

Merkel Cells and Permanent Disesthesia in the Oral Mucosa After Soft Tissue Grafts

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Connective tissue grafts are routinely procedures in the treatment of gingival defects. The clinical success of the gingival tissue graft procedures anyway should ensure not only the aesthetic integration between the tissues but also the physiological activity of the graft in terms of sensitivity and immunity because the skin and the mucosae constitute the first natural aspecific borders against pathogens. The aim of this paper was to investigate nervous net recovery after connective graft procedure, in relation with sensorial alteration in the injured area. Results showed that there is a close link among the number of Merkel cells and the alteration of sensations. Merkel cells can be found isolated standing in the basal layer, supposed to have neuroendocrine functions in the epithelia or in larger group not associated with nerves; when found in association with nerves they are named Merkel complexes, acting as slow adapter mechanical receptor. Our data can be explained in two ways: Merkel cells increase as a consequence of tissue injury, a sort of "SOS cells" that secrete neuroendocrine signals to guide tissue healing; as an alternative the presence of the Merkel cells could be read as a derailment of tissue regeneration with the stop of cellular differentiation in the direction of an abnormal proliferation, a sort of mad stem cell.

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In many situations the gingiva needs to be improved to maintain and preserve the integrity and the health of the deep periodontal tissues (Miller, 1985; Freedman et al., 1992; Loe et al., 1992).

Connective tissue grafts are routinely procedures in the treatment of gingival defects (Oates et al., 2003). The coronal-apical flap with connective tissue graft collected from the palate is the most used surgical procedure for gingival recession.

In the tissue grafting surgeries, ischemia causes a cascade of events and mainly the release of a vasoactive peptide, that promotes neoangiogenesis and neovasculogenesis (Hægerstrand et al., 1990). This event is followed by neuroneogenesis, because the newborn nervous fibers follow the new vessels (Kangesu et al., 1998).

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Like a sort of scanner, the oral mucosa analyzes everything through four principal sensorial terminations: the free terminations, the Meissner-like Corpuscles (analogous to those observable in the skin), the glomerous structures and the Merkel complexes (Slominsky et al., 2000). Usually Merkel cells have a round or oval morphology and can be found isolated in larger group not associated with nerves; when found in association with nerves they are named Merkel complexes, acting as slow adapter mechanical receptor. The Merkel cells can be identified by the reaction with antibodies against Protein Gene Product 9.5 (PGP 9.5). These cells stand in the basal layer, supposed to have neuroendocrine functions in the epithelia (Ramieri et al., 1992). The same antibody is able to recognize free sensorial terminations. In the healthy gingival the neural system tunes the inflammation process and the edema (Kondo et al., 1995) in a complex of reactions called "neurogenic inflammation" (Holzen, 1998), sensorial inputs able to control after an injury the haematic flow and the vascular permeability

through the release of vasoactive peptides, such as CGRP (Merhi et al., 1998).

The aim of this paper is to investigate nervous net recovery after connective graft procedure, in relation with sensorial alteration in the injured area.

Materials and Methods

Patient selection and tissue collection

The sample included eight patients (seven female, one male), from 25 to 38 years old (average 30.70 ± 4.37) recruited in the Department of Periodontology, University of Turin Dental School from the November 2006 to July 2007. Two patients (RI and SO) were subjected to one surgery, five patients (TC, RL, PI, BR, and BL) were subjected to two ones, 1 patient (FE) to 3 ones, for a total of 15 surgeries. All the patients were treated for gingival recession. Briefly, a muco-connective flap was elevated in the site of gingival recession; a gingival sample was collected at this stage for time 0 analysis. A connective tissue sample was collected from the palate and used as graft positioned under the muco-connective flap; a palatal sample was used for time 0 analysis of the palate. After 2 months, during plastic finishing surgery of the gingiva, a sample from grafted tissues were collected to challenge the healing processes.

Tissue processing

The biopsy specimens were taken from adult patients during procedures of plastic surgery, and quickly immersed in fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.35) and stored for 24 h at 4°C. Then they were washed in 0.01 M saline phosphate

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buffer (PBS), placed overnight at 4°C and finally they were placed in a 30% sucrose solution in PBS for 3 days at 4°C. The specimens were frozen for cryostat sectioning.

