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Investigation of periodontal ligament reaction upon excessive occlusal load – osteopontin induction among periodontal ligament cells

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Objective: The purpose of the present study was to investigate the reaction of the periodontal ligament to excessive occlusal loading by observing the histological changes and osteopontin induction. The possibility of ligand for receptor activator of nuclear factor κB (RANKL) participation in osteopontin induction was also discussed.

Background: The precise mechanism of periodontal ligament breakdown by excessive occlusal loading remains unclear. We established an experimental model for excessive occlusal loading *in vivo*. Osteopontin is known to be produced upon mechanical loading and is considered to induce the migration of osteoclasts to the resorption site. RANKL is one of the essential factors for osteoclast maturation and induces the constitutive induction of intracellular osteopontin *in vitro*.

Methods: The occlusal surface of the upper left first molars of rats was raised by steel wire bonding in order to induce occlusal trauma. The destruction of the periodontal ligament was observed and the production of osteopontin and RANKL by periodontal ligament cells was detected via immunohistochemistry.

Results: Our model produced wide-ranging destruction of the periodontal ligament. From day 3 to day 7, prominent compression of the periodontal ligament and osteoclast migration were observed at the apical interradicular septum. Osteopontin was detected in some osteoclasts, surrounding fibroblasts, and osteoblasts adjacent to the compression area. RANKL was observed from day 1 to day 7 around the osteoblasts and osteoclasts.

Conclusions: Our model was useful for the detailed investigation of periodontal ligament breakdown during excessive occlusal loading. Although intracellular osteopontin was produced in osteoclasts with intermittent occlusal loading, the role of this protein in the cells was not clear. No correlation between RANKL distribution and osteopontin production in osteoclasts could be found.

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Along with dental plaque induced-inflammation, excessive occlusal force is generally thought to be one of the major etiological factors in periodontal tissue destruction (1–7). Several studies have shown the effect of excessive occlusal load on the periodontal ligament in relation to dental plaque-induced peridontitis (1, 2, 8–12), blood flow (13, 14), and Ruffini endings (15, 16). However, the precise mechanism of periodontal tissue breakdown by excessive occlusal load remains unclear because of the lack of an appropriate *in vivo* experimental model.

In the case of bone, it is well known that mechanical loading induces proliferation and differentiation of bone-derived cells and extracellular matrix synthesis through various macromolecules (17, 18). When a mechanical force is applied to bone, the stress might be detected by certain cells, which then release chemical mediators that induce bone remodeling through autocrine/paracrine mechanisms. These mechano-responsive cells are most likely osteoblasts and osteocytes (18, 19). Similarly, osteoclasts may be the key cells in periodontal tissue destruction upon excessive occlusal loading.

Osteopontin is one of the abundant non-collagenous phosphorylated bone matrix proteins that are known to be produced during mechanical loading (17, 20–23). The relationship between mechanical loading and osteopontin expression has been studied both *in vitro* (24–27) and *in vivo* (28–31). Osteopontin may convert mechanical stress into intracellular signaling as a mechano-transducer (25, 27). In mineralized tissue, osteopontin is thought to be expressed by fibroblasts (32), osteoblastic cells (33–35), and osteoclasts (34, 36). However, these *in vivo* studies examined constant mechanical loading representing physiological tooth movement, rather than intermittent force as excessive occlusal loading. As the forces that cause occlusal trauma are usually intermittent, it is important to observe the tissue responses under such conditions.

The ligand for the receptor activator of nuclear factor κB (RANKL) was also identified as the ligand of RANK. RANKL induces differentiation of osteoclast precursors into mature osteoclasts in the presence of monocyte macrophage-colony-stimulating factor *in vitro*. Thus, RANKL is thought to be essential for osteoclast differentiation and maturation.

In the present study, we established an *in vivo* model for excessive occlusal loading. In previous studies investigating the effects of excessive occlusal loading, periodontal ligament compression was limited and the tissue recovered to the control level rather quickly. In the present study, we raised the bite excessively in order to induce periodontal ligament destruction.

The purpose of the present study was to investigate the histological changes in the periodontal ligament during excessive occlusal loading and to clarify whether osteopontin would be produced in osteoclasts upon intermittent mechanical loading *in vivo*.

