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Effect of cyclic mechanical loading on osteoclast recruitment in periodontal tissue

K. Nozaki[1], M. Kaku[2], Y. Yamashita[3], M. Yamauchi[2], H. Miura[1]

[1]Fixed Prosthodontics, Department of Restorative Sciences, Graduate School of Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan, [2]Dental Research Center, The University of North Carolina at Chapel Hill, NC, USA and [3]Maxillofacial Anatomy, Department of Maxillofacial Biology, Graduate School of Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan

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Background and Objective: It is well accepted that cyclic mechanical loading induces osteoclastogenesis in periodontal tissue, but its molecular mechanisms are not well understood, in part because of a lack of appropriate models. In this study, we investigated a novel device that allows cyclic mechanical loading to be performed in a well-controlled manner. Furthermore, by employing this model, the effect of cyclic loading on osteoclast recruitment in the periodontal tissue was described.

Material and Methods: By using a newly developed device, the cyclic loading of 20 N (reference loading corresponding to the fracture hardness of dietary pellets) and two excessive loadings (i.e. 30 and 40 N) were applied to maxillary right molars in rats for up to 7 d, and osteoclast recruitment in the periodontal tissue was evaluated by analyzing relevant marker proteins using immunohistochemistry.

Results: Osteoclastogenesis was induced by day 3 within alveolar bone subjected to a compression force of 30 N. With both 30 and 40 N loadings, cells that were positive to for tartrate-resistant acid phosphate, receptor activator of nuclear factor-κB ligand and osteoprotegerin were significantly increased in the alveolar bone/periodontal ligament in a time-dependent manner.

Conclusion: A new device was developed that allows various levels of cyclic mechanical loading to be exerted. By using this device in rats, early events of osteoclast recruitment in the periodontal tissues were observed with excessive loadings in a time-dependent manner, indicating the usefulness of this model.

Masaru Kaku, Dental Research Center, The University of North Carolina at Chapel Hill, 4301 Research Commons Building, 79 T.W. Alexander Drive, Research Triangle Park, NC 27709, USA
Tel: +1 919 425 3556
Fax: +1 919 425 3532
e-mail: kakumasaru@gmail.com

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Periodontal ligament is a unique tissue connecting two distinct mineralized tissues – alveolar bone and tooth cementum – providing a means to absorb forces generated by mastication and to maintain the periodontium system. Several clinical observations and studies have suggested that the optimum occlusal loading force is one of the key regulators involved in maintaining this unique feature and structure (1). When a tooth suffers excessive mechanical loading, it starts to become mobilized as a result of structural/compositional changes in the periodontal ligament, resulting in the expansion in width of the periodontal ligament. By contrast, when a tooth does not receive optimum occlusal loading, so-called hypofunction, the disarrangement of periodontal ligament fibers occurs, leading to narrower periodontal ligament width (2).

A series of recent studies have provided insights into the mechanisms of periodontal ligament tissue maintenance regulated by mechanical loading. When mechanical loading is sensed by periodontal ligament cells, bone formation/resorption is triggered by autocrine/paracrine mechanisms (3). Apparently, osteoclast recruitment induced by mechanical loading is mainly regulated by osteoblasts and fibroblasts through their receptor activator of

nuclear factor-κB ligand (RANKL). When these mechano-responsive cells sense mechanical loading, RANKL is secreted to the cell surface and triggers osteoclast differentiation by binding to its receptor [receptor activator of nuclear factor-κB (RANK)] on the osteoclast cell surface (4, 5). This results in the initiation of a series of intracellular signaling events involving nuclear factor-κB, c-Fos and c-jun, which trigger osteoclastogenesis (6). Considering this complex paracrine system that requires osteoblast–osteoclast interaction, an appropriate *in vivo* model would be indispensable for investigating the molecular and cellular events associated with bone formation/resorption in response to mechanical loading.

Osteoprotegerin is another important factor involved in regulating the process of osteoclast differentiation. Osteoprotegerin is a RANK homolog, mainly secreted from osteoblasts and fibroblasts in response to mechanical stimuli, and functions as a decoy receptor for RANKL. Therefore, binding of osteoprotegerin to RANKL inhibits the differentiation of osteoclasts (7, 8). Because of the presence of this dominant negative form of RANKL, the RANKL : osteoprotegerin ratio is commonly used as an index of bone resorption (9–12).

We previously reported histological changes occurring in periodontal tissues following excessive mechanical loading that was applied using a bite-raising model (13). In this model system, periodontal ligament was compressed within 1 d and the recruitment of osteoclasts was observed in 3 d. RANKL was increased in periodontal ligament following excessive mechanical loading. However, in this model, the early events occurring in response to mechanical loading were difficult to assess because of the acute tissue response that induced significant cell death. Therefore, in the present study, we attempted to establish a device that allowed us to exert mechanical loading in a well-controlled manner in order to evaluate the early response of periodontal tissues to mechanical loading. By employing this model, histological changes, osteoclastogenesis, and the levels of RANKL and osteoprotegerin were analyzed.

