Immunohistochemical Demonstration of Keratin 19 Expression in Isolated Human Hair Follicles

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We examined keratins 19 and 8 in extracted human hair follicles using monoclonal antibodies Ks19.1 and CAM5.2, respectively. Ks19.1 reactivity was found in the bulge and infundibulum. Ks19.1(+) cells were dense in the bulge of vellus and intermediate hair follicles. The intact bulge of terminal hair could not be extracted, but the presence of Ks19.1(+) cells was confirmed in transverse sections. Infundibular Ks19.1(+) cells exhibited a dense network pattern of staining in terminal hair follicle, but only a few cells were labeled in vellus and intermediate hair follicles. CAM5.2(+) cells, i.e., Merkel cells, were found in the same locations as Ks19.1(+) cells but were less dense. These patterns of distribution and staining density were not influenced by different phases of hair cycle. Sequential staining of Ks19.1 and CAM5.2 in the same hair follicle demonstrated that the same cells could be reactive for both. However, considering the large number of Ks19.1(+) cells and rather small number of CAM5.2 in the same locations, it was assumed that only a subset of Ks19.1(+) cells are Merkel cells. It was postulated that the bulge area of human adult hair follicles houses embryonic pluripotential cells characterized by stem cells and post-stem cells and that the Merkel cells in the bulge area arise from these immature cells and may play a role in the maintenance and stimulation of this group of immature cells.

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Many studies have been done to identify the location of stem cells in epidermis [1–6], but post-stem cell amplification in the epidermis or in the hair follicle has not been studied. It has been suggested that the structure of keratin 19, lacking the carboxy-terminal nonhelical domain [7,8], may be related to its non-filament-forming properties [9] and that the expression of this keratin may indicate a flexible state of differentiation [8]. It was therefore suggested that keratin 19 could be a marker for an indeterminate state of differentiation and serve, depending upon local demand, as a source of various cell types [8,10]. We previously reported that Merkel cells of the human hair follicle are most concentrated in the bulge and have no significant connection with perifollicular sensory nerve endings, which are located at some distance above and under the epithelial hood, or skirt [11,12]. We assigned a stem cell-stimulating function rather than a tactile receptor function to these Merkel cells [12]. Assuming that keratin 19-positive cells are derived from daughter cells of the stem cells located in the bulge [13], we thought it possible to establish a relationship between stem cells and Merkel cells by staining keratin 19 and simple epithelial keratin, a marker of Merkel cells, in the bulge simultaneously.

MATERIALS AND METHODS

Hair follicle samples were obtained from the margins of excisional skin biopsies from the scalp of a 10-month-old child and the ear of a 70-year-old adult. Three types of hair were included in these samples: vellus, intermediate, and terminal hairs. In this study, we used the following definitions. In general, vellus hair is soft and short, usually not longer than 1 cm, often colorless; it is the general surface hair. Terminal hair is large and coarse, endowed with medulla and pigment, and can vary in length. Intermediate hair is smaller terminal hair. For the preparation of epidermal sheets, the skin was first placed in phosphate-buffered saline (PBS), pH 7.3, at room temperature. Epidermal sheets were obtained by incubating the specimens in 20 mM ethylenediaminetetraacetic acid (EDTA) in PBS for 3–12 h at 37°C and gently separating the epidermis with forceps. The epidermal sheets thus prepared were attached with numerous well-preserved vellus hair follicles of the ear and intermediate and vellus hair follicles of the scalp, but not terminal hair follicles of the scalp. These specimens were observed with a threedimensional immunohistochemical technique described elsewhere by us [11]. The epidermal sheets were immunostained by the avidin-biotin complex (Vector Kit, Vector Laboratories, Burlingame, CA) and diaminobenzidine. Epidermal sheets were fixed with cold acetone for 30 min, washed, incubated for 60 min at room temperature with monoclonal murine antibody CAM5.2 specific for the simple epithelium-type keratin 8 (52.5 Kd) (Becton-Dickinson, San Jose, CA) [14], and washed with PBS solution three times for 10 min. Biotinylated anti-mouse IgG horse serum as secondary antibody and avidin-biotin complex peroxidase as the third reagent were applied for 60 min, and the peroxidase color reaction was developed in the presence of diaminobenzidine. CAM5.2 staining of Merkel cell intermediate filaments was confirmed previously by immunoelectron microscopy [15].

