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Three-dimensional Demonstration of Melanocyte Distribution of Human Hair Follicles: Special Reference to the Bulge Area

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Melanocytes of human hair follicles were histochemically and immunohistochemically examined in two and three dimensions. EDTA-treated extracted anagen vellus and intermediate hair follicles showed that monoclonal murine antibody (MoAb) NKI/beteb-reactive melanocytes were distributed from the infundibulum to the bulb. Melanocytes of the infundibulum and bulb were larger and more strongly stained with MoAb NKI/beteb than those of the middle portion below the sebaceous gland. Latter melanocytes showed less dendricity. Dendritic melanocytes were exclusively observed in the bulb as well as the bulge area of vellus and intermediate hair follicles, where they were variably melanized. In longitudinal and transverse sections of adult human scalp some keratinocytes of the outer root sheath of the bulge area were melanized compared to other parts. This phenomenon was independent of the hair cycle. These findings may be characteristic of bulge melanocytes and/or keratinocytes. ***Key words: NKI/beteb; melanin; dendritic melanocyte.***

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In the late 1950s, Staricco (1) found dopa-negative, amelanotic melanocytes in the outer root sheaths of normal hair follicles, which showed light cytoplasm and dark blue nuclei by dopa-toluidine blue stain. These melanocytes did not synthesize melanins under normal conditions but produced melanins under certain conditions, such as UVR exposure or excision of the epidermis and upper parts of hair follicles by dermabrasion (2–4).

Follicular melanocytes consist of two morphologically and functionally different types; active (dopa-positive) and inactive (dopa-negative). Hair is pigmented only when it grows; the melanogenic activity of follicular melanocytes is closely related to the anagen stage of the hair cycle (5). In catagen, melanin formation stops and remains absent throughout the telogen phase (5). Very little is known about the factors that control melanocyte repopulation of hair follicles during each hair cycle.

By routine staining techniques, such as Fontana-Masson or dopa-oxidase stain, melanocytes are detected only in anagen hair follicles, because these techniques require the presence of active melanogenesis. Even ultrastructurally, the identification of a cell as melanocytes depends on the presence of melanosomes (5). No method to detect dormant melanocytes has been developed.

Recently, several antibodies which react with melanocytic cells have been produced, principally for melanoma detection (6). However, not all markers of melanoma cells are expected to react with normal melanocytes. Antibodies against melanosomal proteins are not expected to stain the dormant melanocytes. NKI/beteb recognizes premelanosome- and melanosome-related antigens of different molecular weights (7, 8). Recent studies using transfection techniques have revealed that the antigens recognized by NKI/beteb are products of the same gene, gp100-c1, which is homologous to the human silver locus gene, Pmel 17 (9). Since monoclonal murine antibody NKI/beteb is the most specific for melanocytes and shows intense melanocyte staining (6, 10, 11), it was attempted in the present study using split-skin preparations to observe the three-dimensional localization of melanocytes, especially putative precursor melanocytes of hair follicles.

MATERIALS AND METHODS

Tissue

Hair follicle samples were obtained from the margins of excisional skin biopsies from the human scalp and face. Three types of hair were included in these samples: vellus, intermediate and terminal hairs. In the present study, we used the following definitions (12). In general, vellus hair is soft and short, usually not longer than 2 cm, often colorless; it is generally surface hair. Terminal hair is large and coarse, endowed with medulla and pigment, and can vary in length. Intermediate hair is small terminal hair.

Longitudinal and transverse sections of an adult human scalp and facial skin were prepared routinely with formalin fixation, paraffin embedding, and hematoxylin and eosin. The presence or absence of melanin granules was confirmed by Fontana-Masson's stain.

