Normal Human Merkel Cells are Present in Epidermal Cell Populations Isolated and Cultured from Glabrous and Hairy Skin Sites

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The Merkel cell is a highly specialized cell that primarily acts as a slowly adapting mechanoreceptor. Merkel cells are scarce in normal skin but can be identified by the expression of distinct keratin filaments. Merkel cells constitute a very unique population and many questions still remain as to their origin, number, proliferative capacity, and functions in cutaneous biology. The dissociation of epidermal cells from skin is a widely used technique to extract and culture keratinocytes. We took advantage of a two-step extraction method to quantify keratin-20-expressing Merkel cells among total cutaneous cells obtained from either hairy or glabrous skin biopsies. Flow cytometry analysis revealed that keratin-20-labeled Merkel cells represent between 3.6% and 5.7% of freshly dissociated basal epidermal cells. No significant differences were seen between samples derived from glabrous palmar and hairy anatomic sites, from children and adult, respectively. We also report on the presence of Merkel cells in primary and first subcultures of epidermal cells indicating their capacity to remain viable after extraction from skin of various anatomic sites. To our knowledge, this is the first demonstration of nontumorigenic human Merkel cells in culture *in vitro*. The persistence of a small number of Merkel cells in culture suggests that, with the development of appropriate culture conditions, these cells could be amplified and further studied to unravel long-standing questions relative to their paracrine function or epithelial origin. *Key words: culture/human skin/keratins/mechanoreceptor/Merkel cells. J Invest Dermatol 120:313–317, 2003*

Skin is the principal barrier protecting us against environmental aggression. As such, this organ is particularly receptive to a wide range of stimuli such as pain, temperature, pressure, and vibration. These stimuli are sensed by many cutaneous sensitive nerves and various tactile corpuscules (Meissner, Ruffini, Pacini) (Holbrook and Wolff, 1987). Merkel cells are specialized skin sensory receptors that contribute to the touch perception. By their strategic localization at the dermo-epidermal junction where most of them are in close contact with dermal sensory nerve endings, the Merkel cell-neurite complex primarily acts as a slowly adapting type I mechanoreceptor (Tachibana, 1995).

The Merkel cell population possesses many interesting features such as coexpression of dense-core neurosecretory granules, keratin (K) filaments, and desmosomal proteins. A reliable method to detect Merkel cells *in situ* is based on their reactivity with antibodies directed against keratin filaments, namely the simple epithelial K8, K18, and K20 (Moll *et al*, 1984; Moll, 1994; Fradette *et al*, 1995). Among these keratins, K20 is now considered the marker of choice for Merkel cell detection as it is the only keratin expressed uniquely by Merkel cells in both fetal and adult skin (Moll, 1994; Moll *et al*, 1995).

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Merkel cells are usually scattered along the dermo-epidermal junction of the epidermis. Within the hair follicle structure, they are mainly found in the outer root sheath layer of the isthmus region and sometimes extend to the bulge area (Lacour *et al*, 1991; Narisawa *et al*, 1993; 1994; Moll, 1994; Fradette *et al*, 1995) where follicular stem cells, as defined by various criteria, have been localized (Cotsarelis *et al*, 1990; Jones *et al*, 1995; Michel *et al*, 1996; Lyle *et al*, 1998; Morris and Potten, 1999). The presence of Merkel cells in proximity to follicular stem cells, combined with the potent neuropeptides contained in their granules, lead to the hypothesis that Merkel cells could be more than tactile receptors and also have a paracrine role in the induction and differentiation of epidermal appendages (Moll *et al*, 1984; Morohunfola *et al*, 1992; Narisawa *et al*, 1992).

Although the distribution and three-dimensional organization of Merkel cells in epidermis and hair follicles have been well described *in situ* (Lacour *et al*, 1991; McKenna Boot *et al*, 1992; Moll, 1994; Narisawa *et al*, 1994), the quantification of minor cell populations remains a difficult task. We took advantage of our isolation procedure that dissociates cells from both the epidermis and the hair follicles (Germain *et al*, 1993; 1997) to quantify Merkel cells in populations of epidermal cells freshly dissociated from human skin.