Material and methods

Experimental excessive occlusal loading model

Eighteen female Wistar/ST rats, 15 weeks old (240–260 g), were examined in the present study. The rats were anesthetized by diethyl ether (Wako, Osaka, Japan) and the occlusal surfaces of the upper left first molar were raised by 1.24 mm using steel wire bonded with methyl-methacrylate resin (Clearfil MEGABOND, Kuraray Medical Inc., Okayama, Japan) (Fig. 1). Three rats served as the control group. The animal protocol was carried out according to the guidelines for animal experimentation of the Tokyo Medical and Dental University (approval number 0030088).

Tissue preparation and histomorphometry

At 1, 3, 5, 7, and 14 days after increasing occlusal height, the rats were anesthetized with diethyl ether and killed. The upper left first molar and surrounding tissues were fixed using 4% formaldehyde for 48 h. After fixation, the bonded steel wire was carefully removed. Tissues were decalcified in 10% ethylene-diaminetetraacetate (EDTA, pH 7.4) for 3 weeks at room temperature and embedded in paraffin according to standard protocol. Mesio-distal serial sections of 4-μm thickness were prepared and stained with hematoxylin–eosin. Morphological changes of the periodontal ligament and its surrounding tissues

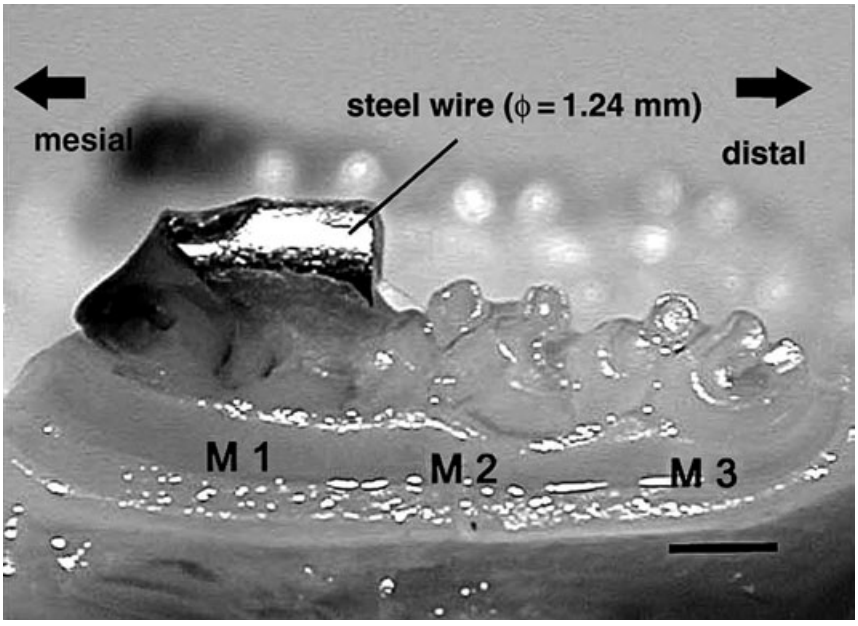

Fig. 1. Steel wire bonded onto the occlusal surface of the left upper first molar (shown upside down). Bar = 700 μm.

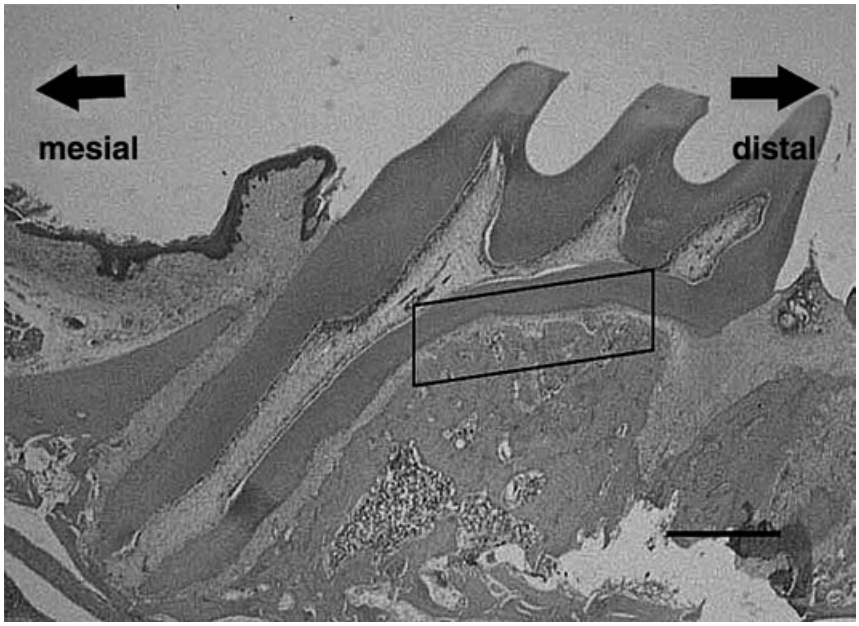

Fig. 2. Mesio-distal section of the upper first molar. The observed area is indicated by the rectangle. Bar = 500 μm.

were observed at the apical interradicular septum under light microscopy (Fig. 2).