Material and methods

Determination of the reference loading force

Studies have reported that the maximal force appears during the first phase of mastication and that it is food/material dependent (14, 15). Thus, we established the fracture hardness of dietary pellet as a reference masticatory bite force. Ten cube-shaped (4 × 4 × 4 mm) pellets were prepared from the standard dietary pellets (CLEA rodent Diet CE-2; CLEA Japan, Inc., Tokyo, Japan) given daily to the rats used in this study.

Custom loading jigs were fabricated to simulate the mastication process of rats. Impressions of the rat right maxillar and mandibular molars were taken using vinyl polysiloxane materials (EXAFINE Regular; GC Co., Ltd, Tokyo, Japan) and cast with silver-alloy using a standard lost-wax technique. The fracture hardness was measured using a universal testing machine (Autograph AGS-H; Shimadzu Co., Ltd, Tokyo, Japan) with the custom loading jigs set at a crosshead speed of 60 mm/min.

Fabrication of the loading device

The experimental device and its schematic diagram are shown in Fig. 1A,C, respectively. The device consists of the following components: a motor unit (IHTY9S40N, C-30Y; Japan Servo Co., Ltd, Tokyo, Japan), a cam, weights, a load cell and a loading jig. The custom loading jig was designed to reach the upper right first molar without disturbance (Fig. 1B). The loading level and frequency were calibrated using a load cell (LMA-A-100N) with an amplifier (PCD-300A) and software (DAS-100A) (all from Kyowa Electronic Instruments Co., Ltd, Tokyo, Japan) (Fig. 1D).

Animal study

The animal use protocol was approved by the guidelines for animal experiments of the Tokyo Medical and Dental University (approval number 0070275). Fifty, 8-wk-old male Wistar/ST rats (250–270 g in weight) were

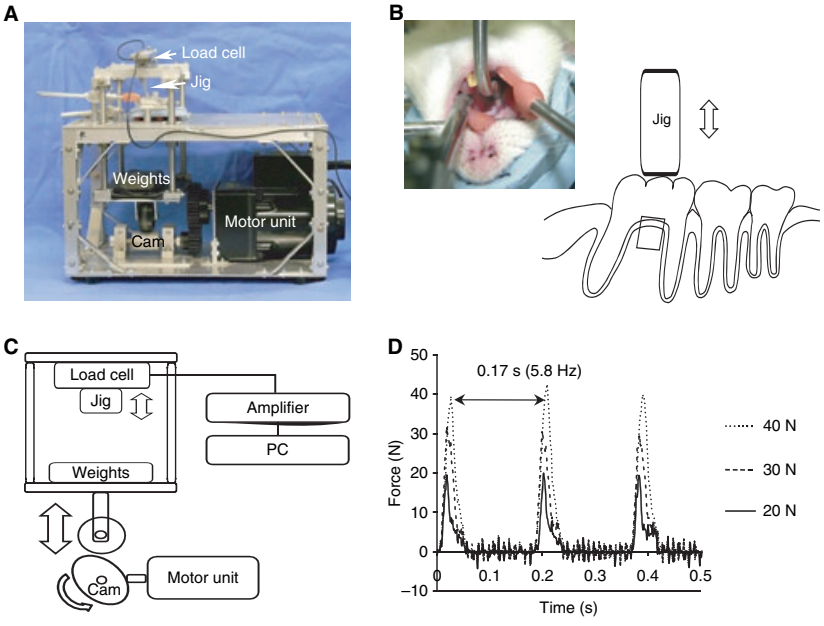

Fig. 1. Schematic figure of the loading apparatus. (A) Lateral view of the cyclic loading apparatus. (B) Occlusal view of a loading jig on the subjected teeth, and a diagram of a loading jig and the right upper first molar. The area for histological evaluation is indicated by a rectangle. (C) Illustration of a loading mechanism. Loading force was controlled with weights, and loading frequency was regulated by a motor unit and a cam. (D) Calibrated force curves at each loading magnitude were detected by a load cell.

used in this study. They were fed with pellets (the same as used for the fracture-hardness test) and water throughout the experimental period. All procedures were carried out under anesthesia (Nembutal; Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan). Maxillary right first molars ($n = 5$) were subjected to cyclic loading of 20, 30, or 40 N (100, 150 or 200% of the reference loading force), at 5.8 Hz for 30 min, daily, for up to 7 d. Five rats that received no loading were used as controls.

