Neuroendocrine cells in Malassez epithelium and gingiva of the cat

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Malassez epithelium has been designated as epithelial cell rests, the biological significance of which is still under debate. This study was designed to analyze Malassez epithelium for the presence of neuroendocrine cells. Gingival tissue was included as a positive control. Using immunohistochemistry, confocal and light microscopy, Malassez epithelium and gingival epithelium from mature cats ($n = 5$) were examined for cells containing the neuropeptides calcitonin gene-related peptide (CGRP), substance P (SP), and vasoactive intestinal peptide (VIP). Both Malassez epithelium and the basal epithelial cell layers in gingival rete pegs regularly displayed cells immunoreactive to CGRP, SP, and VIP. The immunopositive cells were most frequently present in the epithelial cell clusters and strands of Malassez located in the cervical half of the periodontal ligament. Double immunolabeling revealed cellular co-expression of CGRP or SP with VIP, and the neuropeptides were co-localized in the cellular compartments. Labeled cells in both epithelia were occasionally supported by immunoreactive nerve fibers. This study shows that cells immunoreactive to CGRP, SP, and VIP are located within the cat Malassez epithelium. The localization of neuroendocrine cells verifies the diversity of this epithelium and confirms that Malassez epithelium is composed of different cell types, in common with epithelia from other locations. The presence of neuroendocrine cells in Malassez epithelium strongly suggests biological functions of this tissue, and the neuropeptide content may thus indicate endocrine functions of the cells. □ *Calcitonin gene-related peptide; gingiva; Malassez epithelium; neuropeptides; substance P; vasoactive intestinal peptide*

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The developing and mature tooth is surrounded by a continuous, net-like pattern of epithelial cells from the Hertwig root sheath originating from the oral epithelium. The anatomy and distribution of Malassez epithelium within the periodontal ligament are fairly well known from light and electron microscopic studies (1, 2). Malassez epithelial cells are located close to the root cementum over its entire length, and are shown to persist within the periodontal membrane throughout the life of the tooth (3).

A biological function of the Malassez epithelium has been questioned and debated, but there is little information and experimental evidence available. A possible role proposed for Malassez epithelium is its maintaining the width of the periodontal membrane by acting as a hinder for bone in-growth (4). However, this hypothesis has been questioned (5). Stimulation of these cells induces epithelial cell proliferation (6) and promotes inflammatory cell invasion (7), and necrotic pulpal tissues may initiate these cells to form periapical cysts (8).