Preparation of extracted hair follicles

The scalp and facial skin was placed in phosphate-buffered saline (PBS), pH7.3, at room temperature and then incubated in 20 mM ethylenediaminetetraacetic acid (EDTA) in PBS for 3–5 h at 37°C. The epidermal sheets with attached vellus and intermediate hair follicles were gently removed with forceps and rinsed in PBS. They were then fixed with cold acetone for 30 min. Vellus and intermediate hair follicles of the scalp and facial skin were observed three-dimensionally with a light microscope in the wet whole mount. In this experiment terminal hair follicles were excluded, because the portions below the infundibulum were severed.

Immunohistochemistry

The epidermal sheets fixed with acetone were immunostained for monoclonal murine antibody by the labelled streptavidin biotin staining technique (DAKO LSAB Kit, DAKO, K681). The epidermal sheets were incubated for 30 min at room temperature with monoclonal murine antibody NKI/beteb, stained immunohistochemically with biotinylated anti-rabbit and anti-mouse immunoglobulins as secondary antibody for 20 min, and peroxidase- or FITC-conjugated streptavidin as the third reagent for 20 min. The peroxidase color reaction was developed in the presence of DAB and washed with PBS. Immunopositive cells were photographed by a Nikon fluorescence microscope.

RESULTS

Wet-mount specimens

Vellus hair. In extracted anagen vellus hair follicles immunoreactive (ir-) melanocytes were distributed from the infundibu-

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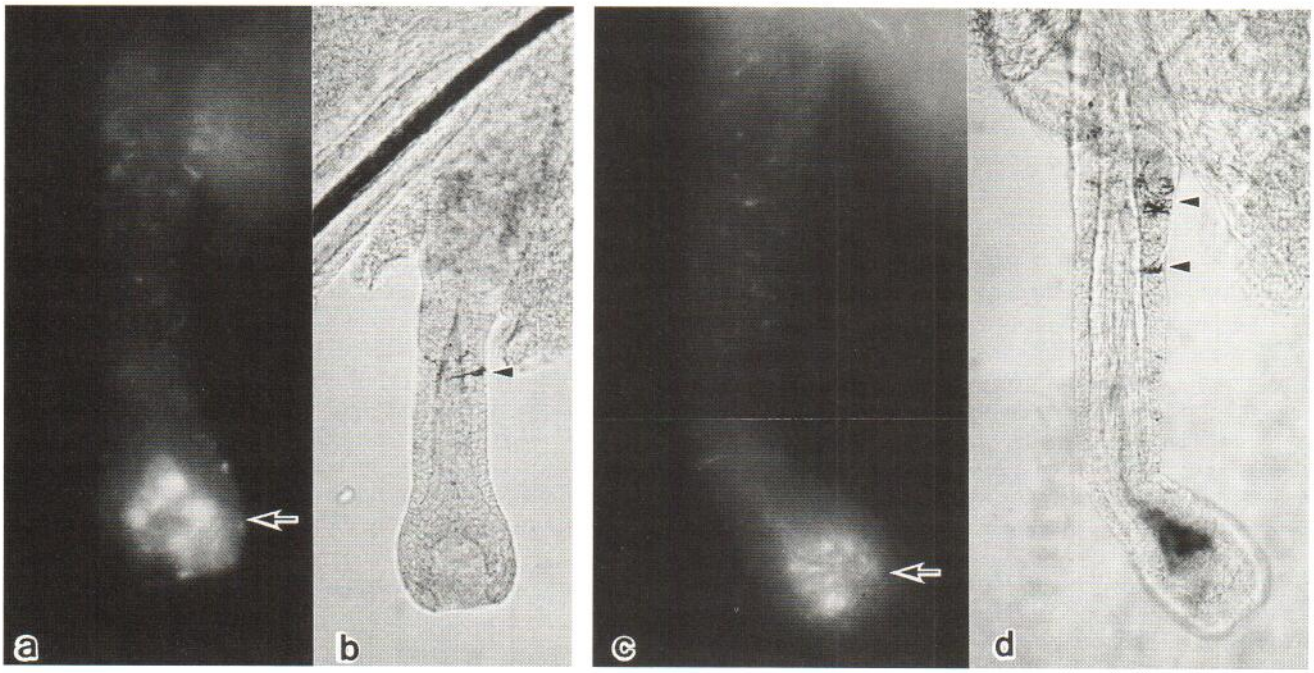

