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Levels of RANKL and OPG in gingival crevicular fluid during orthodontic tooth movement and effect of compression force on releases from periodontal ligament cells *in vitro*

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Dates:
Accepted 30 June 2005

To cite this article:
Orthod Craniofacial Res **9**, 2006; 63–70
Nishijima Y, Yamaguchi M, Kojima T, Aihara N, Nakajima R, Kasai K:
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Structured Abstract

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Objective – To determine the levels of the receptor activator of NFkB ligand (RANKL) and osteoprotegerin (OPG) in the gingival crevicular fluid (GCF) during orthodontic tooth movement. A second objective was to investigate the effect of compression force on RANKL and OPG production from human periodontal ligament (hPDL) cells.

Design – Ten adolescent patients were included. GCF was collected at the distal cervical margins of the experimental and control teeth 0, 1, 24, and 168 h after the retracting force was applied. This*in vitro* study was performed to examine the secretion of RANKL and OPG from hPDL cells following a compression force (0, 0.5, 1.0, 2.0, or 3.0 g/cm^2 for 48 h). Enzyme-linked immunosorbent assay (ELISA) kits were used to determine RANKL and OPG levels in the GCF and the conditioned medium.

Results – GCF levels of RANKL were significantly higher, and the levels of OPG significantly lower, in the experimental canines than in the control teeth at 24 h, but there were no such significant differences at 0, 1, or 168 h. *In vitro* study indicated that the compression force significantly increased the secretion of RANKL and decreased that of OPG in hPDL cells in a time- and force magnitude-dependent manner. The compression-stimulated secretion of RANKL increased approximately 16.7-fold and that of OPG decreased 2.9-fold, as compared with the control.

Conclusions – The results obtained suggest that the changes of amount of RANKL and OPG may be involved in bone resorption as a response to compression force.

Key words: compression force; gingival crevicular fluid; OPG; orthodontic tooth movement; periodontal ligament cells; RANKL

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, both of which are hard tissue, where it serves to anchor the tooth to the alveolus and functions as a cushion between the hard tissues to disperse occlusal and orthodontic forces. Further, PDL fibers are continually being remodeled to adapt to the changing stresses placed on them (1).

During the movement of teeth in the course of 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, the reconstruction of bone associated with periodontal tissues takes place through interactions among osteoclasts, osteoblasts, and fibroblasts.

Mechanical stress from orthodontic appliances is considered to induce cells in the PDL to form biologically active substances, such as cytokines and enzymes, responsible for connective tissue remodeling (3,4). These substances can be monitored non-invasively in humans by following changes in the composition of the gingival crevicular fluid (GCF) during orthodontic tooth movement (5,6). For example, it has been reported that raised levels of prostaglandin E, interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and epidermal growth factor (EGF) occur in the GCF during orthodontic tooth movement (7,8).

The receptor activator of NFkB ligand (RANKL; also called ODF, TRANCE, and OPGL) has been identified recently as a member of the membrane-associated tumor necrosis factor ligand family and is an important regulatory molecule of osteoclastogenesis (9). RANKL is a ligand of osteoprotegerin/osteoclastogenesis-inhibitory factor (OPG/OCIF) and is expressed in the plasma membranes of osteoblasts/stromal cells (10). Most importantly, RANKL induces osteoclast differentiation from hemopoietic precursors and stimulates their bone resorptive activity (11). Ogasawara et al. (12) reported that RANKL was detected in osteoblasts and PDL cells during experimental tooth movement. OPG is a secreted TNF receptor member that functions as a decoy receptor for RANKL, thereby inhibiting these processes and accelerating osteoclast apoptosis (13,14). Thus, the signaling and regulation of the expression of RANKL and OPG may play critical roles in bone remodeling during orthodontic tooth movement. However, little information is available concerning the production of these modulators during orthodontic tooth movement in human subjects.

In the present study, we investigated (a) the levels of RANKL and OPG in the GCF during orthodontic tooth movement, and (b) the effect of compression force on RANKL and OPG production from hPDL cells.

