mM PMSF solution, 1 μl was added to the extracting solution. Specimens were extracted for 3 h at 20°C. For comparative (control) purposes, isolated PHS tissue pieces (6 specimens) and human epidermis were similarly extracted. All hair specimens (controls and cultures) were from the same individual. Human epidermis was obtained by surgery, and was separated from dermal tissue by placing in ice for 10 min, heating in water to 57°C for 5 min, and returning to ice for 1-2 min. Extracts were converted (alkylated) to S-carboxymethyl derivatives (SCM) as follows. Controls were alkylated with 2-[14C] iodoacetic acid (IAA) (Amersham Corp., and cultures with unlabelled

Figure 1. Examples of normal human anagen follicles, freshly plucked (right) and following microdissection to remove IRS and ORS cells (left). Inset (bottom left) shows an example of a lower section (PHS tissue piece used for culture).
IAA (BDH). Alkylated proteins (10 µl) were separated by electrophoresis (2-dimensional) [25] and identified by fluorography on x-ray film for periods at -80°C. For comparative studies these experiments were standardized by using constant numbers of PHS tissue pieces in cultures. Volumes loaded on gels and exposure periods were of similar duration except where stated.

Structural Studies Transmission electron microscopy was performed on cell colonies harvested after culture (30-day culture of 20-h trypsinized cells). With the aid of a binocular microscope (X25), colonies were harvested and washed carefully (3 times) with the above washing solution between pelleting by gentle centrifugation (2,500 rpm for 5 min). These colonies together with controls (human scalp biopsies) obtained by surgery were fixed initially overnight in 0.1 m phosphate-buffered 2.5% vol/vol glutaraldehyde containing 2 mM MgCl₂. After initial fixation, specimens were washed (3 times) for 15-min intervals in 0.1 m phosphate buffer containing 2 mM MgCl₂, followed by postfixation for 2-3 h in 0.1 m phosphate-buffered 1% wt/vol osmium tetroxide. They were then washed 3 times with distilled water, immersed in 0.5% wt/vol aqueous uranyl acetate, left overnight, and rinsed in distilled H₂O. Dehydration was through a graded series of alcohols followed by immersion in propylene oxide and epon. Pre-embedding was performed in a 50:50 mixture of propylene oxide and epon, followed by embedding in epon, initially at 37°C overnight, followed by polymerization at 60°C. Sections were poststained with 0.5% wt/vol uranyl acetate and Reynolds' lead citrate [26].

Immunological Methods Monoclonal antibodies directed against hard keratin intermediate filament proteins (45-50 kD family KF29, K2B9) [27] were used to probe the specificity of proteins formed in culture (10-day cultures). SCM proteins were initially subjected to 2-dimensional electrophoresis as above, then transferred electrophoretically to nitrocellulose membranes using the Western transfer method of Towbin et al [28] before probing with monoclonal antibodies. Antibodies were in ascites fluid, and were diluted 1:400 with PBS containing 1% wt/vol gelatin before use. A rabbit anti–mouse Ig antiserum conjugated to horseradish peroxidase (Biorad Laboratories) was diluted 1:3,000 with the PBS/gelatin solution. The substrate 4-chloro-1-naphthol and H₂O₂ in Tris-buffered saline (pH 7.4) indicated positive antibody binding by producing a purple coloration. Ascites fluid (antibody free) and normal mouse sera were included as controls.

Immunofluorescence microscopy was used to detect the presence of hard keratin proteins in cultured cells. Cultures were initially established on microscope (gelatin coated) coverslips (Thermanox) (PHS tissue pieces, 1-2 h trypsinization, grown on human fibroblasts treated with mitomycin C) and harvested at day 23. These were fixed (30 min at room temperature) and stored in 50% vol/vol ethanol at -20°C. Specimens were prepared for immunofluorescence using monoclonal antibodies (anti-45-50 kD family (KF28, αKI) according to the method described by French and Hewish [27]. Supernatant without antibody was used in controls.

RESULTS

Fig 1 shows a freshly plucked human anagen hair follicle (right) with surrounding outer root sheath (ORS) and IRS tissue present. An example of PHS after dissection (left) and an isolated lower bulb region (inset) is also demonstrated.

Cells obtained after trypsin treatment (15-20 h, 4°C) of PHS tissue (lower bulb) were observed in culture at days 1, 5, 15, and 20. Phase-contrast photomicrographs (Fig 2) show a group of germinal cells (arrow in Fig 2A) on a feeder-cell background (mouse 3T3s) at the beginning of culture. These are similar in appearance to the rounded cortical cell precursors normally observed in sections of PHS tissue [25]. By day 5 (Fig 2B) certain changes were noted in some specimens. On the basis of morphological observations, clusters of original cells had increased in size, apparently forming colonies. By day 15 a proportion of cells within these colonies appeared to have elongated shapes (Fig 2C). At day 20 (Fig 2D) the colony shown had apparently increased cellular bulk; correspondingly the feeder cells were showing increased degeneration. Fig 2A–D are examples of observations made on the days cited, and are not in all cases the same specimen.