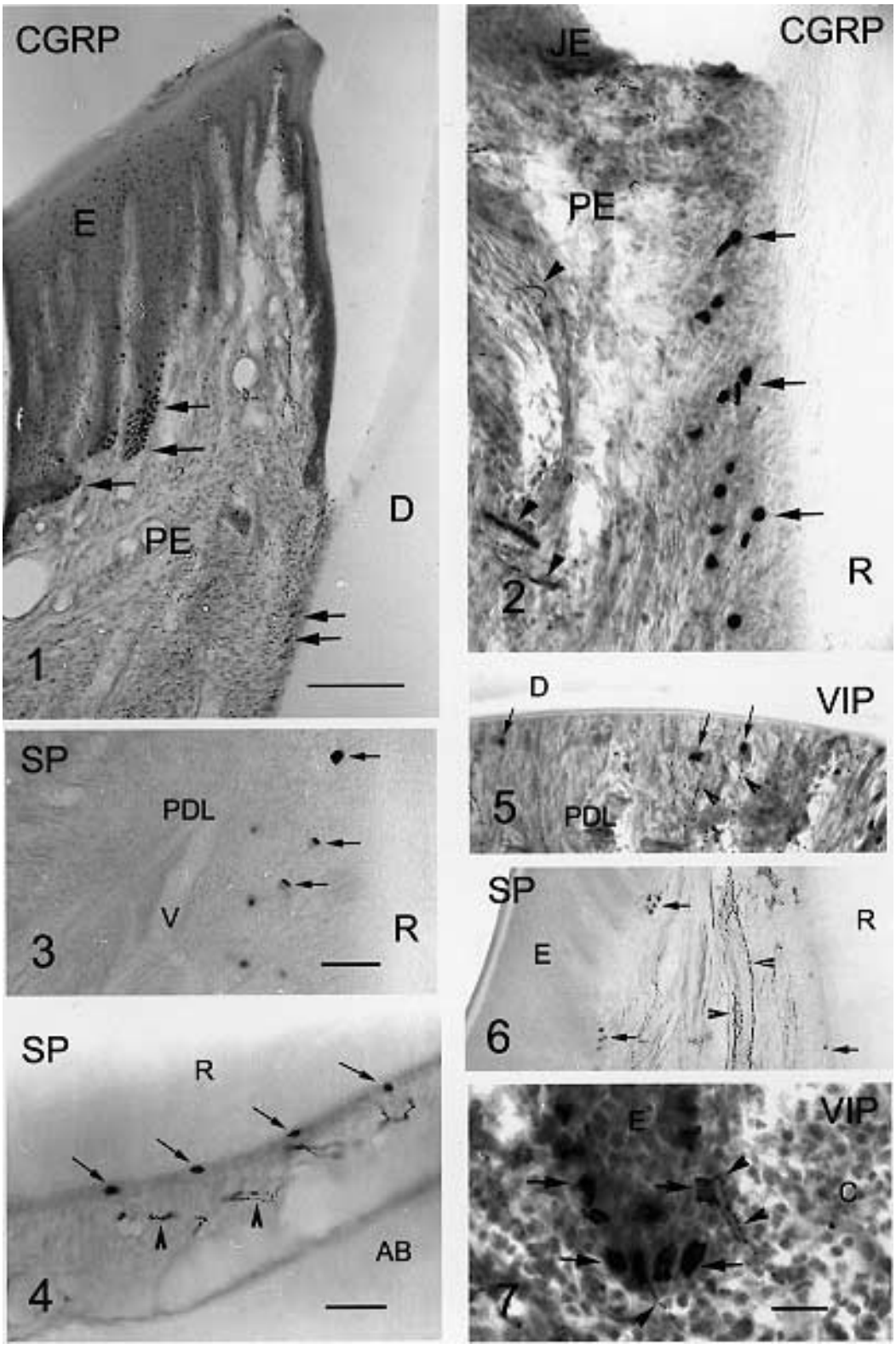
In the basal epithelial cell layers in oral mucosa and gingiva (9–12), some cells are found to express protein gene product PGP 9.5, which is localized in neuroendocrine cells and nerve fibers. Similar to epithelial tissues in skin (13), in larynx and trachea (14), oral epithelium and gingiva are reported to comprise neuroendocrine cells containing the neuropeptides calcitonin gene-related peptide (CGRP), substance P (SP), and vasoactive intestinal peptide (VIP) (16–18).

In contrast to other oral epithelial tissues, the diversity of Malassez epithelial cells has not been investigated previously. The aim of this study was therefore to analyze cat Malassez epithelium for cells immunoreactive (IR) to the neuropeptides CGRP, SP, and VIP. Moreover, it was of interest to establish whether neuroendocrine cells in gingiva and Malassez epithelium share the same types of neuropeptides.

Material and methods

Mature cats ($n = 5$) of both sexes, 18 months of age, were used in this study. The experimental procedures were registered and approved (ECA-97-0049) by the Nihon University School of Dentistry at Matsudo, Japan, and performed in accordance with the recommended guidelines of the Norwegian Experimental Animal Board.