Materials and methods

Experimental subjects

Informed consent was obtained from all subjects, and the study protocol was reviewed by the Board of Nihon University School of Dentistry at Matsudo. Ten orthodontic patients (four men and six women) participated in the study. On average, the male patients were 14.5 years of age (SD: 2.4 years) and the females were 15.4 years of age (SD: 3.1 years). All subjects were in good general health with healthy periodontal tissues; the probing depths were ≤3 mm, and there was no radiographic evidence of periodontal bone loss. Subjects were excluded if they had had antibiotic therapy during the previous 6 months or if they had taken anti-inflammatory medication during the month preceding the start of the study.

Experimental design

The upper first premolars in all subjects were extracted and edgewise brackets (0.018 in. × 0.025 in. slot, Tomy International Inc., Tokyo, Japan) were placed in both arches. In each subject, one upper canine was designated the experimental tooth and the contralateral and opposing canines the control teeth. The experimental canines were retracted along a 0.018 in. archwire with an elastomeric chain (Tomy International Inc.). The chain delivered an initial force of 250 g.

Impressions for study models were also taken at 0, 1, 24 and 168 h, and the distances between the distal contact point of the experimental canines and the mesial contact point of the second premolars were measured with an electronic digital caliper (Max-Cal; Japan Micrometer Mfg. Co. Ltd., Japan) to 0.01 mm. The distances were measured 10 times and the error of the method was determined. The average amount of movement of the experimental teeth over 168 h was 1.6 ± 0.6 mm. No movement of the control teeth was detected.

GCF collection

At each visit the color of the gingivae was recorded, and the plaque was assessed using the Silness and Loe index (15). To avoid contamination of the GCF samples (16), small deposits of plaque were removed with a periodontal probe, and heavy deposits with a sickle scaler. As previous studies of GCF have used samples collected at 0, 1, 24, and 168 h after initiation of tooth movement, the same time intervals were used in the present study (8,17,18).

Using the method of Offenbacher et al. (19), GCF was collected from both experimental and control teeth during the same appointment. The following procedure was used: the experimental/control tooth was gently washed with water, and the gingival area was isolated with cotton rolls (to minimize saliva contamination) and gently dried with an air syringe. Paper strips (Periopaper, Harco, Tustin, CA, USA) were carefully inserted 1 mm into the distal gingival crevice on each experimental and control tooth and allowed to remain *in situ* for 1 min (Fig. 1). After waiting for a further minute, a second strip was placed at the same site for the same length of time. Care was taken to avoid mechanical injury to the gingivae. Following GCF collection, the probing depths and attachment levels of the experimental and control teeth were recorded. Any bleeding following probing was also noted.

The volume of GCF on the paper strip was measured with a Periotron 8000 (Harco) that had been calibrated with human serum. GCF collection was standardized so that the experimental and control sites and different subjects could be compared. After collection the paper strips were stored at −30°C.

For evaluation, the paper strips were placed individually in 100 μl of Tris buffer (12 mM Tris, containing 0.1 M NaCl and 0.05% Tween 20) and then subjected to a vortex thrice over a 30-min period. The strip was then removed and the eluate was centrifuged for 5 min at 3000*g*. The supernatants were separated and frozen at −30°C for use later. Protein concentration in the extract was estimated by the method of Bradford (20), with bovine serum albumin as a standard.

Cell culture

Human PDL fibroblasts were prepared according to a modification of the method of Somerman et al. (21), as described previously (22). Briefly, PDL tissues were taken from the roots of premolars extracted from six healthy young volunteers (three males and three females; 14–16 years old) during the course of orthodontic treatment, after obtaining informed consent

