In Vitro Reconstruction of Neuro-Epidermal Connections

Journal of Investigative Dermatology (2007) **127,** 979–981. doi:10.1038/sj.jid.5700646; published online 7 December 2006

TO THE EDITOR

Sensory neurons are localized in dorsal root ganglia (DRG) (Birder and Perl, 1994). They are associated with glial cells, like Schwann cells, which are important for synaptic plasticity (Allen and Barres, 2005). Synapses between nerve endings and epidermal cells have been shown by confocal scanner laser microscopy and transmission electron microscopy (Merkel, 1875; Hosoi *et al.*, 1993; Hilliges *et al.*, 1995; Hara *et al.*, 1996; Gaudillere *et al.*, 1996; Chateau and Misery, 2004); Through an efferent neurosecretory activity (Ansel *et al.*, 1996; Misery, 1997), they modulate skin properties (Misery, 1997; Steinhoff et al., 2003). Numerous interactions between skin, immunity, and the nervous system allow definition of the neuro-immuno-cutaneous system (Misery, 1997). Because there is no *in vitro* model for studies on neuro-immuno-cutaneous system, we performed a tri-compartmentalized co-culture, each compartment reproducing epidermis, DRG, and spinal cord, respectively, spontaneously connected by neurites. The functionality of these ''synapses'' was assessed by electrophysiological studies.

Epidermal cells were isolated from skin specimens obtained from healthy humans (Bessou *et al.*, 1995). Neurons and glial cells were isolated from DRGs and spinal cords of 2- to 5-day-old Wistar rats (Lindsay *et al.*, 1990; Stucky and Lewin, 1999; Wang and Cynader, 1999). Cultures were performed with glial conditioned medium, as described previously (Bottenstein and Sato, 1979; Wang and Cynader, 1999). Coverslips were sterilized by UV and coated by poly-L-lysine. Two kinds of specific culture dishes were used. A first model was prepared from plastic Petri dishes by drilling three wells connected by channels and attaching a glass coverslip to the outer surface of the dish. A second model consisted of a two-part design, which includes a glass substrate topped by polydimethylsiloxane, including wells and microchannels (Morin *et al.*, 2006).

We performed a tri-compartmented culture with: 1/cells from spinal cords cells (1×10^6 cells/ml); 2/cells from DRGs (1×10^5 cells/ml); 3/epidermal cells (3×10^6 cells/ml). To provide trophic support, a glial feeder layer was added (Wang and Cynader, 1999). Glial conditioned medium was replaced every 2 days.

After 15 days of co-culture, the growth of many neurites, often assembled in bundles, was observed (by phase-contrast microscopy) from the DRG compartment to both equivalents of spinal cord and epidermis and between neurons in the DRG equivalent and neurons.

Immunostainings were performed on co-cultures (first model) after 15 days. Primary antibodies recognized protein gene product (PGP) 9.5 (neurons), cytokeratin (CK, keratinocytes), cytokeratin 20 (Merkel cells), chromogranin A (neuro-secretory granules of Merkel cells), glial fibrillary acidic protein (astrocytes and oligodendrocytes), A2B5 (oligodendrocytes), and myelin basic protein. Neurons and glial cells were assembled in clusters in the spinal cord and DRG compartments and PGP9.5+ nerve endings were seen in the epidermal compartment. Neurites were often surrounded by myelin and associated with Schwann cell-shaped cells.

By confocal scanner laser microscopy, we could recognize in the epidermal compartment, PGP 9.5+ neurons, CK+ keratinocytes, and CK20+ or chromogranin+ Merkel cells. PGP9.5+ nerve fibers were growing from the DRG compartment to the epidermal cells. Double immunostainings revealed overlapping areas, assessing synapse-like structures between nerve endings and keratinocytes and, more frequently, between nerve endings and Merkel cells. Merkel cells often formed Merkel corpuscles (Figure 1a). Contacts of nerve endings with single Merkel cells were also observed