The cats were transcardiacally perfused under deep Nembutal (sodium pentobarbital) anesthesia, 50 mg/kg


CGRP
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b.w. intraperitoneally, with heparinized 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB, pH 7.4. The jaws were excised and postfixed in the same fixative for 48 h. Canines and the molar teeth were demineralized in 4N formic acid and 0.05 M sodium formate, and incisors were demineralized in 10% EDTA containing 7.5% polyvinylpyrrolidone. The specimens were rinsed in phosphate-buffered saline (PBS) for 24 h and saturated in 30% sucrose in 0.1 M PB, pH 7.4, for another 24 h before cryosectioning. Incisors, canines, and molars were cut in a mesiodistal or buccolingual direction, 40 mm thick, on a freezing microtome.

Immunohistochemistry

Avidin-biotin peroxidase (ABC) method. Free-floating sections were incubated for 72 h with rabbit polyclonal antibodies against CGRP (dilution 1:7500, Cambridge Research Biochemicals, Cambridge, UK), SP (dilution 1.4000, Eurodiagnostica, Malmø, Sweden), or VIP (1:4000, Eurodiagnostica). Prior to primary antibody incubation, an appropriate blocking treatment with 0.3% H_2O_2 in methanol, and in 2% normal goat serum (NGS) (Vector Laboratories, Burlingame, CA) in PBS and 0.3% TX, was performed. The antigen-antibody complexes were labeled according to the ABC method (Vector Laboratories, Burlingame, CA) and visualized using 3′3-diaminobenzidine (DAB, Sigma, MO, USA) as the chromogene. To enhance the chromogene reaction, 0.2% $(NH_4)_2$ Ni-$(SO^4)_26H_2O$ was routinely used. Between individual incubation steps, the sections were thoroughly rinsed in PBS. After final rinsing in PBS, the sections were mounted on gelatin-coated slides, dried, and counter-stained in methylene blue/azure II in 1% sodium borate in distilled water, dehydrated and cleared, and cover-slipped with Eukitt (Kindler, Freiburg, Germany).

Fluorescence immunohistochemistry. The primary polyclonal antibodies against CGRP and SP from guinea-pigs (Eurodiagnostica) and the VIP antibody from rabbit were used for double immunolabeling. The sections were pretreated with NGS (1:20 in PBS and 0.3% TX), and thereafter incubated for 24 h in a mixture of antibodies against CGRP (dilution 1:400) and VIP (dilution 1:200), or SP (dilution 1:200) and VIP (dilution 1:200), respectively. After several PBS rinses, the sections were incubated for 2 h in a mixture of fluorescein isothiocyante (FITC)-labeled goat-anti-guinea-pig IgG and tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat-anti-rabbit IgG diluted 1:40 in 1% BSA/PBS and 0.3% TX. The sections were rinsed, air-dried, and mounted in aqueous mounting medium (Vecta-Shield, Vector Laboratories, Burlingame, CA).

Immunocontrols. Standard immunocontrols were conducted regularly, either by omission of the primary antibodies or the biotinylated secondary antibody, or incubation in preabsorbed primary antibody.

Fig. 1. A frontal section from gingiva and the cervical periodontium (PE) of a cat incisor immunolabeled for calcitonin gene-related peptide (CGRP). Cells (arrows) expressing CGRP are located in Malassez epithelium close to the root (D) and in the basal cell layers, mainly in the rete pegs, of gingival epithelium (E). Scale bar = 100 mm.

Fig. 2. A longitudinal section from the cervical periodontium (PE) of a cat canine. A number of CGRP-positive cells (arrows) are displayed in the Malassez epithelium close to the root (R). CGRP-labeled nerve fibers (arrowheads). JE, junctional epithelium.

Fig. 3. Sagittal section from the cervical periodontal ligament (PDL) of a cat canine immunolabeled for SP. SP-positive cells (arrows) are located in Malassez epithelium close to the root (R) surface. V, blood vessel. Scale bar = 50 mm.

Fig. 4. A cross-section from the midroot (R) periodontal ligament immunolabeled for SP. Cells (arrows) immunoreactive to SP are located close to the root (R) surface. A number of nerve fibers (arrowheads) containing SP are shown. AB, alveolar bone. Scale bar = 50 mm.

