Localization of Cathepsin B and L in Rat Periodontal Tissues During Experimental Tooth Movement

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Abstract
Cathepsins are common and well-characterized lysosomal cysteine proteinases involved in tissue destruction under pathological conditions. A recent immunocytochemical study reported that cathepsins B (CAB) and L (CAL) were localized in the periodontal ligament (PDL) of the rat molar. Further, our laboratory demonstrated that the levels of CAB and CAL in gingival crevicular fluid increased during orthodontic tooth movement. The present study focused on the localization of CAB and CAL during experimental tooth movement as well as participation by these cathepsins in the alteration of the periodontal tissues. Tooth movement was performed with the Waldo method, and the time points used for elastic band extraction were 1, 3, and 7 days after tooth movement. To determine localization of CAB and CAL using immunohistochemical analysis was used. Immunoreactivity for CAB and CAL was detected in osteoclasts and PDL fibroblasts. One day after tooth movement, the PDL fiber arrangement became coarse on both sides, and regressive lesions formed while the degree of staining for CAB and CAL increased in those cells as a whole. However, 3 days after tooth movement, many osteoclasts appeared in the compression and tension sides, and the immunoreactivity of CAB and CAL was increased in osteoclasts and fibroblasts. The present study demonstrated that CAB and CAL could be detected in PDL fibroblasts and osteoclasts and were increased in those cells following experimental tooth movement. Therefore, mechanical stress may be involved in extracellular matrix degradation and bone resorption through the stimulation of cathepsins, which is one of the important factors in the periodontal tissues.

Keywords:
periodontal ligament, osteoclast, cathepsins B and L, tooth movement

Introduction
Periodontal tissue has a unique structure because the periodontal ligament (PDL), a typical soft connective tissue, lies between the tooth cementum and alveolar bone, which are hard tissues, where it anchors the tooth to the alveolus and functions as a cushion between the hard tissues to mitigate occlusal force. Further, the PDL fibers are continually being remodelled to adapt to changing stresses placed on them (1).

During the movement of teeth in orthodontic treatment, it is generally agreed that bone resorption on the compression side and bone formation on the tension side change the position of the tooth within the alveolar bone (2). In addition, interactions among osteoclasts, osteoblasts, and fibroblasts produce the reconstruction of bone associated with the periodontal tissues.

The PDL consists of cells and an extracellular matrix and is constantly exposed to mechanical stress in the oral environment. Morphological studies on collagen degradation during remodeling of the PDL have indicated that fibroblasts ingest collagen fibrils and degrade them intracellularly (1). This degradation of native collagen fibrils is dependent on specific collagenolytic enzymes, col-
lagenases, and other matrix metalloproteinases (MMPs), as well as cathepsins, which are lysosomal cysteine proteinases. Resorption of the extracellular matrix occurs in both extra- and intracellular environments (4), and, as an initial step, collagenases act in cellular manner at a neutral pH on collagen fibrils and cleave individual collagen breakdown by lysosomal cysteine proteinases.

Lysosomal cysteine proteinases (e.g., B, H, K, L, and S), members of the cathepsin family of proteinases, contain a serine (e.g., CAG) and aspartyl proteinases (e.g., CAD and CAE), with the nomenclature based on the critical variable amino acid residues at their active sites (5–7). CAB, CAK, CAL, and CAS are each capable of degrading collagen (8), whereas CAH is assumed to be a non-collagenolytic enzyme (9). In a recent study that utilized an inhibitor of cysteine proteinases, important functions of CAB, CAL, and CAK were shown to be involved in the degradation of bone collagen (8, 10–13). Even inside of cells, CAK, a recently identified cysteine proteinase, was abundantly and selectively expressed in osteoclasts and giant cell tumor (osteoclastoma) specimens (14–16). In addition, several studies have reported that CAB and CAL are also distributed and present in osteoclasts while in vitro studies have suggested essential roles for CAB and CAL in bone resorption (17, 18). Further, CAK is thought to be specifically expressed in osteoclasts and to play an important part in osteoclastic bone resorption while CAB and CAL are expressed not only in osteoclasts, but also in PDL fibroblasts (19). In regard to the relationships among CAB, CAL, and PDL cells, Wang (20) reported that these cathepsins were found inside the lysosomal fraction of PDL fibroblasts together with another enzyme, acid phosphatase (ACPase), and Goseki et al. (21) recognized the activities of CAB and CAL in cultured PDL fibroblasts. In other studies, the amounts of CAB and CAL in gingival crevicular fluid were increased with periodontal disease and orthodontic tooth movement (22–24). Thus, a strong participation by CAB and CAL in periodontal tissue metabolism (reconstruction) has been shown; however, the localization and movement by these cathepsins during orthodontic tooth movement have not yet been elucidated.