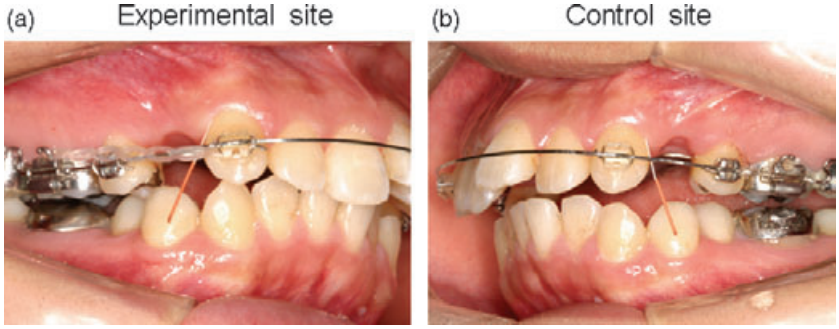

Fig. 1. GCF was sampled at the distofacial angles of the tooth being retracted and at the contralateral control teeth: (a) experimental side; (b) contralateral side.

from the donors, and used according to a protocol reviewed by the Board of Nihon University School of Dentistry at Matsudo. The PDL tissues were placed in 35-mm tissue culture dishes and covered with a sterilized glass coverslip. The medium used was α-MEM (Gibco, Grand Island, NY, USA) supplemented with 100 μg/ml of penicillin-G (Sigma Chemical Co., St Louis, MO, USA), 50 μg/ml of gentamicin sulfate (Sigma), 0.3 μg/ml of amphotericin B (Flow Laboratories, McLean, VA, USA), and 10% fetal calf serum (FCS, Cell Culture Laboratories, Cleveland, OH, USA). The cultures were kept at 37°C in a humidified incubator (Forma CO_2 incubator MIP-3326, Sanyo Electric Medica System Co., Tokyo, Japan) in the presence of 95% air and 5% CO_2. When cells growing from each explant had reached confluence, they were detached with 0.05% trypsin (Gibco) in PBS for 10 min and subcultured in culture flasks. Those cells still attached to the bottom of the flask were discarded to avoid contamination by epithelial cells.

Application of compressive forces

In order to reproduce the conditions of pressure during orthodontic tooth movement, we performed the following *in vitro* experiment, in accordance with the method developed by Kanai et al. (23). hPDL cells were continuously compressed using a uniform compression method as a model of pressure at the site of orthodontic movement (Fig. 2). Previous studies have shown that compressive mechanical stress can be applied by the system utilized in the present experiment (24–26). Static compression force is thought to mimic that found *in vivo* during orthodontic treatment. In the present experiments, the cells were only stimulated once. Briefly, a 30-mm diameter cell disk was placed over a nearly confluent cell layer in the wells of a six-well plate, on top of which was placed a glass cylinder. Compression force was then controlled by inserting lead granules into the cylinder. Prior to the application of the compression force, the cells were pre-incubated for 1 h in culture medium containing 2% FCS, and then they were subjected to 0.5, 1.0, 2.0, or 3.0 g/cm of compression force for 48 h.

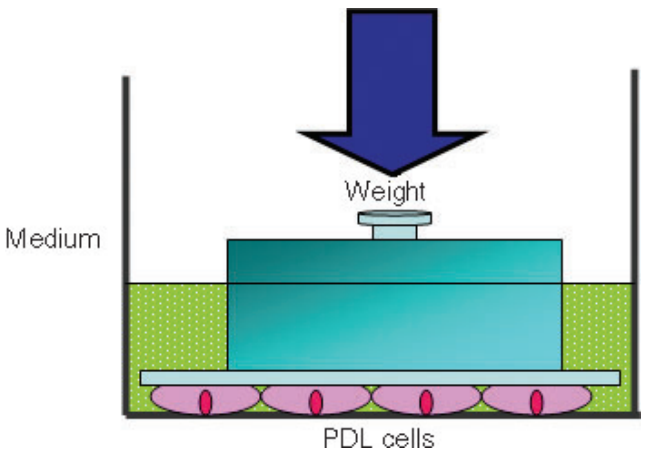

Fig. 2. The method used to apply compressive force. Precultured PDL cells were compressed continuously using a glass cylinder containing different weights. The glass cylinder was placed over a confluent cell layer in one well of a six-well plate. The number of lead granules placed in the cylinder controlled the compressive force.