Fig. 5. Section from an interradicular area of the periodontal ligament (PDL) of a cat molar. Cells (arrows) expressing vasoactive intestinal peptide (VIP) are located within Malassez epithelial islands. Thin, VIP positive nerves (arrowheads) are distributed in the PDL, and some are close to Malassez epithelium. D, dentin.

Fig. 6. Cells (arrows) located in the gingival epithelial rete pegs, Malassez epithelium, and nerve fibers (arrowheads) in the subjacent connective tissues are substance P (SP) immunoreactive. E, gingival epithelium. R, root.

Fig. 7. A number of cells (arrows) expressing vasoactive intestinal peptide (VIP) localized in the basal cell layers of a gingival epithelial rete peg (E). Some cells are supported by VIP containing nerve fibers (arrowheads). C, connective tissues. Scale bar = 25 mm.

Results

Neuropeptide immunoreactive cells were regularly located in Malassez epithelium and in the basal cell layers of gingival epithelium, as demonstrated in the same sections (Figs 1 and 6). The intensity of the labeling varied considerably among the neuropeptide expressing cells in both epithelia, but was generally weaker in Malassez epithelium than in gingiva. In Malassez epithelium the labeled cells were usually located in the cervical half of the periodontal ligament. The regional distribution of labeled cells in gingival epithelium did not conform to any pattern.

Single cells located in the Malassez epithelial strands expressed CGRP (Figs 1, 2, 8a, 8b), SP (Figs 3, 4 and 6), and VIP (Figs 5 and 8c). Both density and distribution of the cells expressing the different neuropeptides within this epithelium were similar and closely situated to the root surface (Figs 1, 2, 4, 5). The labeling of SP was generally weak in immunoreactive Malassez cells.

In the basal cell layers of the gingival epithelium, immunolabeled cells expressing CGRP (Fig. 1), SP (Fig. 6), and VIP (Fig. 7) were found mainly as cell clusters located in the epithelial rete ridges, but were highly variable in number. However, some labeled single cells were regularly present. Occasionally, labeled single cells were also found in the junctional epithelium (not shown).

