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Morphological Changes of Ruffini Nerve Endings in the Periodontal Ligament of Aged Mice

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ABSTRACT Ruffini nerve endings in the periodontal ligament (PDL) of aged mouse maxillary incisors were investigated by means of immunohistochemistry for protein gene product 9.5 (PGP 9.5) at light and electron microscopic levels. C3H/HeSlc mice were sacrificed by perfusion fixation. Frozen sagittal cryostat sections of decalcified maxillary incisors were prepared and stained by anti-PGP 9.5 antibody, followed by biotinylated anti-IgG, to reveal neural elements in the PDL. Apart from the typical Ruffini endings distributed throughout the lingual PDL, club-shaped nerve terminations with few, if any, micro-projections were found among lingual alveolar bone, but not in the lingual PDL of aged mouse incisors. Ultrastructurally, their nerve terminals contained a marked reduction in the number of mitochondria and other cytoplasmic organelles, compared with those in the younger stage. These results illustrated that Ruffini endings in a distinctive area between alveolar bone may be in the hypofunctional stage, causing their structures to undergo a regressive change with aging.

KEYWORDS: Ruffini nerve endings, regression, immunohistochemistry, PGP 9.5.

INTRODUCTION

Periodontal ligament (PDL) is a soft, specialized connective tissue situated between the cementum covering the root of the tooth and the alveolar bone forming the socket wall. In relation to its principal function, PDL undergoes a complex mechanism of development[1] and is composed of collagenous fibers, which have been reported to be the most frequently found structural element in PDL.[2] Periodontal fibroblasts are relatively more active and possess a remarkably higher turnover rate than the fibroblasts in other organs.[3]

Despite its relatively low proportion in PDL, periodontal nerves and endings are spatially arranged to determine the response characteristics of the PDL. Four types of nerve endings, including free endings, Ruffini endings, coiled endings, and encapsulated endings, are found in human PDL.[4] However, only free endings and Ruffini endings are found in rodent PDL, and their histological structures have already been confirmed.[5]

Periodontal mechanoreceptors are involved in the induction of various oral reflexes, which make regular and smooth mastication possible.[6-7] Ruffini endings, as well as free endings, are believed to function as mechanoreceptors[1], and both are found in the PDL of all mammals.[5] Nevertheless, recent physiological studies have shown that there is only one type of periodontal mechanoreceptor.[8-10]

Protein gene product 9.5 (PGP 9.5) is a cytosolic protein and belongs to a family of ubiquitin carboxyl-terminal hydrolases.[11] These hydrolases have modifying effects on the function of T-lymphocyte homing receptors[12], platelet-derived growth factor receptors[13], growth hormone receptors.[14] PGP 9.5 is involved in a variety of cellular biological functions.[15-16] Recent immunohistochemical research has shown that PGP 9.5 is a general marker for nerve and neuroendocrine cells[17-20], and is detected as a cytoplasmic protein contained in central and peripheral neurons.[21] Anti-PGP 9.5 antibody is reported to be useful for demonstrating nerve elements in post-natal developing dental structures[22], and particularly Ruffini endings in the PDL.[23-24]

Rodent incisors continuously erupt and are worn at the incisal edge by attrition throughout life, which makes them useful for studies of dental histogenesis.[25-26] Rodent Ruffini endings represent an appropriate morphology of periodontal mechanoreceptors and are found only in the lingual PDL, which is always in the state of tension.[27] Though recent reports on configurations of these endings in neonatal and adult rodents have been documented[23-24], those in the aged animals are very limited. Hence, it was the purpose of this study to

disclose the histological structures of Ruffini endings in young and aged mice using immunohistochemistry for PGP 9.5 at light and electron microscopic levels.

Materials and Methods

Twelve C3H/HeSlc mice, with ages of 7 and 35 weeks old (n = 6 for each group), were used in this study. They were experimentally handled, according to the instructions of World Health Organization.[28-29] Under a deep anaesthetization with diethyl ether, the mice were perfused through their left ventricles with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Their maxillae were removed *en bloc*, stored in the same fixative solution at 4 °C for 14 hours, and decalcified in 10% ethylene diamine tetra-acetic acid (EDTA)-2Na solution, pH 7.4 at 4 °C for 3 weeks. Then, the decalcified specimens were immersed overnight in 0.01 M phosphate-buffered saline (PBS) containing 30% sucrose solution at 4 °C.