Tissue preparation and histological analysis

After 1, 3 and 7 d of loading, rats were killed and subjected to histological analysis. Target teeth and surrounding tissues were excised and fixed in 4% formaldehyde for 72 h at 21°C. The tissues were then decalcified in 10% EDTA (pH 7.4) for 10 d at 37°C, after which they were embedded in paraffin. The samples were dehydrated in graded ethanol, embedded in paraffin, then cut into 4-μm mesio–distal serial sections and stained with hematoxylin and eosin to assess morphological changes.

Tartrate-resistant acid phosphatase staining Some of the representative sections were subjected to tartrate-resistant acid phosphatase (TRAP) staining, to identify osteoclasts, using the TRAP staining kit (Primary Cell Co., Ltd, Sapporo, Japan) according to the manufacturer's protocol. Sections were counterstained with hematoxylin and analyzed using light microscopy.

Immunohistochemistry In order to detect RANKL and osteoprotegerin, immunohistochemistry was carried out by the avidin–biotin–peroxidase complex (ABC) method using a VECTASTAIN *Elite* ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA). After deparaffinization and dehydration of the sections, antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) for 2 h at 70°C. Then the sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After incubation with normal rabbit serum for 30 min, samples were incubated with primary antibodies in a humidified chamber. The antibodies and conditions used were as follows: goat polyclonal anti-RANKL (C-20, 1:100 dilution) or goat polyclonal anti-osteoprotegerin (N-20, 1:50 dilution) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After washing several times with phosphate-buffered saline, the sections were incubated with biotinylated immunoglobulin G for 30 min, washed several times with phosphate-buffered saline and reacted with streptavidin–horseradish peroxidase conjugated reagent for 30 min. Following three, 5-min washes with phosphate-buffered saline, samples were incubated with 3,3′-diaminobenzidine (DAB; Vector Laboratories Inc.) to visualize the immunoreactivity. Sections were counterstained with hematoxylin and analyzed using light microscopy.

Cell count Representative sections were selected and subjected to digital image analysis using IMAGEJ software (National Institute of Health, Bethesda, MD, USA). Two slides were evaluated from each animal ($n = 5$) and thus a total of 10 samples was subjected to each analysis. The total number of cells, and those positive for TRAP and respective antibodies in the defined areas (500×500 μm), were counted. Because the total number of cells in periodontal ligament constantly changed as a result of mechanical loading, the number of immunopositive cells was assessed relative to the total number of cells. The RANKL/osteoprotegerin ratio was also calculated in each section examined. The statistical analysis was performed by one-way analysis of variance followed by a Tukey-HSD test, with the significance level set at $p < 0.05$.

Results

Fracture hardness of dietary pellets

The fracture hardness of dietary pellets was measured and is shown in Fig. 2. The fracture hardness was 20.4 ± 2.6 N. A few samples showed a different force-deformation pattern, even though the fracture hardness was similar; this was probably because of the nonuniformity of the materials.

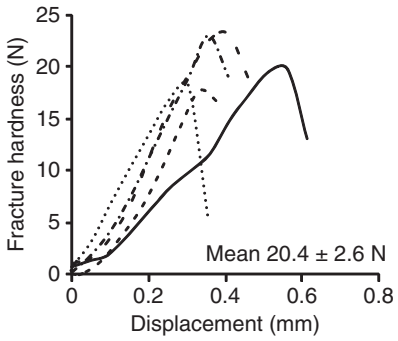

Fig. 2. Fracture hardness of dietary pellets. All samples showed similar fracture hardness, but the force-deformation pattern varied.

Morphological observations

Throughout the experimental periods, neither mobility nor abrasion of the experimental teeth was observed. No inflammatory reaction was shown in the epithelium around the teeth.

Some of our preliminary studies, and our previous report (13), indicated that the inter-radicular septum region showed the most consistent and significant histological changes upon loading. Thus, this area was histologically examined.

Representative hematoxylin and eosin-stained sections are shown in Fig. 3. In the control, cells were evenly distributed in the periodontal ligament, and collagen fibers were observed to run in the mesio–distal direction (Fig. 3J). The alveolar bone surface facing the periodontal ligament was relatively smooth. No histological changes were observed with 20 N loading up to 7 d (Fig. 3A–C). With 30 N loading, no differences were observed on day 1. On days 3 and 7, however, the distribution of cells along the surface of the alveolar bone became sparse (Fig. 3E,F). The direction of collagen fibers changed from mesio–distal to more apical–coronal. After 40 N loading, cells at the bone surface became sparse, even on day 1 (Fig. 3G). On day 7, the number of cells decreased, the changes of fiber