Fig. 1. Extracted anagen vellus hair follicles. *a*) and *c*); NKI/beteb-reactive melanocytes are distributed throughout the entire hair follicles. Immunoreactivity of melanocytes of the bulb (*arrows*) is much stronger than that of other portions above the bulb. *b*) and *d*); dendritic melanocytes (*arrowheads*) are only found in the middle portions. *b*) and *d*) correspond to *a*) and *c*), respectively. *a*) and *c*) show immunostaining for NKI/beteb labeled by FITC, whereas *b*) and *d*) show wet-mount specimens without stain. Three-dimensional views. (Magnification for *a* and $c \times 60$, *b* and $d \times 50$.)

lum to the bulb (Fig. 1 a,c). In some instances, ir-melanocytes were exclusively found below the sebaceous gland, where the bulge area was present (Fig. 2). Melanocytes of the bulb were intensely stained, while those of other portions above the bulb were moderate or weak (Fig. 1 a,c).

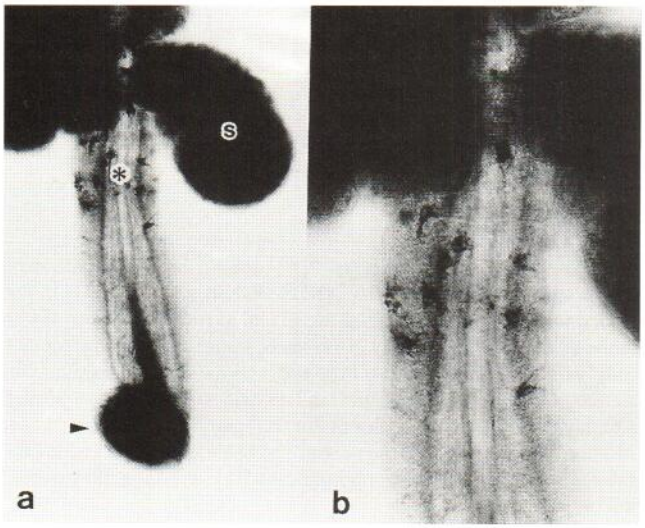

Fig. 2. Extracted anagen vellus hair follicle. *a*); NKI/beteb-reactive melanocytes are exclusively present below the sebaceous gland (*s*), where the presumptive bulge area is present. *b*); high magnification of the area pointed by * in *a*). *Arrowhead* indicates the bulb. NKI/beteb is labeled by peroxidase. Three-dimensional views. (Magnification for $a \times 50$ and $b \times 100$.)

In extracted anagen vellus hair follicles melanization of the bulb was either absent or present (Fig. 1 b,d). A few dendritic melanocytes were exclusively observed in the middle portion below the sebaceous gland (Fig. 1 b,d). In some instances, a few dendritic melanocytes were present in the slightly widened portion indicating the bulge area (Fig. 1 d).

Intermediate hair. In extracted anagen intermediate hair follicles ir-melanocytes were distributed from the infundibulum to the bulb, but fluorescence intensity was different (Fig. 3). Melanocytes of the bulb were strongly stained but those of the portions above the bulb were weak or moderate (Fig. 3 b,c,d).

In extracted anagen intermediate hair follicles the bulb was heavily melanized, while dendritic melanocytes and melanin granules were sparsely scattered in some of the widened portions below the sebaceous glands indicating the bulge areas (Fig. 4).

Sections

Longitudinal sections of an adult human scalp showed that melanin granules stained with Fontana-Masson were densely distributed in the orifice and bulb of anagen terminal hair follicles. Some keratinocytes of the outermost cell layer or sporadically inner layer of outer root sheaths below the sebaceous gland were variably melanized. In the telogen phase the bulge areas were indistinguishable from the clubbed ends. Since melanin granules of the bulge areas were sparse, these were easily overlooked unless Fontana-Masson's staining was applied.