The purpose of the present study is to reveal the localization and movement of CAB and CAL in rat molars during experimental tooth movement using immunohistochemical analysis.

Materials and Methods

Experimental animals and tooth movement

Twenty male Wistar rats, 13 weeks old, were used in the present study, after preliminary breeding for 5 weeks. Experimental tooth movement was performed using the Waldo method (25), with an elastic band (Unitek, No. 400–134) that was inserted between the first and second molars of the maxilla (Fig. 1), after the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (0.1 ml/100 g body weight). Untreated animals were used as the control group, and the experimental and control groups were each composed of 5 rats.

![Fig. 1. Diagram of method used for experimental tooth movement. A piece of elastic band (ELS) is inserted between the upper first molar (M1) and the second molar (M2). Rectangles 1 and 2 indicate the observation area (1, compression side: 2, tension side: M3, the third molar).](image-url)
Tissue preparation

The experimental periods were set at 1, 3, and 7 days after tooth movement. Animals were deeply anesthetized by pentobarbital sodium and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) in a trans-cardial manner, after which the maxilla was immediately dissected and immersed in the same fixative overnight at 4 °C. The specimens were decalcified in 10% disodium ethylenediamine tetraacetic acid (EDTA, pH 7.4) solution for 4 weeks, and then decalcified specimens were dehydrated through an ethanol series and embedded in paraffin. Each sample was horizontally sliced into 6-μm continuous sections and prepared for hematoxylin and eosin (H.E.), tartrate-resistant acid phosphatase (TRAP), and immunohistochemical staining.

Enzyme histochemistry

TRAP staining was done according to the Burstone method (26). The reaction mixture consisted of 5 mg of naphthol AS-MX (Sigma, USA) with an enzymatic substrate, 30 mg of fast red violet LB salt (Sigma, USA) diluted in 25 ml of 0.2 M acetate buffer solution (pH 5.2) containing 50 mM of sodium tartrate. The reaction was conducted for 1 hour at 37 °C and counterstained with hematoxylin.

Immunohistochemistry

Immunohistochemical staining was performed as follows: the sections were deparaffinized and endogenous peroxidase activity was quenched by incubation in 3% H2O2 in methanol for 30 minutes at room temperature (RT). After washing in Tris Buffer Saline (TBS), sections were incubated with the anti-rat CAB rabbit polyclonal (Upstate, USA; working dilution: 1: 200) and the anti-mouse CAL goat polyclonal (SANTA CRUZ, USA; working dilution: 1: 200) for overnight at 4 °C. CAB and CAL were stained using Histofine SAB-PO kits (Nichirei, JAPAN) according to the manufacturer's protocol and subsequently biotinylated. The sections were rinsed with TBS, and final color reactions were performed with the substrate reagent 3,3’-diaminobenzidine tetra-hydrochloride, then they were counter-stained with hematoxylin. As immunohistochemical controls, some sections were incubated in the same way, and then incubated with either non-immune rabbit IgG or 0.01 M PBS alone, instead of the primary antibody.

Results

Histological changes in the periodontal tissues during tooth movement

Control group

Rat PDL specimens were found to be composed of relatively dense connective tissue fibers that ran regularly in a horizontal direction from the cementum toward the alveolar bone. The alveolar bone and root surface were relatively smooth, and very few mononuclear and multinucleate osteoclasts and resorption lacunae were rarely observed on the alveolar bone surface (Fig. 2-A, C). Some of these mononuclear and multinucleate osteoclasts were positive to TRAP (Fig. 2-B, D).

