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Immunohistochemical Demonstration of the Expression of Neurofilament Proteins in Merkel Cells

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The presence of immunoreactive neurofilament proteins has previously been reported in Merkel cell carcinomas but not in normal human epidermal and dermal Merkel cells. We have studied the immunoreactivity of epidermal Merkel cells for neurofilament triplet proteins (68 KD, 70 KD, 160 KD, 200 KD), using epidermal sheets prepared from the plantar skin of human adults, which enabled us to survey large numbers of Merkel cells. Neurofilament protein 200 KD-positive cells were readily identified, while neurofilament protein 68 KD-, 70 KD- and 160 KD-positive cells were largely absent. 200 KD-positive cells in the epidermis were confirmed to represent Merkel cells by a sequential immunoenzyme labeling for the simple epithelial type cytokeratin (No. 8). 200 KD-positive cells were 5.9% of the total number of epidermal Merkel cells. Despite a heterogeneous expression of neurofilament protein subspecies between the normal and transformed Merkel cells, the presence of neurofilament proteins in epidermal Merkel cells may link them to Merkel cell carcinomas. ***Key word: Merkel cell carcinoma.***

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Five classes of intermediate filaments include vimentin, cytokeratins, desmin, neurofilaments and glial filament protein. Each of these has been established as a marker of differentiation in various cells (1–3). The type of intermediate filament defines the origin or function of the cell (4, 5). Recent Merkel cell investigations in our laboratory (6–8), as well as in others (9, 10), strongly support an epidermal origin of Merkel cells. Both normal Merkel cells and Merkel cell carcinomas contain cytokeratins. On the other hand, the neural nature of Merkel cells cannot be ignored, since they are closely associated with nerve endings and express nerve growth factor receptor during a short period of the first trimester when the formation of dermal nerve plexus is guided by them (8). Neurofilaments (NF) are neuronal cytoplasmic intermediate filaments and consist of a triplet of polypeptides with molecular weights of about 70 KD, 160 KD and 200 KD (11). Although NF are said to be absent from rodent epidermal Merkel cells (12, 13) as well as human fetal Merkel cells (10), both the presence and the absence of immunoreactivity for NF have been reported in Merkel cell carcinomas (5, 14–18). Because normal Merkel cells fail to express NF, it has been argued that the carcinomas expressing NF epitopes cannot be derived from Merkel cells. In the present study, we used epidermal sheets instead of vertical sections to observe a large area and chose adult human plantar skin where the Merkel cell population is large. We demonstrated that some Merkel cells are reactive for CAM 5.2, a well-established human Merkel cell marker (8), and simultaneously express the immunoreactivity of NF.

MATERIAL AND METHODS

Normal plantar skin was obtained from the normal margins of excisional skin biopsy specimens from 5 adult patients during routine surgical procedure. The patients had trauma, pigmented nevus and malignant melanoma. The skin was placed in phosphate-buffered saline (PBS), pH 7.3, at room temperature and then incubated in 20 mM ethylenediaminetetraacetic acid (EDTA) in PBS overnight at 4°C. The epidermal sheets were gently removed with forceps and rinsed in PBS. They were then fixed in Zamboni's fixative overnight at 4°C and washed at 4°C for 24 h in several changes of PBS. Other epidermal sheets were fixed with cold acetone for 30 min.

The epidermal sheets fixed with Zamboni's fixative or acetone were immunostained for monoclonal murine antibodies using the avidin-biotin-peroxidase complex (ABC) (VectorKit, Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB) coloration technique. The two fixatives were equally good. The following antibodies were used: (i) monoclonal murine antibodies neurofilament 68 KD (NR4) (SIGMA), 70 KD (CHEMINON, Temecula, CA), 160 KD (NN18) (SIGMA) and 200 KD (N52) (SIGMA); (ii) monoclonal murine antibody CAM 5.2 specific for the simple epithelial type cytokeratin No. 8 (52.5 KD) (Becton-Dickinson, San Jose, CA) (19). CAM 5.2 has been established as a suitable marker for Merkel cell since it labels all cutaneous Merkel cells at light and immunoelectron microscopic levels (7). The epidermal sheets were incubated overnight with primary antibodies at 4°C, stained immunohistochemically with biotinylated anti-mouse IgG horse serum as secondary antibody and avidin-biotin-peroxidase complex as third reagent. The peroxidase color reaction was developed in the presence of DAB and washed with PBS. Controls were run by substituting primary antibodies with PBS.

