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Hair shaft elongation, follicle growth, and spontaneous regression in long-term, gelatin sponge-supported histoculture of human scalp skin

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ABSTRACT **In order to better understand the molecular mechanisms of human hair growth control and to test hair growth-modulatory drugs, appropriate *in vitro* models are required. Here, we report the long-term growth, shaft elongation, and spontaneous regression of human hair follicles in histoculture of intact scalp skin. Human scalp skin with abundant hair follicles in various stages of the hair growth cycle was grown for up to 40 days in a gelatin sponge-supported histoculture system at the air/liquid interface. Isolated follicles placed in the gelatin-sponge matrix also supported hair shaft elongation, with the hair follicle cells remaining proliferative and viable for very long periods. Hair shaft elongation occurred mainly during the first 10 days of histoculture of both intact skin and isolated follicles. However, hair follicles were viable and follicle keratinocytes continued to incorporate [^{3}H]thymidine for up to several weeks after shaft elongation had ceased as shown by fluorescent-dye double staining, measured by confocal laser scanning microscopy, and by histological autoradiography of [^{3}H]thymidine incorporation, respectively. Hair follicles could continue their cycle in histoculture; for example, apparent spontaneous catagen induction was observed both histologically and by the actual regression of the hair follicle. In addition, vellus follicles were shown to be viable at day 40 after initiation of culture. In the histocultured human scalp we demonstrated the association of mast cells with anagen follicles and macrophages with catagen follicles, suggesting a role of these cells in the hair cycle. This histoculture technique should serve as a powerful tool for future hair research in the human system as well as a screening assay for compounds that can perturb the hair cycle.**

The clinical, social, and psychological significance of disorders of hair growth and the associated economic aspects are strong incentives to elucidate the control apparatus for follicle growth and cycling. The formation, shedding, and regrowth of pigmented hair shafts result from tightly controlled, developmentally regulated epithelial–mesenchymal–neuroectodermal interactions in a unique miniorgan: for the entire life-span of the mammalian organism, it switches rhythmically from stages of resting (telogen) to stages of growth (anagen) and, via a short regression phase (catagen), back to resting (1). The hair follicle, therefore, can also serve as a fascinating model for studying tissue interactions and tissue remodeling under physiological conditions. It is a still-underappreciated model system for experimental analysis of a plethora of key questions relevant to developmental biology, endocrinology, chronobiology, pigment cell biology, and molecular genetics (1–7).

Despite several decades of hair research, most of the basic mechanisms controlling hair growth and cycling have remained elusive. Since several rodent models are available for studying hair follicle growth *in vivo* and *in vitro* (e.g., refs. 1, 3–13), most progress in hair biology has been made by using rodent systems (1, 3, 4, 6, 8). However, there is a paucity of relevant *in vitro* models for the study of human hair growth. Apart from the obvious relevance of human *in vitro* models for elucidating human hair follicle biology, their development is particularly desirable with respect to the urgent need for safe, reliable, and physiologically relevant screening assays for studying the manipulation of hair growth by candidate drugs for the treatment of hair diseases.

Here, we report the long-term histoculture of human hair follicles growing in intact scalp skin at the air/liquid interface. This technique is based on our previously described system for the histoculture of mouse skin and mouse hair follicles (14, 15), which employs collagen-containing gelatin sponges for tissue culture (16–18). For comparison, some isolated human scalp hair follicles were also histocultured on gelatin sponges. Since long-term viability, follicle cell proliferation, hair shaft elongation, and follicle regression can be studied in gelatin sponge-supported histoculture, it has significant potential as an experimental tool for hair research in the human system.