Immunocytochemistry

Specimens were serially cut at 25 µm thickness with a Leica CM1900 cryostat and processed by the biotin-avidin method. Sections from recipient site (attached gingiva), donor site (palate), and gingival connective tissue graft (GCTG), of several patients were always stained together, so that between-assays variance could not cause systematic group differences. Sections were washed in PBS containing 0.2% Triton X-100 for 30 min and then treated to inhibit endogenous peroxidase activity with a solution of PBS containing methanol/hydrogen peroxide for 20 min (16, Streefkerk, 1972). Sections were incubated for 30 min with normal goat serum (Vector Laboratories, Burlingame, CA) and incubated overnight at room temperature with mouse anti-human collagen IV (DakoCytomation, Milan, Italy) diluted 1:100, or mouse anti-human smooth muscle actin/HRP (DakoCytomation) diluted 1:200, or rabbit anti-human PGP 9.5 (Ultraclone, Yarmouth, UK) diluted 1:20000 in PBS, pH 7.3–7.4, containing 0.1–0.2% Triton X-100. A biotinylated anti-mouse (for collagen and actin) or anti-rabbit (for PGP 9.5) secondary antibody (Vector Laboratories) was then used at a dilution of 1:200 for 60 min at room temperature. The antigen-antibody reaction was revealed by incubation with avidin-peroxidase complex (Vectastain ABC Kit Elite, Vector Laboratories) for 60 min. The peroxidase activity was visualized with a solution containing 0.187 mg/ml 3,3-diaminobenzidine (Sigma, Milan, Italy) and 0.003% hydrogen peroxide in 0.05 M Tris-HCl buffer pH 7.6. The reaction was blocked by rinsing the sections in distilled water. Sections were air dried, cleared in xylene and cover slipped with Entellan (Merck, Milan, Italy).

The specificity of the commercial antibodies used were tested by the factories. Moreover, we have performed the following controls in our material: (a) the primary antibody was omitted or replaced with an equivalent concentration of normal serum (negative controls); (b) the secondary antibody was omitted. In these conditions, the sections were totally unstained.

To improve the identification of structures, some sections were stained with hematoxylin and eosin histochemistry.

Sections were examined and photographed using a Nikon Eclipse 80i microscope connected to a Nikon DS-Fi digital video camera.

Sensorial tests

After 12 months all the patients have been tested for anesthesia or paresthesia in the host areas where tissue graft procedures have been performed. A blinded examiner carried out two types of tests:

Epicritical stimulation test. To challenge surface sensations, a puncture, performed with a 30 g pressure calibrated top-fencing probe, was led in the area of grafted tissues. The supposed alteration of epicritical sensation was confirmed by the test of discrimination of two different top-fencing probes challenging the mucosa contemporarily at a distance of 10 mm. The patients were asked for positive or negative answers. The mucosa areas positive to this tests have been labeled with ink spots.

Protopaternal stimulation test. To challenge deep sensations, a puncture with metal top fencing probe was led up to bleeding. The patients were asked for positive or negative answers. The mucosa areas positive to these tests have been labeled with ink continuous lines.

Results

Histological observation of gingival and palatal mucosa

At time 0 all palatal mucosa sections, showed an extremely variable (0–60) number of Merkel cells; gingival mucosa sections, at the contrary, showed no or a low number (<3–4 elements for section) Merkel cells; 2 months after the surgery, in 10 out of 15 gingival samples, an increasing of the

Merkel cells number has been observed. In the 60% (6 of 10) 10 or more Merkel cells were detected in every section. With some exceptions for palatal sample, these Merkel cells did not constitute cell-neurite complexes and had no connection with nervous system, as observed using PGP 9.5 antibody (follows). No relation between the number of Merkel cells in the gingival sample at 2 months after the surgery and the number in the palatal donor site at time 0 has been observed (Table 1).

Immunocytochemical evaluation of re-innervation

The antiserum against PGP 9.5 used in this study showed several neuronal elements in all specimens. In particular, section derived from gingival recipient site show several thick nerve bundles in the connective tissue, many fibers that run parallel near the epithelium-connective junction and few thin nervous fibers that enter the epithelium and run to the epithelial surface as free nerve endings in the lamina propria (Fig. 1A–C).

In the palatal donor site there are many nervous fibers both in the deep and superficial connective tissue. The antiserum against PGP 9.5 stained also nervous fibers in the thick wall of big blood vessels. Occasionally Merkel cell-neurite complexes and Meissner's corpuscles were observed (Fig. 1D–F).

