The Appearance, Density, and Distribution of Merkel Cells in Human Embryonic and Fetal Skin: Their Relation to Sweat Gland and Hair Follicle Development

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The density and distribution of Merkel cells in human embryonic and fetal skin were studied using an immunolabeling technique on epidermal and dermal sheets obtained by ethylenediamine tetraacetic acid separation. Merkel cells were identified by the known cytokeratin markers CK20 and CK18. Merkel cells showed CK20 immunoreactivity as early as 56 d estimated gestational age (EGA) in the palmar epidermis (133.11 ± 44.27 cells/mm^2). The density increased rapidly, reaching a maximum of more than 1400 cells/mm^2 at 80–90 d EGA. At this stage, the cells became distributed along the primary epidermal ridges. In the palmar epidermis of fetuses older than 100 d EGA, the distribution of Merkel cells showed the same pattern, but the density then decreased gradually. Merkel cells were not observed in ductal and glandular portions of eccrine sweat glands. In the epidermal sheets of hairy skin, a few cells were first seen in the fetus at 75 d EGA. At 100 d EGA, only a few Merkel cells were observed, mostly in the hair pegs and bulbous hair pegs. In the older fetus, ring-like arrangements and aggregates of Merkel cells were prominent in the infundibulum and bulge of hair follicles, respectively. Merkel cells were both globular and dendritic in shape. The ratio of dendritic to globular cells increased gradually until the period of highest Merkel cell density in both the glabrous and hairy skin. All Merkel cells located in the dermis were globular in shape. In accord with the results obtained, we postulate that Merkel cells may have some functional role in the formation and proliferation of eccrine sweat glands and hair follicle anlagen in developing skin. *Key words: CK20/skin appendage. J Invest Dermatol 104:411–416, 1995*

Among the non-keratinocytes of epidermis, only Merkel cells have characteristics of an epithelial nature: desmosomal contacts with surrounding keratinocytes [1] and the expression of simple epithelial-type cytokeratins [2]. The melanocytes are derived from neural crest and migrate into the embryonic epidermis before 50 d estimated gestational age (EGA). Langerhans cells are bone-marrow–derived and also enter the epidermis of the embryo. Overwhelming data now support the concept that Merkel cells are of epidermal keratinocyte origin [2–6].

Merkel cells are concentrated in the epidermal ridges of developing epidermis [2,7] and in the outer root sheath of developing hair follicles [8]. Immunoreactive Merkel cells are highly concentrated in the bulge and infundibulum of the developing hair follicle [9] and in the proximal nail fold of developing digital skin [10].

Merkel cells are neuroendocrine cells of the skin that are commonly thought to function as slowly adapting mechanoreceptors [11]. Many studies have revealed the paracrine function of Merkel cells. Epidermal Merkel cells produce nerve growth factor [12] and dermal Merkel cells express nerve growth factor receptor before making connection with immunoreactive nerves [13]. These findings suggest that epidermal Merkel cells are targets for sensory nerves [12] and that dermal Merkel cells contribute to development of the cutaneous nerve plexus in the upper dermis [13]. In support of these roles, Merkel cells react positively with antibodies that recognize vasoactive intestinal polypeptide [14], substance P [15,16], met-enkephalin [17], chromogranin A [15], calcitonin gene-related polypeptide [15], neuron-specific enolase [18,19], and bombesin [19]. The above studies concerning distribution and paracrine function suggest a role for Merkel cells in the formation of skin appendages such as eccrine sweat gland, hair follicle, nail, and nerves in the developing skin.

Recently, CK20, a major cytoskeletal polypeptide of human intestinal epithelium and uroepithelial cells [20], was detected as a prominent component and specific marker of Merkel cells in the skin [21,22]. It is expressed in Merkel cells much earlier than are other simple epithelial-type cytokeratins [21].

Our objectives were as follows: 1) to determine when Merkel

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Abbreviation: EGA, estimated gestational age.

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cells first appear in the skin, by immunocytochemistry using CK20 antibody in epidermal-sheet preparations and by electron microscopic observation; 2) to measure the density of Merkel cells in the epidermis of palmar skin; and 3) to observe the distribution patterns of Merkel cells in relation to the development of eccrine sweat glands and hair follicles. Epidermal and dermal sheets were prepared from embryonic and fetal skin (56–163 d EGA) and immunostained with Merkel-cell–specific cytokeratins, CK18 and CK20.

MATERIALS AND METHODS

Skin Samples Skin was obtained from the palms and hairy skin of 32 human embryos and fetuses, ranging from 56 to 163 d EGA as determined from maternal data and measurements of foot and crown-rump lengths. Samples that were 60 d or younger were designated as embryonic, whereas those older than 60 d were termed fetal. Skin samples were collected by the Central Laboratory for Human Embryology at the University of Washington under the direction of Dr. Alan Fantel. The specimens were obtained in accord with Department of Health, Education, and Welfare policies and the University of Washington Human Subjects Review Board.