Histological sections were subjected to digital image analysis. The periodontal ligament area and the length of cementum facing the periodontal ligament at the apical interradicular septum were measured using image analysis software (Win ROOF, Mitani Corp., Fukui, Japan). For the analysis, representative sections from each animal were selected (three sections per experimental period). The average width of the periodontal ligament at the apical interradicular septum was calculated as follows: average width of the observed periodontal ligament = area of the periodontal ligament/length of cementum facing the periodontal ligament.

Means and standard deviations were calculated for each experimental period. These data were compared across the experimental period by one-way ANOVA ($p < 0.05$).

Immunohistochemistry

In order to detect the production of osteopontin and RANKL, immunohistochemistry was carried out via the avidin–biotin–peroxidase complex (ABC) method using a VECTASTAIN ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA). Sections were deparaffinized by xylene and graded ethanol, and then treated with 20 μg/ml Proteinase K (Roche Diagnostics, Mannheim, Germany) for 10 min. In order to prevent endogenous peroxidase activity, the sections were incubated for 30 min in 0.3% H_2O_2/methanol. The sections were then treated with 0.1% blocking serum albumin to prevent non-specific binding and incubated with primary antibody for 30 min. Rabbit polyclonal anti-osteopontin (LSL Co., Ltd, Tokyo, Japan) and goat polyclonal anti-RANKL (c-20) (sc-7627, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used. The dilution of primary antibodies used was osteopontin (1 : 6000–8000) and RANKL (1 : 100). After being washed with phosphate-buffered saline several times, the sections were incubated with biotinylated IgG for 30 min and subsequently with streptavidin–horseradish peroxidase for 30 min. Following several washes with phosphate-buffered saline, 3,3′-diaminobenzidine (Funakoshi Co., Ltd, Tokyo, Japan) substrate was applied. As a negative control, non-immune serum was used instead of primary antibody.

After the immunostaining, tartrate-resistant acid phosphatase (TRAP) staining was carried out using a TRAP staining kit (Hokudo Corp., Sapporo, Japan) in order to identify the osteoclasts and mononuclear precursors. All experimental procedures were carried out at room temperature. Sections were counterstained with hematoxylin and observed under light microscopy. osteopontin-positive/negative osteoclasts were counted for each experimental period.

Results

Morphological observations

Macroscopically, the mobility of the upper left first molar was observed in the mesio-distal direction after 7 days and in the bucco-lingual direction after 9 days. No inflammatory reaction was observed at the epithelium around the respective teeth throughout the experimental period.

Representative histological observations are shown in Fig. 3. In the control, the periodontal ligament maintained a constant width and main fibers ran across the cementum and the alveolar bone in an orderly manner (Fig. 3a). A thin layer of acellular cementum was observed around the root furcation. A few osteoclasts characteristic of bone remodeling were observed at the inner side of the alveolar bone. Blood vessels in the periodontal ligament were localized mostly on the alveolar bone side and proximal to the blood vessels in the alveolar bone.

One day after the application of the excessive occlusal loading, the periodontal ligament was compressed at the apical interradicular septum, and periodontal ligament fibers ran almost parallel to the cementum (Fig. 3b). The width of the periodontal ligament became irregular and relatively narrow in some areas. Blood vessels were moderately compressed and blood cells could clearly be identified.

