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Effects of compression force on fibroblast growth factor-2 and receptor activator of nuclear factor kappa B ligand production by periodontal ligament cells *in vitro*

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Background and Objective: Mechanical stress by an orthodontic appliance induces biologically active substances. Fibroblast growth factor is a multifunctional cytokine that has various effects on fibroblast cells, and fibroblast growth factor-2 plays an important role in remodeling of the periodontal ligament. The receptor activator of nuclear factor kappa B ligand (RANKL) is an important protein involved in osteoclastogenesis and we recently reported that RANKL levels were increased by compression force *in vitro*. In the present study, we investigated the effects of compression force on fibroblast growth factor-2 and RANKL production by human periodontal ligament cells.

Material and Methods: Compression force (0.5–4.0 g/cm^2) was applied to human periodontal ligament cells for 0–24 h. The amounts of soluble RANKL (sRANKL) and fibroblast growth factor-2 were measured using an enzyme-linked immunosorbent assay, whereas mRNA levels were determined by the reverse transcription-polymerase chain reaction. Furthermore, anti-fibroblast growth factor-2 was added to the cell culture media and we measured the release of sRANKL and fibroblast growth factor-2 by enzyme-linked immunosorbent assay.

Results: Compression force induced higher levels of sRANKL and fibroblast growth factor-2 in both a time- and magnitude-dependent manner. Treatment with anti-fibroblast growth factor-2 inhibited the release of sRANKL.

Conclusion: Fibroblast growth factor-2 may be partly involved in osteoclastogenesis during orthodontic tooth movement.

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Periodontal tissue has a unique structure, as the periodontal ligament, 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 to disperse occlusal and orthodontic forces. Furthermore, periodontal ligament 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 periodontal ligament to form biologically active substances, such as cytokines and enzymes, which are responsible for connective tissue remodeling (3,4). Previously, our laboratory reported that human periodontal ligament cells produced prostaglandin E_2, interleukin-1, interleukin-6 and cathepsins B and L under mechanical stress *in vitro* (5–8).

Basic fibroblast growth factor (fibroblast growth factor-2) is a component of the bone matrix and plays a role in regulating bone remodeling (9–11), and both osteoblasts and osteoclasts express the receptor for fibroblast growth factor-2 (12). Fibroblast growth factor-2 is produced by cells of the osteoblastic lineage that accumulate in the bone matrix and acts as an autocrine/paracrine factor for various types of bone cells (13–16), whereas it has been shown to have variable regulation of the proliferation and differentiation of osteoblastic cells, thereby modulating bone formation (17–19). In addition, fibroblast growth factor-2 has been reported to stimulate bone resorption in bone organ cultures and osteoclastogenesis in a mouse bone marrow culture (20–22).

The receptor activator of nuclear factor kappa B ligand (RANKL) was recently identified as a member of the membrane-associated tumor necrosis factor ligand family and an important regulatory molecule of osteoclastogenesis (23). RANKL is a ligand of osteoprotegerin/osteoclastogenesis-inhibitory factor and is expressed in the plasma membranes of osteoblasts/stromal cells (24). Most importantly, RANKL induces osteoclast differentiation from hemopoietic precursors and stimulates the bone-resorptive activity of osteoclasts (25), and it was also revealed that tumor necrosis factor-α convertase converts membrane-bound RANKL to soluble RANKL (sRANKL) and its osteoclastogenesis activity is attenuated (26–28). Recently, we reported that RANKL levels were increased in human periodontal ligament cells by compression force *in vitro* (29). Furthermore, fibroblast growth factor-2 was shown to stimulate RANKL in a mouse macrophage-like cell line, C7 (30). However, little information is available concerning the production of fibroblast growth factor-2 and RANKL in human periodontal ligament cells in response to mechanical stress. In the present study, we investigated the effects of compression force on fibroblast growth factor-2 and RANKL production by human periodontal ligament cells.