In this paper, we determined the percentage of Merkel cells present in freshly dissociated populations of epidermal cells from glabrous and hairy anatomic sites using immunohistochemical staining and flow cytometry. No significant difference in the number of Merkel cells was observed between the palmar/plantar, breast, and scalp anatomic sites investigated. We also

examined the presence and fate of Merkel cells in primary and first subcultures of epidermal cells derived from these anatomic sites. We report for the first time on the presence of normal human Merkel cells *in vitro*, among keratinocyte monolayer cultures.

MATERIALS AND METHODS

Tissues and epidermal cell extraction method Human glabrous skin from children (palmar/plantar; mean age ± SD 1.6 ± 0.5 y) or hairy skin biopsies from adults (breast 40.6 ± 12.4 y and scalp 46.5 ± 8.2 y) were obtained after plastic reconstructive surgery. Skin (3–9 cm^2) biopsies were incubated overnight at 4°C or for 3 h at 37°C in a 500 μg per ml thermolysin solution (Germain *et al*, 1993). The epithelium (epidermis and hair follicles) was separated from the underlying dermis with fine forceps and the epidermal cells were dissociated with trypsin for 15 min at 37°C. The freshly isolated epidermal cell suspensions were seeded for cell culture and/or fixed with 70% cold ethanol for flow cytometry analysis.

Cell culture, immunochemistry, and flow cytometry analysis Epidermal cells were plated on a mouse irradiated 3T3 fibroblast feeder layer in culture flasks (27,000 cells per cm^2) or on coverslips (80,000 cells per cm^2) and cultured in complete Dulbecco's modified Eagle's HAM supplemented with 10% Fetal Clone II serum (Hyclone, PDI Bioscience, Aurora, Ontario, Canada) (Rheinwald and Green, 1975; Germain *et al*, 1993; Michel *et al*, 1996). For the labeling and enumeration of Merkel cells in first subculture (P1), primary cultures (P0) grown in tissue culture flasks were trypsinized and cells were seeded on coverslips for the subculture. Ethanol-fixed cultured cells (10 min at –20°C) were labeled by indirect immunofluorescence as described previously (Michel *et al*, 1996) using the antibodies listed below. Each coverslip was examined and K20-labeled cells were carefully enumerated. Cell nuclei were counterstained with Hoechst reagent 33258 (Sigma, Oakville, Ontario, Canada). Negative controls consisted of omission of the primary antibody during the labeling reaction. Flow cytometry analysis was carried out as previously reported (Michel *et al*, 1996). Briefly, freshly isolated cells were labeled with anti-K20 or VM2 antibodies and then analyzed on a FACScan (Becton Dickinson, San Jose, CA) with 20,000 events acquired for each sample. The cell population was gated to exclude false positive differentiated cells and cell debris (channels 80–200 were kept, forward scatter). Each biopsy was analyzed multiple times and the number (n) of biopsies were $n = 8$ for child palmar skin, $n = 5$ for adult breast skin, and $n = 4$ for adult scalp skin. The antibodies used included mouse monoclonal anti-human K20 (IT-Ks 20.10, American Research Product, Belmont, MA) and mouse monoclonal anti-α3 integrin subunit conjugated to fluorescein isothiocyanate (FITC) for direct labeling (VM2, clone #HB-8530 ATCC, Manassas, VA). A phycoerythrin-conjugated goat anti-mouse secondary antibody was used for detection of K20 (Jackson, West Grove, PA). Negative controls consisted of omission of the primary antibody during the labeling reaction. FITC-conjugated mouse anti-KLH (Becton Dickinson) or 1% bovine serum albumin in phosphate-buffered saline were used as negative controls for direct staining experiments. HT-29 cells (ATCC) were used as positive controls as these cells express K20. Cells were observed under a Nikon Optiphot microscope equipped with epifluorescence or with a Bio-Rad MRC-1024 confocal imaging system equipped with a krypton–argon laser beam and mounted on a Nikon Diaphot-TMD. Series of 0.1 μm optical sections were obtained with the 60 × objective of the confocal microscope.