In order for us to verify the coexpression of NF and cytokeratins on normal Merkel cells, a sequential immunoenzyme labeling procedure was carried out using epidermal sheets. First, the epidermal sheets were immunostained with CAM 5.2 as a marker for Merkel cells, followed by goat anti-mouse immunoglobulin conjugated to FITC. Immunopositive cells were photographed by a Nikon fluorescence microscope. Antibody complexes were eluted with glycine-hydrochloric acid buffer (pH 2.2) (20). After the removal of immunofluorescence was confirmed, an ABC method against NF was carried out on the same epidermal sheets. No cross-reactivity between the developing reagents of the second immunostaining and the antibody of the first was observed in control sections.

The numerical frequency was estimated by counting CAM 5.2 (+) and NF (+) Merkel cells per cm^2.

RESULTS

On the epidermal sheets of plantar skin numerous CAM 5.2-reactive Merkel cells were strongly stained (Fig. 1). They had an oval, round, polygonal, bipolar, or triangular appearance with occasional short dendrites. They were not regularly distributed but grouped in various arrangements and linear distribution (Fig. 1).

Cells with NF 200 KD immunoreactivity were sparsely observed (Fig. 2). NF 160 KD, 70 KD and 68 KD immunoreactivi-

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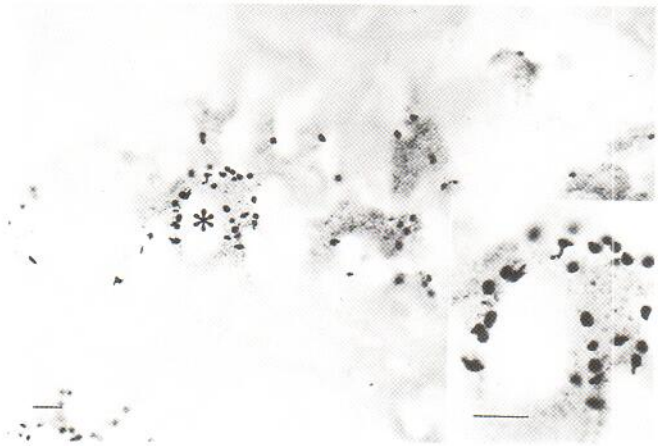

Fig. 1. Distribution pattern of CAM 5.2-reactive Merkel cells on an epidermal sheet of plantar skin of a human adult. Ir-Merkel cells are irregularly grouped. *Insert*: high magnification of the area indicated by * in the main picture. Merkel cells appear as round to polygonal cells with variable dendrites. Three-dimensional views stained with CAM 5.2. Same specimen as used for Figs. 2 and 3. *Bars*, 100 μm.

ties were found only in a few cells. The distribution pattern of CAM 5.2-reactive Merkel cells was similar to that of NF 200 KD-positive cells.

A sequential immunostaining was carried out on the epidermal sheets of plantar skin, where Merkel cells are numerous and easy to find. All NF 200 KD-reactive cells were also positive for CAM 5.2, while CAM 5.2-reactive Merkel cells did not necessarily express NF 200 KD immunoreactivity (Fig. 3). NF 200 KD is expressed in approximately 5.9% of CAM 5.2-positive cells. The staining reaction of NF 200 KD was weaker than that of CAM 5.2; there is a possibility that some of the weakly stained NF 200 KD-positive cells were missed. NF 68 KD, NF 160 KD and NF 200 KD were detected in 0.16%, 0.24% and 5.9% of CAM 5.2 (+) epidermal Merkel cells, respectively.

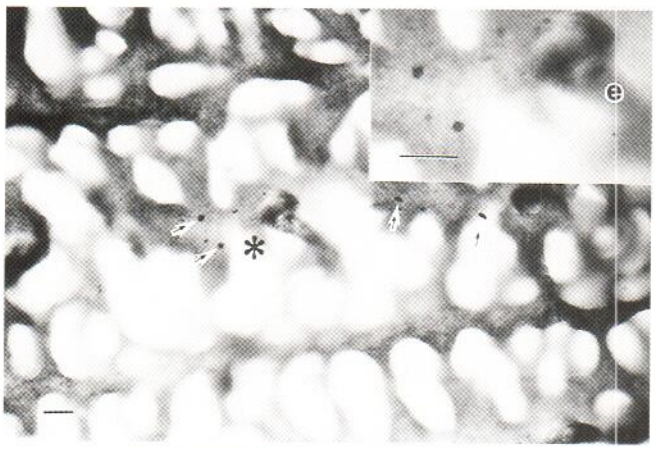

Fig. 2. Distribution pattern of NF (200 KD) immunoreactivity on an epidermal sheet of plantar skin of a human adult. NF (200 KD) immunoreactive cells (*arrows*) are sparsely distributed. *Insert*: high magnification of the area indicated by * in the main picture. *e*; eccrine ridge. Three-dimensional views stained with NF (200 KD). Same specimen as used for Figs. 1 and 3. *Bars*, 100 μm.