MATERIALS AND METHODS

Preparation of Human Scalp Skin Tissue and Isolation of Human Follicles. Human scalp skin tissue taken from autopsy or facelift surgery was prewashed in Earle's minimum essential medium with penicillin G, streptomycin, amphotericin B, tetracycline, amikacin, chloramphenicol, and gentamicin for 30 min. The epithelial surface of the scalp skin was then cleaned with 70% ethanol after the outgrowing hair shafts were shaved. Intact scalp skin (2×2 cm^2) was cut by a 2-mm Acu-punch or surgical blade along the direction of hair growth. The additional subcutaneous fat was removed carefully with a scalpel without injury to the hair follicle bulbs.

Intact individual human anagen hair follicles were isolated with a scalpel. The isolated follicles were dissected at the infrainfundibular level without epidermis or sebaceous gland tissue, but with a narrow band of perifollicular connective tissue.

Histoculture of Human Scalp Tissue and Isolated Human Follicles. We have developed a method (14–17) based on the work of Leighton (18) to histoculture various tumor and normal tissues on gelatin sponges. Small pieces of intact human scalp tissue (described above) were explanted with the epidermis up at the air/liquid interface and dermis-down on $1 \times 1 \times 1$-cm pieces of collagen-containing gelatin sponge (Gelfoam, Upjohn) that had been prehydrated for at least 4 hr with culture medium (Eagle's minimum essential medium

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Abbreviation: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5 (and 6)-carboxyfluorescein acetoxymethyl ester.

plus 10% fetal bovine serum and gentamicin at 50 μg/ml). The cultures were maintained at 37°C in a gassed incubator with 95% air/5% CO_2.

Confocal Microscopy. The confocal microscopy system consisted of an MRC-600 confocal imaging system (Bio-Rad) mounted on a Nikon Optiphot using a 10× PlanApo objective.

Fluorescence Microscopy. For standard microscopy, a Nikon fluorescence microscope, equipped with fluorescein and rhodamine cubes, was used.

Fluorescent-Dye Labeling of Live and Dead Cells. Viable cells were selectively labeled with the dye 2',7'-bis(2-carboxyethyl)-5 (and 6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), which is activated to fluorescence by nonspecific esterases present only in living cells (14). Nonviable cells, whose plasma membranes are leaky, were labeled with propidium iodide, a dye that enters only cells with nonintact membranes (14). Since the emission spectra of these two dyes were different they could be used simultaneously on the same specimen. Both dyes were used at a concentration of 15 μM. The double-dye-treated cultures were analyzed by fluorescence and confocal microscopy within 30 min of staining.

[^{3}H]Thymidine Labeling of Proliferating Cells. The cultures were incubated with [^{3}H]thymidine (4 μCi/ml; 1 μCi = 37 kBq) for 3 days, washed with phosphate-buffered saline, fixed with buffered 10% formalin, and processed for autoradiography as described (14, 15). After exposure to the photographic emulsion and fixation, slides were stained with hematoxylin/eosin and analyzed under epi-illumination polarization so that replicating cells could be identified by the presence of silver grains over their nuclei, visualized as bright green in the epipolarization system (for details, see refs. 14 and 15).

Histology and Hair Growth Measurement. Tissue fixed in buffered 10% formalin was processed for routine histology and stained with hematoxylin/eosin or Giemsa reagent according to standard procedures. The length of hair shafts was measured by a ruler from the dissection-microscope photomicrographs.

Immunohistochemical Staining of Macrophages. We used a Dako LSAB (labeled streptavidin–biotin) kit for immunohistochemical staining of macrophages in histocultured human scalp tissue. The primary antibody was mouse anti-human macrophage monoclonal antibody DAKO-CD68 (DAKO, Carpinteria, CA). First, the deparaffinized tissue sections were bathed in 0.05 M Tris buffer (pH 7.6) for 5 min. Serial incubations were then performed with hydrogen peroxide, blocking serum, primary antibody (1:100), linking antibody, peroxidase-conjugated streptavidin, and 3-amino-9-ethylcarbazole substrate solution for 5–10 min each. The sections were then lightly counterstained with Mayer's hematoxylin and mounted with liquid glycerol gelatin (DAKO). Negative control was observed by replacing the primary antibody with phosphate-buffered saline.