On day 3, the periodontal ligament was tightly compressed. Consequently, cells could barely be observed among the periodontal ligament fibers. The periodontal ligament fibers ran almost

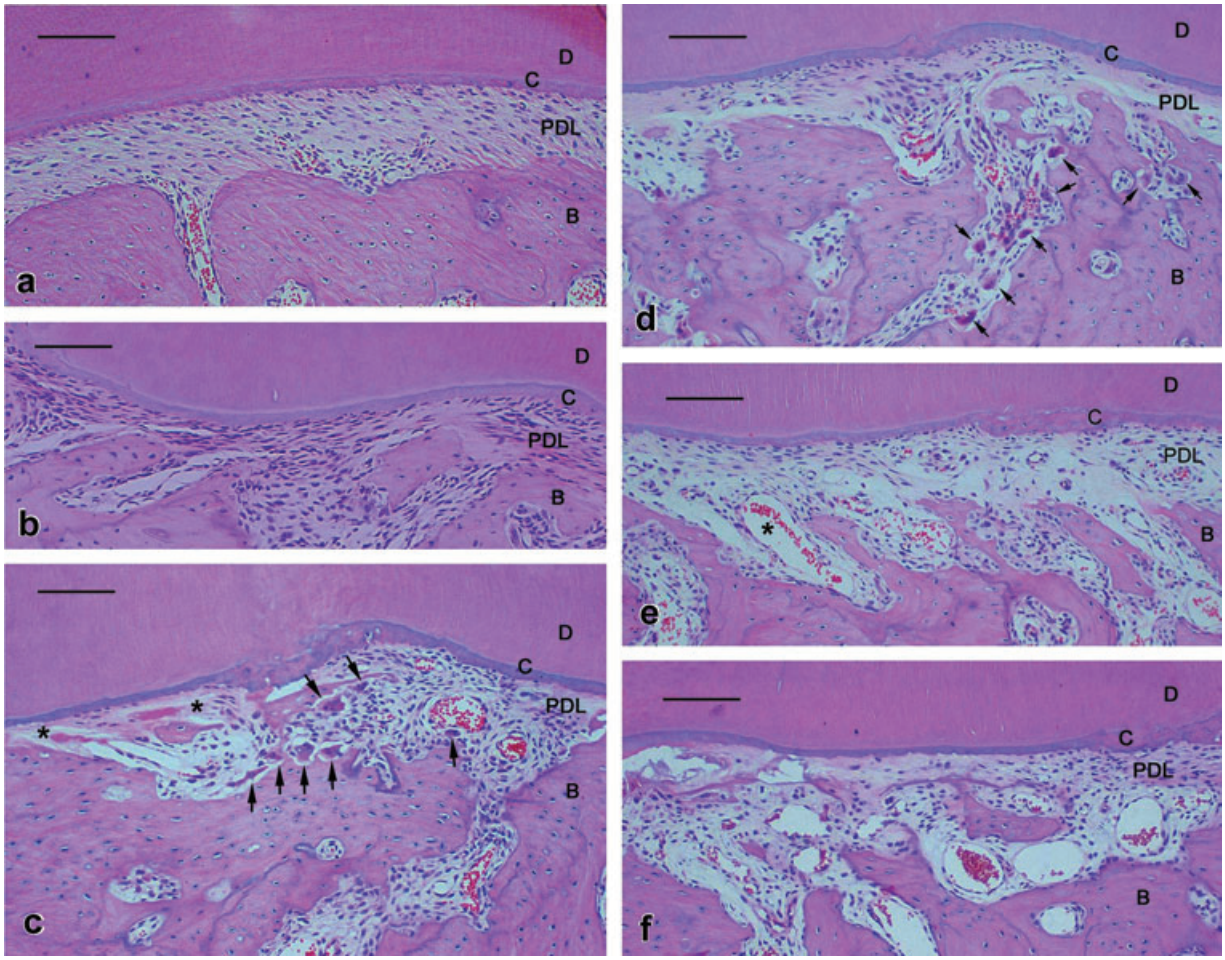

Fig. 3. Mesio-distal sections of rat periodontal ligament stained with hematoxylin-eosin. (a) Control: periodontal ligament had constant width, and fiber arrangement is in order. (b) Day 1: periodontal ligament became compressed, and fibers run almost parallel to the dentin. (c) Day 3: periodontal ligament compression became enhanced so that individual fibers were barely identifiable (*). Osteoclasts were observed on alveolar bone adjacent to the compressed area (arrowheads). (d) Day 5: compressed area became wider. Osteoclasts were observed in a wide area of alveolar bone (arrowheads). (e) Day 7: periodontal ligament compressions started to recover. (f) Day 14: periodontal ligament width was recovered to the control level. Expansion of blood vessels was observed (*). B: bone; C: cementum; D: dentin; PDL: periodontal ligament. Bar = 200 μm.