Light and Electron Microscopy Full-thickness skin from each specimen was processed for light and electron microscopy and immunohistochemistry. Samples for immunolabeling were excised immediately and frozen in 100% ethanol cooled on dry ice. For histology and electron microscopy, samples were fixed in one-half strength Karnovsky's fixative, rinsed in 0.1 M sodium cacodylate buffer, and post-fixed for 1 h in 2% OsO_4. They were dehydrated through graded alcohols into propylene oxide and embedded in Polybed 812 by standard procedures. Frozen skin was cut into sections 6–8 μm thick for immunostaining. Semi-thin and thin sections were cut from the plastic-embedded samples and stained with Richardson's stain for light microscopy and with saturated uranyl acetate and lead citrate for electron microscopy.

Preparation of Epidermal and Dermal Sheets Skin from human embryos and fetuses was dissected from the palms and hairy regions of the body, removed of subdermal tissue, and rinsed in cold phosphate-buffered saline, pH 7.3. Epidermal and dermal sheets were obtained by incubating the skin in a buffered 10-mM ethylenediamine tetraacetic acid solution, pH 7.3, for 1–2 h in a shaker at 37°C. The samples were then immersed in cold phosphate-buffered saline, and the epidermis was gently teased away from the dermis with forceps. The separated sheets were rinsed twice in cold phosphate-buffered saline and fixed in Zamboni's fixative (2% paraformaldehyde with picric acid) for 10 min, then rinsed again in phosphate-buffered saline and used immediately in labeling experiments.

Immunohistochemistry

Sections: Frozen sections were fixed in acetone for 10 min, and antibody localization was performed using the avidin-biotin immunoperoxidase technique. The immunostained sections were counterstained with hematoxylin.

Epidermal and Dermal Sheets: Immunoperoxidase staining was performed in suspension on lightly fixed epidermal and dermal sheets. Saponin (0.01%) was added to all antibody and wash solutions to enhance penetration of antibody. The epidermal and dermal sheets were immersed in 0.3% H_2O_2 and in 0.3% H_2O_2 with 0.1% sodium azide [23], respectively, to block endogenous peroxidase activity. Labeling of extraneous antibodies was blocked by 2% goat and 0.5% horse serum. The sheets were then incubated with monoclonal antibody IT-Ks 20.3 against CK20 (1:10–15 dilution, IBL Research, Cambridge, MA) and/or monoclonal antibody Ks 18.04 against CK18 (1:20 dilution, IBL Research) in 1 mg/ml bovine serum albumin/Tris-saline (0.1 M)/saponin, pH 7.6, overnight at 4°C. Indirect immunoperoxidase stain was performed using biotinylated horse antibodies against mouse IgG (1:200 dilution, Vector, Burlingame, CA). The reaction product was developed in diaminobenzidine. The stained sheets were mounted flat on glass slides in Glycergel. For a negative control, the primary antibody was omitted or normal mouse serum (1:1000 dilution) was substituted for the primary antibody.

Cell Density and Size Measurements The numbers of Merkel cells were determined on epidermal sheets by counting CK20-immunoreactive cells on a computer monitor connected to a light microscope using the National Institutes of Health image program (Bethesda, MD). All focal planes within an epidermal sheet were examined for the presence of immunoreactive Merkel cells. To eliminate bias, only the cells touching the left and upper borders were counted; cells touching the right and lower borders were not counted. Fields were rejected if the tissue was torn or folded or did not completely cover the screen. Merkel cell densities relative to epidermal surface area were determined by counting the number of labeled cells in 10 randomly chosen areas from each epidermal sheet, except for small embryonic samples, which do not have 10 fields in total. In each epidermal sheet of the younger samples, the densities were determined in all the possible areas. To measure the cross-sectional area of individual Merkel cells, the boundary of the individual cell body was traced in the graphic tablet connected to the computer. The size of 50 cells was measured in an individual specimen. The trend of Merkel cell size according to fetal age was tested using a simple linear regression test.

RESULTS

Identification of Merkel Cells in the Embryonic and Early Fetal Period Merkel cells were identified by electron microscopy based on characteristic dense core granules, and by immunohistochemistry based on CK18 and CK20 reactivity. In epidermal- and dermal-sheet preparations, both cytokeratin antibodies clearly demonstrated Merkel cells. Anti-CK20–positive Merkel cells were observed in the palmar epidermis as early as 56 d EGA **(Fig 1*A*)**, whereas CK18-positive Merkel cells were identified by 67 d EGA. CK20 immunoreactivity was seen only in the Merkel cells. CK18 immunoreactivity was observed weakly in periderm and basal keratinocytes as well as in Merkel cells, although the staining intensity was stronger in Merkel cells. Compared to CK18, CK20 immunostaining was stronger and more diffuse throughout the cytoplasm. Therefore, we used skin samples immunolabeled with antibody to CK20 to evaluate density, distribution, and size of Merkel cells.

