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Immunohistochemical localization of elastin, fibrillins and microfibril-associated glycoprotein-1 in the developing periodontal ligament of the rat molar

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Background and Objective: Elastic system fibers are a major component of the periodontal ligament, but little information is available about their detailed composition or the mechanism of elastogenesis in the developing periodontal ligament. The purpose of this study was to investigate immunolocalization of elastin, fibrillins and microfibril-associated glycoprotein-1 (MAGP-1) in the developing periodontal ligament of the rat molar.

Material and Methods: Frozen sections of demineralized as well as non-demineralized periodontal ligament of Wistar rats of various ages from 19 days to 7 weeks were incubated with anti-elastin, anti-fibrillin-1 and -2 and anti-MAGP-1 antibodies followed by peroxidase-conjugated secondary antibodies. After incubation with diaminobenzidine solution, immunoreaction products were observed with a light microscope.

Results: In the developing periodontal ligament of 19-day-old rats, fibers immunopositive to elastin were not present, but fibers positively stained for fibrillin-2 and MAGP-1 were widely distributed throughout the ligament. The latter fibers were arranged in the apico-occlusal direction along with blood vessels. In 3-week-old rats, fibers stained for elastin were observed for the first time in the apical region of the ligament. The number and distribution pattern of these elastin-positive fibers was basically the same as those in rats aged 5 and 7 weeks. In contrast, fibrillin-2- and MAGP-1-positive fibers were more extensively distributed in the ligament, and their pattern of distribution was comparable to that of reported oxytalan fibers. Fibrillin-1 was, however, not detected either in demineralized sections or in non-demineralized sections, indicating its absence in periodontal ligament.

Conclusion: Elastin expressed in the periodontal ligament assembled into elaunin fibers in the vicinity of blood vessels. Both fibrillin-2 and MAGP-1 are structural components not only of the elastin-associated microfibrils but also of elastin-free microfibrils, with possible roles in elastogenesis and in periodontal ligament homeostasis.

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The periodontal ligament is a complex, vascular and highly cellular connective tissue localized between the tooth and alveolar bone, and serves as a cushion against occlusal forces during mastication (1). A major constituent of the ligament is parallel bundles of collagen fibers attached to the root of the tooth and to alveolar bone. An additional component of the ligament is the network of elastic system fibers (2,3). In a recent study in our laboratory, elastic system fibers in the periodontal ligament of the rat molar were immunohistochemically characterized at light microscopic and ultrastructural levels (4). The results showed that elastin-positive fibers were localized in the vicinity of blood vessels in the apical region of the ligament and were confirmed to be elaunin fibers, that is, an immature form of elastic fibers. No mature elastic fibers were found in the ligament.

During elastogenesis, microfibrils play a crucial role in providing scaffolding for the deposition of tropoelastin and in determining the orientation of the elastic fibers (5). Connective tissue microfibrils are known to be composed of heterogeneous macromolecules including fibrillins, microfibril-associated glycoproteins (MAGPs), proteoglycans, fibronectin, vitronectin and amyloid (6). Fibrillin-1 and -2 are large glycoproteins ($\sim$ 350 kDa) rich in cystein and reported to be the main components of extracellular microfibrils (7,8), and also of defective gene products in Marfan syndrome (9) and congenital contractural arachnodactyly (10), The expression of fibrillin-1 and -2 is differentially controlled but they are expressed simultaneously during development of elastic tissues (11). Fibrillins and MAGPs, among other components, were reported to be the most important components for the assembly of elastic fibers (12,13). In the periodontal ligament, the greater part of the elastic system fibers are elastin-free microfibrils (oxytalan fibers) and, as described above, elaunin fibers were localized within a very restricted area of the ligament. However, the detailed composition of elaunin fibers and the process of elastogenesis are not yet fully understood. In this study, localization of elastin, fibrillins and MAGP-1 in the developing periodontal ligament of the rat molar was observed immunohistochemically. The results show the importance of these components in the assembly of elastic system fibers and for the maintenance of the homeostasis of periodontal ligament.