Table 1. Change in periodontal ligament width

		Day				
	Control	1	3	5	7	14
Width	147.55	*44.83	*59.29	*107.08	156.47	158.33
SD	1.48	1.48	11.67	10.14	9.78	41.64

*Statistically significant with respect to the control.
SD, standard deviation.

parallel to the cementum at the apical interradicular septum (Fig. 3c). In some areas, no bundles of periodontal ligament fibers could be identified and extreme reduction in the eosin staining occurred. Osteoclast migration was observed at the apical interradicular septum, adjacent to the compressed tissue and alveolar bone. There were no odontoclasts on the surface of the cementum, despite severe compression. Blood vessels disappeared from areas in which severe compression was observed, although dilated blood vessels were observed in the surrounding areas.

On day 5, the prominent compressed areas became wider. At this stage, osteoclasts were widely observed in the alveolar bone at the apical interradicular septum (Fig. 3d).

On day 7, the arrangement of the periodontal ligament fibers was still irregular and the number of cells within the fibers remained small (Fig. 3e). Along the edge of the alveolar bone, a small, but dramatically decreased, number of osteoclasts were still observed. Dilation of the blood vessels became more enhanced and lacunae of the alveolar bone surface

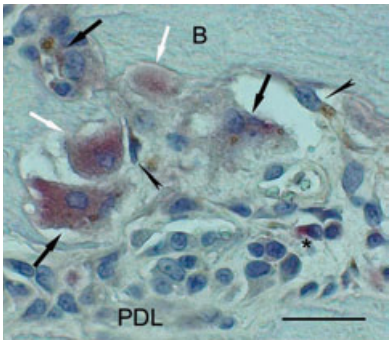

Fig. 4. Double stained with anti-osteopontin serum and tartrate-resistant acid phosphatase (TRAP) on day 3. Black arrow: osteopontin-positive osteoclasts; white arrow: osteopontin-negative osteoclasts; black arrowhead: osteopontin-positive osteoblasts; *: osteoclast precursor; B: bone; PDL: periodontal ligament. Bar = 20 μm.

were mostly occupied by the expanded blood vessels.

On day 14, the width of the periodontal ligament recovered to the control level. The arrangement of periodontal ligament fibers was almost the same as that of the control, although compressed bundles of periodontal ligament fibers were still present. Pieces of isolated alveolar bone were observed as a result of acute bone resorption in the interradicular septum area (Fig. 3f). The number of osteoclasts was decreased to the control level. Alveolar bone surface lacunae were still occupied by dilated blood vessels.

Throughout the experimental period, there were no obvious changes in osteoblast population or location in the observed area.

Periodontal ligament width

Mean changes in periodontal ligament widths and standard deviations are shown in Table 1. The width of the periodontal ligament was tremendously reduced at day 1 and remained significantly smaller ($p < 0.05$) until after day 5. At day 7, the periodontal ligament width became almost the same as that of the control.

Immunohistochemical observations

The results of the anti-osteopontin/TRAP double staining of the day 3 specimen are shown in Fig. 4. In the control, osteopontin was detected on the surface of the dentin facing the dental pulp (predentin) and the cortical layer of the alveolar bone (osteoid) (data not shown). There was no osteopontin expression in fibroblasts, osteoblasts, or osteoclasts. A small number of osteopontin-positive osteocytes existed in the interradicular septum area. RANKL was not detected at this stage.

One day after treatment, no osteopontin-positive cells were revealed by immunohistochemistry. On day 3, osteopontin was detected in a few osteoclasts and surrounding cells

