

The Nerve-dependency of Merkel Cell Proliferation in Cultured Human Fetal Glabrous Skin

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Merkel cells are thought to function as slowly adapting mechanoreceptors and are known as targets for sensory nerves. However, the nerve-dependency of Merkel cells remains controversial. In this respect, some investigators have found inter-regional differences between hairy and glabrous skin and others have shown intraregional differences within denervated rat touch domes. Differences between species have also been reported.

This study was performed to determine whether Merkel cells proliferate *in vitro* in the absence of the systemic factors, blood vessels and the intact nerves in human skin. Suspension organ culture was performed using fetal digits to investigate their *in vitro* proliferation. Merkel cells and cutaneous nerves were identified using antibodies to cytokeratin 20 and protein gene product 9.5 (PGP 9.5), respectively. Fetal digits of 56-82 day gestational age were cultured in serum free medium in a high O₂ (45%) environment. Tissues were harvested before starting culture (D0) and 1,4,7,14, 28d after culture. Merkel cells were observed in the volar pads and dorsal nail matrices at D0. After 28d of suspension organ culture, digits looked healthy structurally and the number of Merkel cells had increased. However, PGP 9.5-immunoreactive nerves were markedly diminished after 1 day of culture and almost disappeared after 4 days.

Merkel cell proliferation *in vitro* suggested that Merkel cell development is probably nerve-independent in human fetal glabrous skin.

Key Words: Merkel cell, neural dependency, cytokeratin 20, PGP 9.5, suspension organ culture

INTRODUCTION

Merkel was the first to observe that the cells,

which now carry his name, are often found in close association with intra-epidermal sensory nerve endings.¹ Merkel cells are thought to function as slowly adapting mechanoreceptors² and are known to be targets for sensory nerves.³ This relationship between Merkel cells and nerve endings was demonstrated using double labeling method.^{4,5} However, the nerve dependency of these cells remains controversial.⁶⁻⁹ Some investigators have shown interregional differences between hairy and glabrous skin^{7,8,10} and other have shown intraregional differences within denervated rat touch domes.⁹ Differences between species have also been observed in various vertebrates such as rats, cats, and opossum.^{6,11}

Regeneration of human Merkel cells was observed in cultured keratinocyte grafts derived only from sole skin, and not from the skin of axilla, groin or scalp.⁷ These reports suggested that Merkel cell differentiation may be body-site dependent and that Merkel cell development is independent of neural induction.

The specific aim of this study was to determine whether Merkel cells proliferate *in vitro* without systemic factors, blood vessels and intact nerves in human skin. Suspension organ culture (SOC) was performed using fetal digits to investigate the *in vitro* proliferation of Merkel cells.

MATERIALS AND METHODS

Skin samples

Tissues were obtained from human embryos

Received February 20, 2001

Accepted April 3, 2001

*This work was supported by faculty research grant of Yonsei University College of Medicine for 1994.

and fetuses, ranging from 56-82 days of EGA as determined from maternal data and measurements of foot and crown-rump lengths. Skin samples were collected by the Central Laboratory for Human Embryology at the University of Washington under the direction of Dr. Alan Fantel, and all specimens were obtained in accord with DHEW policies and the policies of the University of Washington Human Subjects Review Board.

Tissue preparation

Full thickness skin from each specimen was processed for immunohistochemistry. Harvested tissues samples and D0 standards were immediately frozen in 100% ethanol cooled on dry ice for the immunolabeling of anti-cytokeratin 20. Frozen samples were stored at 70°C until use. For PGP 9.5 immunolabeling, samples were fixed in 2% paraformaldehyde, dehydrated through graded alcohols (after rinsing in 10% sucrose solution in PBS) and embedded in paraffin.