Density and Distribution of Merkel Cells in the Skin Merkel cells were observed in palmar epidermis as early as 56 d EGA (133.11 $\pm$ 44.27 cells/mm^2) **(Fig 1*A*)**. The density increased rapidly, reaching a maximum in the epidermis of the fetus at 80–90 d EGA (more than 1400 cells/mm^2) **(Fig 2)**. The cells were distributed irregularly in the palmar epidermis of the embryo and early fetus, with no apparent pattern **(Fig 1*A,B,C*)**. In sections of early fetal skin, Merkel cells were observed in the suprabasal or basal layer. Beginning around 80 d EGA, Merkel cells were distributed primarily along the newly formed primary epidermal ridges **(Fig 1*D,E,F*)**, with few cells evident in the inter-ridge areas. In the fetus older than 100 d EGA, the density of Merkel cells was decreased and the cells were confined to the primary epidermal ridges. Merkel cells were not seen in the inter-ridge areas or in the secondary epidermal ridges. In palmar epidermis of the 137-d and 163-d fetus, in which eccrine sweat glands and ducts were well formed, the Merkel cells remained in a linear arrangement along the epidermal ridges and were not found in either the ductal or glandular portion **(Fig 1*F*)**. In sections, Merkel cells also were observed mostly in the ridges, and a few dermal Merkel cells were seen **(Fig 1*G*)**. The density of Merkel cells decreased further with increasing age.

In the dermal sheet of palmar skin, we observed a few anti-CK20–positive Merkel cells as early as 81 d EGA **(Fig 3*A*)**. The dermal sheet of palmar skin from fetuses older than 100 d EGA contained many globular Merkel cells distributed in a linear pattern, following the apparent traces of epidermal ridges **(Fig 3*B*)**. In the dermal sheets prepared from 117-, 137-, and 163-d EGA palmar skin, globular Merkel cells were scattered, showing no specific pattern and a reduced density compared to the 101-d specimen **(Fig 3*D*)**. In sections, all dermal Merkel cells were located in the upper dermis close to the dermoepidermal junction **(Fig 1*G*)**. Some junctional Merkel cells were also observed **(Fig 3*C*)**.

A few Merkel cells were first seen at 75 d EGA in the epidermal sheets of hairy skin **(Fig 4*A*)**. They were mostly related to developing hair germs. In the fetus at 80–90 d EGA, more Merkel cells were observed in hair germs and pegs **(Fig 4*B*)**. In the skin of the 101-d EGA fetus, Merkel cell aggregates were seen mostly in the infundibulum **(Fig 4*C,D*)**. A few scattered Merkel cells also were observed in the other parts of follicular and interfollicular areas. In the epidermis of the fetus at 117 d EGA, Merkel cell aggregates were seen in the infundibulum of bulbous hair pegs in trunk skin **(Fig 4*E*)** and in the infundibulum and bulge of more

