SHORT COMMUNICATION

Immunohistochemical Demonstration of Cathepsins B and L in the Periodontal Ligament (PDL) of the Rat Molar

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Introduction

The periodontal ligament (PDL) is not only necessary for supporting the teeth in their sockets and for buffering stress to them, but it is also of great importance during orthodontic tooth movement. The PDL consists of cells and an extracellular compartment of fibers dispersed within the extracellular matrix. The fibers are continually remodeled to adapt to the changing stresses placed on them[3]. Morphological studies on collagen degradation during remodeling of the PDL have indicated that fibroblasts ingest collagen fibrils and intracellularly degrade them[23]. Under normal physiological conditions characterized by an equilibrium between their synthesis and degradation, the intracellular pathway forms the major route for PDL collagen digestion[4]. Deporter and Ten Cate[5] reported that collagen-containing profiles in the fibroblasts were positive for acid phosphatase (ACPase) and alkaline phosphatase (ALPase) activity. Furthermore, Yajima[6] reported that cultured human gingival collagen-secreting fibroblasts displayed weak ACPase activity, while fibroblasts phagocytosing collagen showed strong ACPase activity.

Collagen fibrils taken up by fibroblasts are theorized to be degraded in lysosomes by cathepsins[4], which are the major peptidases activated in acidic compartments and perform important roles in the digestion of the extracellular or cytoplasmic proteins and in the activation of lysosomal enzymes[7-11]. In particular, since cathepsins B, L, N, S and K are able to degrade collagen[12-19] they are referred to as collagenolytic cathepsins. Cathepsin K, which has an especially strong collagenolytic activity[18], is an osteoclast specific enzyme[19] and is considered to play a major role in the collagen degradation in bone resorption.

Furthermore, in regard to the relationship between cathepsins and PDL cells, Wang[20] reported that cathepsins B, D, H and N were inside the lysosomal fractions of the PDL fibroblasts together with the other enzymes such as ACPase, and Goseki, et al.[21] reported cathepsins B and L activity in cultured PDL fibroblasts. Several morphological investigations attempted to localize the relationship between cathepsin B and the fibroblasts both in vitro[22-24] and
in vivo^{25}. Tanaka, et al.^{24} also reported an enzyme histochemical activity for cathepsin B in PDL fibroblasts, and Everts, et al.^{27} and Van Noorden, et al.^{28} showed that cathepsin inhibitors caused an increase in the collagen-containing lysosomes in cultured periodontal fibroblasts. These previous studies strongly suggest that collagenolytic cathepsins, especially cathepsins B and L are involved in the intracellular degradation of collagen fibrils taken up by fibroblasts. Thus, the aim of the present study was to provide evidence of the immunohistochemical reactivity of cathepsins B and L in lysosomes degrading collagen fibrils within PDL fibroblasts.

Materials and Methods

1. Tissue preparation

All experiments were reviewed and approved by the Niigata University School of Dentistry Intramural Animal Use and Care Committee prior to the study.

Eight-week-old male Wistar rats were used. After being anesthetized by the inhalation of diethyl ether and an intraabdominal administration of sodium pentobarbiturate (30 mg/kg) (Dainabot, Osaka, Japan), the animals were perfused with a fixative through the left ventricle. A mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.06 M cacodylate buffer (pH 7.3) was used for the immunohistochemistry, and a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.06 M cacodylate buffer (pH 7.3) was used for the ultrastructural observations. Dissected maxillae were immersed in the same fixative overnight at 4°C, and decalcified in 4.13% EDTA (pH 7.3) for 30 days at 4°C.

After decalcification, some specimens were dehydrated with a graded series of ethanol, and then embedded in paraffin. Seven micrometer-thick sagittal serial sections were made using a sliding microtome. The other specimens were sliced parallel to the occlusal surfaces at a thickness of 70 µm using a DTK-1000 Microslicer (DSK, Kyoto, Japan).