Suspension organ culture (SOC)

Fetal tissues ranging from 56 to 82d EGA were obtained, rinsed and dissected in sterile PBS supplemented with penicillin G (300 U/ml, Gibco, Gaithersburg, MD, USA), streptomycin sulfate (300 µg/ml, Gibco, Gaithersburg, MD, USA) and fungizone (100 µg/ml). Each digit was bisected with a sterile razor blade proximal to distal interphalangeal joint. All manipulations were performed with sterile instruments and solutions in a laminar flow hood. The medium consisted of Dulbeccos Modified Eagles Medium (DMEM-F12, Gaithersburg, MD, USA), which contained 15 mM HEPES buffer, L-glutamine, bovine serum albumin (6 mg/ml), 0.1 mM nonessential amino acid, penicillin G (75 U/ml), streptomycin (75 mg/ml) and fungizone (1.25 mg/ml). Samples were individually floated in each well of 12 well culture cluster dishes (Costar, Cambridge, MA, USA) with 1.5-2 ml of medium in each well. Cultures were maintained at 37°C in a Queue 2700 series dual chamber incubator with an atmosphere of 45% O₂ and 5% CO₂ and N₂. The medium was changed every 2-3 day. Samples were harvested after

culturing for 1,4,7,14, and 28 days.

Immunohistochemistry for digit SOC

To demonstrate the presence of anti-CK20 immunoreactive Merkel cells, immunolabeling was carried out using the avidin-biotin immunoperoxidase and the diaminobenzidine coloration technique. 8 mm thickness frozen sections were cut and fixed in acetone for 5 min.

Sections were immersed in 0.3% H₂O₂ to block endogenous peroxidase activity, and extraneous antibodies labeling was blocked by 2% goat and 0.5% horse serum. The sections were then incubated with anti-CK20 antibody (1:20 dil., IBL Research, Cambridge, MA, USA) in 1 mg/ml BSA/Tris-saline (0.1M) pH 7.6 for 1 hour at room temperature, and then incubated with biotinylated horse antimouse IgG for 30 min as the secondary antibody. Samples were washed with Tris-saline and serum between the applications of primary and secondary antibodies. The slides were then incubated with avidin-biotin-peroxidase complex for 30 min, and the reaction product was developed in diaminobenzidine (DAB). The stained sections were mounted flat on glass slides in Glycergel. As a negative control, primary antibody was either omitted or substituted by normal mouse serum.

Immunolabeling was carried out using alkaline phosphatase technique to demonstrate PGP 9.5 immunoreactive nerves. 8 mm paraformaldehyde fixed paraffin sections were prepared and deparaffinized. The labeling of extraneous antibodies was blocked by 2% goat serum, and the sections were then incubated with polyclonal PGP 9.5 antibody (1:14,000 dil., Accurate Chemical) in 1 mg/ml BSA/Tris-saline (0.1 M) pH 7.6 for overnight at 4°C and stained with biotinylated goat anti-rabbit IgG (1:400 dil., Vector, Burlingame, CA, USA) for 30 min as secondary antibody. Samples were washed with Tris-saline and serum washes between the application of the primary and secondary antibodies. After rinsing with Tris-saline, the slides were incubated with avidin-biotin-alkaline phosphatase complex reagent for 30 min. The reaction product was developed in alkaline phosphatase substrate solution under microscopic observation. The stained sec-

tions were mounted flat on glass slides in Glycergel. The primary antibody was either omitted or substituted with normal rabbit serum as a negative control.

Electron microscopy

Tissue samples were pre-fixed in one-half strength Karnovskys fixative, post-fixed in 2% OsO₄, dehydrated and embedded in Polybed 812 (Polysciences Inc., Warrington, PA, USA). The thin sections were stained with uranyl acetate and lead citrate and examined in a JOEL 1200 EXII transmission electron microscope at 80 kV/Zeiss EM 902A at 50kV.

RESULTS AND DISCUSSION

Distribution of Merkel cells and nerves in the human fetal digits before starting culture

Merkel cells were observed in the dorsal section of the digit, including the dorsal nail matrix (Fig. 1, A and B) and the volar pad (Fig. 1, C and D), but not in the ventral nail matrix or the nail bed,

which was in agreement with a previous report.¹² PGP 9.5-immunoreactive nerves were observed in the dermis and the periosteal areas (Fig. 2A). Nerves increased and distributed more closely to the epidermis in older fetuses. In the nail bed, nerves were located more so in the deeper dermis than in the other areas of the digits.