Overall, in the gingival area, 2 months after connective tissue graft, there is a diminution of the PGP 9.5 immunoreactivity. There are less free nerve endings in the lamina propria, and few nerve bundles in the connective tissue. Moreover, in these specimens it is possible to observe many clusters (20–30 elements for section) of non-innervated Merkel cells and several Merkel cell-neurite complexes (Fig. 1G–J). Free nervous terminations were found reduced in the graft sites, when compared with donor site or the host site before the surgery. Actually, no tactile corpuscles have been observed in the specimens, before and after the surgery.

Sensitive tests

Twelve months after the surgery, in the 50% (7 out of 15) of the surgical sites, patients showed difficulties to identify and recognize stimuli carried on the gingiva in those sites. These alterations were divided into two groups: paresthesias (Fig. 2), with the only loss of the epicritical sensation and anesthetics (Fig. 3), with the loss of both epicritical and protopaternal sensations. In five sites patients developed complete anesthesia and in one site only paresthesias after surgeries.

All the samples coming from patients who developed sensorial alterations, showed an increased number of Merkel cells at 2 months with respect with the same site challenged at time 0. In the five sites where after 12 months it has detected a complete anesthesia, the number of Merkel cells for section was equal or bigger than 10 for section. In the case where it has been possible to identify only the loss of epicritical sensation was detected a slight increase of Merkel cells, with a final number of about 5 for section. On the contrary, in 1 site (FE1°) Merkel cells increased up to 20 cells for section, the highest number observed in this experiment; nevertheless the surgical area was found positive both to epicritical and protopaternal sensation. The results coming from the histological analysis on the tissue sample and the outcomes of the sensorial tests performed on the same patient are summarized in Table 1.

Discussion

The soft tissue grafts are surgical procedures orientated to root coverage in periodontal surgery. Unfortunately there is no entire comprehension of the physiological mechanisms leading to the epithelial-connective wound healing. In the present study we demonstrate that sensorial alterations occur also after complete root coverage and clinical success of the surgery.

TABLE I. Surgeries, cytochemistry and clinical tests

Patient	Free nerve endings	Connective fibers	Epith-conn. junction fibers	Merkel cells	M. cells numbers	Merkel c.-neurite complexes	Vessels	Sensorial tests	
								Epicritical	Protopatinal
BR(I ^o) 1	++	++	++	+	3-4	-	-		
BR(I ^o) 2	++	++	++	+++	15-20	-	-		
BR(I ^o) 3	+++	++	++	++	15	-	-	NEG	NEG
BR(II ^o)1	+	++	-	+	3	-	-		
BR(II ^o)2	+	+	++	+	3	-	-		
BR(II ^o)3	-	-	-	-	-	-	-	POS	POS
TC(I ^o)1	+	+	+	-	-	-	+		
TC(I ^o)2	-	+	-	-	-	-	++		
TC(I ^o)3	-	+	-	++	10	-	-	NEG	NEG
TC(II ^o)1	+	+	+	+	3-4	-	-		
TC(II ^o)2	+	+	+	++	10-15	-	++		
TC(II ^o)3	-	+	+	++	10-15	-	+	NEG	NEG
RL(I ^o)1	-	+	-	+	2-3	-	-		
RL(I ^o)2	-	+	+	+	5-6	-	-		
RL(I ^o)3	-	+	+	++	10-15	-	-	NEG	NEG
RL(II ^o)1	+	+	-	-	-	-	-		
RL(II ^o)2	++	++	++	+++	15-20	++	++		
RL(II ^o)3	+	+	+	+	5-6	-	-	NEG	POS
PI(I ^o)1	-	+	+	+	2-3	-	-		
PI(I ^o)2	+	+	+	-	-	-	-		
PI(I ^o)3	-	+	-	+	2-3	-	-	POS	POS
PI(II ^o)1	+	+	-	-	-	-	-		
PI(II ^o)2	-	+	++	+++	15-20	++	+++		
PI(II ^o)3	-	+	-	-	-	-	-	POS	POS
FE(I ^o)1	+	+	+	+	2-3	-	-		
FE(I ^o)2	-	+	+	+++	50-60	+	-		
FE(I ^o)3	+	+	-	+++	15-20	+	+	POS	POS
FE(II ^o)1	-	+	+	+	2-3	-	-		
FE(II ^o)2	+	++	++	+	5-6	+	++		
FE(II ^o)3	-	+	+	+	2-3	-	-	POS	POS
BL(I ^o)1	+	+	-	-	-	-	+		
BL(I ^o)2	+	+	+	+	3-4	-	+		
BL(I ^o)3	+	-	-	-	-	-	-	POS	POS
BL(II ^o)1	+	-	-	-	-	-	-		
BL(II ^o)2	-	+	+	+	4-5	-	-		
BL(II ^o)3	-	-	++	+	3-4	-	-	POS	POS
FE(III ^o)1	-	+	+	+	-	-	-		
FE(III ^o)2	+	+	+	++	10-15	+	-		
FE(III ^o)3	-	+	+	+	5-10	-	+	NEG	NEG
RI1	+	+	+	-	-	-	-		
RI2	-	+	+	+++	20-30	-	+		
RI3	+	-	-	+	2-3	-	+	POS	POS
SO1	++	++	++	+	4-5	-	-		
SO2	+	+	++	+++	40-50	-	-		
SO3	+	+	+	+	5-10	-	+	NEG	NEG