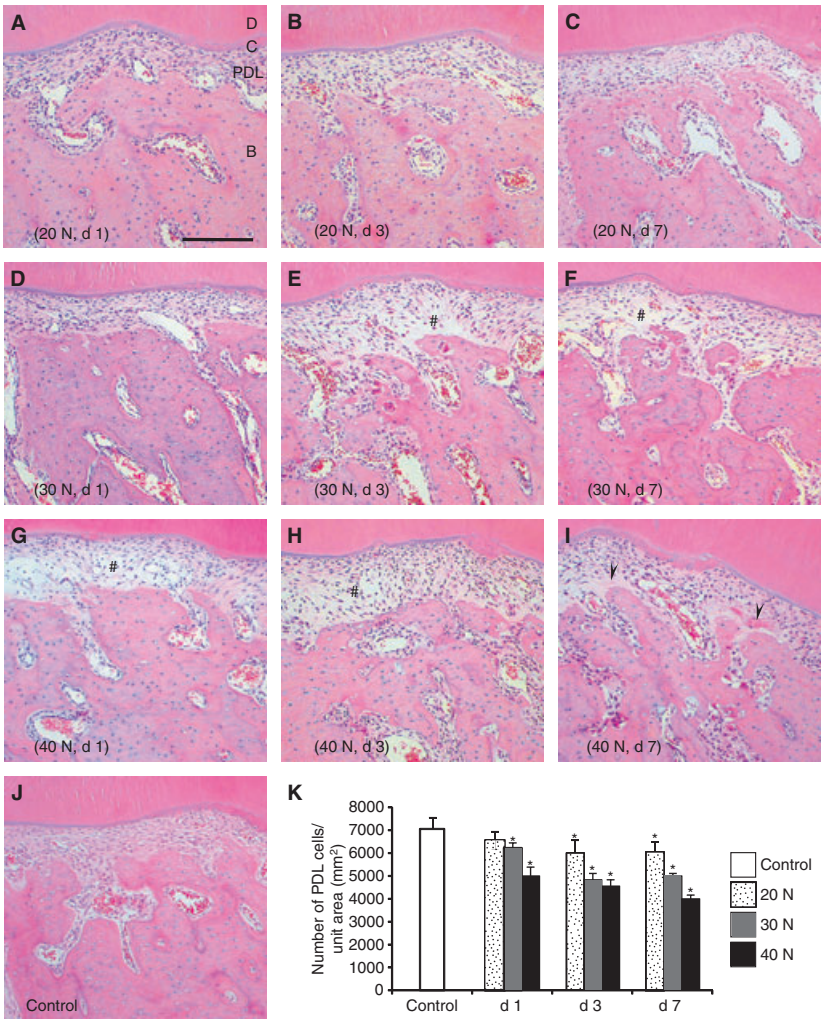

Fig. 3. Mesio–distal sections of rat periodontal tissue, stained with hematoxylin and eosin after cyclic loading. Periodontal ligament cells were evenly distributed in control sections and in sections subjected to 20 N loading (A–C, J). Areas with sparse cells (#) were observed in periodontal ligament, 3 and 7 d after 30 N loading (E and F), and 1 and 3 d after 40 N loading (G and H). Hyalinized tissue (arrow head) was observed only at 7 d after 40 N loading (I). The number of periodontal ligament cells was significantly decreased after 30 and 40 N loading. *Statistically significant with respect to the control. (K). B, bone; C, cementum; D, dentin; PDL, periodontal ligament. Bar = 200 μm.

direction became more apparent and some hyalinized tissues were observed (Fig. 3I). The cell numbers in periodontal ligament were counted and are shown in Fig. 3K. There was a slight decrease in the cell number after 20 N loading, but the decrease was clearly more significant after 30 and 40 N loading and it was time-dependent.

TRAP-positive cells

Representative TRAP-staining sections are shown in Fig. 4. No TRAP-positive multinuclear cells were detected inside the periodontal ligament throughout the experimental period. In the 20 N loading groups, only a few TRAP-positive osteoclasts were observed up to day 7 in the alveolar bone, which was similar to the control samples (Fig. 4A–C,J). However, after 30 and 40 N loading, the number of TRAP-positive osteoclasts in alveolar bone was increased on day 3 and further increased on day 7 (Fig. 4D–I). The number of TRAP-positive cells was counted and is shown in Fig. 4K. No significant increase was detected on day 1 but, subsequently, significant increases were seen with 30 and 40 N loadings in a loading-dependent and time-dependent manner.

