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Mechanical force augments the anti-osteoclastogenic potential of human gingival fibroblasts *in vitro*

S-H. Kook[1,2], Y-O. Son[3], Y. Choe[1], J-H. Kim[3], Y-M. Jeon[1], J-S. Heo[3], J-G. Kim[1], J-C. Lee[1,3]

[1]Laboratory of Cell Biology, Department of Orthodontics, School of Dentistry and Institute of Oral Biosciences, Chonbuk National University, Chonju, Korea, [2]Division of Biological Science, Chonbuk National University, Chonju, Korea and [3]Department of Bioactive Materials and Research Center of Bioactive Materials, Chonbuk National University, Chonju, Korea

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Background and Objective: The cellular response of human gingival fibroblasts to a mechanical force is considered to be primarily anti-osteoclastic because they produce relatively high levels of osteoprotegerin. However, there is little information available on the effects of compression force on the production of osteoprotegerin and osteoclastic differentiation by these cells. In this study, we examined how mechanical force affects the nature of human gingival fibroblasts to produce osteoprotegerin and inhibit osteoclastogenesis.

Material and Methods: Human gingival fibroblasts were exposed to mechanical force by centrifugation for 90 min at a magnitude of approximately 50 g/cm^2. The levels of osteoprotegerin, receptor activator of nuclear factor-κB ligand (RANKL), interleukin-1β and tumor necrosis factor-α were measured at various time-points after applying the force. The effect of the centrifugal force on the formation of osteoclast-like cells was also determined using a co-culture system of human gingival fibroblasts and bone marrow cells.

Results: Centrifugal force stimulated the expression of osteoprotegerin, RANKL, interleukin-1β and tumor necrosis factor-α by the cells, and produced a relatively high osteoprotegerin to RANKL ratio at the protein level. Both interleukin-1β and tumor necrosis factor-α accelerated the force-induced production of osteoprotegerin, which was inhibited significantly by the addition of anti-(interleukin-1β) immunoglobulin Ig isotype; IgG (rabbit polyclonal). However, the addition of anti-(tumor necrosis factor-α) immunoglobulin Ig isotype; IgG1 (mouse monoclonal) had no effect. Centrifugal force also had an inhibitory effect on osteoclast formation.

Conclusion: Application of centrifugal force to human gingival fibroblasts accelerates osteoprotegerin production by these cells, which stimulates the potential of human gingival fibroblasts to suppress osteoclastogenesis. Overall, human gingival fibroblasts might have natural defensive mechanisms to inhibit bone resorption induced by a mechanical stress.

Jeong-Chae Lee, Department of Bioactive Materials and Research Center of Bioactive Materials, Institute of Oral Biosciences, Chonbuk National University, Chonju 561-756, Korea
Tel: +82 63 2704049
Fax: +82 63 2704049
e-mail: leejc88@chonbuk.ac.kr

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The aim of orthodontic treatment is to relocate abnormally positioned teeth. This is achieved by applying a prolonged mechanical force to the tooth, which causes biophysical changes to the fibroblasts presented in the periodontal tissue. Human periodontal tissue consists of three types of fibroblastic cells, namely gingival fibroblasts, periodontal ligament fibroblasts and osteoblasts (1).

Periodontal ligament fibroblasts play the most important roles in the processes of bone remodeling and tooth movement by an orthodontic force through the production of various active cytokines and enzymes, including receptor activator of nuclear factor-κB ligand (RANKL) (2), osteoprotegerin

(3), interleukin-1β and tumor necrosis factor-α (4). In particular, RANKL, a ligand of osteoprotegerin, induces osteoclastic differentiation from hemopoietic precursors and stimulates their bone-resorptive activity (5). By contrast, osteoprotegerin, a decoy receptor for RANKL, inhibits osteoclastogenesis and accelerates osteoclast apoptosis (6). Therefore, the balanced expression of RANKL and osteoprotegerin in the periodontal ligament may play important roles in bone remodeling during orthodontic tooth movement.

It has been suggested that in addition to periodontal ligament fibroblasts, human gingival fibroblasts play regulatory roles in an orthodontic force-induced tooth movement. However, the response of human gingival fibroblasts to a mechanical force is different from that of periodontal ligament fibroblasts. Periodontal ligament fibroblasts can induce the formation of osteoclast-like cells in a RANKL-dependent manner in co-culture of periodontal ligament fibroblasts and human peripheral blood mononuclear cells (2) or mouse bone marrow cells (7), even though they also can inhibit this process by producing osteoprotegerin (8). Unlike periodontal ligament fibroblasts, the response of human gingival fibroblasts to mechanical force is assumed to be primarily anti-osteoclastic. Indeed, the interleukin-1α-stimulated osteoprotegerin mRNA expression in human gingival fibroblasts was greater than that in periodontal ligament (9). It was also reported that lipopolysaccharide-stimulated human gingival fibroblasts inhibit monocyte differentiation into osteoclasts through the production of osteoprotegerin (10). This means that human gingival fibroblasts can produce relatively higher levels of osteoprotegerin than RANKL. Therefore, we postulated that when human gingival fibroblasts are exposed to a mechanical force, they suppress the formation of osteoclastic-like cells, which might be mediated by the production of a high level of osteoprotegerin. However, there is little information available concerning the production of these molecules within human gingival fibroblasts in response to a mechanical force.