I = gingiva time 0; 2 = palate time 0; 3 = gingiva time I.

In the histological analysis performed after 2 months important differences can be underlined for the neural net: the histological analysis evidenced the presence of many non-innervated Merkel cells in the specimens of gingival area where connective tissue graft was performed. Cluster of non-innervated Merkel cells were previously described by Ramieri et al. (1992) in keratinized human mucosa (palate and attached gingiva). These cells were seen only occasionally and in a few numbers in the specimens derived from the gingival recipient site at time 0 but were always present in the specimens derived from the palatal donor site and in the 85% of the samples those obtained from the tissue graft after 60 days as this experiment demonstrated.

Merkel cell in the epidermis has generally been regarded as a mechanoreceptor which detects tissue deformation and subsequently releases some neurotransmitters to nerve endings (Haeberle et al., 2004). In addition for Merkel cells it has been hypothesized a role as neuroendocrine cutaneous cells with an unclear origin due to the characteristics of both epidermal and neuroendocrine cells (for a recent review of this argument see Boulais and Misery, 2007; Lucarz and Brand, 2007): actually they derived from the neural crest (Szeder et al., 2003) but at the same time they show important feature of modified

keratinocyte. It has been reported too an increased presence of Merkel cells after skin injuries and within grafts of cultured epidermis (Tachibana et al., 1983; Compton et al., 1990): these cells appeared during the third week of the regeneration or healing process and were never innervated. The proliferation of these cells seems to involve a differentiation from their precursor that may reside in the dermis. In a recent study it was postulated that non-innervate Merkel cells in the skin could be undifferentiated or immature elements (Uchigasaki et al., 2004). Moreover, Narisawa et al. (1992) shown that in the human embryos Merkel cells express nerve growth factor (NGF) receptor just before epidermal innervations suggesting a role in this physiologic process.

In our observation we found numerous non-innervated Merkel cells in the specimens derived from the graft and in the same time we found less innervations respect of specimens derived from donor and recipient sites. It is possible that the increase of immunoreactive Merkel cells observed in the graft is a surgical consequence: the insertion of the graft causes an interruption of existing vessels and nervous fibers. In the graft we have observed less free ending fibers respect of the original recipient site; it is possible that these new Merkel cells were immature elements derived from dermis precursor after the