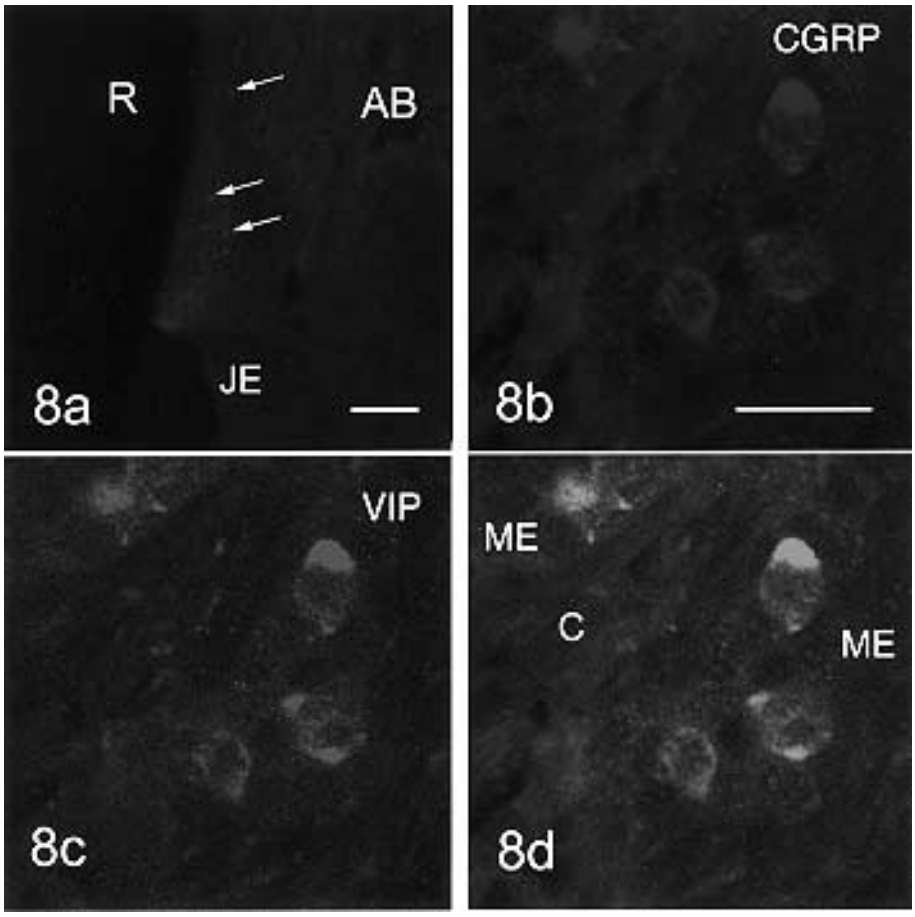

Fig. 8. Confocal images of a longitudinal section from the periodontal ligament of a cat canine, double-labeled with calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) antibodies. Fig. 8a demonstrates cells (arrows) expressing CGRP in Malassez epithelium in cervical periodontal ligament. Fig. 8b shows higher magnification of labeled cells (arrows) in Fig. 8a. CGRP are localized to the cell periphery. Fig. 8c. VIP is expressed in the same cells shown in Fig. 8b. Fig. 8d. Merging of the confocal images in b and c demonstrates coexpression of CGRP and VIP, and co-localization of the peptides in labeled cells located in Malassez epithelium (ME). AB, alveolar bone. C, connective tissues. JE, junctional epithelium. R, root. 8a, Scale bar = 100 mm. 8b, Scale bar = 20 mm.

The morphology of the CGRP-, SP-, and VIP-expressing cells in Malassez epithelium (Figs 3, 8b, c, d) and in gingival basal cell layers (Fig. 7) was similar. Some cells were roundish in shape, while others clearly demonstrated cytoplasmic extensions (Figs 7 and 8b, c, d). The neuropeptides were mainly excentric located in the cell cytoplasms and in the cell spikes or extensions (Figs 3, 7, and 8b, c, d), although immunoreactivity was expressed along the entire cell periphery. Furthermore, CGRP and SP (not shown) were co-expressed and even co-localized with VIP (Fig. 8d) within cells in both Malassez and gingival epithelium.

Some of the IR cells in both epithelia, as shown in Malassez epithelium in Fig. 5 and in gingival epithelium in Fig. 7, were closely supported by neuropeptide-containing nerve fibers.

Discussion

This study provides clear evidence for the presence of neuroendocrine cells in Malassez epithelium, in accordance with neuroendocrine cells located in gingival epithelium. Furthermore, the neuroendocrine cells in cat Malassez tissues contain and co-express CGRP, SP, and VIP neuropeptides that are all localized in neuroendocrine cells in other epithelia. Accordingly, these findings confirm that Malassez epithelium is composed of different cell types in common with epithelia from other oral tissues and the digestive tract (14, 15, 17, 18). The demonstration of neuroendocrine cells in Malassez epithelium thus strongly supports our suggestions that this tissue may have biological functions and significance in maintaining homeostasis in the tooth supporting structures.