In the present study, we examined the effects of a mechanical force on the production of RANKL and osteoprotegerin from human gingival fibroblasts by applying a centrifugal force to these cells using a horizontal microplate rotor. We also investigated the effects of tumor necrosis factor-α and interleukin-1β on the force-mediated production of RANKL and osteoprotegerin. In addition, the effects of centrifugal force on the formation of osteoclast-like cells were studied using a co-culture system of human gingival fibroblasts and mouse bone marrow cells.

Material and methods

Chemicals and laboratory ware

Unless specified otherwise, all chemicals and laboratory ware were obtained from Sigma Chemical Co. (St Louis, MO, USA) and SPL Life Sciences (Pochun, South Korea), respectively.

Cell culture

Human gingival fibroblasts were obtained from three healthy male individuals (20–30 years of age) who had their third molar extracted, and those gingival fibroblasts were cultured according to methods described previously (11). Written, informed consent for use of the gingival tissues was obtained from all donors. This study was approved by the Review Board of Chonbuk National University Hospital. Briefly, the gingival tissues were finely dissected, and single-cell suspensions were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and antibiotics (100 IU/mL of penicillin G and 100 μg/mL of streptomycin) in 100-mm culture dishes. All the cultures were maintained at 37°C with a humidified gas mixture of 5% CO_2/95% air and switched to a fresh batch of the same medium every 3 d. When the cells had reached confluence, they were subcultured for several passages and spread onto various types of flat-bottomed culture plates before application of the the centrifugal force. All experiments were carried out at the fifth to seventh passages.

Application of centrifugal force

A mechanical force was applied to the human gingival fibroblasts by centrifuging the culture plates at approximately 50 g/cm^2 for various periods of time (0–90 min) using a horizontal microplate rotor (Universal 32 R; Tuttlingen, Germany), as described previously (12,13). The applied force calculation was based on the following equation (13): $P = (m \times r \times r.p.m.^2 \times \pi^2)/(A \times 9.8 \times 900)$, where P = kg pressure per cm^2 of cells, m = mass of medium (g), r = radius (0.12 m), $r.p.m.$ = revolutions per minute and A = area of contact between the medium and the cells (cm^2).

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted from the control or force-applied human gingival fibroblasts according to the manufacturer's instructions (SV Total RNA Isolation System; Promega, Madison, WI, USA). Reverse transcription and PCR amplification were performed using an Access RT-PCR System (Promega) according to the manufacturer's protocol. The primer sequences used to amplify bone-active substances were as follows: osteoprotegerin (253 bp), 5′-GAG TGT CTA TAC TGC AGC CC-3′ (forward) and 5′-TGT TTT CTA CAG GGT GCT TT-3′ (reverse); RANKL (233 bp), 5′-TGG TTC CCA TAA AGT GAG TC-3′ (forward) and 5′-AGG GTA TGA GAA CTT GGG AT-3′ (reverse); tumor necrosis factor-α (128 bp), 5′-CTG GTA TGA GCC CAT CTA TC-3′ (forward) and 5′-GCA ATG ATC CCA AAG TAG AC-3′ (reverse); and interleukin-1β (247 bp), 5′-TTC TTT CCC TTC ATC TTT GA-3′ (forward) and 5′-GCC ACA GGT ATT TTG TCA TT-3′ (reverse). The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (190 bp) with the sequences 5′-AAC CTG CCA AAT ATG ATG AC-3′ (forward) and 5′-ATA CCA GGA AAT GAG CTT

GA-3′ (reverse), was used as the control. The PCR was performed for 30–35 cycles at 94°C for 30 s, at 50–55°C for 30 s and at 72°C for 1 min in a DNA Thermal cycler (MJR PTC-1000; GMI Inc., Ramsey, MI, USA). The PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. Band intensity was calculated using a gel imaging system (model; F1-F2 Fuses type T2A, Bio-Rad, Segrate, Italy). The PCR amplifications were repeated in at least three independent RNA preparations.

Measurement of osteoprotegerin and RANKL protein levels

When the human gingival fibroblasts had reached approximately 95% confluence in a 96-multiwell plate, the culture medium was replaced with fresh medium and the cells were exposed to the centrifugal force for 90 min as described above. At various time-points after force application, the culture medium was collected and analyzed for the osteoprotegerin and RANKL levels using enzyme-linked immunosorbent assay (ELISA). ELISA for the detection of human osteoprotegerin (BI-20402; Biomedica, Vienna, Austria) and human RANKL (BI-20422H; Biomedica) was performed according to the manufacturer's instructions.

Immunostaining

Human gingival fibroblasts grown in a 96-multiwell culture plate were exposed to the centrifugal force. At various time-points, the cells were fixed with 4% paraformaldehyde for 30 min. The fixed cells were washed with phosphate-buffered saline and treated with osteoprotegerin monoclonal antibody (SC-8468; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:100 dilution for 1 h. After washing with phosphate-buffered saline, the cells were exposed to fluorescein isothiocyanate-conjugated secondary antibodies (Santa Cruz Biotechnology). Finally, the cells were incubated in a 0.1 μg/mL solution of 4′-6′-diamidino-2-phenylidol (DAPI) for 1 min and observed using fluorescence microscopy (Axioskop 2; Carl Zeiss, *Göttingen,* Germany).