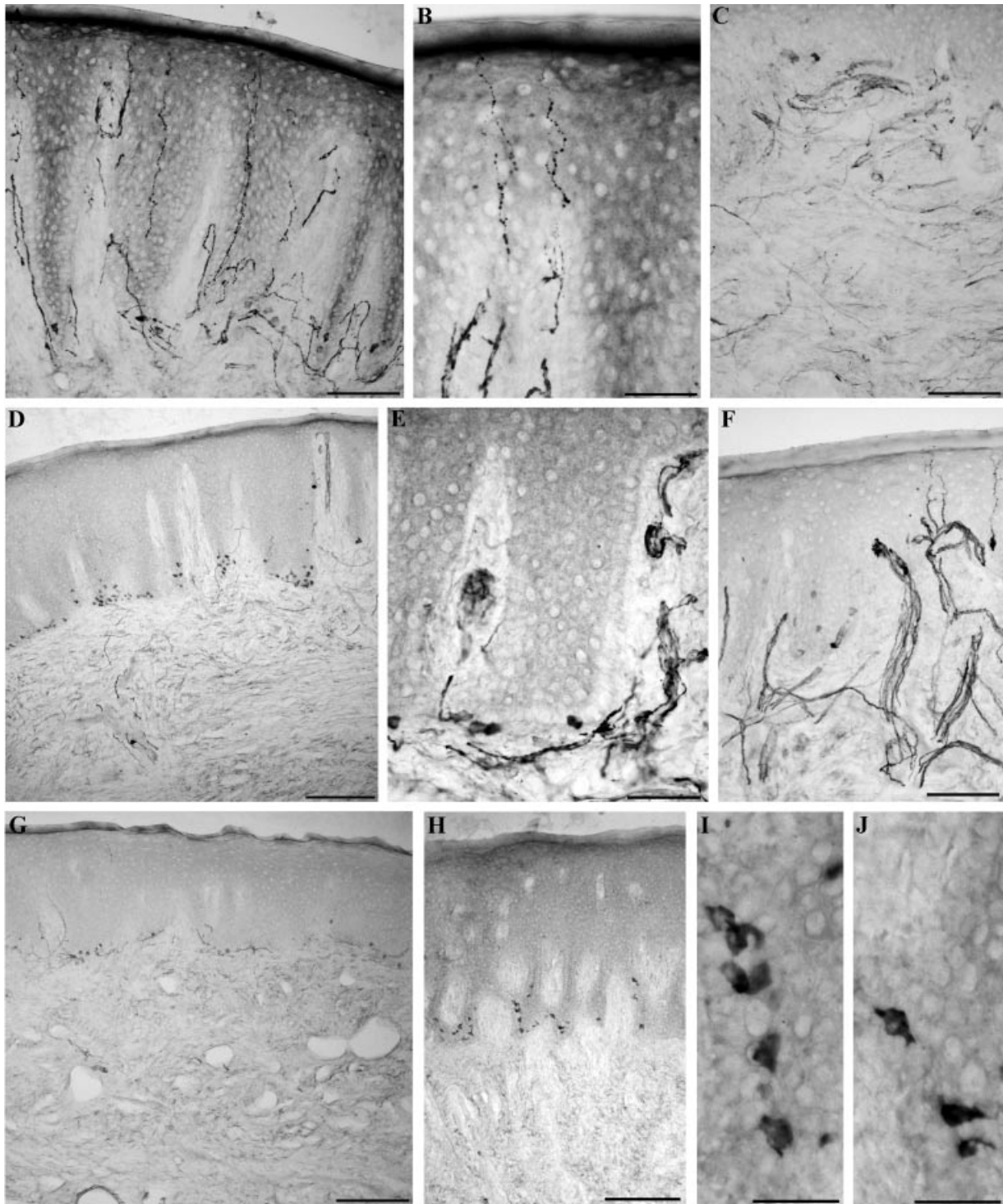


Fig. 1. PGP 9.5 immunocytochemistry. **A:** Recipient site at low magnification; it is possible to note numerous PGP 9.5-immunoreactive fibers in both the connective tissue and the epithelium. **B:** High magnification of the epithelium of the recipient site, showing free ending PGP 9.5 immunoreactive fibers. **C:** Recipient site at low magnification; there are some PGP 9.5-immunoreactive fibers in the connective tissue. **D:** Donor site at low magnification; note numerous Merkel cells at the epithelium-connective junction. **E:** Donor site; Meissner's corpuscles receptor. **F:** Donor site; several thick nerve bundles in the connective tissue. **G:** Graft at low magnification; note the presence of Merkel cells and few nervous fibers. **H:** Graft; note the numerous non-innervated Merkel cells. **I,J:** Enlargement of (H) Magnification bars: 200 μm for (D,G,H); 100 μm for (A,C,F); 50 μm for (B,E); 30 μm for (I,J).



Fig. 2. Disesthetic area (stripes) surrounded by healthy area (spotted) in the recipient site 12 months after surgery. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



Fig. 3. Anesthetic area (no marker) surrounded by healthy area (spotted) in the recipient site 12 months after surgery. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

surgical procedure; possibly, they produce neurotransmitters to improve neurites to reach again the epithelium of the mucosa.

The sensitive tests confirmed alterations of the epicritical and protopatal sensations. All the sites (14) whose gingival

samples sections showed enhancing of free Merkel cells showed sensorial alteration. There is a close link between the number of Merkel cells and the alteration of sensations. It is important the data that no every patient exhibited in sensitive alteration but only the ones whose histological analysis said Merkel cells increased.