Changes of Merkel cells and nerves after Suspension organ culture (SOC)

Even after 28 days of SOC, digits looked healthy structurally and the number of Merkel cells had been maintained or increased (Fig. 1, B and D) compared to original(D0) digit samples. During the culture period, the distribution of Merkel cells was similar to that of D0 digits. PGP 9.5-immunoreactive nerves were diminished after 1d of SOC (Fig. 2B) and almost disappeared after 4d. PGP 9.5-immunoreactive nerves were hardly observed after 28d (Fig. 2C). Ultrastructurally, Merkel cells maintained their characteristic morphology with intact granules after SOC.

A suspension organ culture system was developed,^{13,14} which permits long term maintenance, growth, morphogenesis, and differentiation of explants of human embryonic and early fetal skin

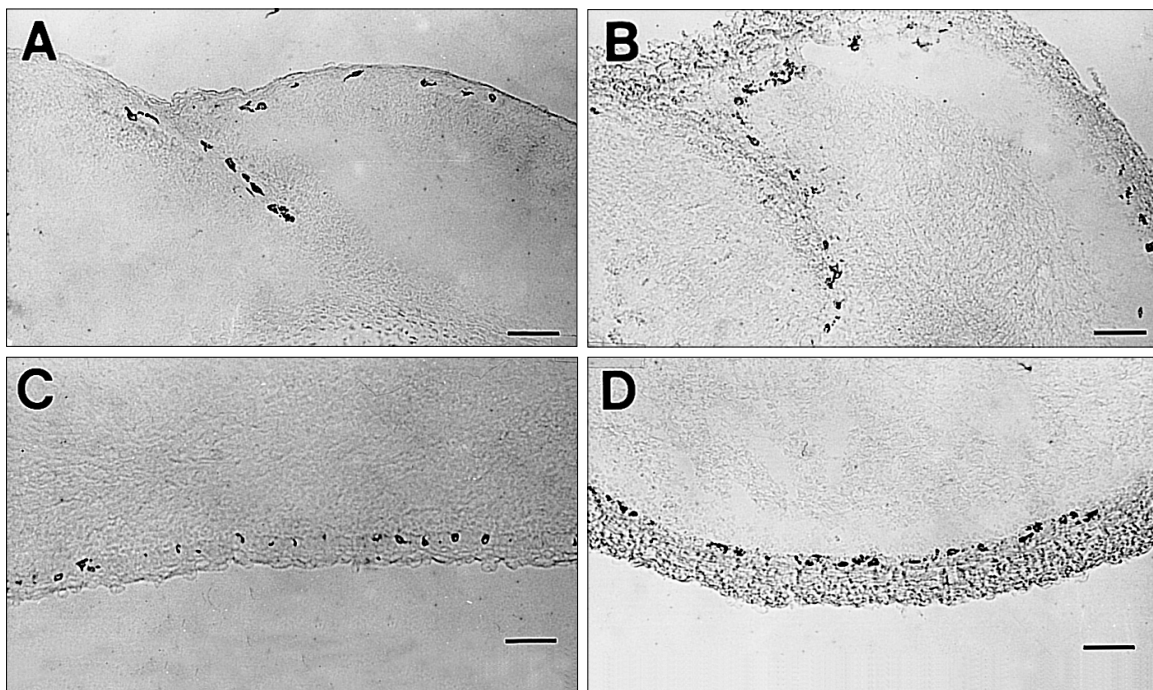


Fig. 1. Merkel cells labeled with anti-CK20 antibody in the digit of a 78d gestational aged fetus. Merkel cells are shown in the dorsal nail matrix, epidermis of dorsal part of the digit (A,B) and the epidermis of volar pad (C,D) before (A,C) and after 28 days (B,D) of suspension organ culture. Bars, 50 μ m.