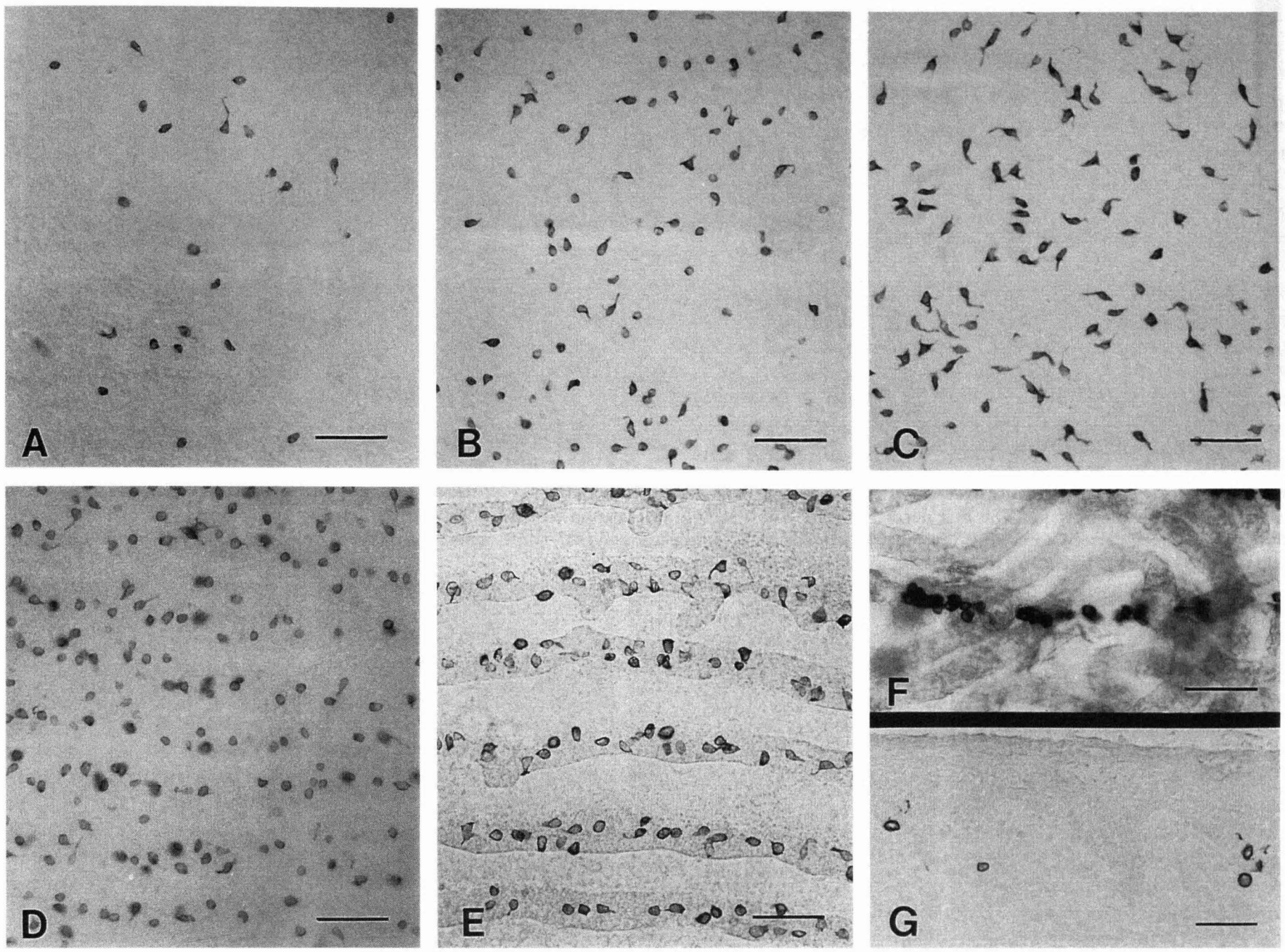

Figure 1. Density and distribution of Merkel cells labeled with anti-CK20 antibody in epidermal sheets and sections of palmar skin. Merkel cells are distributed irregularly in the epidermis of the embryo and younger fetus (*A-C*). Beginning at about 80 d EGA, Merkel cells are distributed along the newly formed primary epidermal ridges (*D-F*). Merkel cell distribution in the ridges is confirmed in sections (*G*). No Merkel cells are seen in the secondary ridges (*E*) or in the ductal or glandular portion of eccrine sweat gland (*F,G*). EGA corresponds to 56 d in *A*, 67 d in *B*, 75 d in *C*, 84 d in *D*, 101 d in *E*, and 163 d in *F* and *G*. *Bars*, 50 μm.

advanced hair follicles in dorsal hand skin **(Fig 4*F*)**. Merkel cell aggregates formed ring-like arrangements in the infundibulum and dense groups in the bulge. In contrast to the earlier follicle stages, these Merkel cell aggregates were present in almost all the follicles. Samples from the fetus at 137 and 163 d EGA showed the same pattern of Merkel cell distribution; however, the number of Merkel cells in the bulge decreased. Only a small number of Merkel cells was observed in the interfollicular areas (data not shown). We confirmed the distribution of Merkel cell aggregates in the infundibulum **(Fig 4*G*)** and bulge **(Fig 4*H*)** using sections of skin. A small number of individual Merkel cells was scattered in the interfollicular areas.