Taken together these data can be explained in two ways: as first hypothesis, when there is a problem in tissue regeneration, Merkel cells increase as a consequence, a sort of "SOS cells" that secrete neuroendocrine signals to guide tissue healing; the reduction of free nervous terminations and sensorial alteration observed and the relation between the seriousness of these alterations and the number of Merkel cells confirm the failure of this attempt to restore the healing process. The case of FE (I^o) in this way can be explained with a succesful attempt obtained through extensive cellular proliferation (20 cells/section). As second hypothesis the presence of the Merkel cells could be read as a derailment of tissue regeneration with the stop of cellular differentiation in the direction of an abnormal proliferation, a sort of Mad stem cell. The Merkel cell as progenitor cell of cellular components related with sensitive function of the epithelia is a fascinating vision that need to be better and further investigated.

Literature Cited

- Boulais N, Misery L. 2007. Merkel cells. *J Am Acad Dermatol* 57:147–165.
- Compton CC, Regauer S, Seiler GR, Landry DB. 1990. Human Merkel cell regeneration in skin derived from cultured keratinocyte grafts. *Lab Invest* 63:233–241.
- Freedman AL, Salkin LM, Stein MD, Green KA. 1992. 10-year longitudinal study of untreated mucogingival defects. *J Periodontol* 63: 71–72.
- Haeberle H, Fujiwara M, Chuang J, Medina MM, Panditrao MV, Bechstedt S, Howard J, Lumpkin EA. 2004. Molecular profiling reveals synaptic release machinery in Merkel cells. *Proc Natl Acad Sci USA* 101: 14503–14508.
- Hægerstrand A, Dalsgaard CJ, Jonzon O, Larsson O, Nilsson J. Calcitonine gene-related peptide stimulates proliferation of human endothelial cells 1990. *Proc Natl Acad Sci USA* 87: 3299–3303.
- Holzen P. 1998. Neurogenic vasodilatation and plasma leakage in the skin 1998. *Gen Pharmac* 30: 5–11.
- Kangesu T, Manek S, Terenghi G, Gu XH, Navsaria H, Polak J, Green C, Leigh I. 1998. Nerve and blood vessel growth in response to grafted dermis and cultured keratinocytes. *Plast Reconstr Surg* 101: 1029–1038.
- Kondo T, Kido MA, Kiyoshima T, Yamaza T, Tanaka T. 1995. An immunohistochemical and monastral blue-vascular labelling study on the involvement of Capsaicin-sensitive sensory innervation of the junctional epithelium in neurogenic plasma extravasation in the rat gingiva. *Anchs Oral Biol* 40: 931–940.
- Löe H, Arenud A, Boysen H. 1992. The natural history of periodontal disease in man. Prevalence, severity and extent of gingival recession. *J Periodontol* 63: 489–495.
- Lucarz A, Brand G. 2007. Current considerations about Merkel cells. *Eur J Cell Biol* 86: 243–251.
- Merhi M, Disting GJ, Khalil Z. 1998. CGRP and nitric oxide of neuronal origin and their involvement in neurogenic vasodilatation in rat skin microvasculature. *Br Jo Pharmacol* 123: 863–868.
- Miller PD. 1985. A classification of marginal tissue recession. *Int J Periodontics Restorative Dent* 5: 8–13.
- Narisawa Y, Hashimoto K, Nihei Y, Pietruk T. 1992. Biological significance of dermal Merkel cells in development of cutaneous nerves in human fetal skin. *J Histochem Cytochem* 40: 65–71.
- Oates TV, Robinson M, Gunsolley JC. 2003. Surgical therapies for the treatment of gingival recession. A systematic review. *Ann Periodontol* 8: 303–320.
- Ramieri G, Panzica GC, Viglietti-Panzica C, Modica R, Springall DR, Polak JM. 1992. Non-innervated Merkel cells and Merkel-neurite complexes in human oral mucosa revealed using antiserum to protein gene product 9.5. *Arch Oral Biol* 37: 263–269.
- Szeder V, Grim M, Halata Z, Sieber-Blum M. 2003. Neural crest origin of mammalian Merkel cells. *Dev Biol* 253: 258–263.
- Tachibana T, Sakakura Y, Ishizeki K, Iida S, Nawa T. 1983. An experimental study of the influence of sensory nerve fibers on Merkel cell differentiation in the labial mucosa of the rabbits. *Arch Histol Jpn* 46: 469–477.
- Uchigasaki S, Suzuki H, Inoue K. 2004. Merkel cells in the vellus hair follicles of human facial skin: A study using confocal laser microscopy. *J Dermatol* 31: 218–222.