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Optimal compressive force accelerates osteoclastogenesis in RAW264.7 cells

TAKAKO HAYAKAWA[1,2], YOSHITAKA YOSHIMURA[2], TAKASHI KIKUIRI[3], MINO MATSUNO[2], TOMOKAZU HASEGAWA[4], KUMU FUKUSHIMA[1], KENJIRO SHIBATA[1], YOSHIAKI DEYAMA[2], KUNIAKI SUZUKI[2] and JUNICHIRO IIDA[1]

Departments of [1]Orthodontics, [2]Molecular Cell Pharmacology and [3]Pediatric Dentistry, Hokkaido University Graduate School of Dental Medicine, Sapporo, Hokkaido 060-8586; [4]Department of Pediatric Dentistry, Faculty of Dentistry, Tokushima University, Tokushima 770-8504, Japan

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Abstract. Mechanical stress produced by orthodontic forces is a factor in the remodeling of periodontal ligaments (PDLs) and alveolar bone. It has been reported that the expression of a number of cytokines associated with osteoclastogenesis is upregulated when compressive forces act on osteoblasts and PDL cells. The present study investigated the effects of compressive forces on the formation of osteoclasts from the macrophage cell line RAW264.7. Compressive forces on osteoclasts were exerted using layers of 3, 5, 7, 9 or 14 glass cover slips on the 4th day of culture for 24 h. The number of osteoclasts was determined by counting the number of cells positive for tartrate-resistant acid phosphatase staining. Osteoclastogenesis advanced rapidly on days four and five. The number of osteoclasts with >8 nuclei peaked when the force of 7 slips was applied, which was therefore regarded as the optimal compressive force. Alterations in the expression of osteoclast-associated genes are associated with changes in the differentiation and fusion of macrophages in response to compressive forces; therefore, osteoclast-associated genes were assessed by reverse transcription quantitative polymerase chain reaction in the present study. The mRNA expression of osteoclast-associated genes increased significantly after 3 h of optimal compression, whereas mRNA expression increased after 24 h in the control group. These findings suggested that osteoclastogenesis of macrophages was accelerated when an optimal compressive force was applied.

Correspondence to: Dr Yoshitaka Yoshimura, Department of Molecular Cell Pharmacology, Hokkaido University Graduate School of Dental Medicine, Kita 13 Nishi 7, Kita-Ku, Sapporo, Hokkaido 060-8586, Japan
E-mail: yoshi@den.hokudai.ac.jp

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Introduction

The purpose of orthodontic treatment is to achieve optimal occlusion for an individual. Orthodontic forces generate mechanical stress and are a factor that brings about the remodeling of the periodontal ligament (PDL) and alveolar bone (1,2). The PDL contains ample vascular and cellular connective tissue, including Sharpey's fibers, fibroblasts, osteoblasts, osteoclast precursors and osteoclasts, and surrounds the roots of the teeth. Osteoclast precursors and osteoclasts are present on the alveolar bone.

Previous studies have revealed the response of osteoblasts and fibroblasts to various types of mechanical stimulation. Mechanical stimuli, including tensile force (3-5), compressive force (6,7), fluid flow (8), hydrostatic pressure (9), rotation-associated stress (10) and other stimuli (11,12), are influential factors in bone remodeling. Orthodontic forces comprise tensile and compressive forces, and lead to a marked compression of the region of the PDL which they are applied to (13).

Tensile forces exerted using a Flexercell tension system suppressed osteoclast differentiation and fusion (4,5). Furthermore, when compressive forces were applied to osteoblasts and PDL cells, essential factors that promote osteoclast differentiation and activation, including prostaglandin E_2 (PGE_2), interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, receptor activator of nuclear factor κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (11,12), were upregulated. Numerous studies have reported that applying such forces leads to secretion of cytokines associated with bone resorption and formation (14,15).However, it has remained elusive whether direct compressive forces regulate osteoclastogenesis.