Electron microscopy Epidermal cell cultures dissociated from scalp skin were fixed with formaldehyde (1%, 30 min at room temperature) and methanol (10 min at –20°C), labeled with anti-K20, and revealed with peroxidase using the Ultra HRP detection system (ID Labs, London, Ontario, Canada) and incubation with 3-3′-diaminobenzidine (Sigma). Cells were then postfixed with 2.5% glutaraldehyde, washed with 0.1 M cacodylate buffer, and postfixed with 1% OsO_4 for 30 min. Regions containing Merkel cells were identified and embedded in LRWhite. Thin sections stained with uranyl acetate and lead citrate were observed with a JEOL 1200 EX transmission electron microscope.

Statistical analyses A nonparametric Mann–Whitney test was used for statistical analyses. In all statistical tests, the confidence interval was set to 95% ($p < 0.05$).

RESULTS

Quantification of K20-positive Merkel cells among cutaneous cells dissociated from glabrous and hairy anatomic sites The two-step epidermal cell isolation method designed for the isolation of keratinocytes free of fibroblasts and routinely used for massive cell amplification for the production of epithelial sheets suitable for grafting (Green *et al*, 1979; Germain *et al*, 1993) also allowed extraction of Merkel cells from epidermis as well as hair follicles (Fradette and Germain, data not shown). To quantify Merkel cells, the percentage of K20-expressing cells among the epidermal cells freshly dissociated from glabrous (palmar/plantar) or hairy (breast, scalp) skin biopsies was determined. Flow cytometry analysis confirmed that Merkel cells represent a minor population of the total epidermal cells, with 1.5% ± 0.5% of K20-expressing cells for samples isolated from children glabrous skin and 1.6% ± 0.9% for hairy skin biopsies from adults (**Fig 1**). To ensure adequate comparisons between the dissociated cell populations obtained from different body sites, the percentage of basal cells was determined for each sample by labeling with the VM2 antibody, which recognizes the α3 integrin subunit expressed by basal cells of the epidermis and outer root sheath of hair follicles (Kaufmann *et al*, 1989; Jones *et al*, 1995; Michel *et al*, 1996). The percentages of freshly dissociated cells that express K20 relative to the percentage of basal cells found in the same sample were 3.6% ± 0.6% for child palmar skin ($n = 8$), 5.7% ± 2.4% for adult breast skin ($n = 5$), and 4.8% ± 3.8% for adult scalp skin ($n = 4$). No statistical difference in the percentage of Merkel cells extracted from hairy skin biopsies from adults or from child glabrous skin biopsies was observed.

Human Merkel cells are present within cultures of epidermal cells *in vitro* By seeding dissociated epidermal cells at high density on glass coverslips with a 3T3 feeder layer, we have been able to detect Merkel cells in plantar epidermal cultures by immunostaining with K20 (**Fig 2*A***). The K20-expressing cells were usually seen as single cells, located between keratinocyte colonies. The scarce K20-labeled cells were brightly stained and exhibited abundant bundles of keratin filaments in their cytoplasm, as observed by confocal microscopy (**Fig 2*G***). With increasing culture time, the morphology of K20-labeled cells changed from roundish (days 1–2) to more flattened (days 3–8). Sometimes, short processes extended from the periphery of the cells (**Fig 2*A,C,D***). After 8 d of primary culture onto glass coverslips, Merkel cells showed no sign of nuclear disorganization characteristic of apoptosis. Merkel cells were not only identified within the primary cultures derived from palmar skin of young children but also in cultures established from adult breast and scalp skin (**Fig 3*A***). Their numbers were variable, even