Immunohistochemical observations

Results of immunohistochemistry with anti-RANKL serum are shown in Fig. 5. Representative results from the 40 N loaded groups are shown in Fig. 5A–D and the ratio of RANKL-positive cells : total cells in periodontal ligament is shown in Fig. 5E. In the control, RANKL was detected mostly in the cells adjacent to the bone surface and only a few RANKL-positive cells were detected inside the periodontal ligament (Fig. 5D). There was no increase in the number of RANKL-positive cells in the 20 N loading groups (Fig. 5E). After 30 N loading, the number of RANKL-positive cells was increased on days 3 and 7. After 40 N loading, an increase in the number of RANKL-positive cells was observed from day 1 and further increased thereafter. These RANKL-positive cells were mainly located in the areas where cells were sparse. RANKL was also detected in osteoclasts inside the alveolar bone. A few RANKL-positive osteocytes were detected, but the number was not changed in any of the experimental groups (data not shown).

Results of immunohistochemistry with anti-osteoprotegerin serum are shown in Fig. 6 for periodontal ligament and in Fig. 7 for the inner region of alveolar bone. Representative images of periodontal ligament from the 40 N loading groups are shown in Fig. 6A–D and the ratio of osteoprotegerin-positive cells : total cells is shown in Fig. 6E. In the control, osteoprotegerin-positive cells were detected mainly at the bone surface, similarly to RANKL. On day 1, there was no significant difference among control, and 20 and 30 N loading groups. A slight increase was observed on day 3 only with 40 N loading. Osteoprotegerin was clearly detected in loading-induced osteoclasts in alveolar bone. We also noted that osteoprotegerin-positive osteocytes were often detected in the inner region of alveolar bone but were relatively sparse in the

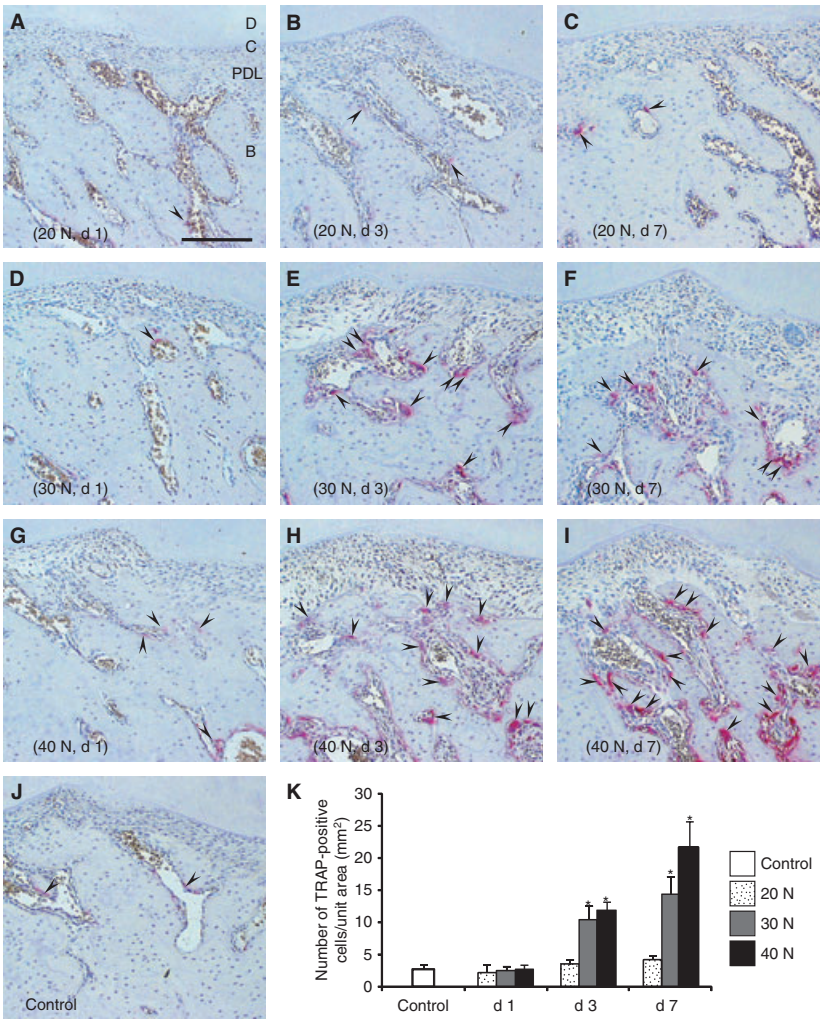

Fig. 4. Mesio–distal sections of rat periodontal tissue stained with tartrate-resistant acid phosphatase (TRAP). There was no increase in the number of TRAP-positive cells following 20 N loading (A–C). With 30 and 40 N loading, TRAP-positive cells were increased in time-dependent and loading-dependent manners (D–I). Recruitment of TRAP-positive cells was observed within the alveolar bone but not in periodontal ligament. The number of TRAP-positive cells was significantly increased on day 3, and thereafter, as a result of 30 and 40 N loading (K). *Statistically significant with respect to the control. Arrow head, TRAP-positive osteoclasts; B, bone; C, cementum; D, dentin; PDL, periodontal ligament. Bar = 200 μm.

area near the periodontal ligament (Fig. 7).