Osteoclasts are derived from the monocyte/macrophage lineage and are formed by the fusion of mononuclear precursors. RANKL is an essential factor for osteoclast differentiation and activation (16). RANKL is secreted by osteoblasts and stromal cells, and is bound to RANK (14). RANK is induced by M-CSF in osteoclastogenesis, and is an essential factor for the formation of mature osteoclasts (17). The murine macrophage cell line RAW264.7 expresses RANK and can differentiate into osteoclasts after

RANKL stimulation without M-CSF (5). RANKL signaling induces the master transcription factor nuclear factor of activated T cells c1 (NFATc1). NFATc1 is an essential regulator for numerous osteoclast-specific genes associated with differentiation, fusion and bone resorption (18).

Therefore, the aim of the present study was to develop a method for applying compressive forces to RAW cells and to investigate their osteoclastogenesis induced by applying various magnitudes of force for various periods of up to 24 h.

Materials and methods

Cell culture. The murine monocyte/macrophage cell line RAW264.7 (TIB-71™; American Type Culture Collection, Manassas, VA, USA) was used as osteoclast precursors. RAW264.7 cells have been shown to differentiate into osteoclast-like cells in the presence of RANKL (5). Cells were cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA) and 66.7 μg/ml kanamycin sulfate (Meiji Seika, Tokyo, Japan) at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Cells were seeded onto 100-mm standard dishes (BD Biosciences, Franklin Lakes, NJ, USA) and cultured overnight. 12-mm diameter glass cover slips (slips; Fisher Microscope Cover Glass; Thermo Fisher Scientific, Waltham, MA, USA) was placed into the wells of a 24-well culture plate (BD Biosciences). For osteoclast differentiation, RAW264.7 cells were transferred into the 24-well culture plates at $1.0x10^4$ cells/well and were cultured in α-minimum essential medium (α-MEM; Wako) supplemented with 10% heat-inactivated FBS, 2 μM L-alanyl-L-glutamine (Wako), 284 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 66.7 μg/ml kanamycin sulfate and 50 ng/ml RANKL (Oriental Yeast Co., Ltd., Tokyo, Japan) at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Medium containing these reagents was replaced every other day.

Reversal of cells on glass cover slips. Prior to culture, 15 mm-diameter silicone tubes were cut into 2-mm slices and sterilized in an autoclave (Fig. 1A). RAW264.7 cells were cultured on slips which had been placed on sliced silicone tubes in 24-well culture plates for five days, and the slips were reversed in the same wells filled with culture medium after three days for 48 h, or after four days for 24 h (Fig. 1B). The control group was cultured for the same period without reversing the slips.

Preparation of collagen gels. Acid-soluble collagen solution (Cellmatrix; Nitta Gelatin, Inc., Osaka, Japan), ten-fold concentrated α-MEM (Invitrogen Life Technologies), and reconstruction buffer (2.2 g $NaHCO_3$ and 4.77 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in 100 ml 0.05 N NaOH; Nittal Gelatin, Inc.) were mixed at a volume ratio of 8:1:1, and 10% heat-inactivated FBS, 284 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich) and 2 mM L-alanyl-L-glutamine (Wako) were added in an ice-cold bath kept at <4˚C. This prepared collagen mixture was added to 24-well culture plates at a volume of 500 μl/well, and was subjected to gelation in a CO_2 incubator at 37˚C for 30 min. After the collagen mixture had solidified, it was covered with 50 ng/ml RANKL in culture medium at 1 ml/well.

Application of compressive forces. Prior to the application of compressive forces, RAW264.7 cells were incubated on the slip for three days (Fig. 1C, left). Collagen gel layers were prepared in other 24-well culture plates. Subsequently, osteoclasts on the slips were reversed and placed on top of these collagen gel layers (Fig. 1C, right), and were continuously compressed with layers of 3, 5, 7, 9 or 14 slips (~43 mg/slip) for 24 h, respectively (Fig. 1D). In the control group, the slips with cells attached were reversed onto the collagen gel layer without application of any additional force (Fig. 1C, right).