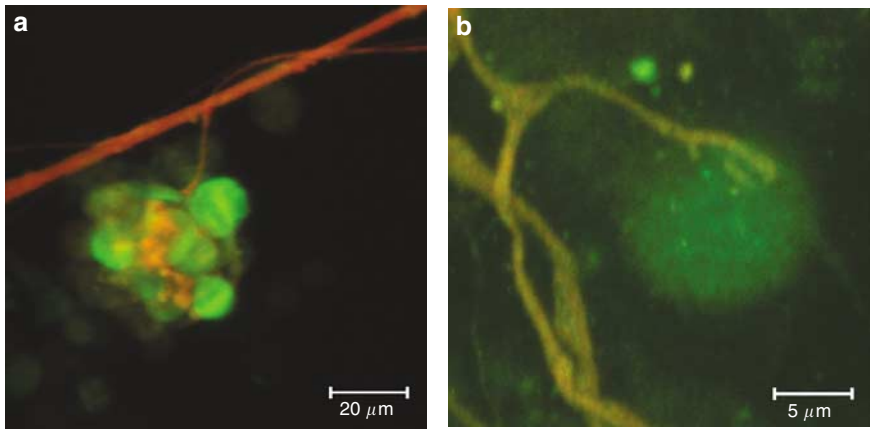

Figure 1. Confocal scanning laser micrography with a double-labeling with anti-CK 20 and anti PGP 9.5 in the epidermal compartment after 15 days of co-culture. (**a**) Merkel cells (green) in contact with PGP 9.5+ nerve endings (red). Merkel cells are assembled around nerve ending and form a Merkel corpuscle. They are associated with nerve endings obtained by division from one axon. (**b**) Merkel cell (green) in contact of nerve fibers (red). An overlap area (yellow) shows the synapse-like structure.

Abbreviations: CK, cytokeratin; DRG, dorsal root ganglia

(Figure 1b). Chromogranin A was localized in neuro-secretory granules from Merkel cells, in front of nerve fibers, like in a synaptic organization.

Transmission electron microscopy observations after 15 days of co-culture (second model) confirmed confocal scanner laser microscopy data. In the epidermal compartment, keratinocytes, Merkel cells, and melanocytes were present. An epithelial organization was assessed by desmosomes. Melanocytes and Merkel cells express dendrites and, respectively, melanosomes and neuro-secretory granules. Nerve fibers were coursing through epidermal cells. They ended in the contact of keratinocytes and Merkel cells.

Electrophysiological measurements were performed after 15 days of co-culture. Electrical activity was recorded from neurites using a macro-patch clamp technique. The signal was recorded via a GeneClamp 500B amplifier. Pipettes were pulled and heat polished from 1.5 mm diameter borosilicate glass with a DMZ – Universal puller. Resistance of the pipettes averaged 1.5 MΩ when filled with recording solution. Currents were low-pass filtered at 5 kHz and digitized at 35 kHz. Giga-seal was checked and leak currents were compensated. The physiological state of the neurite was first checked by recording Na^+ and K^+ currents, confirming the viability of the neurites and the possibility of electrophysiological measurements. Continuous recordings were made in a cell-attached configuration on nerve fiber-like formations allowing the monitoring of spontaneous activity. Heat stimulus was applied by infusing hot medium (37°C for heat stimulation and 45°C for pain stimulation) in the epidermal cell compartment. Electrophysiological recordings were made in the DRG compartment.

Recordings (Figure 2) showed that without external stimulation, no electrical activity could be recorded at 22°C. After heat or painful stimulation, a depolarization was observed and spikes were recorded, corresponding to the triggering of a spontaneous activity. The effects were enhanced with pain stimulation. Ten minutes after stimulation, repetitive electrical activities were still persistent. The spikes progressively disappeared, but could be reinitiated by another heat stimulation, showing the reversibility of the heat effect.

Hence, we have performed an *in vitro* reconstruction of equivalents of spinal cord, DRG, and epidermis connected by neurites. We have obtained a viable