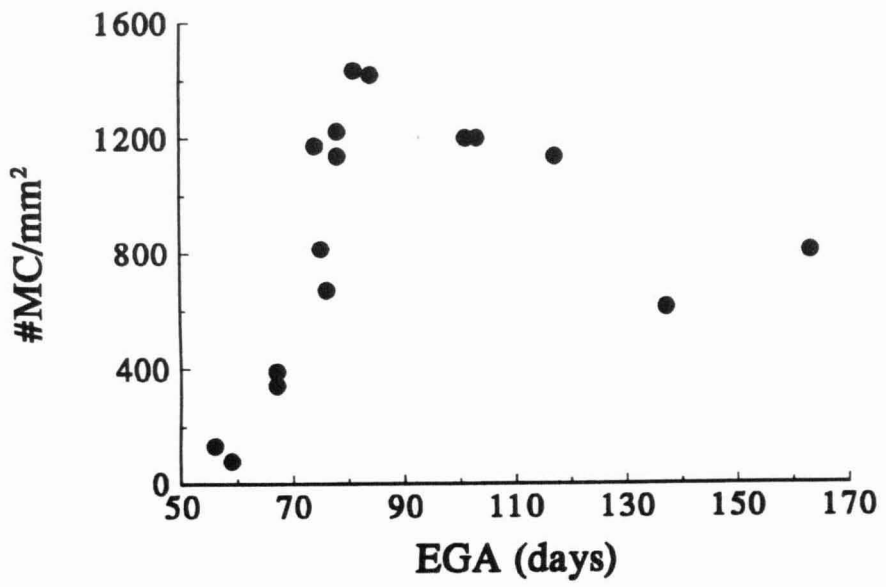

Figure 2. Scattergram illustrating the density of Merkel cells (MC) in embryonic and fetal palmar epidermis at progressive gestational ages (EGA).

Changes in Shape and Size of Individual Merkel Cells Merkel cells were identified as dendritic and/or globular in shape by immunolabeling with antibody to CK20 **(Fig 1)**. The dendritic:globular Merkel cell ratio increased until 80–90 d EGA in the palmar epidermis. In the palmar epidermis of older fetuses (greater than 100 d EGA), more globular Merkel cells were observed **(Fig 1*E,F*)**. In the truncal epidermis at younger than 100 d, most Merkel cells were globular. The small number of dendritic Merkel cells had short dendrites. However, the truncal/scalp epidermis of the 117-, 137-, and 163-d EGA fetuses showed many dendritic Merkel cells. Dermal Merkel cells were mostly globular in both palmar and truncal skin **(Fig 3)**.

The cross-sectional area of individual Merkel cell bodies was measured in the palmar epidermis at different gestational ages. The size was almost constant (60–75 μm^2) in the embryonic and the early fetal period and increased slightly in the older fetus of greater than 100 d EGA (80–90 μm^2). The cell size tended to increase in a statistically significant manner according to progression of the gestational age **(Fig 5)**. The simple linear regression test for this trend was significant, with $t = 5.094$, 14 *df*, and $p = 0.0002$.