Frozen sections, 20 μm thick, were prepared sagittally and serially using a cryostat (Leica CM3000), and collected on poly-L-lysine-coated glass slides (Matsunami, Osaka, Japan). The avidin-biotin-complex (ABC) method was performed after incubating in 0.01 M PBS containing 0.3% Triton X-100 (Sigma Chemicals, St Louis, MO, USA) at room temperature for 15 minutes. The sections were then processed using immunohistochemistry for PGP 9.5. Free-floating 50 μm thick sections were also conventionally prepared for the transmission electron microscopic observation.

Immunohistochemistry for PGP 9.5 at the light microscopic level

Prior to an incubation with the primary antibody, endogenous peroxidase activity and non-specific binding were blocked using 0.3% H_2O_2 in absolute methanol and 2% normal goat serum (Vector Laboratories, Burlingame, CA, USA), respectively. The sections were then incubated with a rabbit polyclonal antiserum against human PGP 9.5 (Ultraclone), diluted 1:10,000 with 0.01 M PBS, in a humid chamber at 37 °C overnight. The sections were subsequently incubated with biotinylated anti-rabbit IgG (Chemicon, CA, USA) and ABC complex, according to the manufacturers' instructions (Vector Laboratories). For the final visualization of immuno-reactive sites, the sections were treated with 0.02% 3,3-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.05 M tris-HCl buffer, pH 7.6. After rinsing, the sections were counterstained with 1% methyl green, dehydrated in ascending graded series of ethanol, cleared in xylene, and mounted with Entellan new (E Merck, Darmstadt, Germany). Specificity of immunohistochemistry for PGP 9.5 was verified by replacing the primary antibody with non-immune rabbit antiserum and by omitting the treatment with anti-rabbit IgG or ABC complex.

Immunohistochemistry for PGP 9.5 at the electron microscopic level

Free-floating 50 μm thick sections were processed for PGP 9.5 immunohistochemistry as described above, except that pre-treatment with 0.3% H_2O_2 in absolute methanol, Triton X-100 and counterstaining were omitted. After light microscopic observation and photography, the sections were post-fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 2 hours, and with 1% osmium tetroxide (OsO_4) reduced with 1.5% potassium ferrocyanide in the same buffer for 2 hours. The sections were then dehydrated through graded series of ethanol, infiltrated and flat-embedded in Epon 812. Ultra-thin sections were cut with a diamond knife, double-stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (Hitachi H-800) at an accelerating voltage of 75 kV.

For ultrastructural observations, some mice were perfused with a mixture of 3% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB, pH 7.4, followed by EDTA decalcification, OsO_4 post-fixation, and embedding in Epon 812.

Results

In all animals, it was found that thick nerve bundles positively immunoreacted to anti-PGP 9.5 antibody entered the lingual periodontal ligament through slits in the lingual bone (Fig 1a, b). Some of them branched toward the incisal and basal directions. The nerve bundles then diverged in a dendritic form, and each of them terminated in dilated bulbs as Ruffini nerve endings. The endings were closely associated with periodontal collagenous fibers restricted to the alveolus-end of the PDL, and were seen throughout a large area of PDL. At high magnification (Fig 2), Ruffini endings possessed irregular outlines with numerous fine micro- or finger-like projections. In addition, some thin nerve endings were also observable. Ultrastructural observation revealed that Ruffini endings consisted of expanded axon terminals filled with numerous mitochondria (Fig 3). The axon terminals were covered by thick Schwann sheaths, and externally surrounded by several layers of basal lamina, which