Preparation of the conditioned medium

A single-cell suspension of human gingival fibroblasts was spread over each well of six-well culture plates at a density of 2×10^5 cells/well. When the cells had reached 95% confluence the existing medium was discarded and replaced with fresh medium and exposed to the centrifugal force of approximately 50 g/cm^2 for 90 min. Forty-eight hours afterwards, the media of the forced or control human gingival fibroblast cultures were collected and centrifuged at 1000 *g* for 10 min to remove the cells and debris. The supernatants were used as the conditioned medium in the osteoclastogenesis assay after determining that there were similar total protein levels in the conditioned media from the human gingival fibroblasts treated with centrifugal force and control human gingival fibroblasts.

Co-culture conditions and tartrate-resistant acid phosphate staining

A single-cell suspension (1×10^5 cells/mL) of human gingival fibroblasts was placed in each well of a 96-multiwell plate at a density of 2×10^4 cells/well. When the cells were confluent (condition that the cells were proliferated and completely occupied the bottom of culture dishes), the mouse bone marrow cells were plated on top of the cultured human gingival fibroblasts at a density of 10^5 cells per well and cultured in RPMI-1640 medium containing 100 nM dexamethasone and 10 nM vitamin D_3, with and without 1 μg/mL of the anti-osteoprotegerin immunoglobulin Ig isotype; goat polyclonal IgG. After co-incubation of the cells for 2 d, they were subjected to centrifugal force as described above. After an additional 5 d of co-incubation, the cultures subjected to centrifugal force and the control cultures were fixed in 4% phosphate-buffered saline-buffered para-formaldehyde and stained with tartrate-resistant acid phosphatase (TRAP). In this study, the bone marrow cells were obtained from 6-wk-old BALB/c mice, according to the methods described previously (14). This experiment was approved by the Chonbuk National University Committee on Ethics in the Care and Use of Laboratory Animals.

Effect of conditioned medium on osteoclastogenesis

Bone marrow cells were collected from the femurs and tibiae of BALB/c mice and resuspended in RPMI-1640 supplemented with 10% fetal bovine serum, antibiotics and 30 ng/mL of murine recombinant macrophage colony-stimulating factor immediately before they were spread onto 100-mm culture dishes. After 3 d of incubation, the cells were collected, resuspended in the culture medium containing 30 ng/mL of macrophage colony-stimulating factor, 20 ng/mL of tumor necrosis factor-α and 100 ng/mL of murine recombinant RANKL, and divided into 96-multiwell plates at a density of 1×10^5 cells per well. At the same time, various percentages (from 10 to 80%, vol/vol) of the conditioned media from the human gingival fibroblasts exposed to centrifugal force or from control human gingival fibroblasts were added into the cultures to determine the effect of conditioned medium on osteoclastogenesis. After 5 d of culture, the cells were processed for TRAP staining, and TRAP-positive multinucleated cells with more than five nuclei were counted as osteoclast-like cells.

Statistical analysis

Unless specified otherwise, all data were expressed as the mean ± standard error. One-way analysis of variance was used for multiple comparisons with SPSS, version 15.0, software. A p-value of < 0.05 was considered significant.

Results

Effect of centrifugal force on osteoprotegerin mRNA expression

The level of osteoprotegerin mRNA expression was determined by RT-PCR at various time-points (0–8 h) after applying a centrifugal force to the cells. As shown in Fig. 1A, osteoprotegerin mRNA was detectable in the

unloaded control cells (unforced cells or negative control cells). When the cells were exposed to centrifugal force, the level of osteoprotegerin mRNA increased dramatically from 30 min and was maintained for up to 8 h after exposure to the centrifugal force. By contrast, the mRNA level of osteoprotegerin had decreased to control levels 16 h after application of the force (data not shown). In addition, there was no a morphological change between the human gingival fibroblasts exposed to centrifugal force and control human gingival fibroblasts (data not shown).

In order to understand in more detail the effect of centrifugal force on osteoprotegerin expression, the cells were subjected to centrifugal force at approximately 50 g/cm^2 for different periods of time and analyzed by RT-PCR 4 h after exposure to centrifugal force. The level of osteoprotegerin mRNA in human gingival fibroblasts was increased by exposing the cells to centrifugal force in a force-dependent manner, such that RNA levels were elevated to 2.5-fold and 4.9-fold in the cells exposed to the centrifugal force for 30 and 90 min, respectively (Fig. 1B).

The stimulatory effect of centrifugal force on osteoprotegerin expression was also examined by staining the cells with anti-osteoprotegerin immunoglobulin Ig isotype; goat polyclonal IgG (Fig. 1C). As shown in the figure, the cellular level of osteoprotegerin protein was increased, in a time-dependent manner, following the application of centrifugal force to the cells.

Effect of centrifugal force on tumor necrosis factor-α and interleukin-1β mRNA expression

Various types of cells produce pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, which are believed to play important roles in bone resorption, bone remodeling and new bone deposition during orthodontic-mediated tooth movement (15–17). Subsequently, the mRNA levels of these cytokines in the human gingival fibroblasts was examined by RT-PCR at set times after exposure to the centrifugal force. As shown in Fig. 2A, the mRNA expression of tumor necrosis factor-α was detectable in the control cells, but no expression of interleukin-1β was detected. The mRNA levels of both tumor necrosis factor-α and interleukin-1β were increased significantly when the cells were subjected to centrifugal force: the mRNA levels of both cytokines had increased within 1 h and remained up-regulated for 8 h after exposure to centrifugal force. When the intensity of the bands was quantified by densitometry, the mRNA levels of tumor necrosis factor-α and interleukin-1β 2 h after exposure to centrifugal force