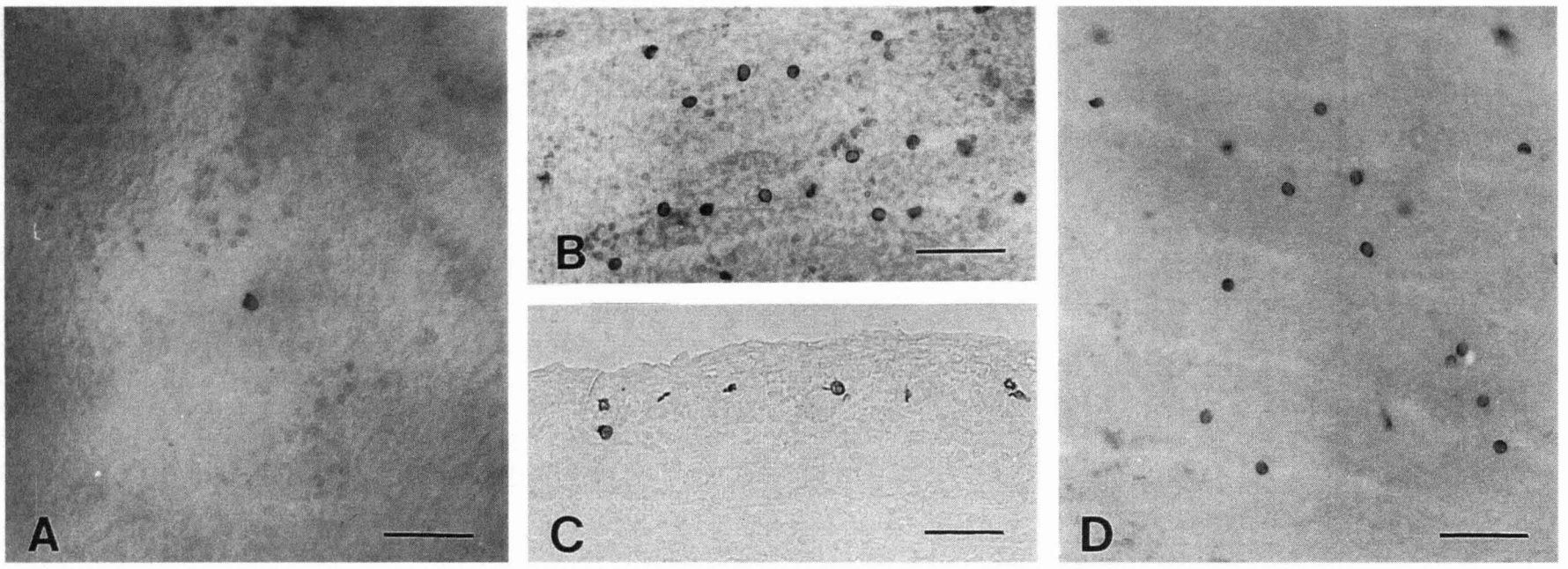

Figure 3. Density and distribution of Merkel cells labeled with anti-CK20 antibody in dermal sheets and sections of palmar skin. All dermal Merkel cells are globular in shape. The first dermal Merkel cell is seen in the fetus at 81 d EGA (*A*). In the fetus at 101 d EGA, globular Merkel cells are distributed in a linear pattern following the apparent traces of epidermal ridges (*B*). Some junctional Merkel cells are observed in sections (*C*). EGA corresponds to 81 d in *A*, 101 d in *B* and *C*, and 163 d in *D*. *Bars*, 50 μm.