Material and methods

Cell culture

Human periodontal ligament fibroblasts were prepared according to a modification of the method of Somerman *et al*. (31), as described previously (32). Briefly, periodontal ligament tissues were taken from the roots of premolars extracted from six healthy young volunteers (three males, three females; 14–16 years old), during the course of orthodontic treatment, after obtaining informed consent from the donors, and were used according to a protocol reviewed by the Ethics Committee of Nihon University School of Dentistry at Matsudo (#04–021). The periodontal ligament tissues were placed in 35-mm tissue culture dishes and covered with a sterilized glass coverslip. The medium used was α-minimal essential medium (Gibco, Grand Island, NY, USA), which was supplemented with 100 μg/mL of penicillin-G (Sigma Chemical Co., St Louis, MO, USA), 50 μg/mL of gentamicin sulphate (Sigma), 0.3 μg/mL of amphotericin B (Flow Laboratories, McLean, VA, USA), and 10% fetal calf serum (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 the cells growing from each explant had reached confluence, they were detached with 0.05% trypsin (Gibco) in phosphate-buffered saline 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 compression force

In order to reproduce the conditions of pressure during orthodontic tooth movement, we performed the following *in vitro* experiments, in accordance with the method reported by Kanai *et al*. (33). Human periodontal ligament cells were continuously compressed using a uniform compression method as a model of pressure at the site of orthodontic movement (Fig. 1).

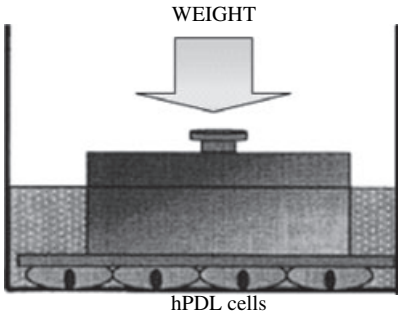

Fig. 1. Method used to apply compression force. Pre-cultured periodontal ligament cells were compressed continuously using a glass cylinder at different weights. The glass cylinder was placed over confluent cell layers in each well of a six-well plate. The number of lead granules placed in the cylinder determined the amount of compression force. hPDL, human periodontal ligament cells.

Static compression force is thought to mimic that found *in vivo* during orthodontic treatment. In the present experiments, the cells were stimulated once. Briefly, a cell disk, 30 mm in diameter, was placed over nearly confluent cell layers in the wells of a six-well plate, on top of which was placed a glass cylinder. Compression force was then controlled by placing lead granules in the cylinder. Before the application of compression force, the cells were pre-incubated for 1 h in culture medium containing 2% fetal calf serum, after which they were subjected to 0.5, 1.0, 2.0, 3.0 or 4.0 g/cm^2 of compression force for 24 h. Previous studies have shown that compressive mechanical stress can be applied by the system utilized in the present experiment (8,29).

Fibroblast growth factor-2, sRANKL and osteoprotegerin levels

Fibroblast growth factor-2 levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN, USA). To clarify the relationship among fibroblast growth factor-2, RANKL and osteoprotegerin production by human periodontal ligament cells under compression force, we examined the effect of incubation with anti-fibroblast growth factor-2. Compression force (4.0 g/cm^2) was applied to human periodontal ligament cells for 24 h in the presence or absence of anti-fibroblast growth factor-2 (R & D Systems). sRANKL production was measured by the sRANKL ELISA kit (BIOMEDICA, Wien, Austria). Osteoprotegerin production was measured by the Human Osteoprotegerin ELISA kit (RayBio, Norcross, GA, USA). Anti-fibroblast growth factor-2 cultures were pretreated with 100 μg/mL of anti-fibroblast growth factor-2 for 1 h before adding compression force.