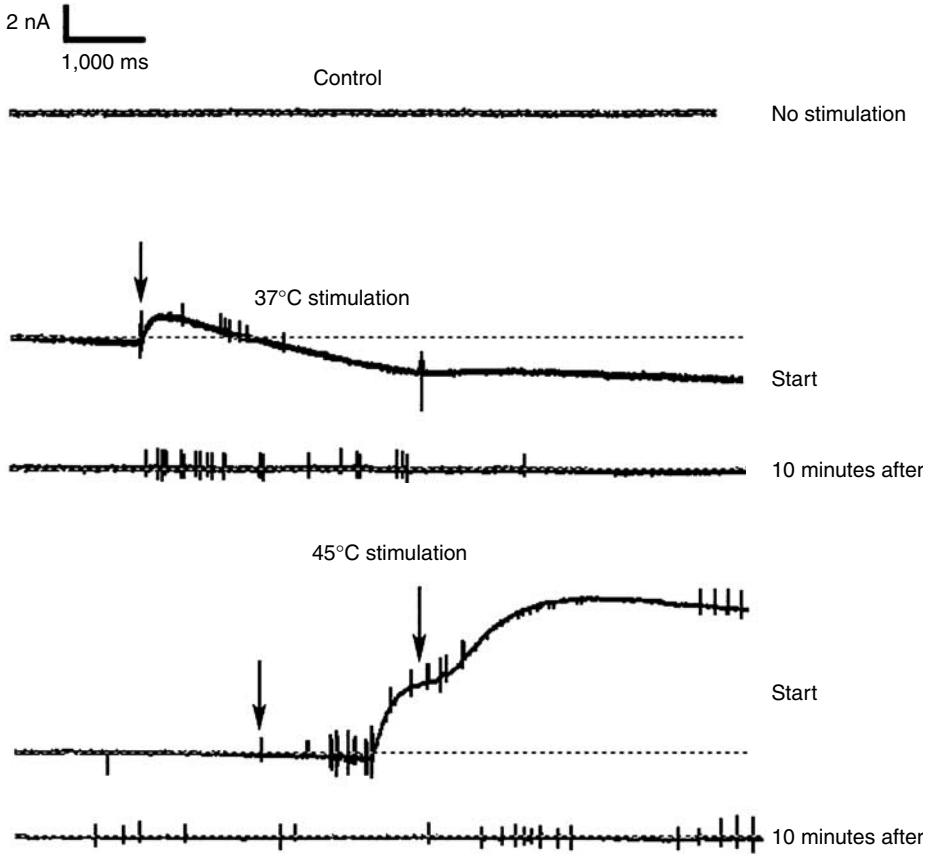

Figure 2. Triggering of spontaneous activity in neurons after heat stimulation of co-cultured keratinocytes. The initial temperature was 22°C. The application of the heat stimulus is indicated by the arrows. Upper trace: control recording showing no spontaneous activity. Middle traces: initial response (start) after stimulation by 100 μl of medium heated at 37°C showing a slow depolarizing current along with the triggering of some spontaneous spikes; after 10 minutes, the slow current vanished, but some spikes still remained. Lower traces: response observed immediately after the application of 100 μl of medium heated at 45°C corresponding to a larger depolarizing current and more numerous spikes. A second application (second arrow) enhances the current. After 10 minutes, a spontaneous activity remained, somewhat more sustained that in the previous stimulation. Recordings were made from neurites by macro-patch technique, in cell-attached configuration.

in vitro culture model of Merkel cells (Gaudillere and Misery, 1994; Moll *et al.*, 2005). To our knowledge, the longest previous culture of Merkel cells was 4 days (Fradette *et al.*, 2003) or 5 days (Vos *et al.*, 1991), whereas Merkel cells were maintained for 15 days of culture in our hands. Other authors had performed co-cultures with keratinocytes (Fradette *et al.*, 2003) or sensory nerve endings (Vos *et al.*, 1991; Shimohira-Yamasaki *et al.*, 2006), but our results suggest that the association of keratinocytes and nerve endings is better.

Until recently, neurons were the only cells that were never included in reconstructed skin or mucosa (Sivard *et al.*, 2004). Gingras *et al.* (2003) have performed a tissue-engineered model mimicking the integration of nerve endings in reconstructed skin but did not show functional synapse-like structures as we did in this study.

Our model of co-culture could be used for studies of the neuro-immunocutaneous system (Misery, 1997) by adding external stimuli, drugs, or cosmetics, and it could be an *in vitro* model of itch (Yosipovitch *et al.*, 2003).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was helped by grants from Coloplast Foundation and French Society of Dermatology. We thank Pr Jean-Luc Carré for his help concerning glial cells and Pr Jean-Paul Leroy for electron microscopy.

Yannick Château[1], Germaine Dorange[1], Jean-François Clément[2], Jean-Pierre Pennec[1], Eric Gobin[3], Laurent Griscom[4], Marie Baudrimont[3], Nathalie Rougier[5], Christophe Chesné[5] and Laurent Misery[1]

[1]Unit of Comparative and Integrated Physiology (EA-3879), University of Western Brittany, Brest, France; [2]Centre of Confocal Scanning Laser Microscopy, University of Western Brittany, Brest, France; [3]Department of Pathology, University Hospital, Brest, France and [4]BIOMIS/SATIE (CNRS UMR 8029), Ecole Normale Supérieure, Bruz, France and [5]Biopredic International, Rennes, France
E-mail: laurent.misery@chu-brest.fr