The epidermal sheets were immunostained by immunofluorescence for keratin 19. Epidermal sheets were fixed in cold acetone for 30 min, washed, and incubated overnight at 4°C with monoclonal murine antibody Ks19.1 specific for cytokeratin 19 (40 Kd) (ICN Immunologicals, Lisle, IL) [16]. Goat anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate was applied, and immunoreactive patterns were photographed with a Nikon fluorescence microscope.

An adult human scalp skin was embedded in paraffin and transverse sections were prepared. Paraffin-embedded specimens were tangentially cut in 6-μm sections and treated with 0.1% pronase (Sigma Chemical, St. Louis, MO) prior to immunostaining [17]. Sections were then incubated with monoclonal murine antibody Ks19.1 overnight at 4°C and stained with biotinylated anti-mouse IgG horse serum as secondary antibody and with
avidin-biotin complex-peroxidase. The peroxidase color reaction was developed in the presence of diaminobenzidine.

To verify the relationship of Ks19.1 and CAM5.2 immunoreactivities in the bulge areas of the vellus hair follicles and the coexpression of Ks19.1 and CAM5.2 on normal Merkel cells in the infundibulum areas of the terminal hair follicles, a sequential immunoenzyme labeling procedure was carried out using extracted vellus hair follicles and transverse sections, respectively. First, extracted vellus hair follicles and the pronase-treated hair follicles, a sequential immunoenzyme labeling procedure was carried out using extracted vellus hair follicles and transverse sections, respectively. Extracted vellus hair follicles and the pronase-treated hair follicles, a sequential immunoenzyme labeling procedure was carried out using extracted vellus hair follicles and transverse sections, respectively. Extracted vellus hair follicles and the pronase-treated

### Results

The distribution patterns of Ks19.1(+) cells and CAM5.2(+) cells in various hair follicles are summarized in Table I.

### Transverse Sections

At the presumptive bulge area below the sebaceous gland, Ks19.1-reactive cells were found either continuously or at intervals in the outermost cell layer of the outer root sheath of terminal hair follicles of the scalp (Fig 1a). Interestingly, morphologic variations of the bulge, such as knob-like swellings and villous projections [15], were also observed with Ks19.1 (Fig 1b). A small number of Ks19.1-positive cells were sporadically found in the inner cell layer of the outer root sheath of terminal hair follicles (Fig 1a). Above or below the level of bulge, Ks19.1 reactivity abruptly ceased. Ks19.1 also decorated the dermal duct and secretory portion of eccrine sweat gland.

### Wet-Mount Specimens

**Vellus Hair:** The bulge areas of most vellus hair follicles of the ear of an adult were strongly stained with Ks19.1 independent of the phases of hair cycle (Fig 2). The intensity of fluorescence obliterated individual cell identity and produced diffuse fluorescent areas (Fig 2). The presumptive bulge areas where hair follicles widen below the sebaceous glands were labeled exclusively with Ks19.1 in the anagen (Fig 2a,b), catagen (Fig 2c,d), and telogen (Fig 2e) phases of vellus hair follicles. In the telogen phase the bulb area became indistinguishable from the clubbed end, where Ks19.1 staining occurred (Fig 2f). The staining intensity was not influenced by the size of hair follicle and hair cycle. A small number (fewer than 20%) of vellus hair follicles of anagen, catagen, and telogen phases were not stained with Ks19.1. In rare instances an intense immunofluorescence for Ks19.1 was observed on one side of the presumptive bulge area of anagen vellus hair follicles (Fig 2a). The staining patterns of Ks19.1 in extracted vellus hair follicles were similar to those of conventional vertical sections from the ear.