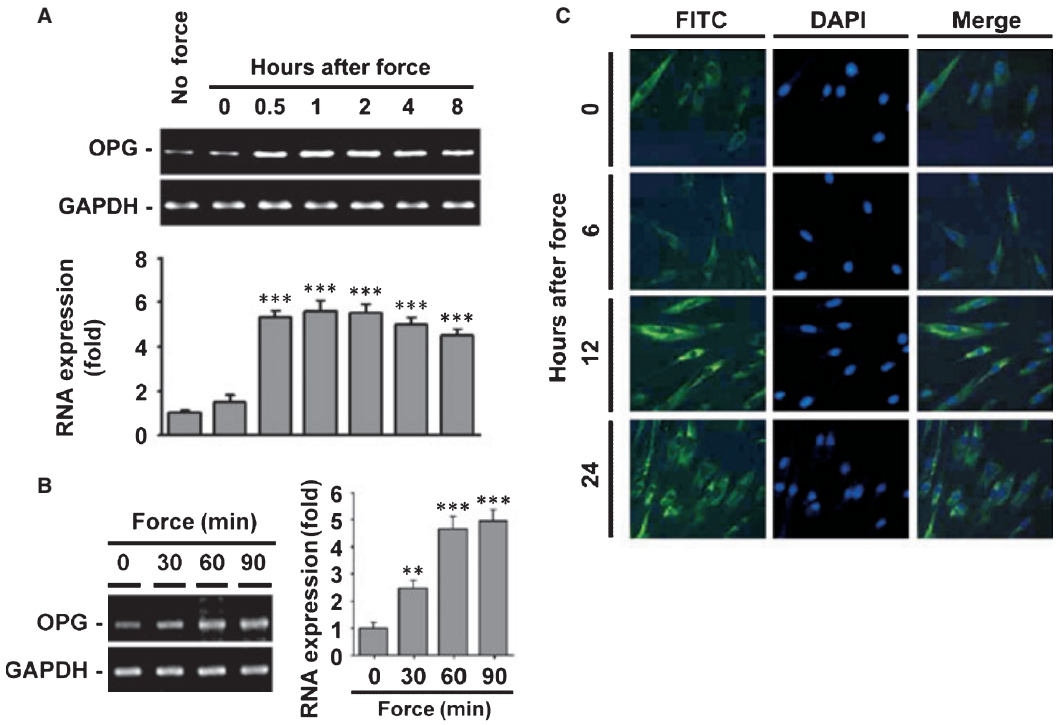

Fig. 1. Effect of centrifugal force on the expression of osteoprotegerin in human gingival fibroblasts. The level of osteoprotegerin mRNA in the human gingival fibroblasts exposed to centrifugal force was measured by reverse transcription–polymerase chain reaction at various time-points (0–8 h) after applying the force for 90 min (A), or at 4 h after applying the force for various periods of time (0–90 min) (B). These results show representative data, and the data from three independent experiments were quantified by densitometry after normalizing the bands to glyceraldehyde-3-phosphate dehydrogenase. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the unloaded control cultures. (C) Human gingival fibroblasts were also stained with anti-osteoprotegerin immunoglobulin at the indicated time-points after applying centrifugal force and then counterstained with a 4′-6′-diamidino-2-phenylidol solution. FITC, fluorescin isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPG, osteoprotegerin; DAPI 4′-6′-diamidino-2-phenylidol.

were 2.5 and 4.8 times higher than that of the unloaded control cells, respectively (Fig. 2B).

Effect of centrifugal force on osteoprotegerin and RANKL secretion

In order to understand the effect of centrifugal force on the production of osteoprotegerin and RANKL by human gingival fibroblasts, the levels of these proteins in the culture supernatants were determined by ELISA. The level of osteoprotegerin in the conditioned medium from the control human gingival fibroblast cultures was 39 ± 4.5 ng/10^6 cells, and this level remained relatively unchanged during the entire experimental period (Fig. 3, upper panel). Centrifugal force increased the level of osteoprotegerin production by human gingival fibroblasts significantly in a time-dependent manner: the osteoprotegerin level increased to 86 ± 5.6 ng/10^6 cells and 132 ± 12.8 ng/10^6 cells 9 and 48 h after applying the force, respectively.

Similarly, the level of RANKL secreted was increased by applying a centrifugal force to the cells (Fig. 3, lower panel). The level of RANKL in the conditioned medium from the control-human gingival fibroblast cultures was approximately 11 ng/10^6 cells. The level of RANKL increased to 46 ± 5.7 ng/10^6 cells at 9 h and remained at this level for up to 48 h after applying the force.

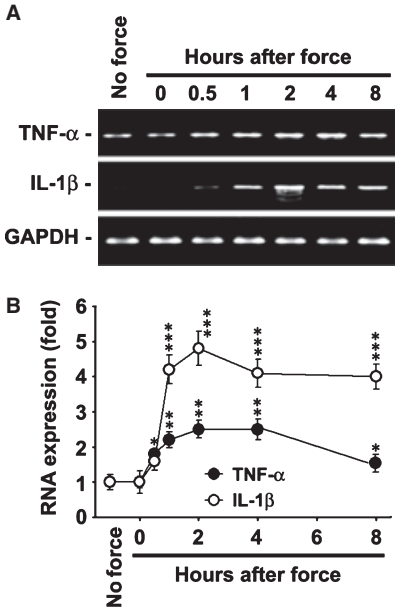

Fig. 2. Effect of centrifugal force on tumor necrosis factor-α and interleukin-1β expression in human gingival fibroblasts. The mRNA levels of these cytokines in the human gingival fibroblasts exposed to centrifugal force were measured by reverse transcription–polymerase chain reaction at the indicated time-points after applying the force. Representative data (A) and the data from three independent experiments were quantified by densitometry after normalizing the bands to glyceraldehyde-3-phosphate dehydrogenase (B). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. the unloaded control cultures. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α.