DISCUSSION

Depending upon the species, Merkel cells have been shown to be immunoreactive for intermediate cytokeratin polypeptides Nos. 8, 18 and 19 (10, 12), neuron-specific enolase (21), chromogranin A (22) and numerous polypeptides such as vasoactive intestinal polypeptides, peptide histidine isoleucine, bombesin and enkephalin (14, 22). No NF epitope has been identified in normal Merkel cells of any species (10, 12, 14). This may be due to the sensitivity of the methods employed by previous workers. On the other hand, NF immunoreactivity was recently found in Merkel cell carcinoma (23, 24). A limited number of Merkel cells may be difficult to detect in vertical sections. The epidermal sheets prepared from human plantar skin contained numerous Merkel cells and enabled us to examine a large number of Merkel cells for immunoreactivity to NF. As a result, the expression of NF 200 KD immunoreactivity was definitely demonstrated in 5.9% of normal Merkel cells. Weakly immunoreactive NF 200 KD (+) cells may have been missed. Coexpression of cytokeratin and vimentin filaments is known to exist but has not been shown in the case of cytokeratin and NF. This is the first report showing that some Merkel cells express immunoreactivity for both.

In a number of neuroendocrine neoplasms, including Merkel cell carcinomas (24), carcinoid tumors (25), pancreatic islet cell tumors (26) and medullary thyroid carcinomas (27), coexpression of NF and cytokeratin has been reported. Since the epidermis is derived from ectoderm closely associated with neuroectoderm, the coexistence of cytokeratin and NF in normal Merkel cells may indicate neural differentiation in a group of cells originally mixed from the neuroectoderm in the germ cells of the epidermis.

It has been reported that NF 68 KD and 160 KD were positive in Merkel cell carcinomas, whereas only a trace amount of NF protein 200 KD was detected by immunocytochemical and bio-

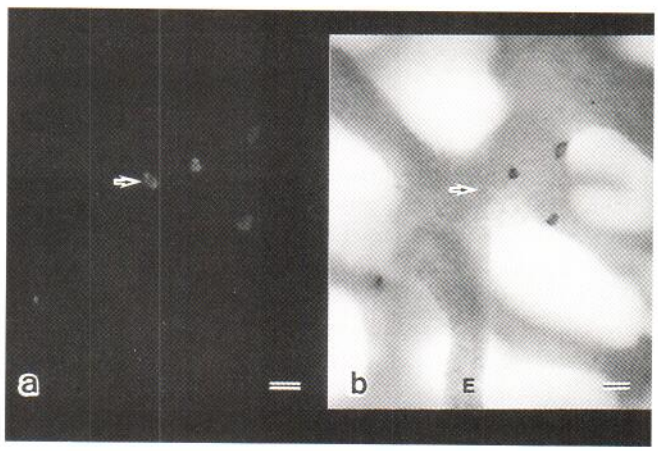

Fig. 3. The same epidermal sheet of plantar skin of a human adult (*a* and *b*) is sequentially stained with immunofluorescence against CAM 5.2 (*a*) and against NF 200 KD (*b*). Among CAM 5.2-reactive Merkel cells (*a*) the majority are simultaneously NF (+) (*b*), while one is CAM 5.2 (+). The Merkel cell indicated by an *arrow* (*a*) is NF (−) (*b*). The photomicrograph shows an area with a large number of 200 KD-positive cells. *E*; eccrine duct. Same specimen as used in Figs. 1 and 2. CAM 5.2 is labeled by FITC and NF 200 KD by peroxidase, respectively. Three-dimensional views (*a* and *b*). *Bars*, 20 μm.

chemical methods (24). This situation is reversed in normal skin, i.e. NF protein 200 KD-positive Merkel cells were easily found, while only a few NF protein 68 KD-, 70 KD- and 160 KD-positive cells were found in the present study. It is postulated that despite a heterogeneous expression of NF subspecies between normal and transformed Merkel cells, the presence of NF in epidermal Merkel cells links them to Merkel cell carcinomas.

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