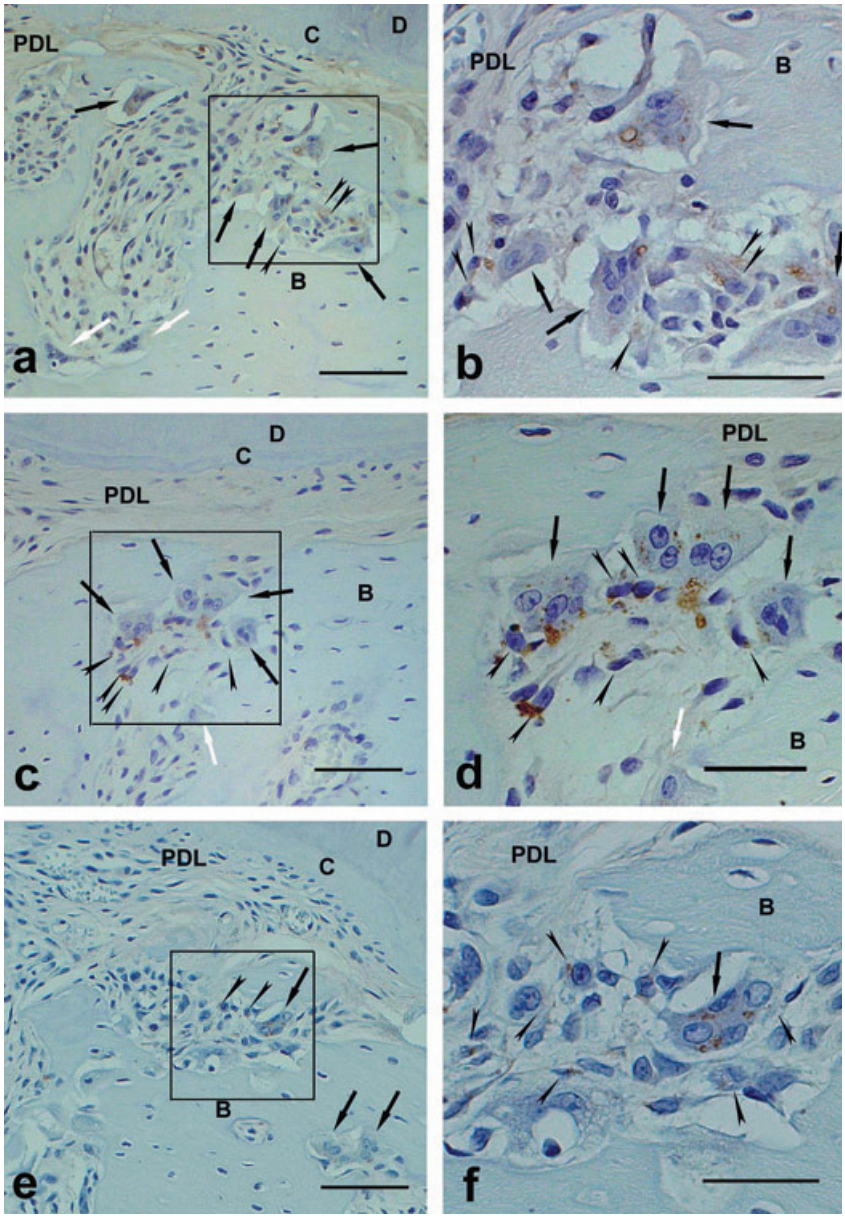

Fig. 5. Mesio-distal sections of rat periodontal ligament stained with anti-osteopontin serum. The rectangle indicates the magnified area in each experimental period. (a, b) Day 3: osteopontin production was observed in osteoclasts (black arrows) and surrounding fibroblasts and osteoblasts (black arrowheads). Note that no osteopontin production was detected in osteoclasts distant from the compressed area (white arrows). (c, d) Day 5: osteopontin was observed in osteoclasts (white arrows). (e, f) Day 7: number of osteoclasts decreased. Osteopontin-positive osteoclasts still existed. B: bone; C: cementum; d: dentin; PDL: periodontal ligament. (a), (c), and (e): bar = 40 μm; (b), (d), and (f): bar = 20 μm.