Experimental groups

After 1 day, the PDL on the compression side showed a decreased width, the arrangement of the fibers and fibroblasts became coarse and irregular, and blood capillaries were pressured (Fig. 2-E) while on the tension side, the collagen fibers became elongated (Fig. 2-G). A few resorption lacunae with TRAP-positive multinucleate osteoclasts were observed on the surface of the alveolar bone on the compression side (Fig. 2-F) while on the tension side, resorption lacunae with TRAP-positive multinucleate osteoclasts were rarely observed (Fig. 2-H). After 3 days, the PDL on the compression side was composed of a coarse arrangement of fibers and expanded blood capillaries (Fig. 2-I) while on the tension side, the collagen fibers were continuously elongated (Fig. 2-K). Many resorption lacunae with TRAP-positive multinucleate osteoclasts appeared on the alveolar bone surface on the compression side while in the fibers of the PDL, many mononuclear TRAP-positive cells were present (Fig. 2-J). Furthermore, on the tension side, some TRAP-positive multinucleate osteoclasts were observed on the alve-
compressive bone surface; moreover, in the fibers of the PDL, some mononuclear TRAP-positive cells were present (Fig. 2–L). After 7 days, on the compression side, fibroblasts in the PDL were increased (Fig. 2–M). On the tension side, the collagen fibers were continuously elongated (Fig. 2–O). Further, on the surface of the alveolar bone on the compression side, bone resorption lacunae with multinucleate TRAP-positive osteoclasts were recognized; however, mononuclear and multinucleate TRAP-positive cells were decreased in comparison with those 3 days after the movement (Fig. 2–N). In addition, the alveolar bone surface on the tension side showed a few resorption lacunae with TRAP-positive multinucleate osteoclasts at 7 days (Fig. 2–P).

**Immunolocalization of CAB and CAL**

**Control group**

The immunoreactivity of CAB and CAL was recognized in osteoclasts, fibroblasts, vascular endothelial cells, and in both the compression and tension sides of the periodontal tissues. The reactivity of CAL was stronger than that of CAB; however, no significant differences were seen between the compression and tension sides (Fig. 3–A, B, C, D).

**Experimental groups**

After 1 day, the PDL on the compression side was composed of a coarse arrangement of fibers with collagen degradation, and the immunoreactivity of CAB and CAL was slightly increased (Fig. 3–E, F).
On the tension side, the collagen fibers were elongated; however, the immunoreactivity of CAB and CAL was unchanged compared with the control (Fig. 3-G, H). After 3 days, in the PDL fibroblasts on the compression side, the immunoreactivity of CAB and CAL was increased compared to the control. The reactivity of CAL was also stronger than that of CAB. Further, the alveolar bone on the compression side contained some osteoclasts that were stained strongly for both cathepsins though the reactivity of CAL was significant stronger than that of CAB (Fig. 3-I, J). On the tension side after 3 days, the collagen fibers were continuously elongated, and some osteoclasts were observed on the alveolar bone surface.

The reactivity of CAL was also stronger than CAB in those cells (Fig. 3-K, L). After 7 days, on the compression side, the degree of CAB and CAL immunoreactivity was continuously increased in the PDL fibroblasts, which also occurred in osteoclasts on the alveolar bone surface (Fig. 3-M, N). On the tension side, the collagen fibers were continuously elongated, and they showed a coarse arrangement while the immunoreactivity of both CAB and CAL was observed, but the degree of immunoreactivity was slightly decreased in comparison with these 3 after the movement (Fig. 3-O, P).
Discussion

The PDL is mainly comprised of type I and III collagens that connect alveolar bone to the roots of teeth. Degradation of collagen fiber occurs through phagocytes by fibroblasts or fibroblasts (27), and ends with degeneration by cathepsins or other types of lysosomal enzymes (28). Thus, cathepsins are known to carry out important work in bone metabolism. Bone resorption is mainly produced by osteoclasts and progresses through two processes, the solubilization of minerals, which occurs by acidification (29, 30), and degradation of the organ matrix, which consists mainly of type I collagen, by proteolytic enzymes, particularly MMPs and lysosomal cysteine proteinases or cathepsins. The present study examined the time-dependent expression of CAB and CAL in the periodontal tissues during experimental tooth movement, in an attempt to clarify their participation in the degradation of bone collagen and elucidate the movement of collagen metabolism in the periodontal tissues.

