Characterization of the peri-implant epithelium in hamster palatine mucosa: Behavior of Merkel cells and nerve endings

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ABSTRACT

The purpose of this study was to investigate the relationship between Merkel cells and nerve elements during tissue regeneration after receiving dental implants. Golden hamsters were divided into 3 groups and titanium alloy implants were fixed in their left-side maxilla through the third palatine ruga. Animals were sacrificed at 1, 2, 3, 4, 5, 6, and 7 days after the implantation and tissues were characterized at the immunohistochemical and morphological levels. CK 20 and PGP 9.5 antibodies which react with Merkel cells and nerve fibers were used. Immunohistochemically, no CK20-positive Merkel cells were seen in the peri-implant epithelium throughout the 7 days. However, starting at day 4, PGP 9.5-positive nerve fibers appeared in the connective tissue, and by day 7, nerve fibers had invaded the more superficial layer of the peri-implant epithelium compared to the mucosa removal control group. At the electron microscopic level, the intercellular spaces of the regenerating epithelium in the mucosa removal control group were small. In contrast, intercellular spaces of the peri-implant epithelium tended to be wide and regenerating nerve fibers invaded those intercellular spaces. In both the mucosa removal control group and the implantation group, the basal lamina and connective tissues regenerated completely. However, clear Merkel cells containing neurosecretory granules were not observed. Taken together, our results indicate that Merkel cells in the hamster palatine mucosa do not regenerate in the peri-implant epithelium. However, regenerative nerve fibers seem to play essential roles as part of the defense and sensory systems around the peri-implant epithelium to compensate for the weakened defense mechanism.

It is well known that Merkel cells play an important role in tissues as a sensory system for pressure (8, 16, 28). Previous studies have revealed that Merkel cells are widely distributed in human oral mucosal tissues including the lip, hard palate and mandibular gingiva (1, 13). A fundamental, ultrastructural study of rodent oral mucosa demonstrated that two types of Merkel cells, with dendritic or roundish shapes, are recognized. Most dendritic Merkel cells are free of innervations while the roundish ones are innervated (26). In addition, Merkel cell-neurite complexes are considered to be slowly adapting type I mechanoreceptors in the oral mucosa of the hamster cheek pouch (28, 29).

The behavior of Merkel cells after denervation has also been elucidated (22), the conclusion being that Merkel cells survive with normal density and appearance in the epidermis after denervation. A study of the behavior of Merkel cells and nerve endings after partial excision of the lip has shown that Merkel cells and nerve fibers are regenerated (25). However, there have been no reports on the behavior of Merkel cells and/or nerve endings in re-
lation to wound healing in oral mucosa.

Many investigators have studied the differences in functions and morphological features between the peri-implant epithelium and the normal junctional epithelium (6, 9–11, 14). Those studies revealed that the intercellular spaces of peri-implant epithelium are wider, which suggests that subsequent defense reactions might be weaker than those of normal junctional epithelial cells. Recently, dental implants have been applied in orthodontic dentistry as anchors or maxillofacial prostheses. However, the distribution of Merkel cells and nerve endings around the peri-implant epithelium in those implant areas has not yet been elucidated.

The purpose of this study was to investigate at the immunohistochemical and ultrastructural levels, the relationship between Merkel cells and nerve elements around the peri-implant epithelium in terms of their regeneration after implantation into the palatine ruga, which contains a number of Merkel cells (12).

MATERIALS AND METHODS

Implants. The screw implants used in these experiments were made from titanium alloy (Ti-6Al-4V), and were 1.3 mm in diameter and 8.0 mm long (Fig. 1). The diameter of the implant used was determined by the width of the third fold in the palatine ruga of each hamster. Before the surgery, each implant was cleaned by ultrasonication in distilled water and by autoclaving at 120°C for 20 min.

Animal treatment. Twenty-one male Syrian golden hamsters, 5 weeks old, and approximately 100 g in body weight, were used. The animals were anesthetized using sodium pentobarbitone (50 mg/kg i.p., Nembutal, Abbott, USA) according to the Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College (approval number 01–63).

1. Mucosa removal group
   Under general anesthesia, both the right and left sides of the third palatine ruga were removed with a modified injection needle (1.20 × 38 mm, Fig. 2). Following surgery, all animals were fed with milk only to avoid solid food effects.