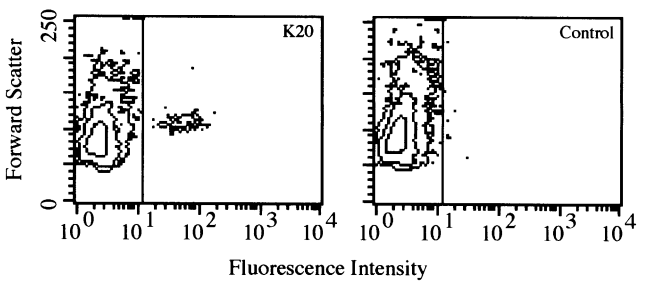

Figure 1. Flow cytometry analysis of K20-expressing cells in a population of epidermal cells extracted from human scalp skin. Flow cytometry data showing that K20-expressing cells represent 1.4% of the total cell population freshly isolated from scalp skin of a 53-y-old woman. Cells were labeled with an anti-K20 antibody and analyzed on a FACScan (Becton Dickinson) as described in *Materials and Methods*. For control, the primary antibody was replaced by 1% bovine serum albumin in phosphate-buffered saline.

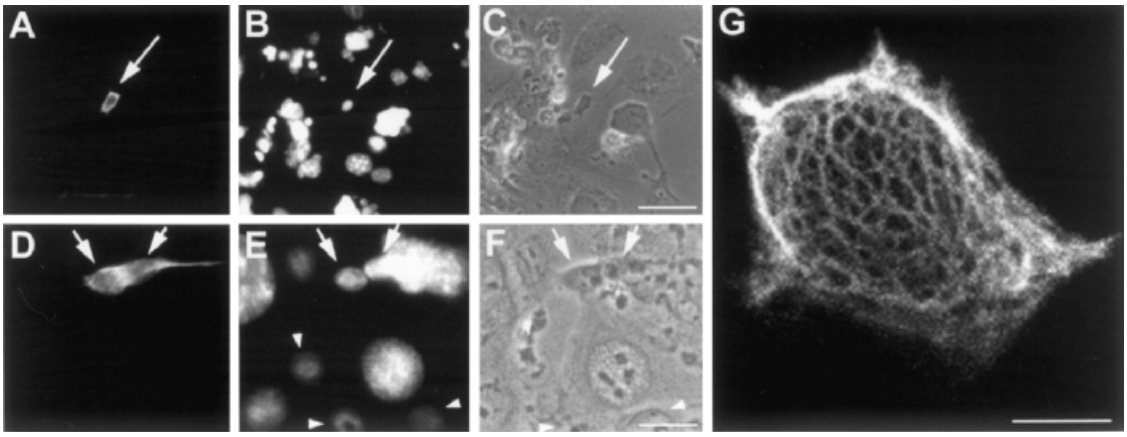

Figure 2. Human Merkel cells are present *in vitro* among monolayer cultures of keratinocytes. Primary cultures (day 5) of epidermal cells derived from plantar skin were stained with an anti-K20 antibody (*A*, *D*, *G*). Human cells (*arrowheads*) are distinguished from the murine 3T3 feeder layer by the uniform appearance of their nuclei revealed by Hoechst staining (*B*, *E*). (*C*), (*F*) are phase-contrast micrographs corresponding to (*A*), (*D*), respectively. (*D*) Doublet of K20-labeled Merkel cells in primary culture of cells derived from finger skin. (*G*) Labeled cells reveal bundles of cytoplasmic K20 intermediate filaments as seen by confocal microscopy. *Scale bars*: (*A*)–(*C*), 47 μm; (*D*)–(*F*), 19 μm; (*G*) 5 μm.