In this experiment, immunoreactivity for CAB and CAL was detected in osteoclasts and fibroblasts; however, those of CAB were weaker than those of CAL. Notably, CAL immunoreactivity was strong in many of the osteoclasts that appeared following tooth movement. Further, many osteoclasts appeared in the compression and tension sides over time with experimental tooth movement while the immunoreactivity of CAB and CAL was reinforced. In the osteoclasts that appeared on day 3, CAL immunoreactivity was especially strong. Previous studies have demonstrated that CAB and CAL are involved in bone resorption by osteoclasts (12). Taken together, those findings and these results suggest that CAB and CAL play important roles in bone resorption in response to tooth movement.

As for fibroblasts, the fiber arrangement in the PDL became coarse on the compression and tension sides with tooth movement, and regressive lesions were formed from the decrease in fibroblasts associated with collagen degradation. However, the degree of staining of CAB and CAL increased in those cells following experimental tooth movement. Our laboratory previously reported that the levels of CAB and CAL increased in gingival crevicular fluid during human orthodontic tooth movement (23, 24) and that CAL activity increased in human PDL cells with tension force in vitro (23). The present results are in agreement with in vitro research results.

In a previous study of lysosomal cysteine proteinases, CAL was shown to be at least 10 times faster than CAB (31). Further, Rifkin et al. (11) found that the activity of CAL in osteoclasts was 25 times greater than that of CAB. In addition, CAL, but not CAB, is generally regulated at the basal level for normal tissue turnover though it is regulated at different levels under specific situations such as tumor genesis (32). Kakegawa et al. (8) reported that bone resorption (pit formation) induced by PTH was suppressed completely by a specific inhibitor of CAL [pig leukocyte cysteine proteinase inhibitor, PLCPI (33)] while osteoclast extracellular deposition was stronger for CAL than for CAB (12). Taken together with the present results, these findings suggest that CAL, rather than CAB, may play a central role in collagen degradation in response to mechanical stress.

CAK, a recently identified cysteine proteinase, is abundantly and selectively expressed in osteoclasts and giant cell tumor (osteoclastoma) (14–16, 34). In more recent studies, CAK mRNA–positive osteoclasts and odontoclasts appeared after experimental tooth movement, and CAK mRNA–positive osteoclasts reached a peak on the compression side after 3 days (35) while CAK mRNA–positive odontoclasts appeared on the compression side on day 6 (36) and day 7 (28). CAK mRNA in osteoclasts has been reported to be 10 times greater than that in CAL and 25 times greater than that in CAB (11, 37). Furuyama et al. (38) also reported that various stimulants (PTH, IL-1α, IL-6, and TNF–α) were able to clarify the activity of CAB, CAL, and CAK. The cysteine protease, CAB, is not activated by stimulants, and CAL activation is adjusted by CAK. Therefore, CAK was synthesized in a constitutive manner as the main cysteine protease, and CAL was synthesized as an inducible cysteine protease in osteoclasts to degrade
type I collagen in combination with CAK. These findings suggest that CAK may be involved in the expressions of CAB and CAL during tooth movement.

During orthodontic tooth movement, it is generally understood that bone resorption on the compression side and bone formation on the tension side change the position of the tooth in the alveolar bone (2). However, Ohba et al. (35) demonstrated that osteoclasts are recognized not only on the compression side, but also on the tension side of the alveolar bone, as CAK mRNA expression was detected in osteoclasts on the compression side of the alveolar bone at 12 hours after force application and the distribution and number of CAK mRNA–positive osteoclasts increased in a time-dependent manner on the compression side. After 3 and 4 days, a marked increase in CAK mRNA–positive osteoclasts was found not only on the compression side but also on the tension side of the alveolar bone in response to tooth movement. In the present study, the immunoreactivity of CAB and CAL in osteoclasts on the compression side was significantly stronger than that in those on the tension side, which showed only a slight expression. These results are in agreement with CAK research results of Ohba et al. (35) and suggest that CAK as well as CAB and CAL participate in bone resorption and collagen degradation during tooth movement.

In conclusion, the present study demonstrated that CAB and CAL could be detected in PDL fibroblasts and osteoclasts and were increased in osteoclasts and fibroblasts following experimental tooth movement. Therefore, mechanical stress may be involved in extracellular matrix degradation and bone resorption through the stimulation of cathepsins, which is one of the important factors in periodontal tissues.

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