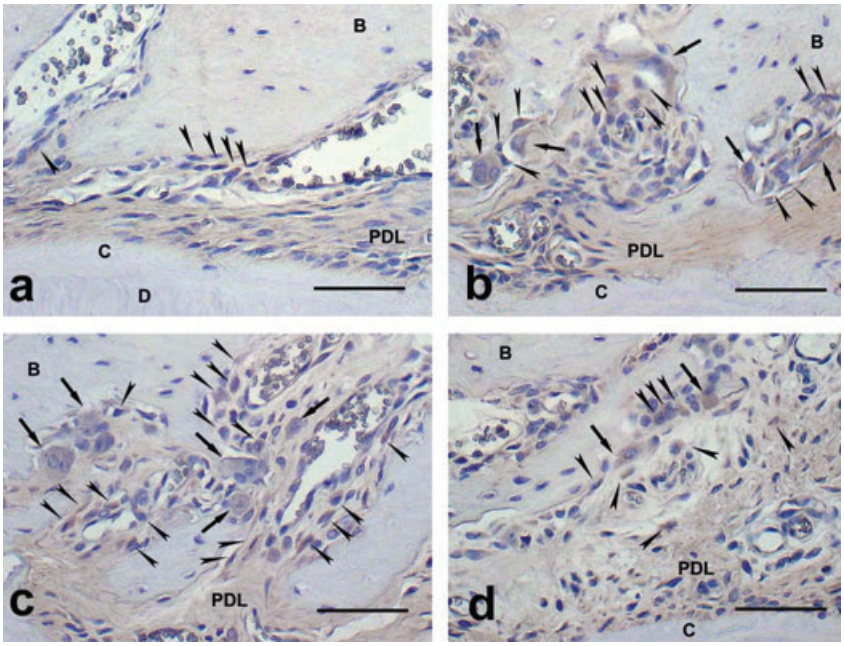

Fig. 6. Mesio-distal sections of rat periodontal ligament stained with anti-RANKL (receptor activator of nuclear factor κB) serum. (a) Day 1: RANKL was observed in a small number of osteoblasts. (b) Day 3: RANKL was observed both in osteoblasts and osteoclasts. (c) Day 5: RANKL was observed on osteoblasts and osteoclasts. (d) Day 7: RANKL was observed in few osteoblasts and osteoclasts. Arrow: RANKL-positive osteoclasts; arrowhead: RANKL-positive osteoblasts; B: bone; C: cementum; D: dentin; PDL: periodontal ligament. Bar = 40 μm.

Table 2. Number of osteoclasts and osteopontin-positive osteoclastss

		Day				
	Control	1	3	5	7	14
Number of OCs	1.67	3.33	32.33	20.67	12.33	6.67
SD	1.15	0.58	1.53	0.58	1.53	1.53
Number of OPN-positive OCs	0.00	0.00	6.00	7.33	2.67	0.67
SD	0.00	0.00	1.00	2.89	0.58	0.58

OCs, osteoclasts; OPN, osteopontin, SD, standard deviation.

(fibroblasts and osteoblasts) at the apical interradicular septum adjacent to the compressed area (Figs 5a and b). There were no osteopontin-positive osteoclasts in region of the alveolar bone. The production of osteopontin was observed in the cytoplasm of each cell. A small number of osteocytes produced osteopontin at the top of the interradicular septum.

One day after treatment, RANKL was detected in osteoblasts at the compressed periodontal ligament area (Fig. 6a). On day 3, RANKL was observed in osteoclasts, adjacent osteoblasts, and in the surrounding matrix, and was also detected in osteoclasts far from the compressed region (Fig. 6b).

On day 5, osteopontin production was still observed in a few osteoclasts and the surrounding cells (fibroblasts and osteoblasts) at the apical interradicular septum. However, at this stage, osteopontin production in osteoclasts became more prominent (Figs 5c and d). Osteopontin was still observed in the cytoplasm of fibroblasts and osteoblasts, but not in the surrounding matrix. The distribution of RANKL-positive cells was the same as that observed on day 3 (Fig. 6c).

Osteopontin-positive cells were still observed at the apical interradicular septum at day 7, but the number of these cells and the production intensity appear to have decreased (Figs 5e and f). RANKL was still present in osteoclasts and the surrounding matrix (Fig. 6d).

On day 14, both osteopontin and RANKL were barely detectable in the site.

The number of osteoclasts and osteopontin-positive osteoclasts at each experimental stage are listed in Table 2. The number of osteoclasts increased dramatically from day 1 to day 7 and remained high on day 14. Osteopontin was observed in a few osteoclasts from day 3 to day 14.

Discussion

The histological changes of the periodontal ligament upon excessive occlusal loading, along with osteopontin and RANKL production by periodontal ligament cells, were investigated herein. Although the detailed mechanisms of orthodontic tooth movement, including the contributions of extracellular matrix (37, 38) and cytokines (39, 40), have been relatively well defined, the mechanisms of periodontal tissue destruction due to excessive occlusal loading remain unclear.

We established an animal model for traumatic occlusion using metal wire. Several studies have investigated periodontal ligament breakdown *in vivo*. However, the methods used in such studies were not appropriate in terms of strength and/or type of load. In most cases, the effect of mechanical loading on the periodontal ligament was investigated under constant mechanical loading. Clinically, it has been shown that the excessive occlusal loading that may cause occlusal trauma is mostly intermittent. Thus, the establishment of an appropriate experimental model in which the loading is both at a high enough level and intermittent is indispensable and allows detailed investigation of periodontal ligament breakdown on mechanical loading. Although previous studies showed less destruction and relatively quick recovery of periodontal ligament, our study demonstra-

ted greater destruction and extensive periodontal ligament change. We believe the experimental model used herein to be accurate.