PHS tissue pieces that had been previously trypsinized (1-2 h, 4°C) and cultured on dermal feeders (mouse and human) showed increased specimen bulk appearing as outgrowths from the germinal region (Fig 3). Typically dissected tissue pieces are shown at the beginning of culture (Fig 3A), and examples of observed changes after 21 days are demonstrated in Fig 3B. In Fig 3B, right the PHS was of full follicular length, whereas the left tissue corresponded to the lower bulb region (Fig 1, inset).

Protein Synthesis in Cultures Labelling extracts (SCM) of PHS tissue after 3, 8, 14, and 17 days of culture (l-[³⁵S]cysteine) (Fig 4) and a control PHS extract (2-[¹⁴C]jodoacetic acid) (Fig 5) are shown as fluorographs after electrophoresis. Extracts of human hair contain two major classes of proteins [29], and these are termed low-sulfur proteins (LSP) and high-sulfur proteins (HSP), resulting from wide variations in cystine (determined as S-carboxymethylcysteine) contents. The PHS extract (control) contains both of these classes of hard keratin proteins, as indicated in Fig 5 (2-dimensional electrophoretic pattern). LSPs appear as individually resolved spots, and possess molecular weights in the range 40,000-60,000, while the HSPs (mostly unresolved) lie on the diagonal in the lower right quadrant and have a wider range of molecular weights.

Culturing PHS tissue in conditioned media (alternative to feeders) avoided problems encountered in separating PHS cells from
Figure 3. Human PHS tissue pieces were isolated by microdissection, treated with a trypsin solution (1–2 h at 4° C) and cultured on human fibroblasts (mitomycin C–treated). An example showing tissue pieces at the beginning of culture is shown in A and after a culture period of 21 days (B). Increased specimen bulk is apparent in the germinal region of B. Phase contrast X 120.

feeder cells. Earlier experiments using feeder cells resulted in significant contamination from labeled proteins of fibroblastic origin. In the fluorographs (Fig. 4A–D), synthesis of both LSP and HSP was apparent. These protein patterns were derived from extracts of PHS tissue cultured in preconditioned media. The proportions of HSP and LSP at day 3 were similar to the control PHS extract (Fig 5). However, at days 8–17, this proportion had changed markedly, after a reduction in HSP synthesis. On days 8 and 14, only trace amounts of HSP (relative to LSP) were detected. By day 17, HSP were virtually absent on these exposures.

Synthesis of LSP was clearly continuous throughout the period of study. Fig 6 details the regions containing IF proteins obtained from a human epidermal extract (Fig 6F), a PHS control extract (Fig 6A), and compares these with similar regions obtained from PHS extracts labeled at days 3 (B), 8 (C), 14 (D), and 20 (E). The intermediate filament (IF) proteins synthesized by PHS cells in culture have yielded the typical hard keratin pattern, and these differed significantly from those obtained with human epidermal extracts in the present electrophoretic system. Minor labeled components of lower molecular weight were observed in the cultured PHS extracts. These were absent in the control PHS extract, and their composition was unknown. Exposure periods on fluorographs were similar for extracts of labeled PHS cultures at days 8, 14, and 20, but were shorter for extracts obtained at day 3. Thus it appears that LSP synthesis decreases after day 3 but plateaus from days 8–20.

Plucked human follicles were also used for assessing feasibility of transporting specimens or holding specimens before culture. In extracts obtained from PHS after holding specimens for up to 48 h at varying temperatures, subsequent fluorographs indicated that both HSP and LSP were synthesized in similar proportions compared with control PHS extracts. The results (not shown) indicated that individual IF components were not as well resolved as those obtained in controlled laboratory experiments.

Ultrastructure of Cultured Cells Transmission electron micrographs (Fig 7) were used to compare sections of cultured PHS cell colonies (15–20 h trypsin at 4° C, 30-day cultures, see Fig 1) with sections obtained from different levels of embedded human follicles (controls). In longitudinal sections of follicle controls, hard keratin IF arrays were deposited intracellularly at the lower levels of developing cortical cells. By about the midregion of the follicle these IF arrays have been infiltrated by a densely stained substance (matrix). The resulting IF/matrix complexes were similar to structures observed in fully developed hair fiber sections (macrofibrils) [19]. Typical macrofibrils are shown in the control follicle section (Fig 7C). In cultured PHS colony sections, both IF arrays (Fig 7A) and macrofibrils (Fig 7B) were observed. The presence of both entities in these cells is indicative of a continuing synthesis and assembly of hard keratin proteins.
Immunoelectrophoretic and Immunofluorescence Studies

Evidence for specific synthesis of hard keratin proteins by cultured PHS specimens was found when monoclonal antibodies were used in immunoelectrophoretic studies. In Fig 8 anti-IF monoclonal antibodies (KF 28) showed positive interaction with extracts of hair (Fig 8A) and cultured PHS tissue (Fig 8B), but were negative when probing extracts of epidermis (human callous) (Fig 8C) or human feeder cells (Fig 8D). The family of IF proteins to which the monoclonal antibodies were directed are shown (arrow) in the control extract (2-dimensional gel) of Fig 5.