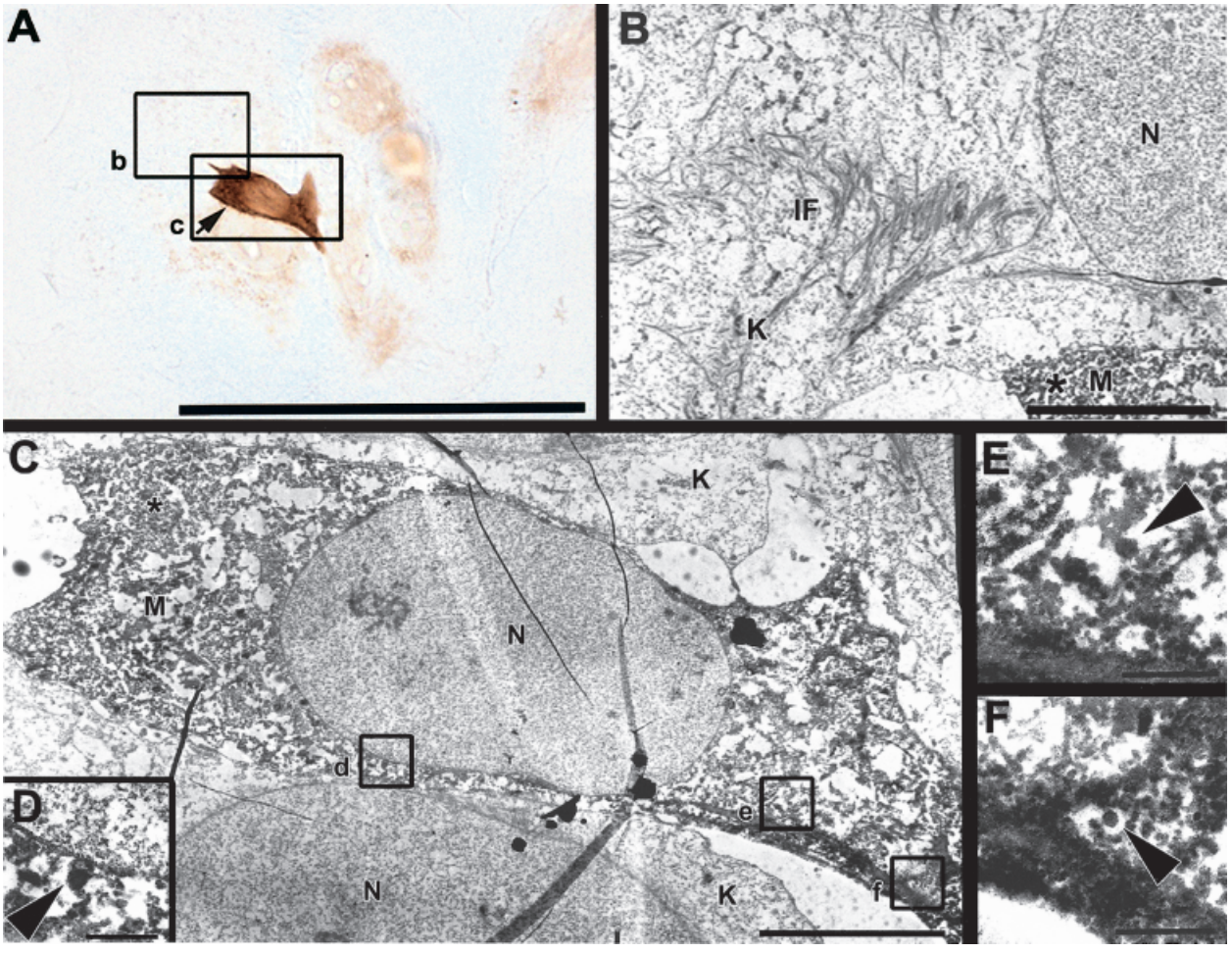

Figure 3. Electron microscopy analysis of K20-expressing cells *in vitro*. Follicular Merkel cells are extracted using the two-step epidermal cell isolation procedure and are present in culture. (*A*) Epithelial cell colony in culture derived from human scalp skin. The electron microscopy images (*B*), (*C*) represent the regions delimited by *b* and *c* on the light microscopy image in (*A*). (*D*)–(*F*) are magnifications of the regions *d*–*f* of (*C*). The cytoplasmic dense peroxidase deposits (asterisk) allowed the identification of K20-expressing cells at the electron microscopy level (*C*). In contrast, large keratinocytes presented a light cytoplasm containing an extensive network of keratin filaments (*B*). The presence of discrete dense-cored neurosecretory granules (*arrowheads*, *D*–*F*) in the cytoplasm of the K20-labeled cell indicates that they are Merkel cells. IF, intermediate filaments; K, keratinocyte; M, Merkel cell; N, nucleus. *Scale bars*: (*A*), 100 μm; (*B*), (*C*), 5 μm; (*D*)–(*F*), 500 nm.