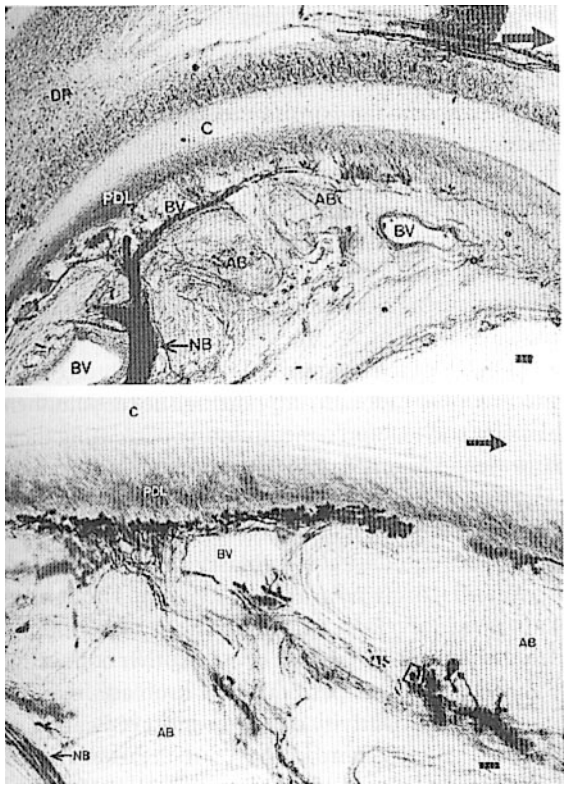

Fig 1. Light micrograph showing distribution of PGP 9.5-immunoreactive nerve elements in the lingual periodontal ligament (PDL) of the incisors of mice with the ages of 7 (a) and 35 (b) weeks old. Large nerve bundles (NB) enter the lingual PDL through slits of the alveolar bone (AB), and branch toward the incisal and basal directions. They terminate as thin free endings or thick Ruffini-like endings. Large arrow indicates the direction of incisal edge, BV: blood vessel, C: cementum, DP: dental pulp. Bar = 200 μm.

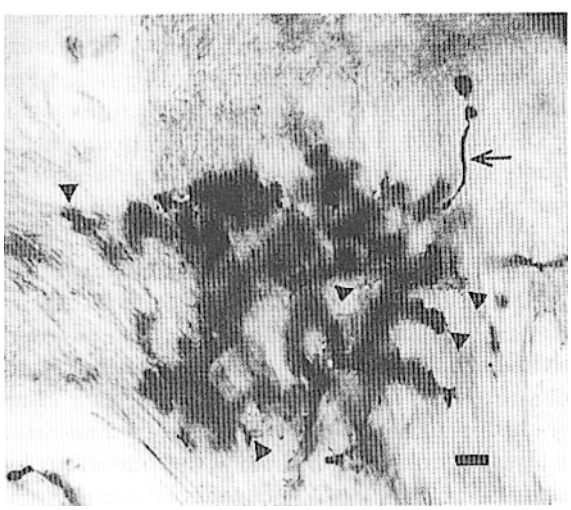

Fig 2. Light micrograph showing a typical Ruffini ending in the lingual periodontal ligament of a 7-week-old mouse. The endings exhibit irregular outlines and possess numerous fine micro-projections (arrowheads). A thin nerve ending is also noted (arrow). Bar = 25 μm.

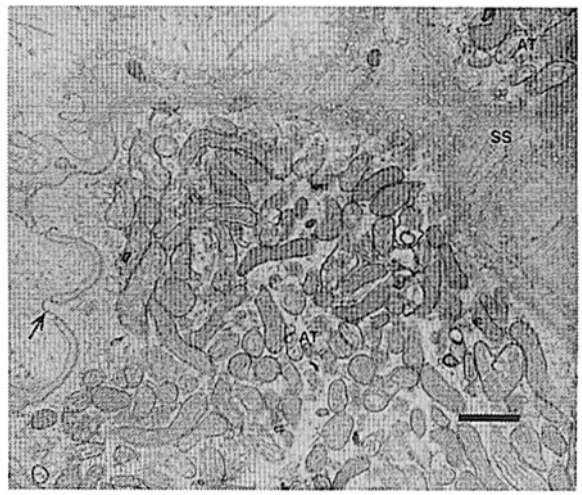

Fig 3. Transmission electron micrograph of typical Ruffini endings in the periodontal ligament of a 7-week-old mouse demonstrating an axon terminals (AT) enclosed with Schwann sheath (SS) and basal lamina. Note a large number of mitochondria in the terminal and an axonal spine (arrow) extending from the terminal to basal lamina through a slit in the Schwann sheath. Bar = 1 μm.

were penetrated by collagenous fibrils. Moreover, some axonal spines were occasionally found extending from the axon terminals through slits in the Schwann sheath.