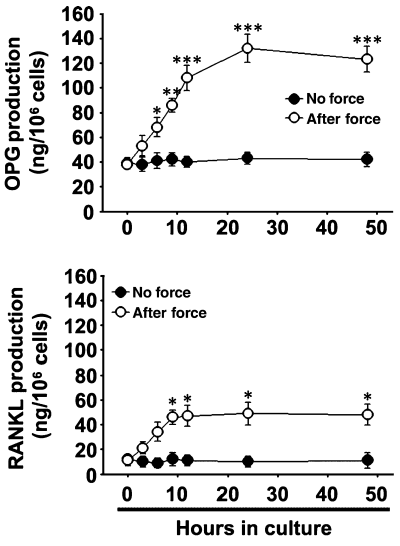

Fig. 3. Effect of centrifugal force on the production of osteoprotegerin and receptor activator of nuclear factor-κB ligand (RANKL) in human gingival fibroblasts. Conditioned medium was prepared from human gingival fibroblast cultures at the indicated time-points after exposure to centrifugal force. The conditioned medium was then processed, by enzyme-linked immunosorbent assay, for the analysis of osteoprotegerin (upper panel) and RANKL (lower panel). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. the unloaded control cultures. OPG, osteoprotegerin.

Effect of tumor necrosis factor-α and interleukin-1β on osteoprotegerin and RANKL secretion

Interleukin-1β and tumor necrosis factor-α are believed to induce inflammation and the destruction of periodontal tissue by acting both directly and indirectly on osteoclasts (18). The expression of RANKL and osteoprotegerin by osteoblasts stimulated by these cytokines might also be responsible for the inflammatory bone resorption (19,20). In addition, the RT-PCR results showed that the centrifugal force up-regulates the mRNA expression of the pro-inflammatory cytokines (Fig. 2). Therefore, this study examined how interleukin-1β or tumor necrosis factor-α affects the level of osteoprotegerin and RANKL production by human gingival fibroblasts (Fig. 4A). As shown in Fig. 4, the addition of interleukin-1β accelerated the production of both osteoprotegerin and RANKL by the human gingival fibroblasts exposed to centrifugal force. For example, the levels of production of osteoprotegerin and RANKL were 130 ± 11.9 and 50 ± 7.2 ng/10^6 cells, respectively, at 24 h after applying the force, which was augmented to 182 ± 8.3 and 98 ± 5.2 ng/10^6 cells, respectively, by adding 10 ng/mL of interleukin-1β to the cells. Similarly, the force-induced production of osteoprotegerin was also facilitated by treating the cells with tumor necrosis factor-α, where the level of osteoprotegerin production was augmented up to 1.52 times by adding 10 ng/mL of tumor necrosis factor-α to the culture, compared with that of the untreated control. By contrast, tumor necrosis factor-α treatment had no effect on RANKL production by the force-applied human gingival fibroblasts.

In order to understand in more detail the stimulatory roles of the pro-inflammatory cytokines on osteoprotegerin and RANKL production by human gingival fibroblasts, the cells were subjected to centrifugal force in the presence or absence of 10 ng/mL of anti-(interleukin-1β) immunoglobulin Ig isotype; IgG (rabbit polyclonal) or anti-(tumor necrosis factor-α) immunoglobulin Ig isotype; IgG1 (mouse

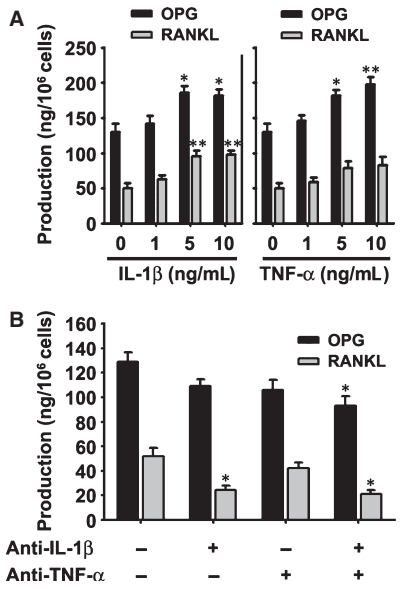

Fig. 4. Effects of pro-inflammatory cytokines on the production of osteoprotegerin and receptor activator of nuclear factor-κB ligand (RANKL) in human gingival fibroblasts. (A) Human gingival fibroblasts were exposed to centrifugal force in the presence and absence of the indicated doses of interleukin-1β or tumor necrosis factor-α. Twenty-four hours after applying the force, the culture supernatants were collected and analyzed using enzyme-linked immunosorbent assay. (B) Human gingival fibroblasts were also subjected to centrifugal force in the presence or absence of 10 ng/mL of anti-(interleukin-1β) immunoglobulin Ig isotype; IgG (rabbit polyclonal) or anti-(tumor necrosis factor-α) immunoglobulin Ig isotype; IgG1 (mouse monoclonal). Twenty-four hours after applying the centrifugal force, the culture supernatants were collected and analyzed using enzyme-linked immunosorbent assay. $*p < 0.05$ and $**p < 0.01$ vs. the untreated control cultures. IL-1β, interleukin-1β; OPG, osteoprotegerin; TNF-α, tumor necrosis factor-α.