Transverse sections of an adult human scalp showed that

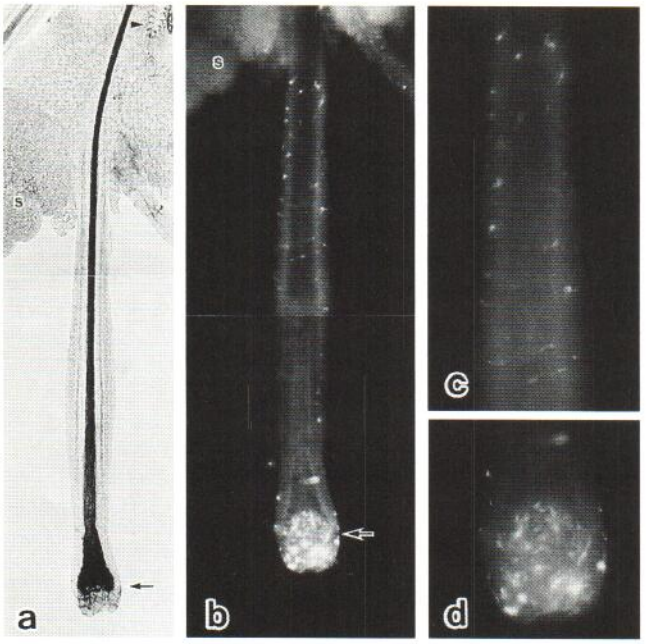

Fig. 3. a); overall view of extracted anagen intermediate hair follicle. *b*); NKI/beteb-reactive melanocytes are seen from the middle portion below the sebaceous gland (*s*) to the bulb (*arrow*). Immunoreactivity of melanocytes of the bulb (*arrow*) is much stronger than that of other portions. *c*) and *d*); high magnification of the areas of the middle portion (*c*) and bulb (*d*) in *b*). *Arrowhead* indicates demodex. *b*), *c*) and *d*) show immunostaining for NKI/beteb labeled by FITC. Three-dimensional views. (Magnification for *a* × 50, *b* × 60, *c* and *d* × 120.)

the orifice and bulb of terminal hair follicles were heavily melanized. Some keratinocytes of the outermost cell layer or sporadically inner layer of outer root sheath of the sebaceous gland level were variably melanized (Fig. 5). The knob-like swellings were sometimes melanized (Fig. 6), as were one of the hallmarks of the bulge (13–15).

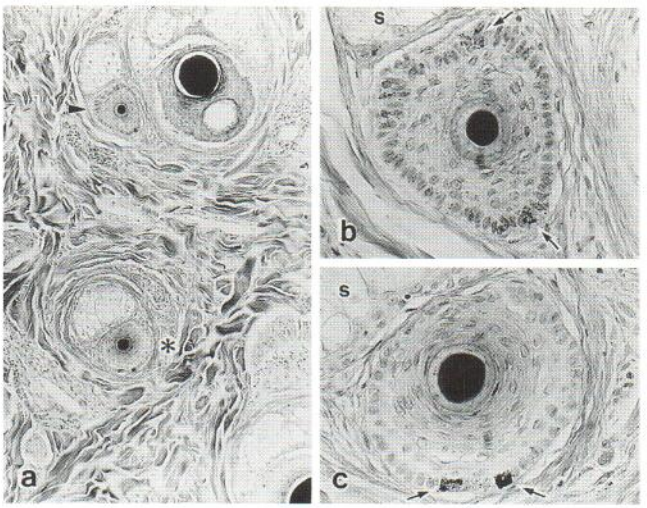

Fig. 5. Transverse section of an adult human scalp. *a*); terminal hair follicles are shown at the level of the sebaceous gland (*s*). *b*) and *c*); high magnification of the areas indicated by *arrowhead* or ※ in *a*). Melanin granules (*arrows*) are distributed in the outermost cell layer of outer root sheath at the level of the sebaceous gland(s). The section has been stained with Fontana-Masson. (Magnification for *a* × 25, *b* and *c* × 100.)