REFERENCES

Allen NJ, Barres BA (2005) Signaling between glia and neurons: focus on synaptic plasticity. *Curr Opin Neurobiol* 15:542–8

Ansel JC, Kaynard AH, Armstrong CA, Olerud J, Bunnett N, Payan D (1996) Skin – nervous system interactions. *J Invest Dermatol* 106:198–204

Bessou S, Surleve-Bazeille JE, Sorbier E, Taieb A (1995) *Ex vivo* reconstruction of the epidermis with melanocytes and the influence of UVB. *Pigment Cell Res* 8:241–9

Birder LA, Perl ER (1994) Cutaneous sensory receptors. *J Clin Physiol* 11:534–52

Bottenstein JE, Sato GH (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc Natl Acad Sci USA* 76:514–7

Chateau Y, Misery L (2004) Connections between nerve endings and epidermal cells: are they synapses? *Exp Dermatol* 13:2–4

Fradette J, Larouche D, Fugere C, Guignard R, Beauparlant A, Couture V *et al.* (2003) Normal human Merkel cells are present in epidermal cell populations isolated and cultured from glabrous and hairy skin sites. *J Invest Dermatol* 120:313–7

Gaudillere A, Misery L (1994) Merkel cell. *Ann Dermatol Venereol* 121:909–17

Gaudillere A, Misery L, Souchier C, Claudy A, Schmitt D (1996) Intimate associations between PGP9.5-positive nerve fibres and Langerhans cells. *Br J Dermatol* 135: 343–4

Gingras M, Bergeron J, Dery J, Durham HD, Berthod F (2003) *In vitro* development of a tissue-engineered model of peripheral nerve regeneration to study neurite growth. *FASEB J*, 2124–6

Hara M, Toyoda M, Yaar M, Bhawan J, Avila EM, Penner IR *et al.* (1996) Innervation of melanocytes in human skin. *J Exp Med* 184:1385–95

Hilliges M, Wang L, Johansson O (1995) Ultrastructural evidence for nerve fibers within all vital layers of the human epidermis. *J Invest Dermatol* 104:134–7

Hosoi J, Murphy GF, Egan CL, Lerner EA, Grabbe S, Asahina A *et al.* (1993) Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. *Nature* 363:159–63

Lindsay RM, Shooter EM, Radeke MJ, Misko TP, Dechant G, Thoenen H *et al.* (1990) Nerve growth factor regulates expression of the nerve growth factor receptor gene in adult sensory neurons. *Eur J Neurosci* 2:389–96

Merkel F (1875) Tastzellen und Tastkorperchen bei den Haustieren und beim Menschen. *Arch Mikrosk Anat* 11:636–52

Misery L (1997) Skin, immunity and the nervous system. *Br J Dermatol* 137:843–50

Moll I, Roessler M, Brandner JM, Eispert AC, Houdek P, Moll R (2005) Human Merkel cells – aspects of cell biology, distribution and functions. *Eur J Cell Biol* 84:259–71

Morin F, Nishimura N, Griscom L, Le Pioufle L, Fujita H, Takamura Y *et al.* (2006) Constraining the connectivity of neuronal networks cultured on microelctrode arrays with microfluidic techniques: a step towards neuron-based functional chips. *Biosens Bioelectron* 21:1093–100

Shimohira-Yamasaki M, Toda S, Narisawa Y, Sugihara H (2006) Merkel cell–nerve cell interaction undergoes formation of a synapse-like structure in a primary culture. *Cell Struct Funct* 31:39–45

Sivard P, Berlier W, Picard B, Sabido O, Genin C, Misery L (2004) HIV-1 infection of Langerhans cells in a reconstructed vaginal mucosa. *J Infect Dis* 190:227–35

Steinhoff M, Ständer S, Seeliger S, Ansel JC, Schmelz M, Luger T (2003) Modern aspects of cutaneous neurogenic inflammation. *Arch Dermatol* 139:1479–88

Stucky CL, Lewin GR (1999) Isolectin B(4)-positive and -negative nociceptors are functionally distinct. *J Neurosci* 19:6497–505

Vos P, Stark F, Pittman RN (1991) Merkel cells *in vitro*: production of nerve growth factor and selective interactions with sensory neurons. *Dev Biol* 144:281–300

Wang XF, Cynader MS (1999) Effects of astrocytes on neuronal attachment and survival shown in a serum-free co-culture system. *Brain Res Brain Res Protoc* 4:209–16

Yosipovitch G, Greaves MW, Schmelz M (2003) Itch. *Lancet* 361:690–4