between samples from a given anatomic site (**Table I**). However, a significantly higher number of K20-expressing Merkel cells was detected in primary cultures derived from finger skin, however, compared to breast or scalp skin ($p < 0.001$) (**Table I**). To determine if Merkel cells were undergoing cell division *in vitro*, coverslips were fixed at various time points of culture and stained by immunocytochemistry; the number of K20-expressing cells was carefully enumerated under the microscope

Table I. Merkel cells are detected in primary and first subculture of epidermal cells derived from different anatomic sites

	Primary culture (P0)			First subculture (P1)	
Skin biopsy	d[a]	d + 2	d + 4	d	d + 2
Finger 1	51.7[b] (11.9) ($n = 6$)[c]	42.8 (7.6) ($n = 6$)	nd	1.3 (0.8) ($n = 6$)	1.3 (1.9) ($n = 6$)
Finger 2	16.3 (6.9) ($n = 4$)	31.3 (11.1)* ($n = 4$)	nd	nd	nd
Breast 1	1.2 (1.0) ($n = 6$)	1.5 (1.3) ($n = 4$)	1.0 (1.4) ($n = 5$)	0 ($n = 5$)	nd
Breast 2	2.5 (1.3) ($n = 4$)	6.8 (2.8)* ($n = 5$)	5.0 (0.0)* ($n = 3$)	0.5 (0.7) ($n = 2$)	2.0 (1.4) ($n = 2$)
Scalp	9.4 (3.8) ($n = 14$)	8.3 (3.6) ($n = 12$)	11.6 (5.3) ($n = 8$)	1.6 (1.2) ($n = 12$)	0.8 (0.8) ($n = 12$)

[a]Labeled cells on coverslips were counted on day 2 or 3 after seeding (d = day) and 2 d later (d + 2).
[b]Mean number of K20-expressing cells per coverslip in primary and first subculture for different anatomic sites. Data are mean ± standard deviation (SD); nd, not determined.
[c]*n*, number of coverslips (22 × 22 mm) analyzed at each time point.
*Values significantly different at d + 2 and d + 4 than at d ($p < 0.04$) for each biopsy.

(**Table I**). A small but significant increase of Merkel cells during primary culture was seen for two of the five cultures examined, namely for cultures derived from finger and breast skin (**Table I**, Finger 2 and Breast 2). Doublets of positive cells such as the one depicted in **Fig 2(*D*)–(*F*)** were observed on rare occasions in these cultures. Merkel cells were present in all subcultures (P1) examined except one (**Table I**). From these observations, it is unclear if the K20-labeled cells identified at passage 1 arose from limited proliferation of Merkel cells or were passively transferred during subculture.

To confirm the identification of Merkel cells, peroxidase-labeled K20-expressing cultured cells (**Fig 3*A***) were processed for electron microscopy. Peroxidase-labeled cells were identified at the electron microscopy level by their cytoplasm being more contrasted due to the labeling (**Fig 3*C***) compared to adjacent keratinocytes (**Fig 3*B, C***). Keratinocytes were round, large, and presented an extensive network of keratin filaments (**Fig 3*B***). The typical polygonal shape of the Merkel cell seen at the light microscopy level (**Fig 3*A***) was identified at the electron microscopy level (**Fig 3*C***). The presence of discrete dense-cored granules in the cytoplasm of K20-expressing cells (**Fig 3*D–F***) indicates that they are Merkel cells.