The RANKL : osteoprotegerin ratio in periodontal ligament was calculated and is shown in Fig. 8. There was no significant difference among control, and 20 and 30 N loading groups. With 40 N loading, the RANKL : osteoprotegerin ratio was higher than that of the control, and of the 20 and 30 N loading groups, at all time-points examined, but the level did not change during the time-course of the study.

Discussion

A number of studies have been carried out to investigate the effect of mechanical loading on periodontal tissues (1). Apparently, both constant and cyclic mechanical loading stimulate bone formation and resorption, although the mechanisms might be different (16, 17). The periodontal tissue response to constant mechanical loading is relatively well described (18–20) because of its simple experimental conditions and the existence of a variety of well-established models (21). We also reported an animal model for the excessive mechanical loading exerted by means of bite-raising (13). Although we successfully induced periodontal tissue changes in response to loading using this model, because of its acute compression, most periodontal ligament cells were severely damaged and disappeared. At present, the response to cyclic mechanical loading remains unclear owing to technical difficulties and to the complexity of conditions such as frequency and profile of wave. In the present study, in an attempt to obtain an insight into the effect of occlusal cyclic loading on periodontal tissues, a new device was developed. Using this device, immunohistochemical analysis was performed to investigate the effect of various levels of cyclical mechanical loading.

The fracture hardness of dietary pellets was used as a reference cyclic loading force for mastication and determined as 20 N. As the fracture hardness is the maximum force exerted during mastication, it represents the highest force value during the mastication process (14, 15). At 20 N loading, no changes were observed in general histology, in the number of TRAP-positive cells, and in RANKL and osteoprotegerin production, up to 7 d. This suggests that 20 N can be considered as a physiological occlusal loading force in our model system. However, 30 and 40 N loading (i.e. 50and 100% greater than the reference force, repectively) induced significant histological changes and additional osteoclast recruitment, probably representing an early response of periodontal tissues to excessive cyclic mechanical loading. By applying cyclic loading using this model, RANKL and osteoprotegerin production was clearly induced in periodontal ligament cells. Furthermore, osteoclast recruitment was observed in a loading-dependent manner. Thus, this novel model could be useful to assess the cellular response in periodontal tissues to various levels of cyclic mechanical loading.

With the application of cyclic mechanical loading, osteoclast recruitment was induced in alveolar bone but not in periodontal ligament. Thus,

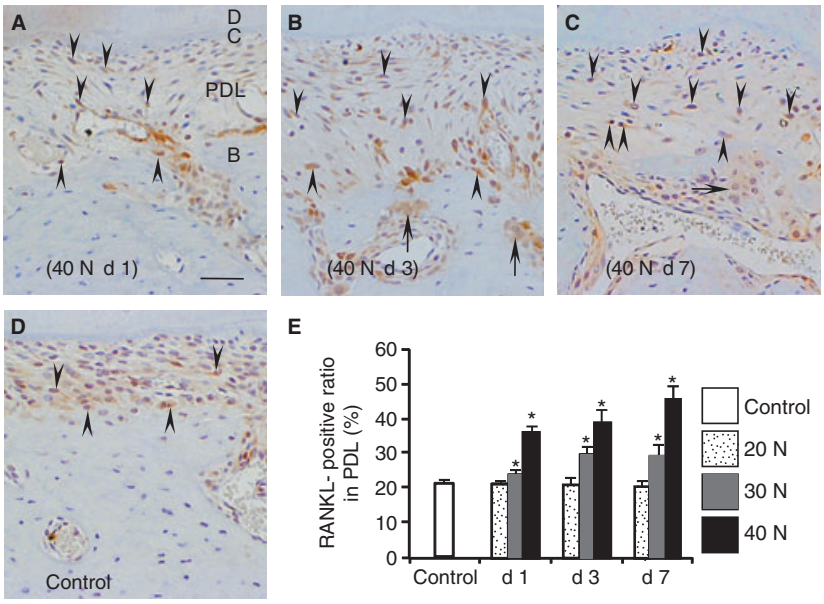

Fig. 5. Mesio–distal sections of periodontal ligament stained with anti-receptor activator of nuclear factor-κB ligand (RANKL) serum. Representative sections obtained from the 40 N loading groups are shown (A–C). RANKL-positive cells were mainly detected in periodontal ligament adjacent to the bone surface, but only a few were detected inside periodontal ligament in the control (D). After loading, the number of RANKL-positive cells was increased around the areas with sparse cells (A–C,E). With 40 N loading, RANKL-positive multinuclear cells were detected at 3 and 7 d (B and C). *Statistically significant with respect to the control; arrowhead, RANKL-positive cell; arrow, RANKL-positive multinuclear cell; B, bone; C, cementum; D, dentin; PDL, periodontal ligament. Bar = 100 μm.