In the PDL of aged mice, lingual alveolar bone was composed of 2 parts, ie, the old (area under the demarcated area in Fig 1b) and the newly formed (area above the demarcated area in Fig 1b) segments. Ruffini endings with the previously recognized structures were detected above the latter part. Additionally, some endings found under that newly formed alveolar bone were trapped in a narrow and small portion of the PDL between the alveolar bone. Such endings possessed a club-shaped configuration with few, if any, micro-projections (Fig 4). Immuno-electron microscopic observation of these endings (Fig 5) revealed a smooth contour and a marked decrease in the number of mitochondria and small vesicles of 30-130 nm in diameter in comparison with those in the young animals.

Discussion

For light microscopy, nerve fibers are commonly revealed by silver impregnation and immunohisto-chemical methods. However, silver provides non-specific stains for other extracellular substances in periodontal ligament (PDL) such as oxytalan and elaunin fibers.[30-31] It is thus considered an inappropriate label for periodontal nerves. On the other hand, the use of immunohistochemistry clearly

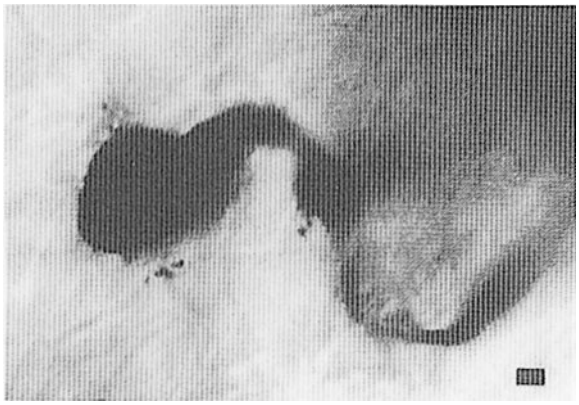

Fig 4. Light micrograph of the demarcated area in Fig 1b, at high magnification showing Ruffini endings which are noticeable only in the 35-week-old mice and exhibit a club-shaped structure with few micro-projections. The endings are situated in a narrow and small portion of the periodontal ligament and among the alveolar bone. Bar = 10 μm.

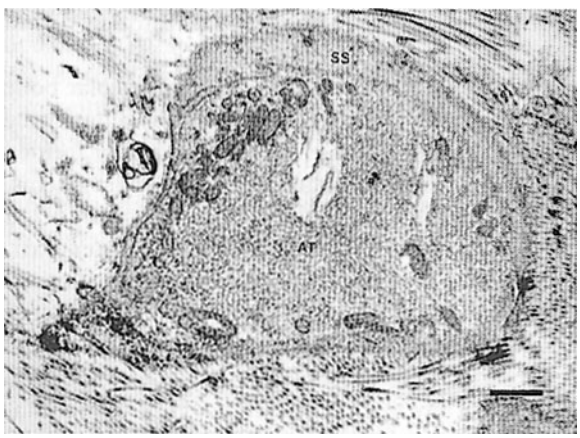

Fig 5. Immuno-electron micrograph of the club-shaped Ruffini endings shown in Fig 4. The axon terminal (AT) possesses a smooth contour and a relative decrease in mitochondrial number. SS: Schwann sheath. Bar = 3 μm.

distinguishes nerve and other components. Recently, periodontal nerve endings have been successfully stained by several markers.[5, 27] Neurofilament protein (NFP) and neuropeptides, which are used as neuronal markers, enable a visualization of some periodontal nerves. Anti-NFP and anti-neuropeptides are suitable and efficient for the detection of A delta and C fibers, respectively.[32] Being within the A beta range[33], Ruffini endings (RE) possess no immunoreactivity for neuropeptides[34-36], and their detailed structures are undetectable by the use of immunohistochemistry for NFP.[5, 24, 37] Protein gene product (PGP 9.5), on the contrary, is distributed in both central and peripheral nerves and is a general marker for nerve and neuroendocrine cells.[17, 20] Since its ability in a revelation of nerves in dental structures[22] and of RE in the PDL[23, 27] has been demonstrated, anti-PGP 9.5 antibody was used in this study to disclose neural elements in the PDL of mouse incisors.