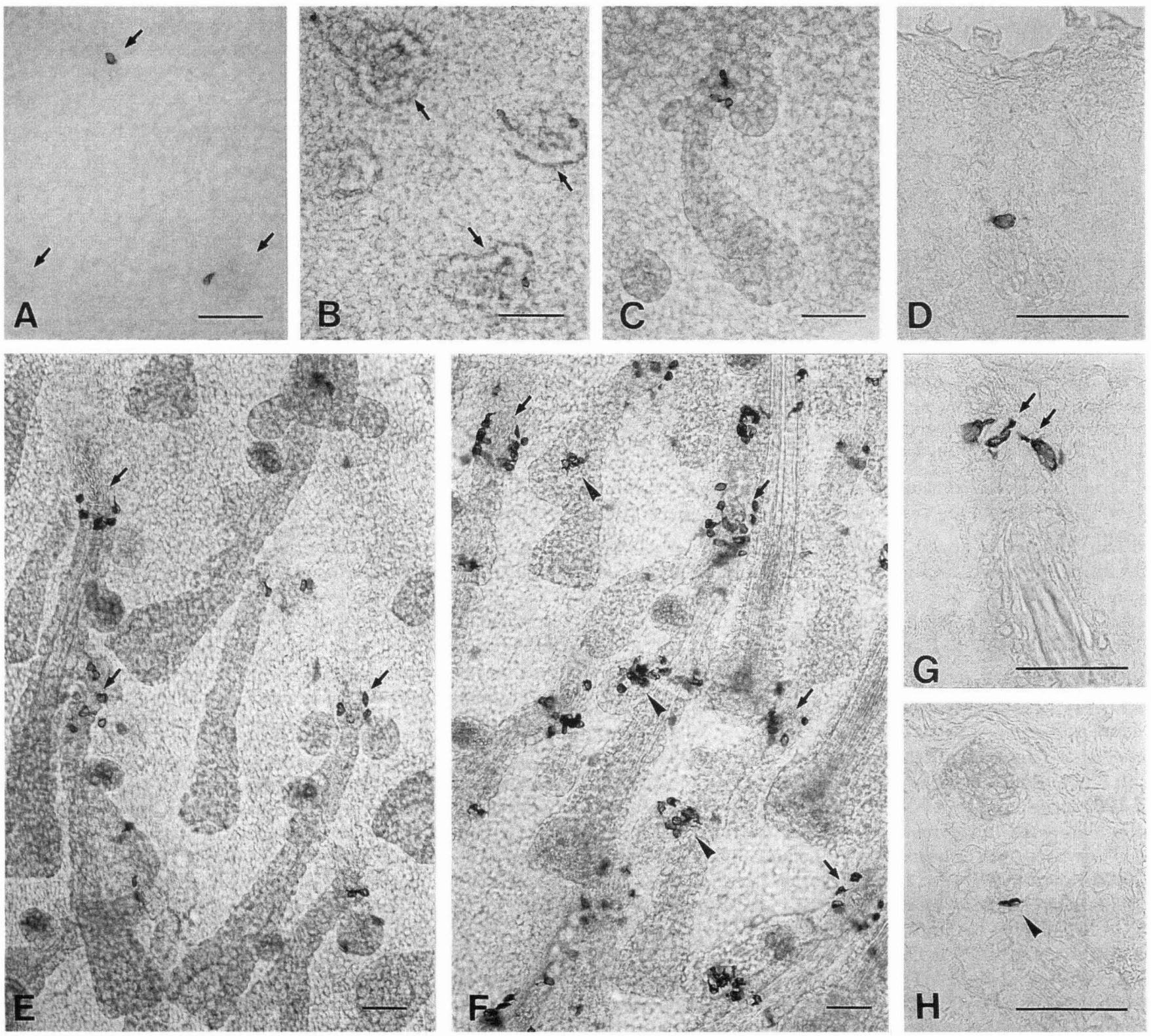

Figure 4. Density and distribution of Merkel cells labeled with anti-CK20 antibody in epidermal sheets and sections of hairy skin. A few cells, mostly related to hair germs (*arrows*), are first seen in the fetus at 75 d EGA (*A*). More Merkel cells are observed primarily in hair germs and pegs (*arrows*) in the 84-d fetus (*B*). In the skin of the fetus at 101 d EGA, Merkel cell aggregates are seen mostly in the infundibulum (*C*). The location is confirmed in sections (*D*). In the epidermis at 117 d EGA, Merkel cell aggregates are observed in the infundibulum (*arrows*) of bulbous hair pegs in trunk skin (*E*) and in the infundibulum (*arrows*) and bulge (*arrowheads*) of advanced hair follicles in dorsal hand skin (*F*). In sections, Merkel cells also are observed in the infundibulum (*G*) and bulge (*H*). *Bars*, 50 μm.

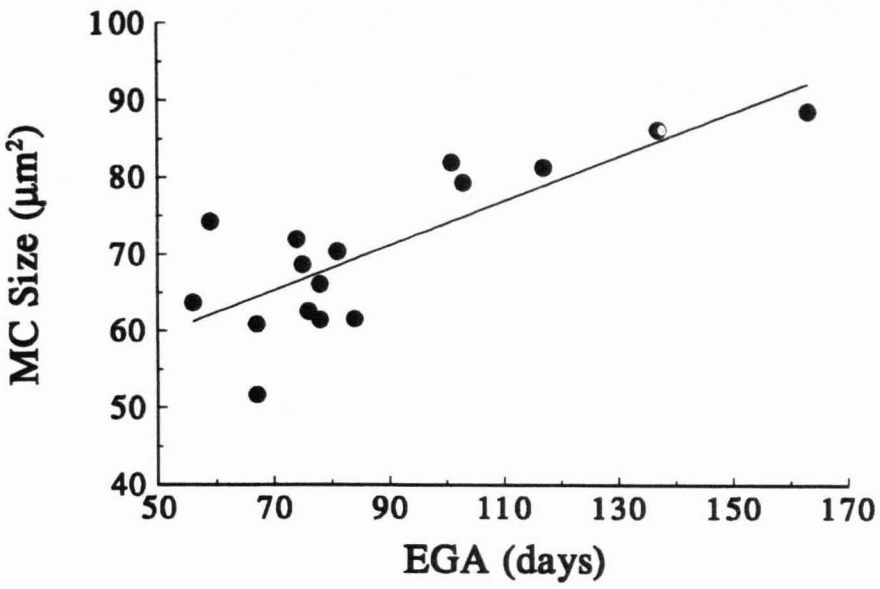

Figure 5. Changes in Merkel cell (MC) size according to the progression of gestational age (EGA). The simple regression test for trend was significant, with t = 5.094, 14 *df*, and p = 0.0002.

DISCUSSION

Simple epithelial cytokeratins, CK8 and CK18, have been used to demonstrate Merkel cells in developing epidermis as early as 10–12 weeks' EGA [2,4,6,13]. However, these cytokeratins are also expressed by basal keratinocytes and periderm [24]. CK20, a major cytoskeletal polypeptide (MW approximately 46 kD) of human intestinal epithelium and uroepithelial cells [20], was detected specifically as a prominent component of epidermal Merkel cells [21,22]. Using CK20 antibody, we observed immunopositive Merkel cells as early as 56 d EGA in palmar epidermis and by 75 d in hairy skin.

Merkel cells are enriched in the glabrous palmar and plantar skin [2,4,7] and in association with hair follicles [8]. Quantitative studies to determine the density of Merkel cells in developing skin are limited. Moll *et al* [2] reported a high density of Merkel cells in plantar skin of human fetuses at 20–24 weeks EGA and considerably decreased density at newborn and adult stages. They suggested that the decrease in Merkel cell density during fetal development may simply reflect the reduced ability of Merkel cells to proliferate. Dermal Merkel cells were first observed at 13 weeks' EGA and increased in number until 17 weeks' EGA, when they represented 17% of total cutaneous Merkel cells [4]. Both of these quantitative studies were performed using serial sections immunostained with antibody to CK18.