monoclonal), and the culture medium was analyzed by ELISA 24 h after applying centrifugal force for 90 min (Fig. 4B). The force-mediated stimulation of RANKL production was inhibited significantly when the cells were treated with anti-(interleukin-1β) immunoglobulin Ig isotype; IgG (rabbit polyclonal). However, the anti-(tumor necrosis factor-α) immunoglobulin Ig isotype; IgG1 (mouse monoclonal) had no effect. By contrast, the level of osteoprotegerin production by the force-applied human gingival fibroblasts was prevented only by treating the cells with both anti-(interleukin-1β) immunoglobulin and anti-(tumor necrosis factor-α) immunoglobulin.

Effect of centrifugal force on osteoclastogenesis

In order to examine the effect of centrifugal force on the formation of osteoclast-like cells, co-cultures with human gingival fibroblasts and mouse whole bone marrow cells were exposed to centrifugal force in the presence or absence of anti-osteoprotegerin immunoglobulin Ig isotype; goat polyclonal IgG, and analyzed by TRAP staining 5 d after the application of force. TRAP-positive cells were detectable only when the co-cultured cells were incubated in the presence of 100 nM dexamethasone and 10 nM vitamin D_3 (Fig. 5A,b, 5B). By contrast, the formation of osteoclast-like cells was significantly increased when the co-cultures were treated with 1 μg/mL of the anti-osteoprotegerin immunoglobulin Ig isotype; goat polyclonal IgG (Fig. 5A,d, 5B). This increase was significantly higher ($p < 0.01$) in the cells not exposed to centrifugal force than in the co-cultures exposed to centrifugal force (Fig. 5B), which suggests that centrifugal force inhibits osteoclastogenesis by stimulating the production of osteoprotegerin by human gingival fibroblasts.

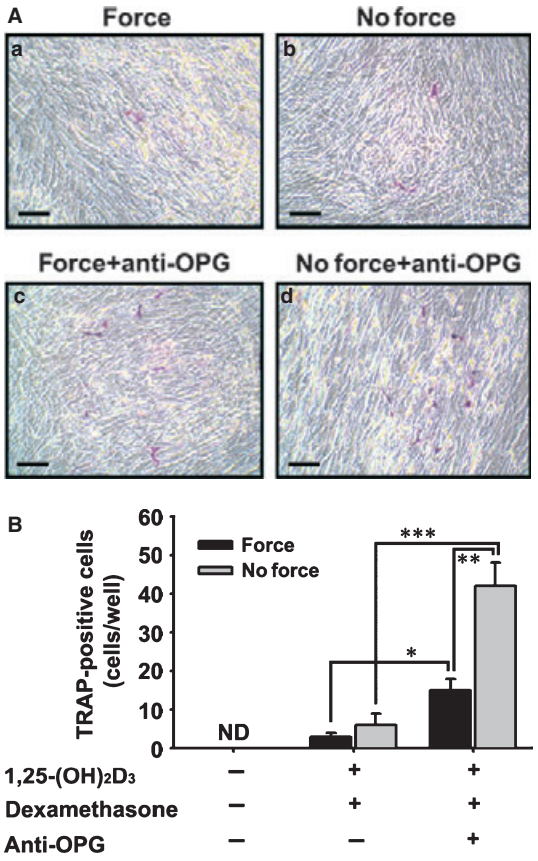

Fig. 5. Effect of centrifugal force on the formation of osteoclast-like cells in a co-culture system with human gingival fibroblasts and mouse bone marrow cells. (A) Human gingival fibroblasts and bone marrow cells were co-cultured in RPMI-1640 supplemented with 100 nM dexamethasone and 10 nM vitamin D_3 with (c,d) and without (a,b) 1 μg/mL of anti-osteoprotegerin immunoglobulin Ig isotype; goat polyclonal IgG. After 2 d of co-incubation, the cells were exposed to centrifugal force (a,c) and analyzed by tartrate-resistant acid phosphatase (TRAP) staining 5 d after applying the force. Bar, 100 μm. (B) The number of TRAP-positive cells was calculated from the data obtained from three independent experiments and is expressed as numbers of cells per well of 96-multiwell plates. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. 1α,25$(OH)_2D_3$, vitamin D_3; ND, not determined; OPG, osteoprotegerin;

Effect of conditioned medium from control or centrifugal force-exposed -human gingival fibroblasts on osteoclastogenesis

In order to confirm the anti-osteoclastogenic effect of centrifugal force through osteoprotegerin production, mouse whole bone marrow cells were cultured in an osteoclastic differentiating medium (RPMI-1640 supplemented with 30 ng/mL of macrophage colony-stimulating factor, 20 ng/mL of tumor necrosis factor-α and 100 ng/mL of RANKL) with and without various concentrations of conditioned medium and/or 1 μg/mL of the anti-osteoprotegerin immunoglobulin Ig isotype; goat polyclonal IgG. As shown in Fig. 6A, multinucleated cells were largely formed in the control osteoclastic differentiating medium cultures. However, the induction of multinucleated cells was inhibited significantly by adding conditioned medium, where the level of inhibition was more apparent when the cells were treated with the conditioned medium collected from the force-applied human gingival fibroblasts than the unloaded control human gingival fibroblasts. When the number of TRAP-positive cells was calculated, the number of multinucleated cells in the control osteoclastic differentiating medium cultures was 175 ± 16 cells in each well of a 96-multiwell plate. This decreased to 54 ± 4 and 16 ± 3 cells when treated with 50% conditioned medium from the unloaded control or force applied-human gingival fibroblasts, respectively (Fig. 6B).