RESULTS AND DISCUSSION

When intact human scalp skin was cultured on gelatin sponge supports at the air/liquid interface, considerable elongation of pigmented and unpigmented hair shafts was observed over time (Fig. 1); the hair shafts grew an average of 0.86 ± 0.18 mm over 5 days and 1.10 ± 0.22 mm over 10 days. This correlated well with extensive proliferation of hair matrix keratinocytes during the first week of histoculture as seen by the cells covered by green grains in the autoradiographs (Fig. 2). The silver grains, which are formed in the photoemulsion over nuclei that have taken up [^{3}H]thymidine, reflect polarized light, which appears green. Follicle viability was assessed by confocal laser scanning microscopy after vital-dye double staining with BCECF-AM and propidium iodide. The vast majority of the follicle cells appeared green, indicating that the cells had cleaved BCECF-AM into the fluorescent form, thereby demonstrating their viability (Fig. 3). The follicle morphology was remarkably well preserved in histoculture, with the outer root sheath, matrix keratinocytes,

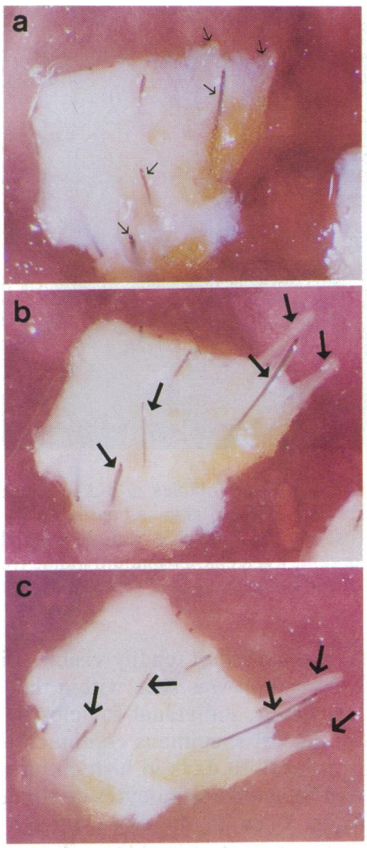

FIG. 1. Hair shaft elongation in gelatin-sponge histoculture of human scalp skin at day 0 (*a*) and after 5 days (*b*) and 10 days (*c*) of histoculture. Arrows point to elongating hair follicles. Note the increasing length of both dark and white hair. (Dissection microscopy, ×6.)

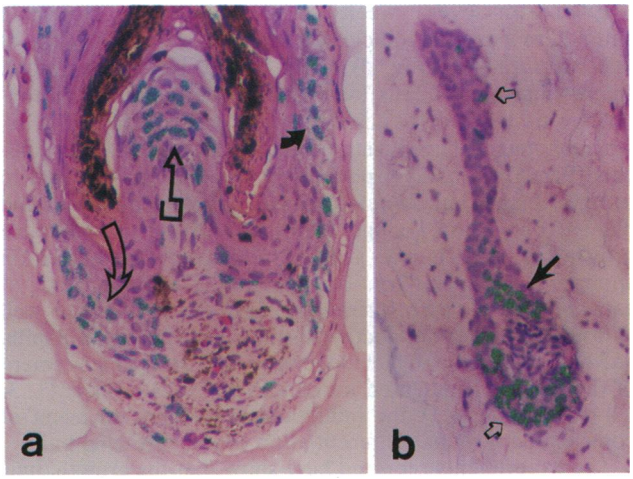

FIG. 2. Autoradiograph of [^{3}H]thymidine incorporation into human scalp hair follicles after 6 days (anagen follicle) (*a*) or 40 days (vellus hair follicle) (*b*) of histoculture. Visualized by epipolarization, silver grains over radiolabeled nuclei reflect the polarized light as green. Note the remarkably preserved morphology of the hair follicles and the high proliferation frequency of the hair matrix cells and the outer-root-sheath follicle keratinocytes. (*a*, ×700; *b*, ×350.)