Melanization of the bulge areas was independent of the hair cycle.

DISCUSSION

The finding that ir-melanocytes are localized in the outer root sheaths from the upper (infundibulum) to lower portions (bulb) of hair follicles is in accordance with previous observations (1, 16, 17). We have added to the evidence for specific localization of dendritic melanocytes in the bulge area. Although ir-melanocytes were distributed throughout the hair follicles, dendritic melanocytes and melanin granules were only

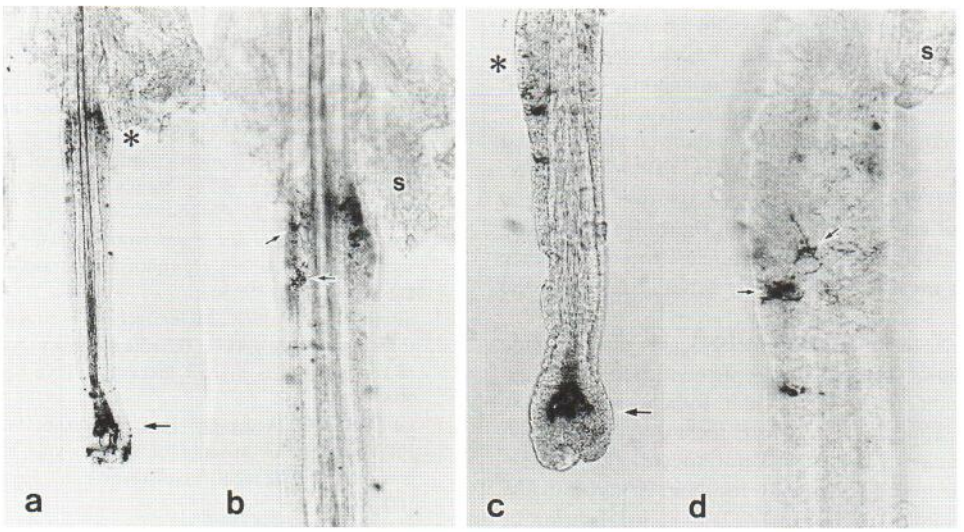

Fig. 4. Extracted anagen intermediate hair follicles. *a*) and *c*); melanization is observed in the bulb (*arrows*) as well as in the slightly widened middle portion (※) below the sebaceous gland (*s*) presumably indicating the bulge area. *b*) and *d*); high magnification of the area pointed by ※ in *a*) and *c*), respectively. The presumptive bulge areas demonstrate a few dendritic melanocytes (*small arrows*) and melanin depositions. Wet-mount specimens without stain. Three-dimensional views. (Magnification for *a* and *c* × 25, *b* and *d* × 50.)