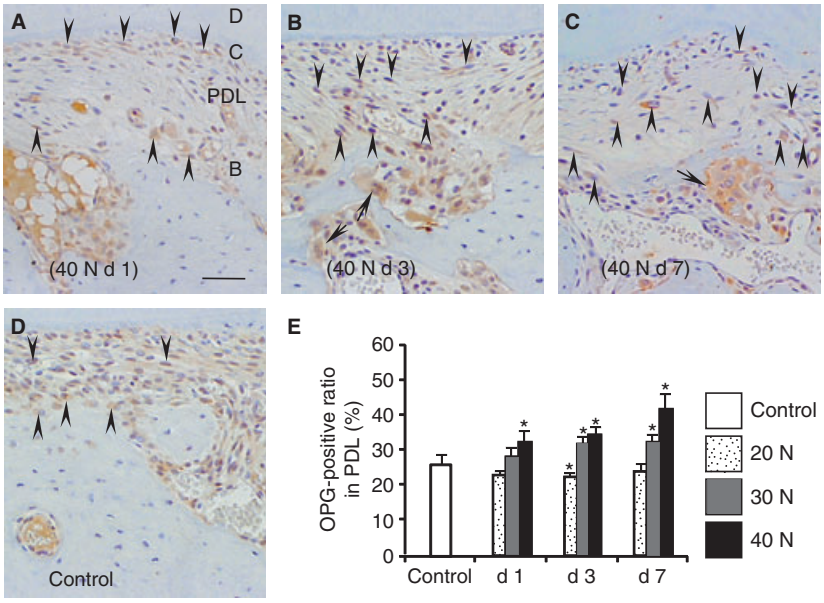

Fig. 6. Mesio–distal sections of periodontal ligament stained with anti-osteoprotegerin serum. A slightly increased number of osteoprotegerin-positive cells were present around the areas with sparse cells (A–C). Osteoprotegerin-positive multinuclear cells were detected after 3 and 7 d (B and C). An increased number of osteoprotegerin-positive cells was observed after 30 and 40 N loading (E). *Statistically significant with respect to the control; arrowhead, osteoprotegerin-positive cell; arrow, osteoprotegerin-positive multinuclear cell; B, bone; C, cementum; D, dentin; OPG, osteoprotegerin; PDL, periodontal ligament. Bar = 100 μm.

those osteoclasts were probably derived from bone marrow, not from the progenitor cells present in periodontal ligament. It has been described that the cell-surface-bound RANKL on osteoblasts directly binds to RANK, a cell-surface receptor on the osteoclast precursor. Subsequently, osteoclast differentiation and maturation are induced. Therefore, it has been postulated that the physical contact between osteoblasts and osteoclast precursors is critical for osteoclastogenesis (22). In addition, the soluble form of RANKL has also been reported and it prolonged the survival of osteoclasts and their resorptive activity (23–25). Recent studies reported that cell-surface-bound RANKL was further cleaved at the ectodomain by certain enzymes, such as matrix metalloproteinase-14 (MMP-14); RANKL then became soluble and was released (26–28). Thus, physical contact between cells may not be necessary for osteoclastogenesis. In our study, osteoclast recruitment was observed spatially far from the periodontal ligament (i.e. within alveolar bone) where RANKL was highly induced. It is possible that the cleaved soluble form of RANKL penetrated into the alveolar bone and then stimulated osteoclastogenesis near the bone marrow where the osteoclast precursors are present.

In our study, both RANKL and osteoprotegerin were detected in multinuclear cells/osteoclasts (Figs 5 and 6). In general, it is believed that RANKL and osteoprotegerin are secreted from osteoblasts and act on osteoclast progenitors, in a paracrine manner, to regulate osteoclastogenesis (3). However, several studies have indicated that mRNA and protein of RANKL and osteoprotegerin can be detected in osteoclasts (29–31). RANKL expressed in osteoclasts may have self-regulatory roles in osteoclast activity and/or survival (29, 30). In addition, osteoprotegerin has a highly basic heparin-binding domain, which facilitates the binding of osteoprotegerin to heparin and heparan sulfate proteoglycans at the cell surface (32, 33). Thus, it is also possible that osteoprotegerin detected in osteoclasts could be derived from osteoblasts,