Material and methods

Animals

Male Wistar rats aged 19 days, 3, 5 and 7 weeks (in 4 groups, each comprising 5 rats) were purchased from JAPAN-SLC (Hamamatsu, Japan) and used in this study. Use and treatment of animals were in accordance with the Guidelines for the Treatment of Experimental Animals of Tokyo Dental College.

Immunohistochemistry

The pre-embedding immunoperoxidase method previously described in detail (4) was used for the immunostaining of the rat (19 days, three, five and seven weeks old) lower molar periodontal ligament. Briefly, under anesthesia with sodium pentobarbital (25 mg/kg, i.p.i, Nembutal, Abbott Lab., North Chicago, IL, USA), animals were perfused with a cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 15 min. Mandibles were dissected out and placed in the same fresh fixative for 6 h at 4°C. They were then demineralized in 10% EDTA for 3 weeks at 4°C, rinsed in 0.01 M phospate-buffered saline (PBS) containing 15–20% sucrose and immersed in Tissue Tek OCT compound (Miles Laboratories, Naperville, IL, USA) followed by quick freezing in liquid nitrogen. Serial sections 15 μm in thickness were prepared by using a Cryostat (Leica, Nussloch, Germany). The periodontal ligament of the lower first molar was sectioned in the direction parallel to either the mesio-distal (longitudinal, right side of mandible was used; 10 sections/per rat) or horizontal plane (left side of mandible; 10 sections from each of the cervical, middle and apical regions/per rat).

In addition to these chemically fixed and demineralized tissues, fresh-frozen sections of unfixed and non-demineralized tissues were also prepared with the method of Kawamoto & Shimizu (14). Briefly, male Wistar rats 7 weeks old (3 rats) were used. The animals were killed by intraperitoneal injection of an overdose of sodium pentobarbitol (200 mg/kg, Nembutal, Abbott Lab., North Chicago, IL, USA), the mandibles were dissected out and rapidly frozen in cooled *n*-hexane and embedded in 5% carboxymethl cellulose (Finetec Co., Tokyo, Japan), followed by quick freezing in cooled *n*-hexane (−75°C). Serial sections 5 μm in thickness were prepared and mounted on polyvinlidene chloride film, and were used for immunohistochemistry.

For immunostaining, tissue sections were at first incubated in 5% normal sheep serum for 5 min to block non-specific binding sites and then incubated overnight with primary antibodies at 4°C. The primary antibodies used were rabbit anti-elastin (AB 2039; Chemicon Inc., Temecula, CA, USA), anti-fibrillin-1 (sc-20,084; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-fibrillin-2 (PR225; Elastin Products Co., Owensville, MO, USA), anti-MAGP-1 (PR315; Elastin Products Co.) polyclonal antibodies and mouse monoclonal anti-fibrillin-1 (MAB 2499; Chemicon). These antibodies were used at 1:100 dilution in PBS. These primary antibodies were confirmed to show cross-reactivity against rat tissue samples (lung and gingiva) by Western blotting (Fig. 1). The method used in Western blotting was the same as that described previously (4). After rinsing with PBS, sections were incubated either with peroxidase-linked fragment antigen binding [$F(ab')_2$] fragment of anti-rabbit immunoglobulin G (IgG; dilution 1:100; Amersham, Biosciences,

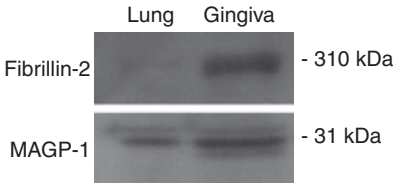

Fig. 1. Western blot in which fibrillin-2 and MAGP-1 are expressed in the rat lung and gingiva.

Buckinghamshire, UK) or anti-mouse IgG (dilution 1:100; Amersham) overnight at 4°C. After rinsing with PBS, they were fixed with 0.5% glutaraldehyde in PBS for 5 min and incubated with 0.02% diaminobenzidine solution (Dojin Chemicals, Kumamoto, Japan) containing 0.003% hydrogen peroxide for 5–7 min at room temperature. Sections, with or without counterstaining, were washed with PBS, dehydrated in a graded series of ethanol concentrations and mounted on coverslips with mounting medium (MP-500, Matsunami Glass Ind, Ltd, Osaka, Japan) for examination with a light microscope (Carl Zeiss, Hallbergmoos, Germany) equipped with a Zeiss Axio Cam HRC digital camera. Each immunostaining procedure was repeated three times. As negative controls, primary antibodies were replaced by non-immunized rabbit serum or PBS alone.