2. Implantation group
   Under general anesthesia, the left side of the third palatine ruga was removed in the same manner as described for the mucosa removal control group. Bone tissue was removed using a 0.8 mm round burr mounted on a dental handpiece, cooled with air. The implantation cavity was then prepared by drilling with a #60 dental K-file. After the debris was washed away with distilled water, the surrounding tissue was wiped off with sterile absorbent gauze. An implant was inserted into the cavity using a modified dental instrument (Fig. 3).

3. Untreated Control group
   The right side of the third palatine ruga without treatment of any kind was used as a normal untreated control sample.
Light microscopy: For observations using a light microscope, animals were sacrificed at 1, 2, 3, 4, 5, 6 and 7 days after the surgery using pentobarbital sodium anesthesia (100 mg/kg). The maxilla were then removed and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer saline (PBS, pH 7.2) for 3 days at room temperature. After the fixation, the tissues were decalcified with 10% tetraboric acid for 10 days at room temperature. The implants of the implantation group were then removed meticulously by mechanical means. The specimens were then dehydrated through a graded series of ethanol before being embedded in paraffin. Paraffin sections, approximately 3 or 4 µm thick, were cut and placed onto silane-coated glass slides, and were stained with hematoxylin-eosin after which the immunohistochemical analyses were performed.

For the immunohistochemical observations, deparaffinized sections were immersed in methanol containing 0.3% H2O2 for 30 min at room temperature, in order to block intrinsic peroxidase activity, and were then washed with PBS. The sections were preincubated with 0.01% citric acid for 20 min at 60°C and 10% normal goat serum at room temperature. These were then incubated overnight at 4°C in a 1:30 dilution of mouse monoclonal antibody for cytokeratin 20 (CK20, Dako, Denmark), which detects Merkel cells, or in a 1:50 dilution of mouse monoclonal antibody for neuron-specific enolase (NSE, Enzo Diagnostics, USA), which detects Merkel cells and nerve fibers. All reaction products were developed by the streptavidin-biotin method using a commercially available SAB kit (HISTFINE SAB-PO, Nichirei, Japan); the presence of peroxidase was visualized after reaction with diaminobenzidine. Finally, the samples were counterstained with hematoxylin, mounted, and coverslipped.

Confocal laser scanning microscopy: In this study, confocal laser scanning microscopy was used because nerve fibers can be detected more clearly than with observation under a light microscope. The tissues were fixed and decalcified in the same manner as described above for light microscopy. The excised maxillae were then immersed in PBS for 12 h at 4°C. The specimens were then immersed into Tissue-Tek OCT compound (Sakura, Japan) and were rapidly frozen in liquid nitrogen (−196°C). Sections, approximately 20 µm thick, were cut using a cryostat (CM1900, Leica, Austria) at −20°C and were immersed in PBS to remove the OCT compound. After preincubation with 0.01 M citric acid for 2 h at room temperature, the specimens were incubated with either a mouse monoclonal antibody against CK20 (diluted at 1:30) or a polyclonal rabbit antibody against protein gene product 9.5 (PGP 9.5, UltraClone, England), which detects nerve fibers (diluted at 1:500). Cell nuclei were labeled by incubation for 2 h at room temperature in a 1:200 dilution of TO-PRO 3 iodide (Molecular Probes, USA). Reaction products were labeled by incubation for 1 h at room temperature in a 1:100 dilution of Alexa 488-conjugated anti-mouse IgG (Molecular Probes, USA) and a 1:100 dilution of Alexa 568-conjugated anti-rabbit IgG. Finally, specimens were observed using a confocal laser scanning microscope (MRC 1024UV, England) with triple frame channels.