2. Immunohistochemistry

The dewaxed and rehydrated paraffin sections were treated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 min at room temperature to inhibit the endogenous peroxidase. After rinsing in PBS, the sections were immersed in PBS containing 5% low-fat milk (skim milk, Snow Brand Milk Products, Sapporo, Japan) for 30 min at room temperature to block the tissue's nonspecific binding sites. After rinsing in PBS, the sections were incubated using the following primary antibodies for 24 h at 4°C: rabbit polyclonal antibody against rat cathepsin B^{29} (0.6 µg/ml), and L^{30} (0.6 µg/ml) and ED 1 monoclonal antibody^{31}(Serotec, Kidlington, UK), which can recognize most of the monocyte-macrophage cell lineage, diluted to 1:300–500. After washing with PBS, the sections were incubated with biotinylated anti-rabbit IgG (Nichirei Inc., Tokyo, Japan) or biotinylated antimouse IgG+IgA+IgM (Nichirei Inc.) for 30 min at room temperature and then further incubated with peroxidase-conjugated streptavidin (Nichirei Inc.) for 30 min at room temperature. For visualization of the immunoreactivity, the sections were immersed in a diaminobenzidine (DAB) (Dojin Chemicals, Kumamoto, Japan) solution containing 0.01% hydrogen peroxide (DAB-H_2O_2 solution) for 2 min at room temperature. After exposure to the DAB-H_2O_2 solution, the sections were counterstained with methyl green, dehydrated with a graded series of ethanol, cover-slipped, and then observed under a Microphot-FXA light microscope (Nikon, Tokyo, Japan).

For the negative control, sections were incubated using non-immune rabbit serum, followed by the biotinylated anti-rabbit IgG.

3. Fine structures

Microsliced sections were postfixed in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4°C, followed by dehydration with a graded series of acetone and subsequent embedding in Poly-bed 812 (Polysciences, Warrington, PA., USA). Ultrathin sections were cut with a MT5000 ultramicrotome (Du Pont Co., Connecticut, USA), and then mounted on copper grids. The sections were stained with 2% tannic acid, uranyl acetate, and lead citrate and then observed under an H-500 transmission electron microscope (Hitachi, Tokyo, Japan).
Fig. 1 PDL light micrographs in the distal part of the mesial root of an upper second molar of a rat. Sagittal serial sections showing immunoreactivity to anti-cathepsin B antibody (a), anti-cathepsin L antibody (b) and ED1 (c).

a and b: Immunoreactivity for cathepsins B and L was detected in multinucleated osteoclasts (OC) adjacent to the alveolar bone surface, in mononuclear cells (arrowheads) lying around the blood vessels (V), and also in many spindle-shaped mononuclear cells (arrows) spreading within the PDL. However, the cathepsin B-immunoreactivity was weak in the osteoclasts.

c: Immunoreactivity to ED1 was detected in the osteoclasts (OC) and in the mononuclear cells (arrowheads) around the blood vessels (V).

d: No immunoreactivity is observed in the negative control section. Bar : 25 μm. B alveolar bone. R root.
4. Immunocytochemistry

Microsliced sections were immersed in 1% BSA-PBS for 4 h at 4°C to block the tissue's nonspecific binding sites, and then incubated with the antibody against rat cathepsins B (3 μg/ml) and L (3 μg/ml) for 3 days at 4°C. After washing with PBS, the sections were incubated with biotinylated anti-rabbit IgG for 24 h at 4°C and then with peroxidase-conjugated streptavidin for 24 h at 4°C. Following fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature, the sections were immersed in a DAB-H2O2 solution for another 10 min at room temperature to visualize the immunoreactivity. Following postfixation in 1% OsO4 in 0.1 M cacodylate buffer for 2 h at 4°C, the sections were dehydrated with a graded series of acetone and then embedded in Poly-bed 812. Ultrathin sections were cut using a MT 5000 ultramicrotome, mounted on copper grids, stained with lead citrate, and then observed under an H-500 transmission electron microscope.