Results

General morphology

In 19-day-old rats, the lower first molars were beginning to erupt into the oral cavity and the roots were continuously elongated in the apical direction. At the apical region, the developing periodontal ligament was observed between the root sheath and alveolar bone. In 3-week-old rats, the roots were further elongated and the root sheath was still present at the tip of the root. In 5- and 7-week-old rats, the whole tooth crown emerged into the oral cavity and reached the occlusal plane. In 7-week-old rats, fully developed roots were observed and were associated with a large amount of cellular cementum at the middle to apical regions of their surface. The mature periodontal ligament was made up of highly ordered bundles of collagen fibers and well-developed blood vessels (micrographs not shown).

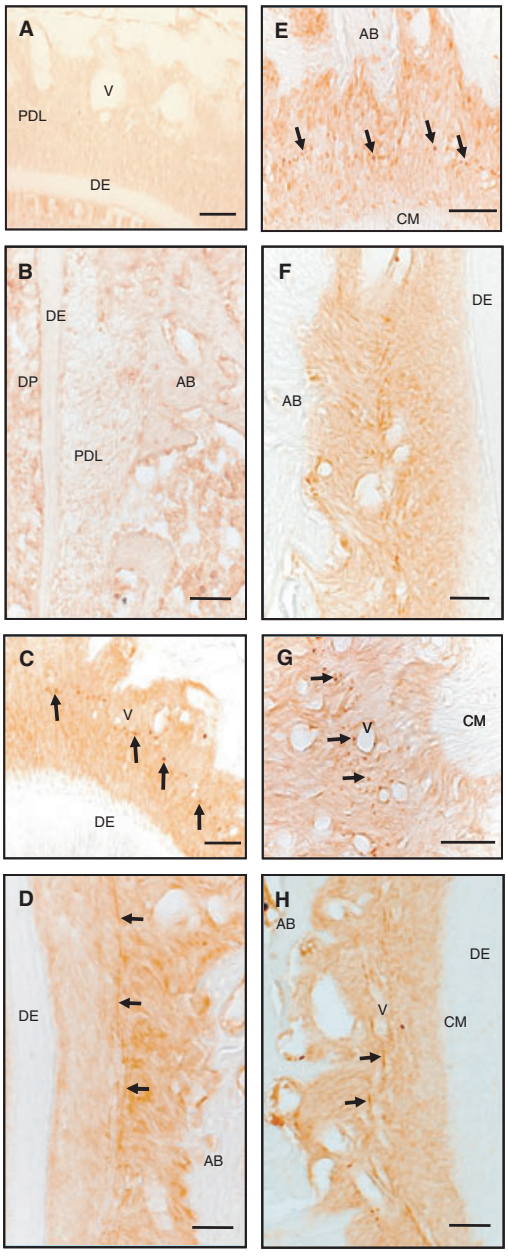

Fig. 2. Immunolocalization of elastin in the developing periodontal ligament of rats aged 19 days (A,B), 3 (C,D), 5 (E,F) and 7 weeks (G,H). (A) Horizontal section. No staining is observed in the periodontal ligament at the growing root. (B) Longitudinal section in which no staining was observed. (C) Horizontal section. Dot-like stains (arrows) are localized near blood vessels. (D) Longitudinal section. Immunostained slender fibers (arrows) are arranged in the apico-occlusal direction. (E) Horizontal section. Fibers (arrows) are localized close to blood vessels in the central area of the ligament. (F) Longitudinal section. (G) Horizontal section. Moderately stained fibers (arrows) are concentrated mainly in the central area of the ligament. (H) Longitudinal section. Arrows indicate elastin-positive fibers localized close to blood vessels. Abbreviations: AB, alveolar bone; CM, cementum; DE, dentin; DP, dental pulp; PDL, periodontal ligament; and V, blood vessels. Scale bars represent 50 μm.