In a physiological functional state, the periodontal ligament is continuously under mechanical and immunological stress. As neuroendocrine cells represent multi-messenger systems that exert a balanced effect in maintaining the tissue homeostasis (19), the neuropeptide-producing cells in Malassez epithelium might thus induce and support various biological responses. Stimulation of neuroendocrine cells results in transient activation of transcription factors like *c-fos* in response to cell depolarization (19), and thus influences the regulation of its own multi-messenger systems. This means that an individual cell can vary the information it transmits under different physiological conditions. Moreover, stimulation of these cells may promote the release of neuropeptides into the PDL, and thereby add local support to the neurogenic signaling from the peripheral nervous system. The neuropeptides CGRP, SP, and VIP may serve various regulating mechanisms that can meet increasing demands in the PDL under physiological as well as pathological conditions. Stimulation of leucocyte chemotaxis, blood flow increase, release of inflammatory mediators and trophic factors are all reported effects exerted by CGRP, SP, and VIP (10, 20, 22–24). Thus, it could be suggested that the neuroendocrine cells in Malassez epithelium take part in and serve several regulating mechanisms in the periodontal ligament.

The local immune cell responses might transiently be modulated by CGRP, SP, and VIP release upon stimulation of the Malassez epithelium, as granulocytes, lymphocytes, and macrophages are equipped with neuropeptide receptors (20–22). These suggestions are supported by recent findings in orthodontically stressed periodontal tissues, where accumulation of immune cells expressing class II molecules was regularly concentrated around enlarged Malassez epithelial cell clusters (7).

CGRP, SP, and VIP are all shown to act as growth factors in vitro, and may have a trophic effect on tissues and cell populations both directly and indirectly, including keratinocytes (23). The neuroendocrine cells may therefore take part in maintaining the epithelial net surrounding the roots and in stimulating epithelial cell proliferation, and might under pathological stimuli promote the confluence of the Malassez epithelium.

Numerous studies have shown the effects of the neuropeptide CGRP, SP, and VIP released from sensory nerves on the increase of blood flow, as shown in oral tissues (10, 24, 25). It could therefore be suggested that neuropeptides from the neuroendocrine cells may add support to a local blood flow increase in the PDL.

So far, the identity of the neuroendocrine cells in Malassez epithelium is not known. Cells containing neuropeptides including SP, CGRP, and VIP (15–17) with a similar morphology, intracellular neuropeptide distribution, and intraepithelial location in oral epithelium and gingiva (26), have previously been classified as Merkel cells (27). Merkel cells are cells with a morphological plasticity that varies from roundish cells to cells with dendrites and cytoplasmic extensions (12). This variation in cell morphology is shown for Merkel cells in oral mucosa (12), and may reflect a heterogene cell function as a response to micro-environmental signals (19). In agreement with a general finding in Merkel cells (16), the intracellular CGRP-, SP-, and VIP-labeling in both epithelia studied was regularly excentrically located within the cells (Fig. 8).

Typical Merkel cells are part of the Merkel-neurite complex related to slowly adapting type-1 mechanoreception (19, 28, 29). Recently, a close relation has been demonstrated between nerve endings and the Malassez epithelium (30). In the present study, labeled cells in both Malassez and gingival epithelia were occasionally closely supported by neuropeptide containing nerve fibers, while other labeled cells were without close neural support. These findings are consistent with Merkel-neurite complexes and non-innervated Merkel cells localized by means of protein gene product (PGP) 9.5 labeling of human oral mucosa (11).

The weak SP labeling of the neuroendocrine cells in Malassez epithelium in this study may be due to the method employed, but this is less likely as the nerve fibers in the same PDL section were strongly SP positive (Figs 4 and 6). Therefore, the weak SP-labeling in the Malassez neuroendocrine cells may rather reflect a low SP content, i.e. the result of a functional state of the cells (19).

In conclusion, our study provides clear evidence that Malassez epithelium contains neuroendocrine cells expressing and co-expressing the neuropeptides CGRP, SP, and VIP. These findings underline the heterogeneous nature of Malassez epithelium in common with gingival and oral epithelium, and with epithelial tissues in general. Moreover, these results strengthen and support the suggestion that Malassez epithelium could have biological functions. Endocrine functions of these cells are strongly suggested owing to their neuropeptide content. Further identification of the neuropeptide-containing cells in Malassez epithelium is currently under investigation.

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