RANKL and OPG determination

RANKL and OPG were measured by commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA). In the*in vivo* study, all samples and standards were assayed twice. Data were reported as the concentrations of cytokine (pg/μl of GCF). In the *in vitro* study, the levels of cytokine present in the media were determined.

Statistical methods

GCF study

Descriptive statistics and statistical analysis were performed using STATISTICA v 5.5 (StatSoft Japan Inc., Tokyo, Japan). Mann–Whitney *U*-tests were used to compare the means of the groups (Fig. 3).

In vitro study

Values are shown as the mean ± standard deviation (SD). Statistical significance was determined using Tukey–Fisher's test (Figs 4 and 5). Data were subjected to two-way ANOVA and then indicated in the results (Figs 4 and 5).

Results

GCF study

Clinical parameters

In all patients, plaque accumulation was minimal throughout the study and gingival health was excellent. Furthermore, probing depths remained less than 2 mm at all times throughout the experimental period, and there was no gingival bleeding on probing.

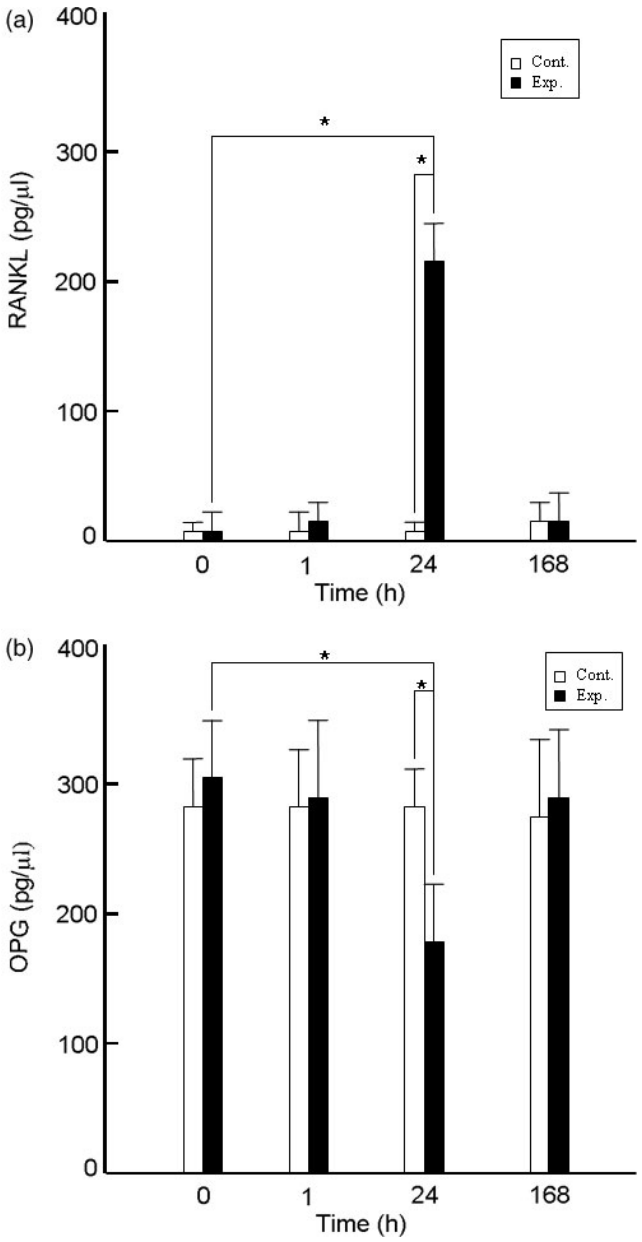

Fig. 3. RANKL (a) and OPG (b) concentrations in the GCF during orthodontic tooth movement. Cont., contralateral control teeth; Exp., experimental teeth (*$p < 0.001$).