Immunohistochemistry

Elastin In 19-day-old rats, no immunostaining for elastin was observed in the developing periodontal ligament (Fig. 2A,B; note that all tissues shown in Figs 2–4 are demineralized tissues unless indicated otherwise). Elastin-positive fibers were first detected in the ligament of two 3-week-old rats, but the other three rats did not show the staining. The same result was obtained after the staining was repeated three times. Elastin-positive fibers were mainly

localized near the blood vessels at the apical region of the distal root in the form of dot-like structures as observed in horizontal sections (Fig. 2C). In longitudinal sections, positively stained slender fibers were seen running along the surface of the root (Fig. 2D). In 5- and 7-week-old rats (Fig. 2E–H) both the distribution and the number of elastin-positive fibers were approximately the same as those of 3-week-old rats (Fig. 2C,D). Again, most of these elastin-positive fibers were localized in the close vicinity of blood vessels at the apical region of the root.

Fibrillin-1 and -2 No detectable immunostaining for fibrillin-1 was observed in the periodontal ligament at any of the developmental stages in either demineralized or non-demineralized sections. Therefore, only the results of immunostaining for fibrillin-2 are described in detail.

After immunostaining for fibrillin-2, positively stained dot-like structures (cross-sections of stained fibers) were widely distributed throughout the developing ligament at the cervical, middle and apical levels of the root of 19-day-old rats (Fig. 3A). In the apical region, densely immunostained entities with a helical appearance were often seen among blood vessels or near the developing root (Fig. 3B). In 3- (Fig. 3C,D) and 5-week-old rats (Fig. 3E,F), the number of stained fibers increased. In longitudinal sections, many fibers were oriented in the apico-occlusal direction along blood vessels (Fig. 3D), and some became altered in their course away from the apico-occlusal direction towards the enamel–cement junction and were inserted into cementum (Fig. 3F). In 7-week-old rats, the pattern of distribution of stained fibers in the periodontal ligament was similar to that of 5-week-old rats. In horizontal sections, a large number of fibers, including thicker fibers, were intensely stained for fibrillin-2 and localized mainly at the central area of the ligament (Fig. 3G; non-demineralized, fresh-frozen section). In the periodontal ligament of interradicular bone, positively stained fibers were extended from the surface of the alveolar bone to the root cementum (Fig. 3H).