Hard keratin protein synthesis was assessed in dividing cells using fluorescence microscopy incorporating anti-IF protein monoclonal antibodies (KF 28). When phase-contrast and UV images were compared (Fig 9), fluorescence was observed in PHS tissue pieces, and often in outgrowths from the bases of these pieces (as observed previously in Fig 3B). Background fluorescence of feeder cells was virtually absent. The fluorescence observed in outgrowths was usually less frequent than that found in the original tissue pieces.

DISCUSSION

An important prerequisite to developing model systems for studying hair growth in vitro requires careful consideration of the starting tissue or cells. Certain salient features of mammalian hair follicles such as histologic complexity and relatively small sizes are obvious limitations to cell culture studies [1–8]. Indeed many previous attempts to formulate in vitro cell-free systems [9–15], cell, or tissue culture techniques intended to specifically study hair shaft development [1–3,8] have encountered these problems. When initial cultures contained probable and unknown mixtures of IRS, ORS, epidermal, and dermal cells (in addition to PHS cells), different growth rates often caused confusion and inhibited development of optimal growth conditions.
Incorporation of radiolabeled cysteine into cultures was used for demonstrating synthesis of human hair keratin proteins. The classes of keratin proteins normally observed in extracts of human hair include LSP and HSP [29]. LSP compose the IF moiety [20–22], while HSP are located in the matrix [19]. In PHS tissue pieces synthesis of LSP and HSP was observed, indicating both maintenance of cellular function (up to 20 days) and the purity of cells in culture. It was also possible to hold specimens in transit for up to 2 days before culture. Current results suggested that synthesis of HSP was markedly reduced after 3 days in culture. The LSP were diminished in production after 3 days, but relative to synthesis of HSP, continued to be observed on fluorographs in significant quantities throughout the experimental period. PHS cultured extracts obtained at days 3, 8, 14, and 20 showed protein patterns similar to hard keratin control extracts (PHS tissue), and these were different from patterns obtained with human epidermal extracts. The minor labeled components observed in fluorographs obtained from PHS extracts after culture were of apparent lower molecular weight than keratin IF components, but their identity was not established.

Sections of colonies containing PHS cells (30-day cultures) examined by transmission electron microscopy were shown to contain intracellular IF arrays and fibrillar structures (macrofibrils) similar to those observed at various levels of developing cortical cells in situ. At the beginning of human hair formation, presumptive cortical cells contain hard keratin IF arrays [16,17], which later in the process become mixed with a densely stained substance (HSP) to form macrofibrils [19]. The presence of these typically hair-like structures in cultured PHS cell colonies indicates that hard keratin macromolecular complexes form in vitro. This conclusion was further supported by the absence of keratohyalin granules normally found in epidermal cultures [23].

The immunoelectrophoretic studies showed that anti-hard keratin IF protein monoclonal antibodies reacted with LSP obtained in cultured PHS cell extracts. These proteins (labeled with [35S]cysteine) produced by PHS cells in culture, indicated a specific synthesis of hard α-keratin proteins. Although the antibodies presently used showed a broad specificity for hard keratin proteins [27], none were found to react with epidermis in sections used for immunofluorescent studies. In determining whether keratin proteins were synthesized by original PHS cells (those in initial specimens) and/or by new cells formed in culture, fixed tissue (20-day cultures) was used in immunofluorescent studies. Cells from the original tissue were found to contain hard keratin IF proteins. In outgrowths associated with the original tissue, fluorescence was variable in intensity and distribution, indicating marginal evidence for keratin synthesis in these regions.

The culture systems described present a new approach to avoiding the problems previously encountered in attempts to develop model systems for hair growth. Demonstration of specific synthesis and assembly of hard keratin proteins indicates their success in maintaining cell function. In this regard they have the capacity for monitoring certain cell processes, including the kinetics and sequence of keratin formation in vitro. Additionally, the capacity to transport hair follicle specimens should be of value in these and other follicle studies. With further refinement, this new approach to culturing PHS cells also shows potential for use in understanding the growth and differentiation characteristics of hard keratinizing epithelia, which is ultimately aimed at elucidating the mechanisms controlling hair growth.

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REFERENCES

19. Fraser RDB, MacRae TP, Rogers GE: Keratins: Their composition, structure and biosynthesis, edited by Kugelmas Springfield, IL, Charles C Thomas 1972