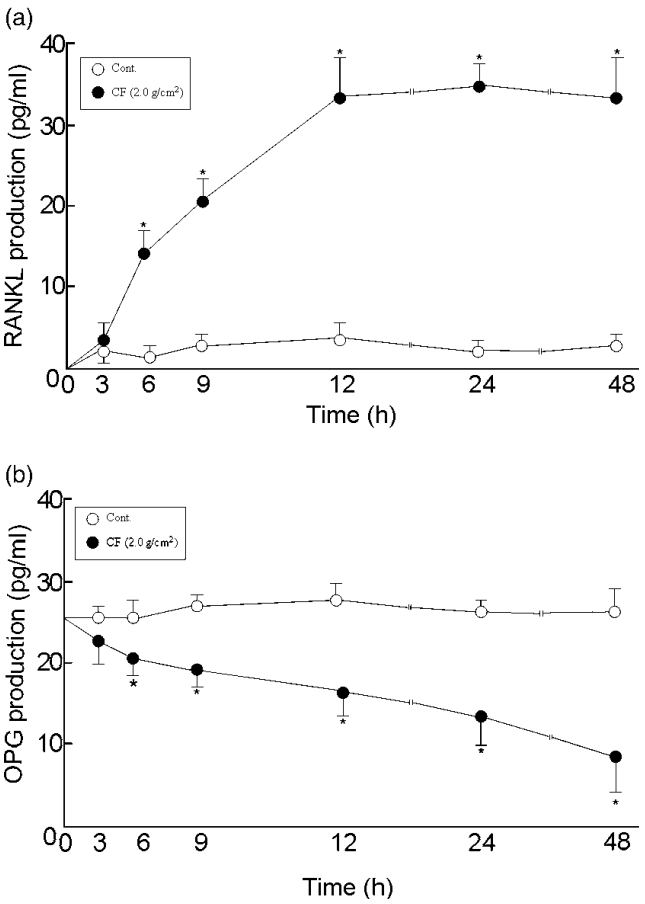

Fig. 4. Effects of incubation time on the secretion of RANKL (a) and OPG (b) in compressed hPDL cells. The secretions of RANKL and OPG were assayed as described in section "Materials and methods". Data are expressed as the mean ± SD of six cultures. RANKL was increased and OPG was decreased by compressive force (2.0 g/cm^2), and the pattern was time-dependent ($p < 0.001$, by two-way ANOVA). Significant difference from the corresponding control at each incubation time: *$p < 0.001$.

As GCF volume has been shown to be correlated with inflammatory state the mean volumes of GCF taken from both paper strips were compared (27). There was no significant difference in the mean volumes of GCF at 24 h between the experimental teeth (mean: 0.44 ± 0.05 μl) and the control teeth (mean: 039 ± 0.05 μl). In addition, the mean volume of GCF collected from the experimental teeth were similar to the mean volume of GCF collected from the contralateral control teeth (mean: 0.41 ± 0.04 μl) in the same subject.

GCF levels of RANKL and OPG

The mean RANKL values for the experimental teeth were significantly higher than the mean RANKL values at the control sites after 24 h (RANKL: experimental site, 219.3 ± 26.9 pg/μl; control sites, 7.7 ± 5.8 pg/μl; OPG: experimental site, 176.9 ± 42.3 pg/μl; control sites, 280.8 ± 30.8 pg/μl). While the mean OPG values for the experimental teeth were significantly lower than the mean OPG values at the control sites after 24 h, there were no significant differences between the experimental and control sites at 0, 1, and 168 h (Fig. 3).

In contrast to the changes in RANKL and OPG levels, there were no significant differences in total protein levels over the experimental period (data not shown). There was no significant difference in total protein at the experimental site after 24 h and at baseline (base-