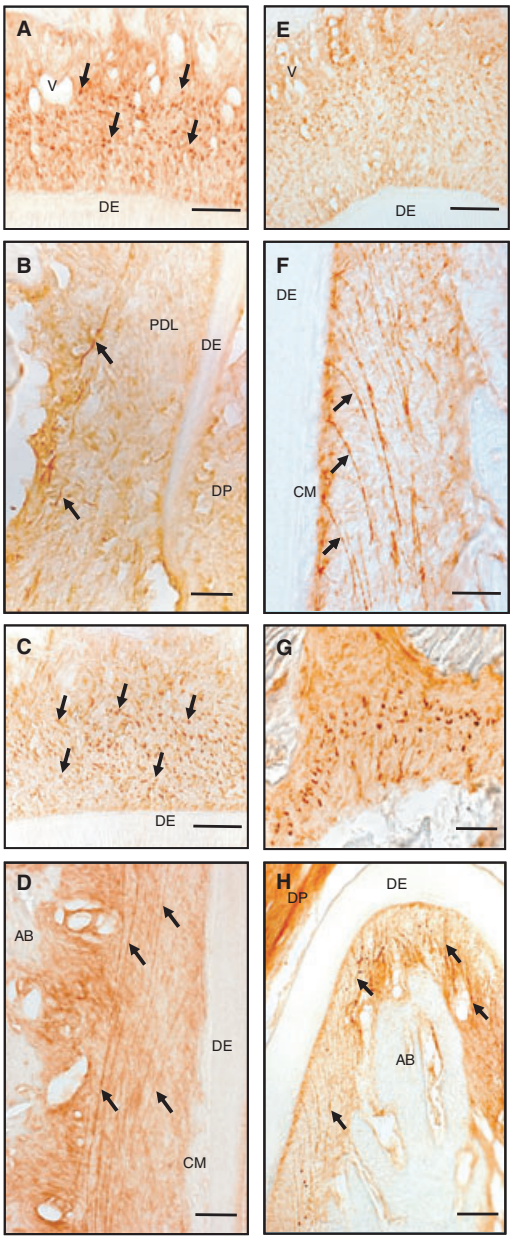

Fig. 3. Immunolocalization of fibrillin-2 in developing periodontal ligament of rats aged 19 days (A,B), 3 (C,D) 5 (E,F) and 7 weeks (G,H). (A) Horizontal section. Immunostained dot-like structures (arrows) are localized throughout the middle area of the ligament at the distal root. (B) Longitudinal section. Positively immunostained fibers (arrows) with a helical appearance are also observed in the apical region. (C) Horizontal section. There are more immunostained structures (arrows). (D) Longitudinal section. A number of stained fibers (arrows) are oriented in the apico-occlusal direction. (E) Horizontal section. (F) Longitudinal section. The tips of fibrillin-2-positive fibers (arrows) are inserted into cementum. (G) Horizontal section of non-demineralized tissue. Immunostaining for fibrillin-2 is improved compared with that of demineralized tissues (compare with Fig. 3E). (H) Longitudinal section. Arrows indicate immunostained fibers in periodontal ligament of interradicular bone. Abbreviations as in legend to Fig. 2. Scale bars represent 50 (A-G) and 100 μm (H).

Microfibril-associated glycoprotein-1 After immunostaining for MAGP-1, both the pattern of distribution of stained fibers and the intensity of staining were basically the same as those of fibers immunostained for fibrillin-2. A number of immunostained dot-like structures in horizontal sections

(Fig. 4A) and elongated fibers in longitudinal sections (Fig. 4B) were observed in the ligament of 19-day-old rats. In 3- and 5-week-old animals, the intensity of staining for MAGP-1 throughout the ligament was similar to that for fibrillin-2 (Fig. 4C–F). In longitudinal sections, at the cervical and middle regions the fibers were scattered throughout the ligament. A number of thicker fibers were localized close to blood vessels (Fig. 4D,F). In 7-week-old rats, a large number of fibers ran in the apico-occlusal direction along blood vessels and also along the surface of the root (Fig. 4G; non-demineralized, fresh-frozen section). The number of MAGP-1-positive fibers in the apical region was slightly smaller than that in 5-wk-old rats (micrograph not shown). In the periodontal ligament of interradicular bone, well-developed fibers were running between the alveolar bone and the root (Fig. 4H).

The results of observations of the distribution of fibers immunostained for elastin, fibrillin-2 and MAGP-1, respectively, in the periodontal ligament of the rat molar, are summarized in Fig. 5.

No immunoreaction products were observed in any control sections (data not shown).