The addition of anti-osteoprotegerin immunoglobulin Ig isotype; goat polyclonal IgG blocked the conditioned medium-mediated decrease in the number of TRAP-positive cells, as expected (Fig. 6C). As shown in Fig. 6, the number of multinucleated cells in the osteoclastic differentiating medium culture was reduced to 16 ± 3 cells by supplementing them with 50% conditioned medium from the force-applied human gingival fibroblasts. The number was increased significantly to 78 ± 13 cells ($p < 0.05$) by adding the anti-osteoprotegerin immunoglobulin Ig isotype; goat polyclonal IgG to the cultures. These results indicate that human gingival fibroblasts have the potential to inhibit osteoclastogenesis through the production of osteoprotegerin, which is further accelerated by the application of a centrifugal force.

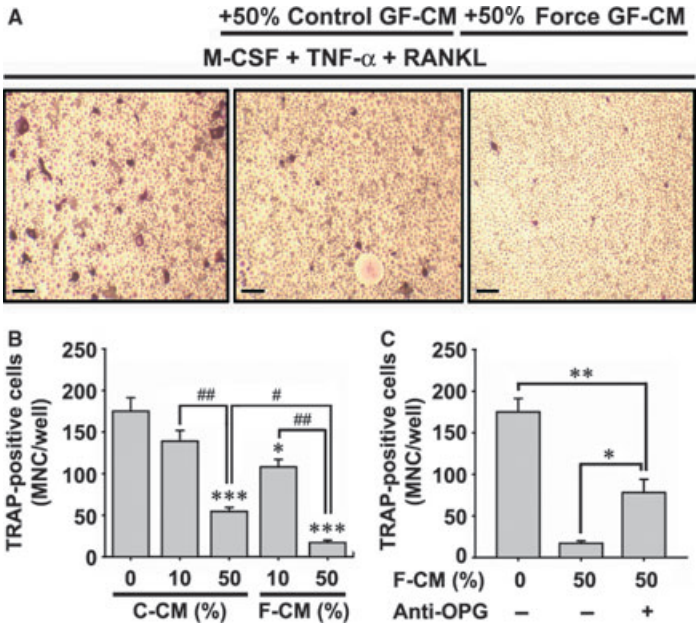

Fig. 6. Effect of conditioned medium collected from the centrifugal force-applied human gingival fibroblast cultures on the formation of multinucleated cells. (A) Mouse bone marrow cells were cultured in an osteoclastic differentiating medium with and without the indicated concentrations (%) of conditioned medium from the force-applied or control human gingival fibroblast cultures. After 5 d of incubation the cells were processed for tartrate-resistant acid phosphatase (TRAP) staining. Bar = 100 μm. (B) The number of multinucleated cells with more than five nuclei was counted as osteoclast-like cells. $*p < 0.05$ and $***p < 0.001$ vs. the untreated control cultures. $p < 0.05$ and $^{\#\#}p < 0.01$ indicate significant differences between the experiments. (C) Mouse bone marrow cells were also cultured in osteoclastic differentiating medium with 50% conditioned medium from the force-applied -human gingival fibroblast cultures and/or 1 μg/mL of the anti-osteoprotegerin immunoglobulin Ig isotype; goat polyclonal IgG. After 5 d of culture, the cells were processed for TRAP staining, and the number of multinucleated cells was counted, as described above. $*p < 0.05$ and $**p < 0.01$. C-CM, conditioned medium collected from control human gingival fibroblasts; F-CM, conditioned medium collected from -human gingival fibroblasts exposed to centrifugal force; Forced GF-CM, conditioned medium from human gingival fibroblasts exposed to centrifugal force; GF-CM, conditioned medium from human gingival fibroblasts; M-CSF, macrophage colony-stimulating factor; MNC, multinucleated cells; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; TNF-α, tumor necrosis factor-α.

Discussion

The results show that human gingival fibroblasts naturally produce high levels of osteoprotegerin, and that the production of osteoprotegerin is further enhanced by applying centrifugal force to the cells. The findings also show that centrifugal force stimulates the capacity of human gingival fibroblasts to inhibit the formation of osteoclast-like cells by producing a relatively high osteoprotegerin to RANKL ratio. Although several studies have suggested that human gingival fibroblasts could have anti-osteoclastogenic potential (9,10,21), this study reports for the first time the accelerating effect of centrifugal force on the human gingival fibroblast-mediated suppression of osteoclast formation.