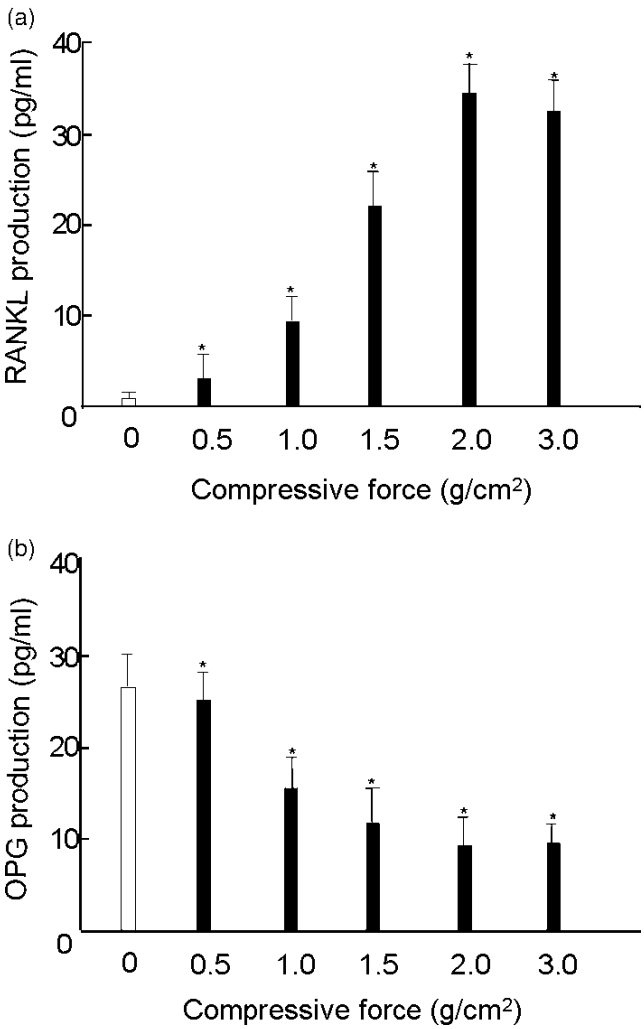

Fig. 5. Effects of compressive force on the secretion of RANKL (a) and OPG (b) in hPDL cells. PDL cells were incubated with or without the compressive force (2.0 g/cm^2) for 12 h, and the secretions of RANKL and OPG were assayed as described in section "Materials and methods". Data are expressed as the mean ± SD of six cultures. RANKL was increased and OPG was decreased by compressive force in a magnitude-dependent manner ($p < 0.001$, by two-way ANOVA). Significantly different from the control at compressive force: $^*p < 0.001$.

line: 31.3 ± 6.8 μg/μl; 24 h: 28.6 ± 8.9 μg/μl), and also no significant difference in total protein at the experimental and the control sites after 24 h (experimental: 31.3 ± 6.8 μg/μl; control: 27.1 ± 9.0 μg/μl).

In vitro study

Evaluation of compression force

The effect of time and compression force in hPDL cells was examined. When hPDL cells were treated with a continuous compression force (2.0 g/cm^2) for 3, 6, 9, 12, 24, and 48 h, the secretions of RANKL was significantly increased in the stress (+) group in a time-dependent manner, in contrast to the stress (–) group. In contrast, the secretion of OPG was significantly decreased in a time-dependent manner. Further, the secretions of both after 6 h of compression were significantly changed in the stress (+) group than in the stress (–) group ($p < 0.001$ by Tukey–Fisher's test) (Fig. 4).

As shown in Fig. 5, when compression force ranging from 0.5 to 2.0 g/cm^2 was applied to hPDL cells for 12 h, the secretions of RANKL showed significant increases at all compression forces as compared with the control ($p < 0.001$), in a force-dependent manner up to 2.0 g/cm^2 ($p < 0.001$, two-way ANOVA), while the secretions of OPG showed significant decreases at all compression forces as compared with the control ($p < 0.001$), in a force-dependent manner up to 2.0 g/cm^2 ($p < 0.001$, two-way ANOVA). A comparison between each data set revealed that the secretions were significantly changed in hPDL cells subjected to compression force at all four magnitudes as compared with that in the control cells ($p < 0.001$ by Tukey–Fisher's test) (Fig. 5).

Discussion

We found increased level of RANKL and decreased level of OPG, but not total protein, in GCF samples collected from areas adjacent to teeth undergoing orthodontic tooth movement. Twenty-four hours after initiating an orthodontic force the mean total levels of RANKL and OPG were to approximately 28.5-fold and 0.63-fold, respectively, of the total levels of RANKL and OPG in GCF collected from the control teeth. By 168 h, however, the levels of RANKL and OPG had returned to approximately baseline levels. We attribute this return in RANKL and OPG to our experimental design, which did not provide for a continuous and consistent force over the entire experimental period of 168 h (Fig. 3).