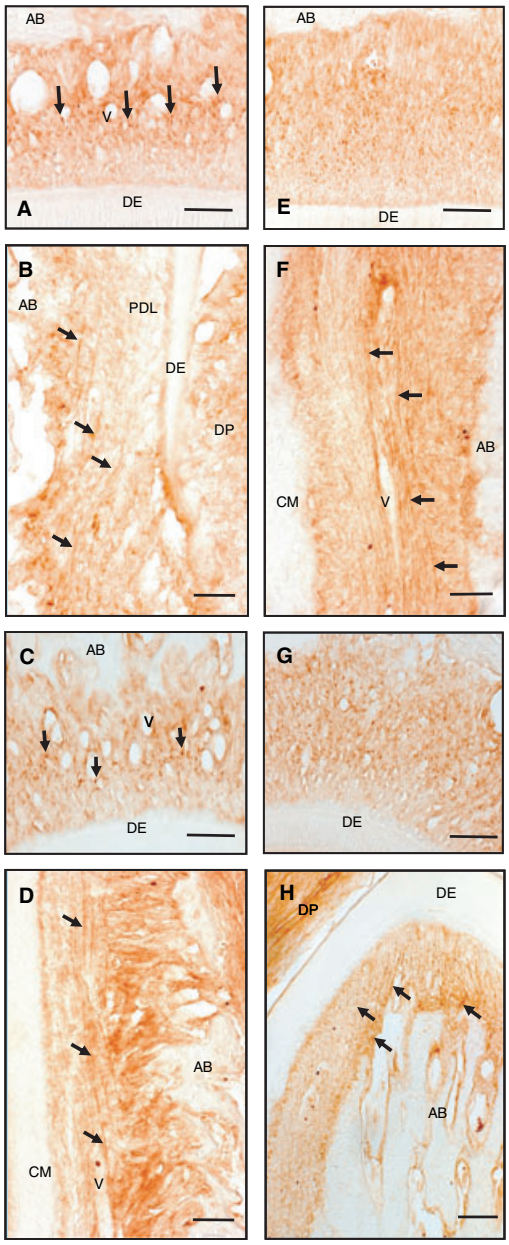

Fig. 4. Immunolocalization of MAGP-1 in developing periodontal ligament of rats aged 19 days (A,B), 3 (C,D), 5 (E,F) and 7 weeks (G,H). The pattern of distribution of immunostained fibers is similar to that of fibrillin-2. (A) Horizontal section. A number of stained dot-like structures (arrows) are localized mainly close to blood vessels. (B) Longitudinal section. Immunostained fibers (arrows) are localized at the apical region. (C) Horizontal section. There are more immunostained structures (arrows). (D) Longitudinal section. Stained fibers (arrows) are arranged in the apico-occlusal direction. (E) Horizontal section. (F) Longitudinal section. Arrows indicate parallel arrangement of fibers. (G) Horizontal section of non-demineralized tissues. (H) Longitudinal section. Arrows indicate immunostained fibers. Abbreviations as in legend to Fig. 2. Scale bars represent 50 (A-G) and 100 μm (H).

Discussion

In the present study, elastin-positive fibers were shown to appear first at the apical region of the periodontal ligament of the molar of 3- or 5-week-old rats and were not seen in 19-day-old rats. The distribution of these fibers and the intensity of immunolabeling for elastin were similar to those reported in our previous study, in which 7- to 8-week-old rats were used (4). These results indicate that the expression of elastin (in the form of elaunin fibers) in the periodontal ligament begins when a tooth reaches the stage of functional occlusion. Therefore, it may suggest a correlation between the occurrence of elaunin fibers and mechanical occlusal forces. However, there is a lack of direct evidence supporting such a relationship and, according to the finding of this study that the number and intensity of elastin-positive fibers remained approximately the same in 3- to 7-week-old rats, such a relationship is still not confirmed and is the subject of further studies. As to the origin of elastin, a previous study showed preferential localization of elaunin fibers close to fibroblasts in the vicinity of blood vessels in the periodontal

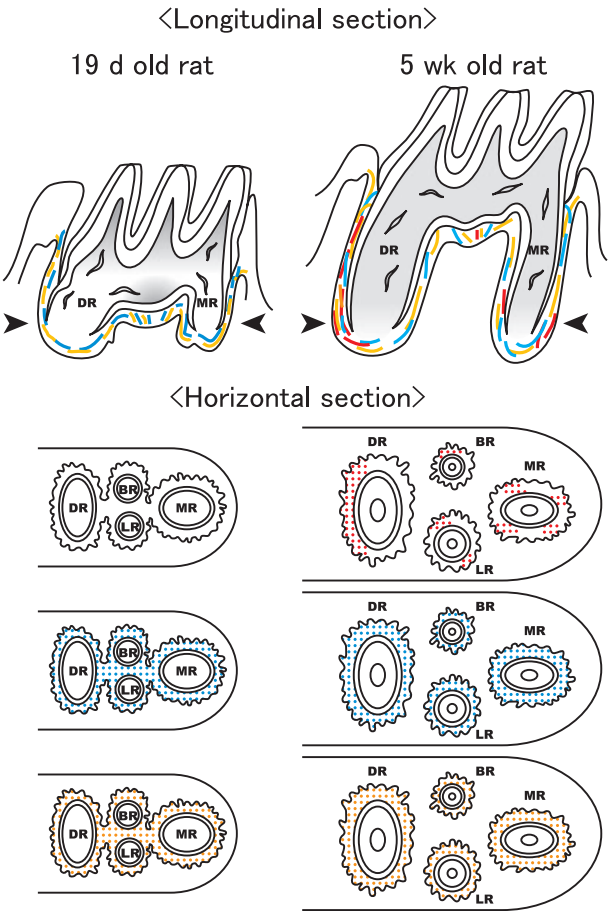

Fig. 5. Schematic drawing of distribution of elastin (red), fibrillin-2 (blue) and MAGP-1 (yellow) in developing periodontal ligament of the rat molar. Horizontal sections were cut at the apical level indicated in the longitudinal sections (arrowheads). Abbreviations: BR, buccal root; DR, distal root; LR, lingual root; and MR, mesial root.