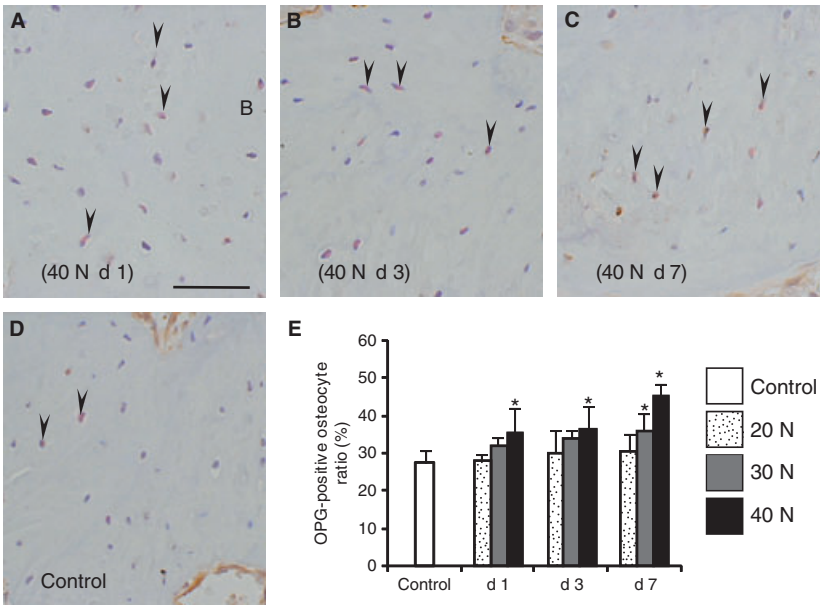

Fig. 7. Mesio–distal sections of the inner region of alveolar bone stained with anti-osteoprotegerin serum (A–D). The number of osteoprotegerin-positive osteocytes was increased with 30 and 40 N loading (E). *Statistically significant with respect to the control; arrowhead, osteoprotegerin-positive osteocyte. Bar = 100 μm. OPG, osteoprotegerin.

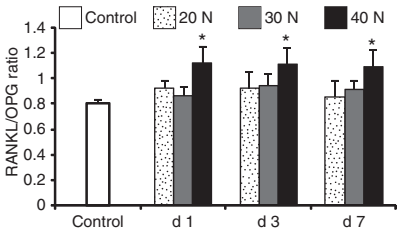

Fig. 8. Receptor activator of nuclear factor-κB ligand (RANKL) : osteoprotegerin ratio in periodontal ligament cells. Only after 40 N loading was the RANKL : osteoprotegerin ratio significantly increased (*), although it was not changed during the time-course of the experiment. OPG, osteoprotegerin.

forming a complex with osteoclast cell-surface proteoglycans.

Several *in vitro* studies have revealed some of the potential mechanisms of osteoclastogenesis through RANKL and osteoprotegerin production under cyclic mechanical loading (34–37). Osteoclast differentiation and maturation are regulated by a balance between RANKL and osteoprotegerin (38, 39). The cyclic tensile force inhibited osteoclastogenesis through osteoprotegerin induction, whereas the concentration of RANKL was not changed in human periodontal ligament cells (36). Interestingly, the cyclic compressive force up-regulated the expression of RANKL, but down-regulated the expression of osteoprotegerin, thus inducing osteoclastogenesis in human periodontal ligament cells (40). In the case of human osteoblasts, the cyclic compressive force appeared to up-regulate osteoprotegerin but down-regulate RANKL (41). It is probable that different cell types and experimental conditions (compressive vs. tensile) are responsible for the different responses. In the present study, when excessive cyclical mechanical loading (i.e. 30 and 40 N) was applied, the numbers of both RANKL-positive cells and osteoprotegerin-positive cells were increased and the RANKL : osteoprotegerin ratio became high with 40 N loading. However, osteoclast recruitment was observed with both 30 and 40 N loading. In addition to RANKL and osteoprotegerin, a number of local factors in a specific microenvironment probably contribute to osteoclastogenesis *in vivo*. For instance, tumor necrosis factor-alpha can induce the formation of osteoclasts in the absence of RANKL (42). Clearly, further studies, by modifying the experimental conditions and by analyzing other molecules related to osteoclastogenesis, are warranted to elucidate the mechanisms by which osteoclasts are recruited in the periodontal tissues upon cyclic mechanical loading.

In conclusion, we have developed a device that can be used as a tool to investigate the molecular/cellular events of periodontal tissues *in vivo* in response to various levels of cyclical mechanical loading. By using this model, we detected increased numbers of cells that are immunopositive to RANKL or osteoprotegerin and subsequent osteoclast recruitment in loading- and time-dependent manners. Further analysis, by modifying the loading conditions, may help to elucidate the mechanisms of dynamic changes of periodontal tissues in response to occlusal loading.

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