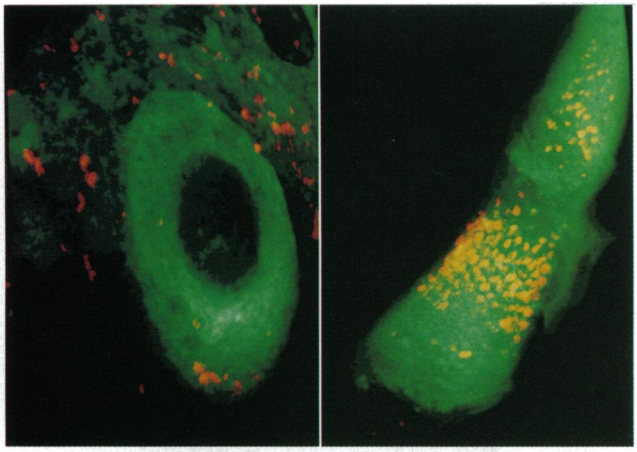

FIG. 3. Viability of hair follicles in 8-day gelatin-sponge histoculture of intact human scalp skin, as assessed by confocal laser scanning microscopy of vital-dye double-stained histocultures [BCECF-AM (green), live cells; propidium iodide (red), dead cells]. Vast majority of follicle cells appear green and are therefore alive. (×375.)

dermal papilla, and hair shaft readily visible (Fig. 2). Primarily, hair shaft elongation was seen within the first 5 days of histoculture. However, individual follicles in histocultured scalp skin from facelift specimens continued to form longer hair shafts for at least 10 days in histoculture. Yet follicles were viable, and follicle keratinocytes continued to proliferate much longer, even at 40 days, though proliferation occurred predominantly in cells of the outer root sheath (Fig. 2).

While 14–25% of follicles grown in intact skin showed hair shaft elongation within the first week of histoculture, 73% of isolated follicles explanted on the gelatin sponges exhibited hair shaft lengthening. These follicles had been dissected at the infrainfundibular level; no epidermis or sebaceous gland tissue was present, only a narrow band of perifollicular connective tissue (see Fig. 6). However, the growth rate of the follicles in the intact skin histocultures was an average of 0.86 ± 0.18 mm for 5 days, while the isolated follicles grew an average of 0.49 ± 0.06 mm for 5 days, suggesting the importance of extrafollicular cell types in hair growth.

Even after several weeks of histoculture of intact scalp skin, there was little follicle degeneration. Mast cells (Fig. 4) and macrophages (Fig. 5) could readily be demonstrated. Interestingly, mast cells localized preferentially to the connective tissue directly adjacent to anagen follicles (Fig. 4), whereas macrophages were prominent around catagen follicles (Fig. 5). Notably, spontaneous regression appeared to occur in anagen hair follicles that had either been isolated and planted on gelatin sponges (Figs. 6 and 7*b*) or that were growing in intact human scalp skin histocultured on gelatin sponges (Figs. 5 and 7*a*). The generally accepted criteria of catagen—follicle bulb shrinkage, increasing distance as well as formation of a connective tissue strand between papilla and bulb, cessation of melanogenesis, and upward movement of the zone of pigmented keratinocytes in the follicle (1, 3, 19)—clearly suggest the induction of catagen, an event of coordinated terminal differentiation of the epithelial bulb.

The experiments reported here show that the growth, hair shaft elongation and pigmentation, and regression of human hair follicles growing in intact skin can be followed over an extended period of time with histocultured scalp skin on gelatin sponges at the air/liquid interface (Fig. 1). This system makes it possible to study the biology of mechanically or enzymatically undisrupted human hair follicles in their natural tissue environment under *in vivo*-like conditions. On the other hand, isolated follicles can be cultured for several weeks on gelatin sponges.