ligament, and the possibility that fibroblasts are the site for the production of elastin was suggested (4). It was shown that cultured fibroblasts of human periodontal ligament expressed tropoelastin mRNA, and its level increased as a result of applied pressure in a time-dependent manner (15). In this study, elaunin fibers were observed in the close vicinity of blood vessels, and fibroblasts in the area of blood vessels could be the possible site of synthesis and secretion of elastin when they are exposed to mechanical stress. Connective tissue microfibrils 10–12 nm in diameter are composed of a number of glycoproteins and are organized into tissue-specific architectures. They provide a scaffold for the deposition of tropoelastin monomers and play a crucial role in elastogenesis (5). Fibrillin-1 and -2 are reported to be the principal structural components of elastic fiber-associated microfibrils, and they have distinct but overlapping patterns of expression (8). Distribution of fibrillin-1 and -2 in human embryonic and early fetal tissues has been demonstrated (16), and both fibrillins were widely distributed in various human anlagens. However, it was found that in the anlagen of certain organs, such as kidney, liver and rib notochord, fibrillin-1 and -2 were distributed differently. These authors suggested different roles of fibrillin-1 and -2 during development of these structures.

In our previous study (4), the presence of fibrillin was demonstrated in the periodontal ligament of the rat molar, and the pattern of distribution of fibrillin-positive fibers was found to be basically the same as that of the reported distribution of oxytalan fibers (17). However, in our previous work it was not clear whether the antibody used recognized fibrillin-1 or fibrillin-2. In this study, it was confirmed that fibrillin-2, and not fibrillin-1, was a component of both elastin-free and elastin-associated microfibrils in the developing periodontal ligament. In this study, sectioning of fresh-frozen, non-demineralized tissues according to the method of Kawamoto & Shimizu (14) was also done to examine the effect of chemical fixation and demineralization of tissues on immunostaining. The results showed that elastin, fibrillin-2 and MAGP-1 were more intensely stained in sections of fresh-frozen tissues compared with sections of demineralized tissues. This may indicate that the processing of tissues affects immunodetection of these components. An *in vitro* study using recently developed DNA microarray technology, fibrillin-2 mRNA was found to be intensely expressed in cultured fibroblasts of human periodontal ligament, and its expression was 5.6-fold more intense than that of the fibroblasts of the gingiva (18). This reported result is in accord with and supports the results of immunohistochemical staining in this study.

In the mouse embryo, fibrillin-2 is expressed earlier in development, and it may be important, mainly in the formation of elastic fibers, in the early stages of elastogenesis, whereas fibrillin-1 may play its role at a later stage of elastogenesis (19,20). Therefore, microfibrils containing fibrillin-2 and occurring in the developing periodontal ligament may play a role in the process of the deposition of elastin. The reason for the failure of immunodetection of fibrillin-1 is not clear but it could be that: (1) fibrillin-1 was absent in periodontal ligament at this stage of development; (2) alternatively, it was present but in too small an amount to be detected with the method used in this study; or (3) the antibody was not appropriate. The reason may be the first possibility, because it was proposed (19) that fibrillin-1 and -2 have different roles, and the latter has the role of regulation of assembly of elastic fibers in its early stages while

fibrillin-1 mainly provides force-bearing support. Therefore, fibrillin-2 may be expressed at first during assembly of elastic fibers, and then fibrillin-1 would appear later.

A recent study showed that developing arteries and neural crest EGF-like (DANCE) (also referred to as fibulin-5) is deposited onto microfibrils and promotes assembly and cross-linking of tropoelastin along microfibrils, suggesting the importance of this component in the molecular mechanism of elastogenesis (21). For further characterization of the periodontal ligament, immunostaining of the ligament for DANCE is currently in progress in our laboratories.