Epidermal sheets have been used to observe the density and distribution of Merkel cells [9,25], melanocytes [26], and Langerhans cells [27]. These preparations allow one to map the three-dimensional distribution of Merkel cells in relation to adjacent cells or structures [9] and to count the immunoreactive Merkel cells in different planes of epidermis, thereby permitting a more accurate determination of cell density. Immunolabeled sections also were studied to confirm the spatial relations of cells.

Although tissue processing can alter the dimensions of samples and affect the measurement of cell density [28], our samples were fixed briefly (10 min) and were not subject to dehydration. Any shrinkage would be expected to be minimal.

The temporal relation between the high concentration of Merkel cells and the sweat gland primordia in the initial stage of development may suggest a functional relation or may reflect the highly proliferative potential of epidermis and Merkel cells in the primary epidermal ridges. We observed the highest Merkel cell density in the palmar skin of fetuses at 80–90 d EGA, when primary epidermal ridges are developing in the palms and soles [21].

A high concentration of Merkel cells in the bulge and infundibulum of the hair follicle was first demonstrated in the fetus as early as 16 weeks' EGA [9]. In the present study, Merkel cell distribution in the developing follicles correlated with the stage of follicle development. Merkel cells were few in germs and hair pegs. More than half of the follicles lacked Merkel cells until 105 d EGA, but by 117 d EGA, Merkel cell aggregates were remarkable in the infundibulum of almost all the hair follicles in trunk skin and were observed in both the infundibulum and bulge of the structurally more mature follicles of dorsal hand skin. Therefore, we propose that Merkel cells appear first in the infundibulum and then in the bulge, where they may be involved in active proliferation of cells in those sites or in the development of adjacent structures, such as perifollicular nerves or the arrector pili muscle. Narisawa *et al* [25] reported that the highest concentration of follicular Merkel cells occurred in the bulge of both anagen and telogen follicles of adult skin, where they might stimulate stem cell activity during early anagen. Our results, showing a greater concentration of Merkel cells in the bulge at 117 d EGA and a decreasing concentration in the older fetus, support the idea that Merkel cell function may be more related to active proliferation in initiation of the bulge, possibly through a paracrine interaction with adjacent cells.

Merkel cells in the epidermis [29] and nail fold [10] may act as "target structures" for ingrowing nerve fibers. These interactions may be related to the release of nerve growth factor from Merkel cells [12]. Merkel cells in the bulge or their products may serve as attractants for the growing arrector pili muscle, which contains peripheral nerve fibers [9,25]. Dermal Merkel cells are thought to play an inductive role in the development of the cutaneous nerve plexus in the upper dermis [13]. The disappearance of dermal Merkel cells during later fetal development might reflect the loss of paracrine functions [2]. Merkel cell granules contain vasoactive intestinal polypeptide [14] and substance P [15,16], which stimulate proliferation of keratinocytes [30] and fibroblasts [31], respectively. Thus, it is possible that Merkel cells that are localized in highly proliferative areas of developing skin could have a paracrine influence on the initiation of sweat gland primordia and proliferation of infundibulum and bulge of the hair follicle. The stimulus for localization in these sites is unknown.

Prominent, thread-like dendrites up to 20 μm in length were observed in fetal epidermal Merkel cells but were rare in adult Merkel cells [2]. It was suggested that the dendrites provide communication among the clustered Merkel cells of a glandular ridge during fetal stages of development. In the present study, epidermal Merkel cells were most dendritic in the period when the density was high, both in palmar and in hairy skin. This supports the idea that Merkel cell dendricity is related to active proliferation, with a high mitotic rate and/or activation of paracrine functions in the areas where skin appendages are forming and differentiating.

Changes in the size and shape of other dendritic cells in skin have been reported. In embryonic and fetal epidermis, melanocytes were most dendritic at 80–90 d EGA, when the density was high and mitosis occurred in keratinocytes of all epidermal layers [32]. Langerhans cells were small and truncate in embryonic skin and became larger and more dendritic with increasing gestational age [27]. Newly influxed Langerhans cells in postnatal epidermis also appeared to be much smaller and rounder [33,34]. All the dermal Merkel cells that we observed were globular in shape and located mostly in the upper dermis, suggesting that they had migrated from the epidermis to the dermis.

In conclusion, Merkel cell aggregates in primary epidermal ridges, the infundibulum, and the bulge of hair follicles in the bulbous–hair-peg stage might have an important functional role or may simply reflect active proliferation of Merkel cells and keratinocytes in those areas during the initial development of the sweat gland and hair follicle. Further studies are needed to clarify the role of Merkel cells related to the local formation, growth, and differentiation of skin appendages such as hair follicles, eccrine sweat glands, and nerves. Studies on the production of tissue hormones, cytokines, or growth factors by Merkel cells and the proliferative potential of Merkel cells might be helpful to unveil new functions of this peculiar dendritic cell of the skin.

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