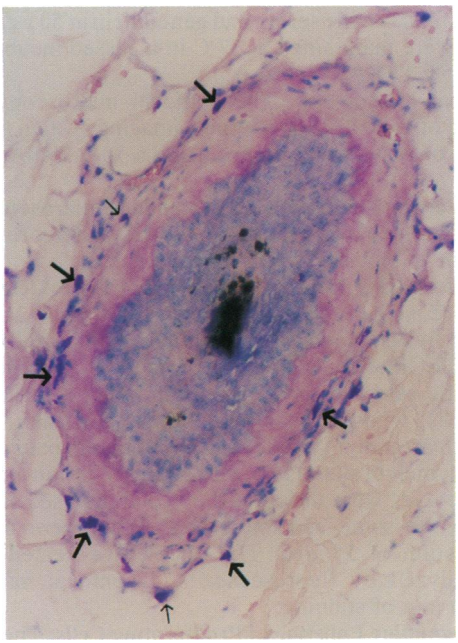

FIG. 4. Giemsa staining of human scalp skin histocultured on gelatin sponge for 10 days, showing perifollicular mast cells (horizontal section through follicle). Note the close association of the mast cells and the anagen follicles. (×650.)

Previously reported explant methods for culturing human follicle cells (e.g., refs. 20–22) have permitted the study of isolated outer-root-sheath keratinocytes *in vitro*. Since these populations of follicular cells are grown in isolation from the complex epithelial–mesenchymal–neuroectodermal interactions that govern hair growth, pigmentation, and cycling (1–7), this explant technique has evident limitations as a tool for addressing the key question of hair growth regulation. Also, outer-root-sheath keratinocytes are probably not the cells most relevant to hair shaft formation and shedding as well as to follicle regeneration and regression (1, 4).

Philpott *et al.* (23), in contrast, have reported the culture of intact human follicles and their response to epidermal growth

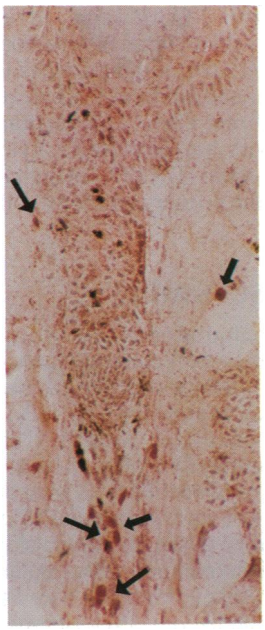

FIG. 5. Immunohistochemical detection of perifollicular macrophages in intact human scalp skin histocultured 10 days on gelatin sponge. Note the close association of the macrophages with catagen follicles (arrows). (Standard microscopy, ×335.)

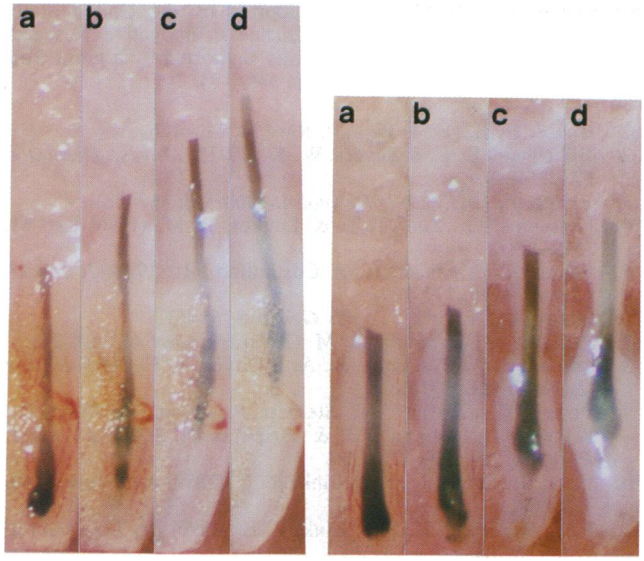

FIG. 6. Two representative examples of spontaneous regression of isolated human scalp hair follicles in gelatin-sponge histoculture, suggestive of the onset of catagen. (*a*) Zero time. (*b*) Five days. (*c*) Ten days. (*d*) Fourteen days. Note the formation of club-shaped hair bulb and the movement of the hair follicle out of the follicle as model of hair loss. (Dissection microscopy, ×15.)