There are two functionally different types of fibroblasts in the dental anatomy – human gingival fibroblasts and periodontal ligament fibroblasts – which are known to have dual effects

(i.e. they can induce the formation of osteoclast-like cells and they can inhibit osteoclast formation) (7,21). However, human gingival fibroblasts are primarily different from periodontal ligament fibroblasts with regard to their osteoclastogenic potential because human gingival fibroblasts induced transparently fewer TRAP-positive cells in a co-culture with peripheral blood mononuclear cells than periodontal ligament fibroblasts (21). This difference is believed to be induced by the different functions of these fibroblasts in response to extracellular stimuli. In particular, periodontal ligament fibroblast-mediated osteoclast formation is fundamental for remodeling the alveolar bone, which occurs throughout life but is particularly active during orthodontic tooth movement (22). By contrast, human gingival fibroblasts are essential for maintaining the gingival connective tissue and play an important role in protection against oral pathogens. Therefore, we speculate that the response of human gingival fibroblasts to a mechanical force is primarily anti-inflammatory and anti-osteoclastic, even though human gingival fibroblasts can also induce osteoclastogenesis in a co-culture system, as found in this study. This is supported by a report showing that lipopolysaccharide stimulates osteoprotegerin expression by human gingival fibroblasts, which decreases the induction of osteoclastogenesis by these cells (10).

In addition to osteoprotegerin and RANKL, many pro-inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1α, interleukin-1β and interferons are believed to play important roles in the pathogenesis of periodontitis. These cytokines can cause inflammation and destruction of the periodontal tissue, as well as the resorption of alveolar bone, by acting both directly and indirectly on the cells in the periodontal tissue (23). In particular, the interleukin-1 family is one of the most potent cytokines associated with inflammatory bone resorption in periodontitis (24) and is known to increase the level of RANKL expression by osteoblasts (20,25). In addition to the interleukin-1 family, the expression of RANKL and osteoprotegerin by osteoblasts stimulated with tumor necrosis factor-α might be responsible for the inflammatory bone resorption that is also induced by the affected osteoclasts (18). It has been reported that lipopolysaccharide stimulates the expression of osteoprotegerin and RANKL in periodontal ligament fibroblasts through the induction of tumor necrosis factor-α and interleukin-1β (4). Both tumor necrosis factor-α and interleukin-1β up-regulate the expression of osteoprotegerin and RANKL in osteoblastic cells (19,20). Similarly, our present findings showed that centrifugal force stimulates the production of both tumor necrosis factor-α and interleukin-1β by human gingival fibroblasts, and supplementation with these cytokines augments the production of osteoprotegerin and RANKL. This led us to postulate that centrifugal force also facilitates osteoprotegerin and RANKL expression indirectly by stimulating the production of tumor necrosis factor-α and interleukin-1β by human gingival fibroblasts. However, it is possible that in contrast to interleukin-1β, tumor necrosis factor-α does not stimulate directly the production of osteoprotegerin and RANKL induced by centrifugal force. This is because supplementation of the centrifugal force-stimulated-human gingival fibroblast cultures with a neutralizing antibody against tumor necrosis factor-α does not inhibit the production of osteoprotegerin and RANKL, even though the level of mRNA expression specific to tumor necrosis factor-α was significantly increased by the centrifugal force. More detailed experiments will be needed to determine the precise roles of the pro-inflammatory cytokines on osteoprotegerin and RANKL production by human gingival fibroblasts.

This study also examined, using two culture systems, how a centrifugal force affects the formation of osteoclast-like cells. The results clearly showed that the anti-osteoclastogenic action by the production of osteoprotegerin is a primary phenomenon of human gingival fibroblasts, which is further stimulated by centrifugal force. These results are similar to a report showing that conditioned medium obtained from lipopolysaccharide-exposed human gingival fibroblasts inhibits significantly the formation of TRAP-positive cells, where osteoprotegerin plays a key role (10). Kanzaki *et al*. reported that local osteoprotegerin gene transfer to palatal periodontal tissue partially inhibited RANKL-mediated osteoclastogenesis induced by mechanical stress, thereby preventing experimental tooth movement (26). Taken together, our present findings and previous results strongly suggest that osteoprotegerin plays critical roles in the anti-osteoclastogenesis stimulated by a mechanical force in gingival tissue. Notwithstanding, we also assume that in addition to osteoprotegerin, there are other factors associated with the force-induced acceleration of anti-osteoclastogenic activity by human gingival fibroblasts. This is because the treatment with a neutralizing antibody against osteoprotegerin did not completely abolish the inhibitory effect of conditioned medium on the formation of osteoclast-like cells. In addition, when the bone marrow cells were cultured in ODM supplemented with 5 μg/mL of anti-osteoprotegerin immunoglobulin Ig isotype; goat polyclonal IgG, the force-mediated decrease of TRAP-positive cells was not completely blocked (data not shown). Furthermore, it is important to consider that osteoclastogenic activation in alveolar bone remodeling by mechanical stimuli may be controlled, at least in part, by various types of proteins, such as hormones (27,28), growth factor (29) and cytokines (9,16,17,30). More detailed study will be needed to determine the precise mechanism(s) involved in the anti-osteoclastogenic potential of human gingival fibroblasts and its stimulation after centrifugal force.

In summary, our present data suggest that human gingival fibroblasts naturally produce high levels of osteoprotegerin, which is accelerated by the application of a centrifugal force to the cells. In addition, centrifugal force facilitates the inhibitory potential of human gingival fibroblasts on the formation of osteoclast-like cells by

stimulating osteoprotegerin production. Interleukin-1β up-regulated by the applied force in human gingival fibroblasts is believed to be closely related to the accelerating effect of centrifugal force on anti-osteoclastogenesis. Overall, this study suggests that human gingival fibroblasts can inhibit osteoclast formation and have a defensive mechanism to inhibit bone resorption in a mechanical force.

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