Even though 6- to 8-week-old rats are commonly used in this type of experiment, we used 15-week-old rats in order to account for the fact that periodontal bone loss caused by excessive occlusal loading is usually clinically observed among elderly people. Previous studies have reported the delay of biological responses to orthodontic stimuli (41, 42) and the decrease in osteopontin mRNA expression (43) in older rats. In the present study, constitutive production of osteopontin in osteocytes was also low and did not increase, even with the application of excessive occlusal loading. Still, the periodontal ligament was extensively destroyed in our model, suggesting that this model is suitable for determining osteopontin production in osteoclasts upon excessive occlusal loading *in vivo*.

There have been a number of *in vivo* reports of osteopontin production in osteocytes upon mechanical loading (28–31). The major cells producing osteopontin during physiological tooth movement are osteocytes, which are thought to play a major role in mechano-transduction (28–30). However, as mentioned previously, almost all of these studies applied orthodontic force according to the Waldo method (44) to induce osteopontin production. Interestingly, although constant orthodontic force resulted in osteopontin production in alveolar osteocytes, our model did not show inducible osteopontin production in the cells. This is probably due to the intermittent loading in our model. Even though the degree of bone resorption by intermittent force was less than that by continuous force (45), both constant and intermittent loads result in bone resorption, indicating that osteoclasts are recruited through different mechanisms. The fact that osteoclast recruitment is not delayed in the case of intermittent loading (45) supports this hypothesis.

Osteopontin is considered to interact with $\alpha_v\beta_3$ integrin of osteoclasts and is key in the development and migration of osteoclasts (46). In our findings, osteopontin-positive osteoblasts and fibroblasts were detected in periodontal ligament adjacent to the prominent compression area. This may support the current hypothesis that osteopontin expressed by osteoblasts and fibroblasts may induce the migration of osteoclasts to the resorption site. However, the osteopontin production of these cells was almost simultaneous with the bone resorption. Moreover, we could not find secreted osteopontin around these cells, even though osteopontin has generally been recognized as a secreted protein. The intracellular form of osteopontin is considered to promote osteoclast formation (47). In our findings, intracellular osteopontin was clearly detected in some osteoclasts adjacent to the prominent compressed periodontal ligament. These facts suggest that osteopontin function at this stage might be associated with the mechano-response of osteopontin-positive cells themselves. Osteopontin might have functions other than anchoring molecules against osteoclasts.

RANKL was detected around the osteoblasts and osteoclasts during bone resorption. RANKL is one of the factors that is essential for osteoclast maturation. It is secreted from osteoblasts and differentiates osteoclast precursors into mature osteoclasts (48). *In vitro* osteoclast culture systems stimulated by RANKL show constitutive expression of intracellular osteopontin in osteoclasts (49). This suggests the possibility of the involvement of RANKL in the production of intracellular osteopontin in osteoclasts. However, in our study, RANKL was observed in both osteopontin-positive and osteopontin-negative osteoclasts, indicating that the production of osteoclastic intracellular osteopontin *in vivo* might not be induced directly by RANKL, but rather by a different pathway upon mechanical stress.

It has been reported that not only bone resorbing osteoclasts, but also those distant from the bone surface were producing osteopontin in the case of osteoarthritic femurs (34). Although several studies have demonstrated that osteoclasts produce osteopontin (34, 36, 50), whether osteoclasts really produce osteopontin still remains controversial (36, 51). Our results clearly indicate osteopontin production by osteoclasts in the periodontal ligament, but not in the distant region of the alveolar bone upon mechanical loading. This result also suggests that there might be several pathways of osteopontin induction in osteoclasts *in vivo*.

Osteopontin has been reported to promote bone matrix formation as well as bone resorption (52). In the present study, we showed the possibility of osteopontin production in osteoclasts as a mechano-responsible molecule upon intermittent mechanical loading. Of course, we should consider this in the context of complicated bone or periodontal ligament biology. Further detailed investigation, including the possibility of osteopontin contribution to periodontal tissue regeneration, is necessary.

Acknowledgement

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