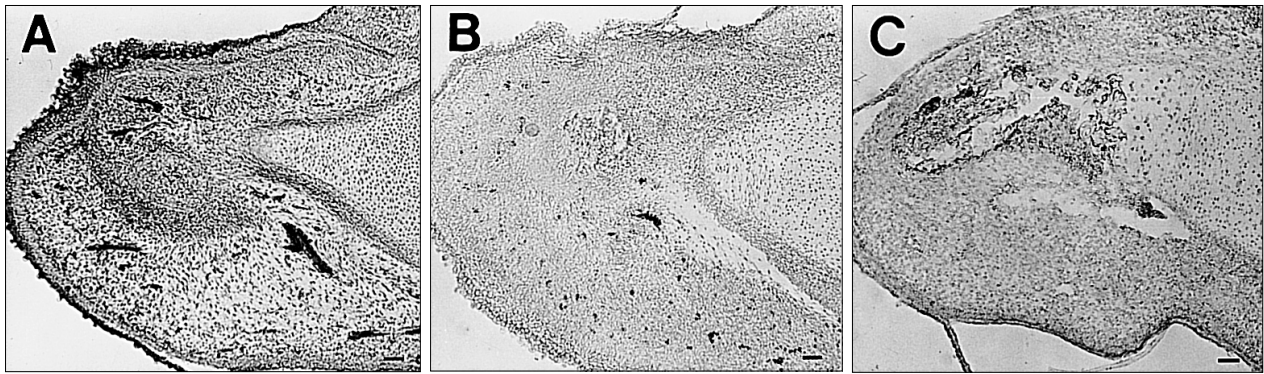


Fig. 2. Nerves labeled with PGP 9.5 antibody in the digit of 82d gestational aged fetus. PGP 9.5 immunoreactive nerves are shown in the dermis and periosteal areas (A), which start to decrease after 1d (B) and disappeared after 28d (C) of suspension organ culture. Bars, 50 μ m.

(60-85d). Ultrastructural studies of samples that have been maintained for 30d revealed an intact dermal-epidermal junction and persistent attachment structures (hemidesmosomes and anchoring fibrils). The dermal and matrix cells were maintained and the epidermis was found to undergo differentiation. The culture system also permits several aspects of follicle morphogenesis to occur depending upon the age of the explanted tissue. Therefore, we used this system to evaluate the neural dependency of Merkel cell development *in vitro*.

In the vertebrate, the development and maintenance of most of the specialized sensory receptors are critically dependent upon the presence of sensory nerves.⁸ Although Merkel cells are thought to function as slowly adapting mechanoreceptors, and are known as targets for sensory nerves, the nerve-dependency of Merkel cells remains an issue of controversy. Some investigators have shown interregional differences between hairy and glabrous skin. Mills et al⁸ observed the relationship between denervation¹⁻⁴ days after birth and Merkel cell development in rat skin. Merkel cells were found to differentiate normally in the paw pads, in the absence of their nerves. Other studies using the glabrous skins of cats,⁶ salamanders¹⁵ and *Xenopus*³ observed normal Merkel cell differentiation after denervation. In the touch domes of hairy skin, however, denervation prevented the differentiation of the normal Merkel cell population and this was associated with the disappearance of most of the Merkel cells.⁸ Nurse et al⁹ showed intraregional

differences within denervated rat touch domes. A quantitative study using denervated rat touch domes showed a progressive reduction in the number of Merkel cells to 40% of that present at the time of denervation.⁹ It was suggested that the two different populations of cells, one labile and the other stable, coexist in the same location. Hartschuh and Weihe⁶ observed Merkel cells in hairy and glabrous skin after denervation in cats. Merkel cells did not disappear from either area even 12 weeks after denervation. Differences between species have also been reported, for example, the maintenance of Merkel cells depends on an intact nerve supply in cats and opossums, but not in rats.¹¹

Studies on the nerve-dependency of Merkel cells using human skin are very limited. Cultured keratinocytes were grafted to full-thickness wounds in 20 pediatric patients treated for massive burns or giant congenital nevi.⁷ Merkel cells were identified only in cultured keratinocyte grafts derived from sole skin and regenerated Merkel cells were never innervated. These reports suggested that Merkel cell differentiation may be body-site dependent and that Merkel cell development and maintenance is independent of neural induction. In our recent study using confocal laser scanning microscopy, we observed that Merkel cells in human fetal glabrous skin are rarely innervated in contrast to Merkel cells in hairy skin (data to be published). These results support the idea that Merkel cell development and maintenance probably be nerve-independent in glabrous skin.

On the basis of this study, which observed Merkel cell proliferations *in vitro*, it appears that that Merkel cell development is probably nerve-independent in human fetal glabrous skin.

ACKNOWLEDGEMENT

The authors acknowledge the expert assistance of Mr. Robert A. Underwood for the preparation of photographs.

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