Reverse transcription-polymerase chain reaction (RT-PCR)

We extracted RNA from human periodontal ligament cells using an RNeasy mini kit (Qiagen Co., Tokyo, Japan), following the manufacturer's protocol. RNA was amplified using an RT-PCR kit and we obtained 40 μL of purified total RNA. Total RNA was converted to cDNA using ReverTra Ace (Toyobo, Co., Osaka, Japan). PCR amplification was performed using KOD Dash (Toyobo, Co.) in a thermal cycler (PTC-0200 DNA Engine; MJ Research, Inc., Waltham, MA, USA). After a hot start, the samples were denatured at 98°C for 20 s and then the primer was annealed at 55–60°C for 2 s and extended at 74°C for 30 s for 25–30 cycles. PCR primers for fibroblast growth factor-2 and α-actin were purchased from Sigma Genosys Co. (Hokkaido, Japan), and designed with reference to the cDNA sequences reported for fibroblast growth factor-2 and α-actin. The primers were designed as follows: fibroblast growth factor-2, 5′-GGTG AAACCCCGTCTCTACA-3′ and 5′-T CTGTTGCCTAGGCTGGACT-3′; α-actin, 5′-GGACTTCGAGCAAGAG ATGG-3′ and 5′-AGCACTGTGTTG GCGTACAG-3′. The PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide staining under ultraviolet light illumination. The differences between the PCR products were quantified according to the luminescence values. The relative intensities were measured by using NIH IMAGE software (NIH, Bethesda, MD, USA).

Statistical methods

Values are shown as the mean ± standard deviation. Data were analysed by a Mann–Whitney *U*-test and by two-way analysis of variance.

Results

Evaluation of compression force

The effects of compression force and time on human periodontal ligament cells were examined. When human periodontal ligament cells were treated with continuous compression force, the secretion of fibroblast growth factor-2 was increased in both a time- and magnitude-dependent manner, in contrast to the control group, and the level at 4.0 g/cm^2 of force for 24 h was significantly higher ($p < 0.001$, Mann–Whitney *U*-test).

When compression force ranging from 0.5 to 4.0 g/cm^2 was applied to human periodontal ligament cells for 24 h, the levels of fibroblast growth factor-2 and sRANKL were significantly increased as compared with the control ($p < 0.001$), in a magnitude-dependent manner ($p < 0.001$, two-way analysis of variance). A comparison between each data set revealed that the levels were significantly changed in human periodontal ligament cells subjected to compression force at all five magnitudes, as compared with the control cells ($p < 0.001$, Mann–Whitney *U*-test) (Fig. 2). Osteoprotegerin release was

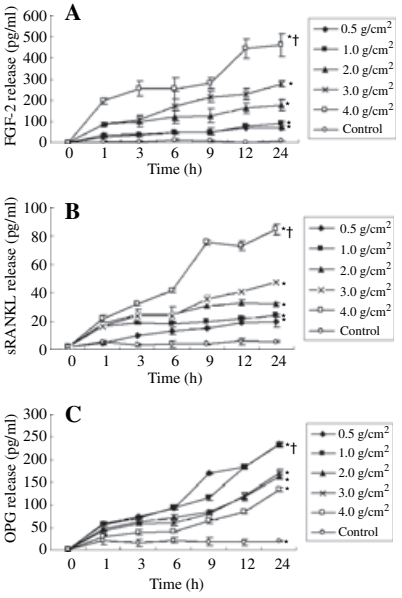

Fig. 2. Effects of duration of compression force on fibroblast growth factor-2 (A), soluble receptor activator of nuclear factor kappa B ligand (sRANKL) (B) and osteoprotegerin (C) production by human periodontal ligament cells. Compression force increased the production of fibroblast growth factor-2 and sRANKL, but decreased the production of osteoprotegerin from human periodontal ligament cells in both a time- and magnitude-dependent manner ($p < 0.001$; two-way analysis of variance). *$p < 0.001$, significantly different from the corresponding control at each incubation time. †$p < 0.001$, significantly different from other conditions. Control: 0.5–3.0 g/cm^2. FGF-2, fibroblast growth factor-2; OPG, osteoprotegerin.

also increased in a time-dependent manner, but the secretion was higher at low magnitude than at high magnitude ($p < 0.001$, two-way analysis of variance) (Fig. 2).