Transmission electron microscopy: For observation using an electron microscope, the tissues of euthanized animals were fixed by intracardiac perfusion with Karnovsky’s fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde dissolved in 0.24 M Sörensen’s phosphate buffer, pH7.2) at room temperature. Each maxilla with an implant was removed and immersed in the same solution for 3 days. The tissues were decalcified in 10% EDTA for 10 days at room temperature. The implants of the implantation group were then removed meticulously by mechanical means. The tissues were cut at an angle parallel to the implant and were post-fixed with 2% osmium tetroxide solution (OsO4) for 2 h at 4°C. The tissues were then dehydrated through a graded series of ethanol before being embedded in epoxy resin (Epon 812, TAAB, England). Ultrathin sections, approximately 70 or 80 nm thick, were cut using an ultramicrotome (Reichert Ultracut UCT, Leica, Austria) and were stained with uranyl acetate and lead citrate. These sections were then observed using a transmission electron microscope (H-7100, Hitachi, Japan).

RESULTS

Untreated Control Group
1. Light microscopic observations
Merkel cells were recognized as clear cells in the basal cell layer of the stratified keratinized squamous epithelium (Fig. 4a), and were positive for CK20 (Fig. 4b). NSE reactivity was seen both in CK20-positive cells and in nerve fibers in the connective tissue (Fig. 4c).

2. Confocal laser scanning microscopic observations
Fig. 4 Light microscopic observations of normal third palatine ruga. **a:** H-E staining. Clear cells in the basal layer of an epithelial rete ridge are evident (arrowheads). **b:** Immunohistochemical staining for CK20. CK20-positive Merkel cells are observed in the basal layer (arrowheads). **c:** Immunohistochemical staining for NSE. NSE-positive fibers (arrowheads), which are just beneath the basal cell layer, and Merkel cells (arrows) can be seen.

Fig. 5 Confocal laser scanning microscopic picture using anti-CK20 and anti-PGP 9.5. PGP 9.5-positive fibers (red) extend from the connective tissue to the CK20-positive cells (green) located at the basal cell layer which make close contact with each other.

Fig. 6 Electron micrograph of a Merkel cell. Many neurosecretory granules 80 to 120 nm in diameter (arrowheads) can be observed in the cytoplasm, forming synapse structures with nerve fibers (N). MC: Merkel cell
Merkel cells and nerves in peri-implant epithelium

PGP 9.5-positive fibers extended from the connective tissue to CK20-positive cells making close contact (Fig. 5). Some free nerve fibers were also observed which had invaded the epithelium.

3. Transmission electron microscopic observations
Most Merkel cells had a roundish shape with lobular nuclei. These cells possessed numerous neurosecretory granules, 80 to 120 nm in diameter, which tended to accumulate at synaptic sites of the cytoplasm. The cytoplasm of Merkel cells was electronlucent compared to other types of epithelial cells, and only a few intermediate filaments and organelles in the cytoplasm were observed. Some cytoplasmic processes extruded and were connected to surrounding epithelial cells by desmosomes. These cells formed synapse structures with nerve terminals, which contained large numbers of mitochondria (Fig. 6).

**Mucosa Removal Group**

1. Light microscopic observations
One day after the surgery, the wound surface was covered by fibrinous material under which many erythrocytes and inflammatory cells were seen (Fig. 7a). Two days after the surgery, a small amount of regenerative epithelium had elongated on the wound surface from the surgical margin of the stratified squamous epithelium. At days 3 and 4 after the surgery, the epithelium had almost regenerated but the thickness of the regenerated epithelium was only about half that of normal epithelium. Basal cells in the regenerated epithelium had a flat shape, and no epithelial rete ridges were seen (Fig. 7b). In the connective tissue, dilated capillaries and some inflammatory cells could still be observed. Six and 7 days after the surgery, the shape of the flat basal cells had changed to cuboidal. However, epithelial rete ridges still were not seen, and a cell-rich fibrous connective tissue without inflammatory cells was observed (Fig. 7c).

By immunohistochemical analysis, no CK20-positive cells were observed in the wound area throughout the 7 days (Fig. 7d). One day after the surgery, no NSE-positive fibers were detected in the wound region, but 3 to 4 days after that, NSE-positive fibers were obvious in the connective tissue. Starting 5 days after the surgery, these regenerative nerve fibers appeared beneath the epithelium (Fig. 7e).

2. Confocal laser scanning microscopic observations
Starting 5 days after the surgery, PGP 9.5-positive nerve fibers could be seen in the connective tissue just beneath the basal cell layer. However, CK20-positive cells were not detected in the experimental region throughout the 7 days (Fig. 8).