factor and transforming growth factor β *in vitro*. Though this important study showed the feasibility of human follicle growth *in vitro*, the observations obtained with this culture system may not fully reflect follicular growth characteristics *in vivo*, since individual, mechanically isolated follicles were maintained free-floating in culture medium and were grown in the absence of epidermis, dermis, subcutis, sebaceous glands, and other resident skin cells. Indications are accumulating that the epidermis (11, 12), mast cells (24–26), macrophages (27, 28), or other local immunological factors (2, 10, 29) and the local production of peptide hormones by sebaceous glands (30) might contribute to the regulation of hair growth. Our results indicate that the collagen-containing gelatin sponge matrix is also important in the long-term growth and viability of follicle cells.

The gelatin-supported histoculture system reported here, where follicles grow in intact, full-thickness skin, resembles the physiological situation more closely than any other previously reported technique. This is illustrated by the finding that, even after extended culture periods in histoculture, scalp skin still contains, for example, mast cells (Fig. 4) and macrophages (Fig. 5). In the light of recent findings on the potential significance of mast cell degranulation during anagen development in mice (25, 26), the prominence of mast cells in the connective tissue directly adjacent to the follicle (Fig. 4) further suggests that mast cells play a role in hair growth regulation (see also refs. 24–26, 29).

Considerable elongation of the hair shaft can be observed in histoculture of scalp skin follicles (Fig. 1). While follicle keratinocytes continue to proliferate long after shaft elongation ceases (Fig. 2), it is mainly outer root sheath keratinocytes that proliferate without apparently contributing to hair shaft formation. As mentioned above, hair shaft elongation of isolated human follicles cultured in serum-free William's E medium has been reported by Philpott *et al.* (23), who suggested that this represented active follicle growth and formation of new, keratinized hair shaft. We agree with this view (Fig. 2*a*, for example, shows an autoradiograph of proliferating hair matrix keratinocytes, a cell population that subsequently differentiates into shaft-forming corneocytes). It remains to be excluded convincingly that the elongation of hair shafts *in vitro* is to a significant extent due to the upward movement of a preformed shaft or of epithelial bulb keratinocytes already differentiated prior to the beginning of histoculture. Fig. 6 illustrates that hair shaft elongation can in part be due to upward movement of a preformed hair shaft. Given the profound migratory potential of follicle keratinocytes, this problem requires further analysis, if one wants to use these *in vitro* assays for studying "hair growth" in the sense of *de novo* hair shaft formation.

In the gelatin sponge-supported histoculture system, follicles dissected out of their surrounding skin showed a significantly higher percentage of hair shaft elongation than those grown in their natural skin environment, although the follicles in the intact histocultured skin grew to a greater average length. Though this observation may simply reflect better nutritional conditions for follicles histocultured in isolation, it underscores the significance of studying follicles under conditions as similar to the *in vivo* conditions as possible. It is in line with the previously reported hair growth-inhibitory potential of endogenous antimitotic molecules contained in mouse epidermis (11, 12) and underscores the possibility that epidermal or other perifollicular signals contribute to the regulation of hair growth (11, 12, 29, 30). This histoculture assay can now be used to dissect the relative contribution of