**Fig. 2** Electron micrographs of the PDL fibroblasts containing collagen fibrils.

a: Fibroblasts (FB) within the PDL showing extensive rough endoplasmic reticulum and Golgi complexes. Collagen-containing vacuoles (black frame) can be seen within the cytoplasm. Bar : 3 μm.

b: A magnification of the black frame in Fig. 2a. A collagen-containing vacuole (arrows) within the fibroblasts are seen as elongated electron-dense structures. Bar : 0.5 μm.
Results

1. Immunohistochemistry

In the PDL, immunoreactivity for cathepsins B and L appeared in many spindle-shaped mononuclear cells dispersed within the PDL, in the large multinucleated osteoclasts lying on the alveolar bone surface, and also in mononuclear cells lying around the blood vessels (Figs. 1 a, b). However, the immunoreactivity for cathepsin B in the osteoclasts tended to be weak.

Immunoreactivity to ED1 was noted in the osteoclasts and in the perivascular mononuclear cells in the PDL (Fig. 1 c). These ED1-immunopositive cells were also revealed to be immunoreactive for cathepsins B and L with serial sections (Figs. 1 a—c). No immunoreactivity was observed in the negative control sections (Fig. 1 d).

2. Fine structures

Ultrastructural observations of the PDL revealed fibroblasts with extensive rough endoplasmic

Fig. 3 A fibroblast showing many collagen-containing vacuoles (arrows). Bar : 0.5 μm.

Fig. 4 Collagen fibrils (arrows) within lysosome (Ly). Bar : 0.5 μm.
Cathepsin L-immunoreactivity was detected clearly within the lysosomal structures of the fibroblasts (Figs. 5a, b). Some of the cathepsin L-immunoreactive lysosomes were fused to the spindle-shaped electron-lucent structures engulfed within the cells (Fig. 5b).

Cathepsin B-immunoreaction was detected in the lysosomes close to the collagen fibrils within the cells.
Discussion

Immunoreactivity for cathepsins B and L was detected in osteoclasts lying on the alveolar bone surfaces, in the perivascular mononuclear cells, and in the other mononuclear cells spreading through the PDL. We also investigated the distribution of ED1-immunopositive cells, which are believed to be the monocyte and macrophage lineage cells. In the present study, most of the cathepsin B- or L-immunonegative mononuclear cells were immunonegative to ED1. Taking the cellular constituent of PDL into consideration, the cells without immunoreactivity to ED1 were considered largely fibroblasts.

During ultrastructural observations of the PDL, cathepsin L-immunoreactive lysosomes of some fibroblasts were found to fuse to elongated structures. The elongated structures were identified as collagen according to the observations of fine structure. Thus, a relationship between cathepsin L and intracellular collagen degradation was demonstrated. The synchronous presence of lysosomal immunoreactivity for cathepsin L at the time of intracellular collagen-intake provides conclusive evidence supporting the unequivocal association and explicit role of cathepsins in collagen degradation.

Previous reports have suggested that PDL fibroblasts ingest collagen fibrils and digest them intracellularly. In addition, the involvement of cathepsins B and L in digestion has also been suggested. However, the relationship between cathepsins B and L and the collagen-containing lysosomes is not well clarified. In the present study, immunoelectron microscopy revealed the immunoreactivity of cathepsins B and L in the collagen-containing lysosomes within the fibroblasts. Immunoreactivity for cathepsin K was detected only in osteoclasts, but not in fibroblasts (data not shown). Thus, we suggest that cathepsin K is not concerned with collagen digestion within fibroblasts, and that cathepsins B and L are involved in the intracellular collagen digestion.

In the present study, cathepsins B and L immunoreactivity were seen in the fibroblasts, osteoclasts, and macrophages of the PDL. Fibroblasts are not only responsible for collagen synthesis, but also play a key role in collagen degradation in the PDL, mainly by lysosomes containing cathepsins B and L, and fibroblasts in the PDL carry out an important function in collagen remodeling under normal physiological conditions.

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