As shown in Fig. 3, the levels of PCR products corresponding to α-actin were the same in the six experimental groups, and thus it was considered that the amount of PCR products reflected the level of mRNA. Those of the compression force-stimulated human periodontal ligament cells at each magnitude of force (0.5–4.0 g/cm^2) were more intense than those for the corresponding controls (Fig. 3).

As shown in Fig. 4, when human periodontal ligament cells were subjected to 4.0 g/cm^2 of compression force in the presence of 100 μg/mL of anti-fibroblast growth factor-2, fibroblast growth factor-2 production was suppressed almost completely, whereas sRANKL production was inhibited by ≈49% and osteoprotegerin secretion was inhibited by ≈66%.

Discussion

In order to investigate the mechanism of alteration of fibroblast growth factor-2 in human periodontal ligament cells on the compression side during orthodontic tooth movement, the levels of fibroblast growth factor-2 secreted from human periodontal ligament cells stimulated by various amounts of compression force were measured. We found that compression force significantly increased the secretion of fibroblast growth factor-2 in a time- and magnitude-dependent manner (Fig. 2). Furthermore, fibroblast growth factor-2 mRNA of the compression force-stimulated human periodontal ligament cells at each magnitude (0.5–4.0 g/cm^2) was more intense compared with the corresponding control (Fig. 3).

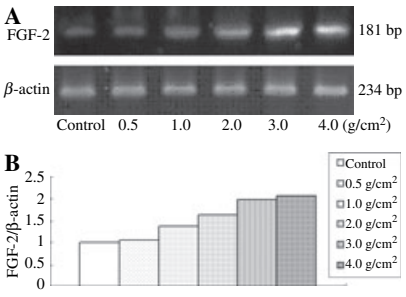

Fig. 3. (A) Human periodontal ligament cells were subjected to different amounts of compression force (0.5–4.0 g/cm^2) for 24 h. Fibroblast growth factor-2 gene expression was up-regulated in a magnitude-dependent manner. (B) Fibroblast growth factor-2 mRNA expression relative to β-actin expression was analyzed using NIH IMAGE software. The band intensities of fibroblast growth factor-2 expression at 0.5 g/cm^2 were assigned a value of 1.

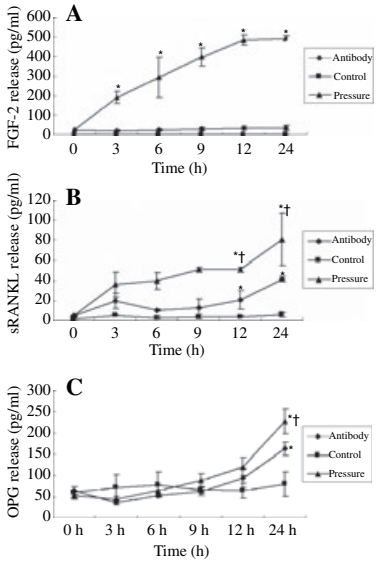

Fig. 4. Inhibitory effects of bFGF antibody on fibroblast growth factor-2 (A), soluble receptor activator of nuclear factor kappa B ligand (sRANKL) (B) and osteoprotegerin (C) production by human periodontal ligament cells under compression force (4.0 g/cm^2, 24 h). The secretion of sRANKL was inhibited by ≈49%, and the secretion of osteoprotegerin was inhibited by 66%. *$p < 0.001$, significantly different from the corresponding control at each incubation time. †$p < 0.001$, significantly different from the antibody group.