Both MAGP-1 and MAGP-2 are additional components reported to be associated with microfibrils, and are widely expressed in the cells of the mesenchyme and connective tissue throughout development (22). Although there is a close similarity between MAGP-1 and MAGP-2, MAGP-2 has more restricted staining patterns during tissue development (23). In the bovine periodontal ligament, immunogold labeling showed the presence of MAGP in the microfibrils of oxytalan fibers (24). The present study demonstrated that MAGP-1 is associated with microfibrils distributing throughout periodontal ligament. It was reported that MAGP-1 is spatially distributed as a component of microfibrils to specifically interact with fibrillins and soluble elastin precursor, tropoelastin (25,26). In addition, MAGP-1 as well as fibrillin-2 were shown, using RNA interference technique, to contribute directly to tropoelastin deposition by suppressing endogenous MAGP-1 and fibrillin-2 (27). These results may confirm the association of fibrillin-2 with tropoelastin in the periodontal ligament as described above. The importance of mediation of an interaction between microfibrils and type VI collagen microfibrils by MAGP-1, and such a role of MAGP-1 in anchoring elastic fibers to the surrounding matrix, has been described (23). In rat periodontal ligament, type VI collagen was found in close association with microfibrils and oxytalan fibers (28).

Based on the results of the study of fibroblasts of the human periodontal ligament by means of DNA microarray analysis (18), it is possible that MAGP-2, more than fibrillin, is the main candidate for being the integral component of microfibrils in the periodontal ligament. The exact role of this component in the periodontal ligament is still not clear. Recently, Lemaire *et al.* (29) suggested that MAGP-2 may regulate early stages of the macroassembly of elastic fibers, including transfer of tropoelastin globules from the cell membrane onto the developing fibers. If this is the case, MAGP-2 in microfibrils in the periodontal ligament may also have a similar role in elastogenesis. Alternatively, MAGP-2 associated with elastin-free microfibrils in the ligament may stimulate expression of type I collagen as suggested in other tissues (30), since turnover of collagen fibers in the ligament is very rapid compared with that occurring in the gingival matrix. In any case, whether MAGP-1 and MAGP-2 are co-localized or differentially localized in the developing periodontal ligament remains to be clarified.

The presence of oxytalan fibers, that is, assemblies of micofibrils, in the periodontal ligament was first reported by Fullmer & Lillies (2). A number of roles of microfibrils were proposed (31), including anchoring, maintenance of elasticity, guideline for cell migration, stabilization of blood vessels and regulation of vascular flow. In view of the close spatial relationship of oxytalan fibers to blood vessels, the concept that oxytalan fibers act as a mechanoreceptive system is particularly compelling.

Recent studies using genetically targeted strains of mice revealed that the tissue-specific architecture of microfibrils in the extracellular space appears to be a structural network that specifies the local concentration and timely release of signaling molecules during morphogenesis and tissue remodeling, as suggested by Ramirez *et al.* (5,32). This idea raised an additional possibility that the oxytalan fibers may serve as a regulator of bone morphogenetic protein (BMP) signaling in periodontal ligament homeostasis and regeneration.

In this study, the localization and distribution of elastin, fibrillin-2 and MAGP-1 were examined in the developing periodontal ligament of the rat molar. Elastin expressed in the periodontal ligament is formed into elaunin fibers in the vicinity of blood vessels. Both fibrillin-2 and MAGP-1 may contribute to elastogensis and homeostasis of the periodontal ligament as structural components of microfibrils, which are associated with or without elastin.

The clinical importance of these findings is to provide valuable fundamental knowledge for the elucidation of the mechanisms of pathogenesis of periodontal diseases or the understanding, for example, of the process of dental implantation or replantation. This, in turn, would be valuable in development of the means of clinical intervention.

In this study, the localization and distribution of three major components were examined in the developing periodontal ligament at the light microscopic level. The next step would be the examination of these components at finer levels in relation to the results obtained in this study; that is, electron microscopy combined with ultrastructural immunolabeling would provide more detailed information for the elucidation of the composition of the periodontal ligament or the mechanism of elastogenesis.

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