DISCUSSION

Merkel cells constitute a minor population of the skin epithelium. We report here that they can be extracted and quantified from all body sites examined whether hair follicles of different sizes are present or not. The first step of the isolation method allows skin separation at the dermo-epidermal junction by thermolysin digestion, resulting in an epithelial portion comprising both epidermis and complete hair follicles that have retained the Merkel cells (Fradette and Germain, data not shown). The second step of this method involves dissociation with trypsin and therefore the formation of a single-cell suspension suitable for quantification by flow cytometry and subsequent culture. Therefore, this methodology allowed us to evaluate the total number of Merkel cells (epidermal and follicular) in biopsies from different anatomic sites. Interestingly, there was no significant difference between the percentages of Merkel cells in dissociated cells derived from hairy skin (adult breast or scalp) compared to glabrous skin (child palmar/plantar), the latter being considered to have a greater density of Merkel cells per square millimeter of basal layer (Lacour *et al*, 1991; McKenna Boot *et al*, 1992). The high number of Merkel cells we obtained from adult hairy skin sites is probably due to the contribution of Merkel cells present in many small vellus hair follicles (breast) and terminal hairs (scalp), which are easily extracted by our technique (Germain *et al*, 1997). As skin presents a complex architecture in which Merkel cells are not distributed regularly, it is difficult to obtain representative quantification from tissue sections. Using flow cytometry for the quantification of Merkel cells among freshly dissociated cells offers the unique advantage of analyzing a large number of cells (isolated from a relatively large skin area: up to 9 cm^2). Our data indicate that follicular Merkel cells are an important contribution to the total number of Merkel cells present in hairy anatomic sites.

In vitro, scarce K20-labeled cells were identified within monolayer cultures of epidermal cells dissociated from either glabrous or hairy skin biopsies. These cells exhibited an organized keratin filament network in their cytoplasm, as shown by confocal microscopy (**Fig 2*G***). Moreover, when these K20-labeled cells were observed by electron microscopy, the presence of discrete dense-cored neurosecretory granules could be seen (**Fig 3*D–F***). The concomitant presence of keratins and neurosecretory granules is the hallmark of Merkel cells *in situ* (Moll *et al*, 1984; Hartschuh and Weihe, 1988; Tachibana, 1995). Although cells from human Merkel cell carcinomas have been successfully cultured (Leonard *et al*, 1993; Moll *et al*, 1994), nontransformed Merkel cells of human origin have never been reported before *in vitro*. To the best of our knowledge, only two other papers on the culture of normal Merkel cells have been published (Vos *et al*, 1991; Fukuda, 1996), in which the cells were isolated from rat skin. In 1996, Fukuda established monolayer cultures of purified newborn rat Merkel cells that survived for more than 2 wk. He also demonstrated that serum was required to prevent apoptotic death *in vitro* (Fukuda, 1996). Previously, Vos and colleagues had established cocultures of embryonic rat Merkel cells with neurons (Vos *et al*, 1991). Interestingly, they reported a significant increase of the quinacrine-labeled rat Merkel cell population over time in culture. In this study, we observed cell doublets, suggesting that proliferation of human Merkel cells could occur and be stimulated under improved culture conditions (**Fig 2*D*, Table I**). Two out of five cultures demonstrated a small but significant increase in the number of K20-expressing cells during the primary culture. Whether this increase is due to the proliferation of Merkel cells or to their generation from precursor cells present within the cultures remains to be determined. It appears that the culture conditions used in these experiments (10% serum, 3T3 feeder layer, presence of keratinocytes) provided an adequate environment to sustain Merkel cell viability. The development of optimized culture conditions for the human Merkel cell population, with regard to their requirements for proliferation and the mechanisms underlying their generation from precursor cells, would bring answers to long-standing questions about the origin of Merkel cells and their ability to undergo mitosis.

Upon further enrichment, the presence of Merkel cells in culture could be beneficial for patients as a high number of Merkel cells in cultured epithelial sheets grafted to extensively burned patients could stimulate re-innervation and help them regain better sensory functions at targeted grafted sites. With the rapid progress made in the fields of tissue engineering (Auger *et al*, 2000), transplantation, and gene therapy, tissue reconstruction using the various cell types found in skin holds great promise and could result in the production of skin substitutes tailored for particular needs and specialized applications.

In conclusion, by the combination of a suitable extraction method using thermolysin and trypsin, adequate detection using K20, and appropriate culture conditions, we have been able to quantify the total number of human Merkel cells (epidermal and follicular) present in a normal skin biopsy and detect them for the first time *in vitro*. Further optimization of culture conditions for human Merkel cells will probably provide a unique *in vitro* model to gain insight into the functions and controversial features of these particular cells.

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