The PDL of mammals is adapted to its predominate function, supporting the teeth in their alveolar sockets and concomitantly permitting them to withstand considerable force during mastication. The orientation and development of PDL fibers are dependent on the masticatory force. Recent studies of rodent periodontal fibroblasts have shown some age- and force-related changes in their configuration.[38-39] Apart from its shortest *in vitro* lifespan when compared with those in other connective tissues[40], the aged periodontal fibroblasts develop more catabolic activity of cathepsin[41], indicating an increase in the periodontal breakdown by the fibroblasts themselves. Additionally, the aged fibroblasts tend to fuse and form multinucleated cells[42], which eventually are involved in phagocytosis and intracellular degradation of incorporated collagenous fibrils.[43] Interestingly, the aged PDL fibroblasts of humans also play a role in destruction of hard tissue. A recent *in vitro* study by Sawa et al[44] has shown that the production potential of osteocalcin, a non-collagenous protein of alveolar bone, is impaired by the aged PDL fibroblasts in culture. In response to both internal and external stimuli, the aged PDL fibers can degrade both themselves and their surrounding structures.

Post-natal morphology of mouse periodontal RE undergoes a complex developmental process, and functional stimuli contribute to their final differentiation.[23] Periodontal nerves possessing an expanded configuration first appear in PDL 4 days after birth. Nakakura-Ohshima et al[24] studied the ultrastructures of the developing RE in post-natal rats and observed that the bulbous portions possess several mitochondria and various kinds of vesicles. They gradually increase their number, and the ones with morphological structures similar to RE in adult rats are noticeable around 7-11 days after birth, the time when eruption of incisors is recognized. During this stage, some parts of the axon terminals extend through the slits of Schwann sheath and form finger-like projections or axonal spines. After a com-

mencement of an anterior occlusion, an increase of large mitochondria, in contrast to a decrease of vesicles, is observed in the axon terminals. In addition, the number and the length of axonal spines are also increased. After an eruption of first molars, the periodontal RE rapidly increase in their number. Before the occlusion between first molars is established, the distribution and density of RE is indistinguishable from those seen in the lingual PDL of adult rat incisors. This post-natal development of RE verifies a close relationship between their configuration and functional forces from occlusion.

In this study, apart from the RE with the mentioned structures, the endings with a marked reduction in the number of mitochondria and other cytoplasmic organelles were observed in aged mice. Obviously, such endings were the extension of the typical RE and their micro-projections seemed to be decreased. Taking part in the periodontal mechanosensation, finger-like projections or axonal spines of RE are intimately associated with collagenous fibers of PDL.[45] The endings with few, if any, projections recognizable in our study were trapped between the alveolar bone, the area of which comprised a small portion of the PDL. Sharpey's fibers, a buffer medium for the occlusal force conveyed from opposing teeth to the PDL[46], are the PDL fibers that locate between cementum and alveolar bone, not among alveolar bone. Ruffini endings that possess an unusual length of projections and an altered ultrastructure are detectable in a hypofunctional PDL area.[47] Such morphology resembles to those observed in this study. Consequently, it is likely that RE in the PDL of aged mice underwent a hypofunctional process and a regressive pattern of their morphology was then induced.

Senile process decreases pain threshold in rat.[48] Park et al[49] noticed an existence of an age-related reduction in the primary neurons of the human vestibular system, possibly resulting in a dysequilibrium recognizable with age in human. In addition, a progressive impairment of mitochondrial complex I and complex IV activities was shown in the cerebral cortices of aged monkeys.[50] In this study, a reduction in the number of micro-projections and mitochondria was disclosed in the RE of aged mice. Since RE serve as mechanosensory receptors in the PDL, it is possible that there might be a functional impairment of the endings observed in the aged animals.

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