3. Transmission electron microscopic observations
Two days after the surgery, several layers had regenerated on the wound surface, but intercellular spaces of the epithelium were extremely wide and a basal lamina could not be detected. Three days after the surgery, a number of epithelial cells with vacuolated structures were observed, and the intercellular spaces between these cells were still wide. Connective tissue was sparse and several erythrocytes were found, but the basal lamina was still not evident (Fig. 9). Four and 5 days after the surgery, the number of epithelial cells with vacuolated structures had decreased. Basal cells were organized and basal laminae were almost complete; hemidesmosomes were observed in these areas. Six days after the surgery, the connective tissue had become dense, basal laminae had regenerated completely, and intercellular spaces revealed normal width. At 7 days after the surgery, regenerative free nerve fiber-like structures were seen in the connective tissue just beneath the basal cell layer. However, no Merkel cells were observed throughout the experimental period (Fig. 10).

**Implantation group**

1. Light microscopic observations
One day after the implantation, a few layers of squamous cells had elongated along the implant surface and many inflammatory cells and erythrocytes were discernible in the connective tissue (Fig. 11a). Two to 4 days after the implantation, stratified squamous epithelial cell layers had elongated deeply toward the connective tissue, and small numbers of inflammatory cells could still be observed in the connective tissue (Fig. 11b). The basal cells of the elongated epithelium had a flat shape, and no epithelial rete ridges were clearly visible. The thickness of the regenerative epithelium was about 1/3 that of normal oral mucosa. Five and 6 days after the implantation, the flattened stratified epithelium had elongated close to the palatal bone but did not come in contact with it. In the connective tissue, a few inflammatory cells could be seen. Seven days after the implantation, the peri-implant epithelium had formed at an acute angle from the oral epithelium, and was oriented parallel to the implant surface. The peri-implant epithelium was composed of 5 to 7 cell layers, and the cell population decreased in the deeper areas. The connective tissue was organized, but no epithelial rete ridges were evident in the peri-implant...
epithelium (Fig. 11c).

By immunohistochemical staining, no CK20-positive cells were detected in the peri-implant epithelium throughout the 7 days after the implantation (Fig. 11d). NSE-positive nerve fibers had appeared in the regenerative connective tissue 3 days after the implantation. They were located just beneath the peri-

implant epithelium at 4 days after the implantation (Fig. 11e).

2. Confocal laser scanning microscopic observations
No CK20-positive cells were seen in the peri-implant epithelium throughout the 7 days after the implantation (Fig. 12a). Four days after the implan-
Merkel cells and nerves in peri-implant epithelium

263
tation, PGP 9.5-positive nerve fibers had become discernible in the connective tissue. Seven days after the implantation, these fibers had invaded the regenerative epithelium (Fig. 12b).

3. Transmission electron microscopic observations
One to 2 days after the implantation fibrous connective tissue was loose and erythrocytes were observed, but the basal lamina could not be recognized. Many epithelial cells with vacuolated structures and tonofilaments were found and intercellular spaces between the epithelial cells were wide (Fig. 13). Three days after the implantation, epithelial cells with vacuolated structures were recognizable.

**Fig. 9** An electron micrograph 3 days after the surgery. Regenerating epithelial cells (EC) contain vacuoles in their cytoplasm (arrowheads); intercellular spaces are wide. A basal lamina is not evident. E: erythrocyte, CT: connective tissue

**Fig. 10** Electron micrograph 6 days after the surgery. The lamina densa and the lamina lucida have regenerated completely. The intercellular spaces have become nearly normal. A regenerating nerve fiber-like structure (arrowhead) is seen in the connective tissue (CT). EC: epithelial cell
in the peri-implant epithelium, and a few tonofilaments were seen in the cytoplasm (Fig. 14a). The connective tissue had matured, a few leucocytes and erythrocytes were detected, and the basal lamina was partly regenerated (Fig. 14b). Four to 5 days after the implantation, intercellular spaces were wide and lymphocyte infiltration was observed. The basal lamina was more clearly visible but was not yet complete. Nerve fibers were evident beneath the basal cell layer (Fig. 15). Six to 7 days after the implantation, the basal lamina had regenerated completely, but the intercellular spaces had a tendency to be wide compared to those of the mucosa removal control group and nerve fibers had elongated into these spaces in the peri-implant epithelium (Fig. 16).