Previous studies have demonstrated that the levels of PGE, IL-1β, IL-6, and TNF-α increased during orthodontic tooth movement as compared to those at control sites after 24 h (8). PGE and these cytokines have all been demonstrated to enhance RANKL mRNA and protein levels, whereas they were shown to inhibit OPG mRNA and protein levels in osteoblasts (28,29). Human PDL cells activated with inflammatory factors such as IL-1β and PGE may directly stimulate osteoclastogenesis through RANKL, which is stimulated to express by these factors (30). These results lend some support to findings of the present study. In contrast, GCF matrix

metalloproteinase (MMP)-8 levels, collected from teeth undergoing orthodontic tooth movement, were significantly higher 4–8 h after application of a force than prior to the initiation of a force (31). This suggests that further studies are needed to determine if the maximum level of RANKL and the minimum level of OPG before 24 h.

Grimaud et al. (32) demonstrated that RANKL/OPG ratio was significantly increased in patients suffering from severe osteolysis compared to the control group and that this imbalance is involved in bone resorption mechanisms. Crotti et al. (33) demonstrated that significantly higher levels of RANKL protein were expressed in periodontal disease. However, OPG protein was significantly lower in periodontal tissues than that in the healthy group (34). In their study of GCF, Vernal et al. (35) demonstrated that the total amount of RANKL in GCF is significantly increased in periodontal disease. It has recently been shown *in vivo* that the ratio of the concentration of RANKL to that of OPG in the GCF was significantly higher in periodontal disease patients than in healthy subjects (36). These results indicate that imbalances in the RANKL/OPG system may play roles in the development of periodontal disease. Taken together, these findings and our present results suggest that RANKL and OPG may be involved in bone remodeling during orthodontic tooth movement.

In order to investigate the mechanism of alteration of RANKL and OPG in GCF on the compression side during orthodontic tooth movement, the secretions of both RANKL and OPG in hPDL cells stimulated by compression forces were examined. We found that compression significantly increased the secretion of RANKL and decreased that of OPG in a time- and magnitude-dependent manner (Figs 4 and 5). Further, compression-stimulated secretion of RANKL was increased approximately 16.7-fold and that of OPG was decreased 2.9-fold, as compared with the control. These results of *in vitro* study appear to reflect the findings of GCF study. Kanzaki et al. (25) reported that RANKL mRNA expression is induced in compressed hPDL cells in a force-dependent manner up to 2 g/cm^2, and in a time-dependent manner for up to 48 h. Furthermore, RANKL upregulation in compressed PDL cells was dependent on PGE_2. These results were similar to the findings of the present study. However, the report also demonstrated that OPG mRNA expression did not change regardless of the amount of compression force or the duration of compression (25). Moreover, Tsuji et al. (37) demonstrated that the protein concentration and the mRNA of OPG were upregulated by intermittent tensile stress using a Flexercell Strain Unit. These confusing results related to OPG may have been due to differences between protein and mRNA or to the different types of mechanical stress. Further studies are necessary to confirm this.

In summary, the levels of RANKL increased and those of OPG decreased in the GCF of the compression side during orthodontic tooth movement. The *in vitro* study showed that the levels of RANKL and OPG changed in hPDL cells in response to a compression force, in a time- and magnitude-dependent manner. Therefore, compression force may be involved in bone resorption through the stimulation of the RANKL/OPG system in the PDL.

Conclusions

1 The levels of RANKL in GCF increased during orthodontic tooth movement. In contrast, the level of OPG decreased.
2 Compression force causes an increase in the secretion of RANKL and a decrease in that of OPG in hPDL cells *in vitro*.
3 The changes in RANKL and OPG may be involved in bone resorption as a response to compression force.

Acknowledgement: This research was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (C:14571969, 14571970).

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