**Intermediate Hair:** Immunofluorescence patterns for Ks19.1 of intermediate hair follicles of a child’s scalp were basically the same as those of vellus hair follicles of adult ear. The newly formed secondary hair germ and the bulb end of early anagen phase follicles were also stained with Ks19.1, but the bulb of mature anagen hair follicles was not. Staining intensity was the same as in the bulge.

**Terminal Hair:** Only the infundibulum areas of terminal hair follicles of the scalp of a child were studied, because the portions below the infundibulum were severed. A densely aggregated or reticular pattern of Ks19.1-positive cells was observed in the infundibulum of the terminal hair follicle (Fig 3a). Ks19.1-positive cells were absent in the infundibulum of vellus and intermediate hair follicles with occasional exceptions.

### CAM5.2-Reactive Cells

Many CAM5.2-reactive infundibular Merkel cells were observed in the scalp terminal hair follicles of a child (Fig 3b). A small number of CAM5.2-reactive Merkel cells were also present within the presumptive bulge areas of anagen (Fig 4a,b) and catagen (Fig 4c) follicles, and the clubbed end of telogen (Fig 4d) vellus hair follicles of the ear of an adult and intermediate hair follicles of the scalp of a child.

### Sequential Immunostains

Sequential immunostaining was carried out on extracted vellus hair follicles and the transverse sections in the infundibulum areas of the scalp skin. In the bulge areas of vellus hair follicles, Ks19.1-positive cells (Fig 5a) were larger in number than CAM5.2-reactive cells (Fig 5b). However, in the infundibulum areas of terminal hair follicles, Ks19.1-reactive cells (Fig 6b) were also positive for CAM5.2 (Fig 6a).

Controls were all negative in the absence of primary antibody. E3 stained the entire hair follicle below the sebaceous gland in each phase of the hair cycle in all types of extracted vellus and intermediate hair follicles.

### Table I. Distribution of Ks 19.1 (+) Cells and CAM5.2 (+) Cells in Various Hair Follicles

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* The reactivities were the same in all phases of the hair cycle.

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NI, not investigated.
**DISCUSSION**

The bulbar region of the hair follicle contains a pool of relatively undifferentiated epithelial cells, termed matrix cells, which give rise to the hair and its surrounding sheaths [20]. It has generally been assumed that matrix cells, through their interactions with the dermal papilla, play a central role in follicular growth and differentiation [21–23]. However, anagen hair will eventually cease to grow, because the hair matrix cells are apparently not endowed with permanent reproductive potential; they are doomed to die after a set number of mitotic cycles. The real regenerative power seems to reside at the end of club hair, where the bulge is housed during the telogen phase. Recently, Cotsarelis et al [13] proposed a new hypothesis that follicular stem cells reside in the bulge instead of in the lower bulb. Stem cells are considered to be relatively undifferentiated, both ultrastructurally and biochemically [13], and their morphologic heterogeneity has been interpreted as indicative of stem cells [24,25].† No one has found a specific immunohistochemical marker for stem cells; the current method for identifying them requires repeated and prolonged pulse labeling with tritiated thymidine to demonstrate label-retaining cells. Monoclonal murine antibody Ks19.1 specific for cytokeratin 19 or a marker of indeterminate differentiation in human skin [8,10,26] may be an alternative to direct demonstration of stem cells and therefore was explored in this study.

Previous studies on expression patterns of Ks19.1 in human skin used conventional sections [27,28]; this method was inadequate to assess a large number of Ks19.1-reactive cells. In this study we used extracted hair follicles to study the expression of Ks19.1 in the whole hair follicle. Using immunofluorescence microscopy, we have observed the intense reaction of Ks19.1 in the presumptive bulge areas, independent of hair types and hair cycles. Ks19.1 staining of transverse sections of terminal hair follicles showed that these fluorescent cells are localized in the outermost layer of the outer root sheath at the level of the bulge.

The morphology of the bulge area of the hair follicle may change in various conditions, such as epidermal regeneration and psoriasis [28]. We described previously normal variations of the bulge, such as knob-like swellings and villous projections of terminal hair follicles.† In this study these structures were found to be stained with Ks19.1.