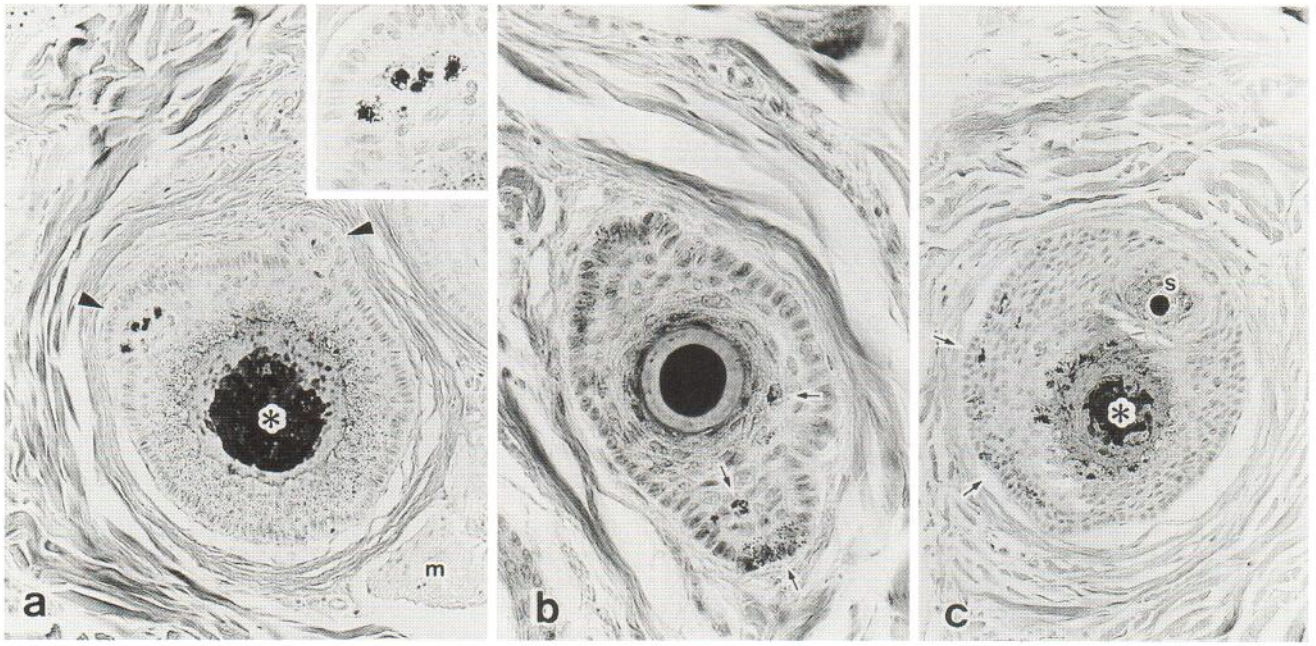

Fig. 6. Transverse sections of an adult human scalp. *a*); knob-like structures (*arrowheads*) of the clubbed end (*) of the telogen phase, *b*); bilateral swelling of the bulge of the anagen phase and *c*); the bulge area composed of clubbed end (*) and secondary hair (*s*) of the early anagen phase. Melanin granules (*arrows*) are present in the outermost layer or inner layer of the outer root sheaths. *Insert* in *a*); high magnificaton of knob-like structure pointed by *right arrowhead* in the main picture. *m*; arrector pili muscle. The sections have been stained with Fontana-Masson. (Magnification for *a* and *c* × 50, *b* and insert in *a* × 100.)

observed in the bulge area. We previously reported a similar phenomenon observed in the bulge area of the human eyebrow (14).

It is difficult to explain how dendritic melanocytes and melanized keratinocytes are concentrated in the bulge areas and why melanin granules are not carried outward through a cell stream movement. The outer root sheath shows more complicated cell kinetics than that of other cell layers (18). Cell movement of outer root sheath cells is poorly understood in comparison with epidermal flow.

Cynomolgus monkey and human palm epidermis showed two structurally distinct populations of basal keratinocytes such as heavily melanized nonserrated and lightly melanized serrated cells (19). Anatomical location, fine structural features and kinetic properties suggested that melanized nonserrated cells represented a stem cell population (19). Moreover, Cotsarelis et al. (20) proposed the bulge activation hypothesis that follicular stem cells were located in the bulge area close to the insertion of the arrector pili muscle. Recent evidence has indicated that most stem cells may actually reside in the bulge (21).

It has been suggested that ORT melanocytes may provide a reservoir of melanocytes and that repigmentation of vitiligo begins in the middle parts of the hair follicles, where new melanocytes first appear (17). More recently, it has been proposed that similar to follicular keratinocytes (20), "stem cells" for follicular melanocytes may reside in or close to the bulge area of the pilosebaceous unit (22). The localization of putative precursor melanocytes remains unknown. Whether the follicular population of melanocytes residing in the bulge behaves in a similar way to the presumptive follicular stem cells is important. Melanization of the bulge area may imply certain biologic characteristics of bulge melanocytes and/or keratinocytes in renewing human hair follicles.

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