Fibroblast growth factors are involved in diverse cellular processes, including chemotaxis, cell migration, differentiation, cell survival and apoptosis (20,34). Fibroblast growth factor-2 is incorporated into the protein matrix of bone (35–37), and was shown to stimulate MC3T3-E1 cell proliferation and differentiation *in vitro* (38). Furthermore, application of fibroblast growth factor-2 increased the rate of bone formation in humans and animals that had bone fractures (39–44). Fibroblast growth factor-2 also has a catabolic effect on bone, as Kawaguchi *et al.* (45) reported that at low concentrations ($\leq 10^{-11}$ M) the cytokine acted directly on mature osteoclasts to resorb bone moderately, whereas at high concentrations ($\geq 10^{-9}$ M) fibroblast growth factor-2 acted on osteoclastic cells to induce cyclooxygenase-2 and potently stimulate bone resorption. In the present study, the concentration of fibroblast growth factor-2 in response to compression force was found to be $\approx 10^{-14}$ M (500 pg/mL) and thus it may contribute to bone resorption. It is generally agreed that bone resorption occurs on the compression side during orthodontic tooth movement (2) and therefore we considered that this phenomenon may be related to the activity of fibroblast growth factor-2.

As for the relationship between fibroblast growth factor-2 and mechanical stress, cyclic mechanical stretch is known to induce fibroblast growth factor-2 expression in human tendon fibroblasts and pulmonary vascular smooth muscle cells. The increased concentrations of fibroblast growth factor-2 after cyclical mechanical stretching may have a positive influence on tendon tissues, by promoting ligament and pulmonary artery healing through stimulation of cell proliferation and differentiation, and matrix formation (46,47). Down-regulation of fibroblast growth factor-2 production from cartilage under compression force has been reported (48). However, in the present study, compression force increased the secretion of fibroblast growth factor-2 from human periodontal ligament cells (Figs 2 and 3). These contrasting findings are not surprising, as compression force produces bone resorption factors, such as prostaglandin E_2 and interleukin-1, from human periodontal ligament cells (4). As a result, alveolar bone is resorbed on the compression side during orthodontic tooth movement (2). Taken together, these findings and our present results suggest that compression force accelerates the secretion of fibroblast

growth factor-2 from human periodontal ligament cells, which may stimulate bone resorption.

Recently, Nakano *et al*. (49) reported that fibroblast growth factor-2 induced the expression of RANKL by rheumatoid arthritis synovial fibroblasts, and the up-regulation of RANKL induced by fibroblast growth factor-2 was inhibited by anti-fibroblast growth factor-2. In addition, fibroblast growth factor-2 has been implicated as a potential inducer of RANKL (30). In the present study, we examined, using anti-fibroblast growth factor-2, whether fibroblast growth factor-2 stimulated by compression force has any effect on the production of RANKL. We found that sRANKL production was inhibited by ≈49% following the addition of anti-fibroblast growth factor-2 to the cultures (Fig. 4). Therefore, fibroblast growth factor-2 stimulates RANKL production by human periodontal ligament cells, at least in part, in response to compression force. Nukaga *et al*. (50) also reported that the expression of RANKL mRNA in human periodontal ligament cells was enhanced by interleukin-1. Therefore, there may be another pathway of compression force-stimulated RANKL in human periodontal ligament cells.

Osteoprotegerin is a member of the tumor necrosis factor receptor family and is known to inhibit osteoclastogenesis and osteoclast function. Yano *et al*. (51) found that fibroblast growth factor-2 inhibited the production of osteoprotegerin by synovial cells from rheumatoid arthritis patients in a dose-dependent manner. In the present study we examined osteoprotegerin production from human periodontal ligament cells under compression force. Compression force increased the secretion of osteoprotegerin in a time-dependent manner, but the secretion was higher at low magnitude than at high magnitude. Fibroblast growth factor-2 inhibition by anti-fibroblast growth factor-2 decreased osteoprotegerin secretion, and it was inhibited by ≈66% (Fig. 4). Therefore, compression force-stimulated fibroblast growth factor-2 modulates the secretion of both RANKL and osteoprotegerin.

In summary, compression force induced higher levels of sRANKL and fibroblast growth factor-2, whereas the inhibition of fibroblast growth factor-2 caused a reduction in the production of sRANKL by human periodontal ligament cells. Our results suggest that compression force-stimulated fibroblast growth factor-2 may be involved in bone resorption in the periodontal ligament during orthodontic tooth movement.

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