**DISCUSSION**

Merkel cells were initially discovered in the epidermis of pig snout skin and are thought to transfer mechanical impulses to the nerve tissue (8, 16, 28). Since the initial discovery, many investigators have studied Merkel cells in the skin, and it is now generally accepted that they exist ubiquitously in vertebrates including birds, fish, amphibians and...
Merkel cells and nerves in peri-implant epithelium

Figs. 13 and 14 illustrate electron micrographs of peri-implant epithelial cells 2 and 3 days after implantation, respectively. Fig. 13 shows a peri-implant epithelial cell (EC) with vacuoles, and no basal lamina is observed. E: erythrocyte, CT: connective tissue. Fig. 14a demonstrates regenerative basal epithelial cells containing vacuoles (arrowheads) and wide intercellular spaces. Fig. 14b depicts the matured fibrous connective tissue with partially regenerated lamina densa (LD) and lamina lucida (LL). EC: peri-implant epithelial cell, CT: connective tissue.

Merkel cells and nerves in peri-implant epithelium

mammals (1, 3, 17, 19, 21, 23, 24, 26, 30, 31). The morphology of Merkel cells in the oral cavity has been investigated in humans and in rats (1, 26), and there have been a few reports on Merkel cells in the touch domes of the hamster cheek pouch (5, 30). In addition, the relationship between nerve fibers and Merkel cells has also been reported (21, 23). However, there have been no reports about the behavior of Merkel cells around dental implants in the palatine mucosa of any kind of animal, although Tachibana and Ishizeki studied Merkel cell behavior related to wound healing in rabbit skin (25). In the palatine mucosa of hamsters, it is known that the number of Merkel cells in the fourth palatine ruga...
is the highest and that the number in the third palatine ruga is the next highest (12). In this study, the third palatine ruga of the hamster was chosen for implantation because there are a number of Merkel cells there and the width of the third ruga is greater than that of the fourth ruga. Concerning the length of the experimental period, Listgarten reported that the junctional epithelium and gingival connective tissue of monkeys had completely regenerated within 12 days after gingivectomy (15). Since in our study, it took only 2 days to cover the wound area after the removal of the oral epithelium of the hamster ruga, we decided that 7 days was a sufficient time period for observation. In addition, Fujii et al. reported that the cryofracture technique is more advantageous for preservation of the implant/tissue in-

**Fig. 15** Electron micrographs 5 days after the implantation. The intercellular spaces of the peri-implant epithelium are still wide, and lymphocyte infiltration (L) can be observed. Regenerating nerve fibers (N) are detected in the connective tissue (CT) just beneath the peri-implant epithelium. EC: peri-implant epithelial cell

**Fig. 16** Electron micrographs 7 days after the implantation. The peri-implant epithelium and connective tissue have regenerated completely. However, the intercellular space is still wide compared to the mucosa removal group. A regenerating nerve fiber (arrowheads) is seen in the peri-implant epithelium. EC: peri-implant epithelial cell, CT: connective tissue
Regeneration capacity of Merkel cells
Many studies have addressed the origin of Merkel cells, and their possible origin from the neural crest or the epidermis has been discussed. For instance, Moll et al. reported that Merkel cells in human skin are post-mitotic cells that are derived from undifferentiated keratinocytes with stem cell characteristics (18). Tweedle demonstrated that Merkel cells could be found in amphibian skin if all presumptive neural tissue, including the neural crest, had previously been removed, and he suggested that Merkel cells are not derived from the neural crest (32). In addition, Tachibana et al. reported the development of peripheral nerve terminals in the subepithelial layer of rat fetus palatine mucosa as it was established (24). They suggested the presence of Merkel cells 4 days after birth and the formation of a Merkel cell-neurite complex. These results suggest that Merkel cells in the skin and oral mucosa may originate from epidermal cells but not from the neural crest. In contrast, Grim and Halata recognized that Merkel cells were evident after chick leg primordia had been grafted onto quail host embryos (7). They suggested that Merkel cells are derived from the neural crest in the case of limb primordia in chick/quail chimeras. The results of the present study show that no Merkel cells are present in the regenerating oral epithelium after wounding or in the peri-implant epithelium after implantation. Although the length of the experimental period seems relatively short in this study, 7 days is a sufficient observation period, since Merkel cells had not regenerated within 3 months after wounding or 14 days after implantation in pilot experiments (unpublished data). However, in contrast to our results, Tachibana and Ishizeki reported that Merkel cells did regenerate after partial resection of the labial skin of rabbits (25). This difference in the regenerative behavior of Merkel cells between the skin and the oral mucosa might be caused by differences in the embryonic environment. The skin contains many appendages, such as sebaceous glands, sweat glands and hair follicles, each of which contains many Merkel cells, whereas no such appendages are formed in the oral mucosa in the fetus. This suggests that the skin in regenerating epithelium contains many stem cells that could differentiate into Merkel cells. In contrast, at regeneration, stem cells of the oral mucosa (the so-called basal cells), might differentiate only into keratinocytes but not into other types of cells.