We have demonstrated that the lower epidermal layers of human fetal epidermis express Ks19.1-reactive cytokeratin at 12 weeks. Ks19.1 expression remains positive in Merkel cells after the epidermal basal cells gradually lose their Ks19.1 expression at 14 weeks [15] and in postfetal life [15]. It is possible that Merkel cells differentiate from the Ks19.1-reactive cell population in fetal and, possibly, adult epidermis. In the bulge of vellus hair follicles, the Ks19.1-immunoreactive cell population was much larger than that of CAM5.2-immunoreactive cells (Fig 2 versus Fig 4; Fig 3a versus Fig 3b; Fig 5a versus Fig 5b). This discrepancy is smaller in the


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**Figure 2.** Ks19.1-reactive cells in vellus hair follicles extracted from the ear. These cells are densely aggregated in the presumptive bulge area below the sebaceous gland (arrows) independent of stages of hair cycle (a, b, anagen phase; c, d, catagen phase; e, telogen phase). In rare instances intense reaction is mainly observed on one side of the presumptive bulge area (a). Arrowheads, the regressing epithelial cords in catagen hairs (c, d). Extracted hair follicles. Bar, 50 μm.

**Figure 3.** Expression of Ks19.1 (a) and CAM5.2 (b) in the infundibulum of terminal hair follicles extracted from the scalp. Dense populations of Ks19.1-positive cells (a) form net-like pattern in two adjacent follicles (arrows), whereas less dense populations of CAM5.2-labeled cells (b) produce dot-like individual cell staining (arrowhead). Bar, 50 μm.
**Figure 4.** CAM5.2-reactive Merkel cells (arrowheads) in the bulge areas below the sebaceous glands (arrows) of vellus hair follicles in anagen (a,b), catagen (c), and telogen (d) phases. Whole mount of vellus hair follicles extracted from the ear. Bar, 50 μm.

infundibulum, where Ks19.1-reactive cells may be equal in number to CAM5.2-positive cells (Fig 6). In fact, some Merkel cells of the infundibulum can coexpress CAM5.2 and Ks19.1 epitopes, as shown in Fig 6. Because the number of Merkel cells as detected by electron microscopy is small in the bulge, CAM5.2-positive cells are probably Merkel cells that coexpress Ks19.1 epitopes, and the majority of Ks19.1-reactive cells may represent daughter cells of stem cells. The expression of Ks19.1 in the follicular Merkel cells suggests that they share some features of indeterminate, multipotential cells.

The bulge areas where the follicular Merkel cells are concentrated were not innervated with prominent perifollicular nerve endings, i.e., with palisading branches under the skirt [11]. The major function of Merkel cells in the bulge may not be to initiate tactile sensation but rather to stimulate stem cells by means of cytokines, i.e., paracrine function to initiate a new anagen phase. The speculation that Merkel cells may secrete cytokines that stimulate growth of skin appendages is consistent with our observation, as well as that of Moll et al [29], that eccrine germs in fetal skin emerge from the sites of the aggregation of Merkel cells in the basal layer. Merkel cells were also found in the upper part of the eccrine germs [30].

In spite of a high mitotic index in the hair bulb, the matrix cells

**Figure 5.** An extracted facial vellus hair follicle in the late catagen phase sequentially stained for Ks19.1 (a) by the avidin-biotin complex-peroxidase method and for CAM5.2 (b) by immunofluorescence. CAM5.2-reactive cells (b) are much smaller in number than Ks19.1-positive cells (a) in the clubbed bulge region. Arrowheads, the regressing epithelial cords. Bar, 50 μm.

**Figure 6.** Transverse section of the infundibulum of the same human scalp terminal hair follicle sequentially stained for CAM5.2 (a) by immunofluorescence and for Ks19.1 (b) by the avidin-biotin complex-peroxidase method. CAM5.2 reactive cells (a, arrow) are also positive for Ks19.1 (b, arrows). Paraffinized specimen. Bar, 50 μm.
did not express Ks19.1 epitopes, suggesting that the daughter cells of these matrix cells begin differentiation immediately instead of staying in an indeterminate state of amplification.

REFERENCES