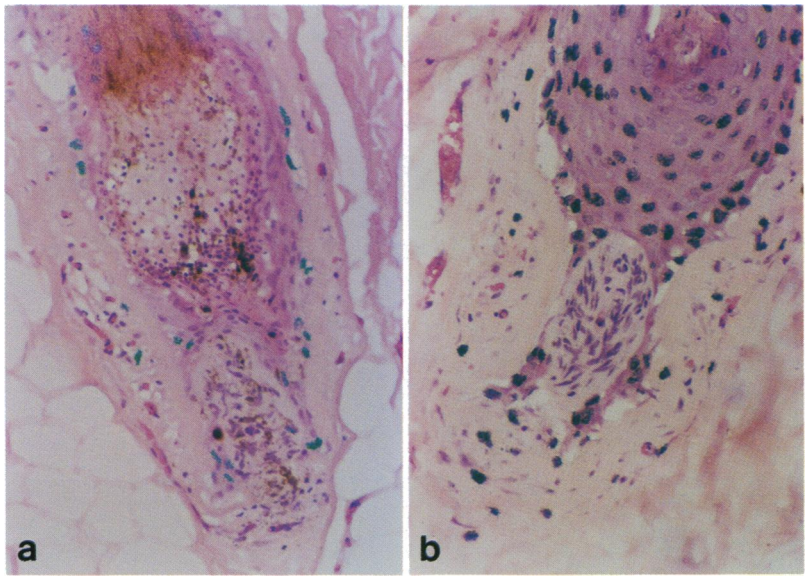

FIG. 7. Autoradiograph of [^{3}H]thymidine incorporation into regressing human scalp hair follicles in gelatin-sponge histoculture. (*a*) Regressing follicle in intact scalp skin histocultured for 6 days. (*b*) Isolated follicle after 5 days of histoculture. Note the increased distance between the dermal papilla and the follicle bulb, and the catagen-characteristic formation of a tissue strand between the retreating, terminally differentiating cells of the bulb and the dermal papilla. (×600.)

epidermis, sebaceous glands, and dermis to the regulation of hair growth.

Due to its extremely high proliferative and metabolic activity and its dependence on a complex balance of nutrients, hormones, and growth factors, the human anagen follicle is exquisitely sensitive to exposure to many drugs—reversible hair loss is among the most common unwanted side effects of a large number of drugs (19). The histoculture of human hair follicles in intact skin (e.g., from autopsies) may, therefore, also serve as a valuable, sensitive, and reliable assay for drug toxicity screening in the human system that would generate more physiologically relevant data than other widely used assays.

The regressive phenomena shown in Figs. 5–7 are suggestive of the induction and development of the catagen stage of the hair cycle and are not likely to represent simple tissue degeneration, since these follicles contain many proliferating keratinocytes and develop the characteristic features of catagen (see ref. 1). The somewhat high proliferative activity for a catagen follicle (Fig. 7) might reflect a histoculture artifact such as escape of selected keratinocyte subpopulations from committment to the terminal differentiation pathway. The catagen-like regression of isolated, free-floating human follicles *in vitro* after addition of epidermal growth factor has been reported (23). Follicle regression in the gelatin-sponge histoculture system did not require the administration of any agents other than those contained in minimum essential medium and fetal bovine serum.

Understanding the clinically important mechanisms of catagen induction and development is a key to more efficient treatment of the vast majority of diseases associated with hair loss (7); shortening of the length of the anagen phase and/or premature induction of catagen characterize androgenetic alopecia (earliest stages), alopecia areata, and diffuse alopecias induced by hormonal or metabolic disorders, malnutrition, systemic disease, and stress (19). Because perifollicular cell populations such as macrophages may play a functionally important role in catagen (7, 27–29), it appears critical to study the anagen–catagen transition of follicles in intact, full-thickness skin. Fig. 5 shows an increase in the number of perifollicular macrophages around a catagen follicle. This is in line with the concept that macrophages may be instrumental to tissue remodeling during catagen (7, 27–29). Human scalp hair follicles histocultured in intact skin can now be employed to study the mechanisms of catagen induction. With the histoculture assay, catagen can be manipulated pharmacologically *in vitro* as an important first step toward the development of drugs that block or retard catagen induction as one strategy to treat alopecia in humans (see ref. 7).

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