Relationship between Merkel cells and nerve fibers
In reviewing the relationship between Merkel cells and nerve fibers, we note that Merkel cells can produce nerve growth factor (NGF) and NGF-receptors (20, 21). Those studies suggest that the NGF produced by Merkel cells probably induces the development of nerve fibers toward the Merkel cells. However, if Merkel cells could induce nerve fiber development in that manner, they should have appeared in our experiments. However, Merkel cells were not found in the regenerated epithelium or in the peri-implant epithelium, which suggests that Merkel cells in the oral mucosa do not play an inductive role in the development of nerve fibers.

Behavior of the peri-implant epithelium and nerve endings
Differences between the peri-implant epithelium and the junctional epithelium of natural teeth are well known (6, 9–11, 14). Fujizeki et al. reported that the peri-implant epithelium is similar to that seen in the oral mucosa, but that it is structurally different from the functional epithelium (6). Titanium-based implant materials could influence the phenotype of oral gingival epithelial cells in different ways (14). For example, Inoue et al. showed that intercellular spaces in the peri-implant epithelium are wider and weaker in defense properties than are those of the junctional epithelium (11). Ikeda et al. demonstrated that a horseradish peroxidase tracer penetrated more easily into the peri-implant epithelium than into normal junctional epithelium, and that some horseradish peroxidase could be detected in the connective tissue under the peri-implant epithelium (10). Those authors also stated that the endocytotic capacity of cells in the peri-implant epithelium is inferior to that of the junctional epithelium. The basis for their conclusion was that the quantity of horseradish peroxidase found in intracellular vesicles and granules of the peri-implant epithelium was less than that found in the junctional epithelium. On the other hand, Buser et al. reported that the peri-implant epithelium is able to regenerate epithelial structures with a peri-implant sulcus and a functional epithelium that is similar to natural teeth (2). Cochran also reported that the epithelial structures which form around implants are similar to the epithelium found around teeth (4). However, Buser et al. showed that there are wide intercellular spaces and transmigrating polymorpho-nuclear leukocytes and lymphocytes in
the junctional epithelium around the implant surface (2). Concerning nerve fibers, Tanaka et al. reported that many nerve fibers containing substance P are present in the middle portion of the junctional epithelium, whereas they are sparse in the oral sulcular epithelium and in the oral epithelium (27). They suggested that substance P, which is released from the nerve fibers into the junctional epithelium, might regulate the endocytotic activity of neutrophils and junctional epithelial cells to form part of the defense network against noxious stimuli.

In this study, intercellular spaces were comparatively wide. A number of regenerative nerve fibers were also evident. These had invaded the superficial layer of the peri-implant epithelium in a manner similar to the invasion into the junctional epithelium as described by Tanaka et al. All of these regenerative nerve fibers possess free endings, and their functions might be a sensory system for pain, touch and pressure (27). We suggest that these free nerve endings play a role not only in the sensory system but also as part of the defense system, even though Merkel cells do not regenerate after implantation.

Taken together, our results show that Merkel cells in the hamster palatine mucosa do not regenerate in the peri-implant epithelium. However, regenerative nerve fibers seem to play essential roles as part of the defense and sensory systems around the peri-implant epithelium: an endocytotic defense mechanism.

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REFERENCES