Tartrate-resistant acid phosphatase (TRAP) staining. After cells were compressed for the indicated times, they were fixed in 10% neutral formalin. They were then washed with distilled water and stained with Fast Red Violet LB Salt (Sigma-Aldrich) in TRAP staining solution [acetate buffer (pH 5.0) (Sigma-Aldrich), naphthol AS-MX phosphate (Sigma-Aldrich) as a substrate, red violet LB (Sigma-Aldrich) as a stain, and 50 mM sodium tartrate (Wako)]. TRAP-positive cells with 2-7 nuclei were considered to be small osteoclasts, and those with >8 nuclei were considered to be large osteoclasts (3,4). Osteoclasts were counted under a light microscope (magnification, x100; Olympus IMT-2; Olympus, Tokyo, Japan) in a 20-mm^2 rectangle within the circular field.

Pit assay. Osteologic™ was used to characterize and quantify osteoclast-mediated bone resorption in order to determine the amount of osteoclastic differentiation. A disc (ϕ12-mm) made of Osteologic™ (BD Biosciences) was coated with calcium phosphate. Osteoclasts were cultured for three days on these discs under normal cell culturing conditions as described above, and discs were reversed onto the top of a collagen gel layer in 24-well culture plates on the fourth day for 24 h as a control group. Another group of reversed osteoclasts was subjected to the compressive force of seven layered slips. On the fifth day, the control and compressed osteoclasts on the discs were rinsed twice with distilled water. Subsequently, 1 ml bleach solution (6% NaOCl and 5.2% NaCl) was added to each well, followed by pipetting up and down to remove cells and incubation for five minutes at room temperature. Discs were washed with 2 ml distilled water three times and were examined by light microscopy (magnification, x100; Olympus IMT-2; Olympus) after drying (19,20). The resorption area was measured using Image J software (v. 1.48a; National Institutes of Health, Bethesda, MD, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Cells were compressed for 1, 3, 6, 12 and 24 h in 24-well culture plates. Total RNA was extracted using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions. Reverse transcription of 1 μg RNA was performed to obtain cDNA. The PCR reaction mixture contained 50 mM Tri-Hcl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 10 mM dithiothreitol, 0.01% Nonidet® P-40 and 50% glycerol. The thermocycling protocol was as follows: Annealing at 30˚C for 10 min, enzyme reaction at 42˚C for 20 min, denaturation at 99˚C for 5 min and cooling at 4˚C. cDNA was amplified using Rever Tra Ace-α FSK-101 (Toyobo, Osaka Japan). Primers (Applied Biosystems, Thermo Fisher Scientific) for

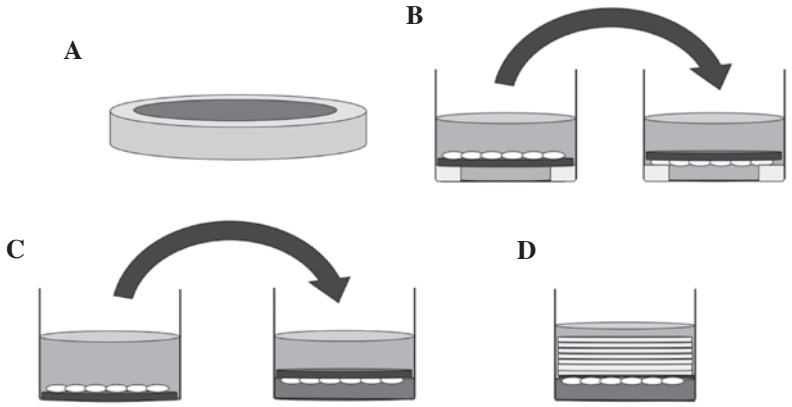


Figure 1. Cell culture. (A) Silicone tubes (15-mm diameter) were cut into 2-mm slices and sterilized in an autoclave. (B) RAW264.7 cells were cultured on 12 mm-diameter glass cover slips. These slips were placed on sliced silicon tubes in 24-well culture plates. The slips with RAW264.7 cells attached were then reversed on the sliced silicone tubes using sterilized forceps to face downwards. (C) Scheme of cell culture methods for applying compressive force. RAW264.7 cells on glass cover slips were cultured in 24-well culture plates. The glass cover slips with cells attached were reversed to face a collagen gel layer at the bottom of the well using sterilized forceps. (D) Reversed cells were continuously compressed by layered slips.

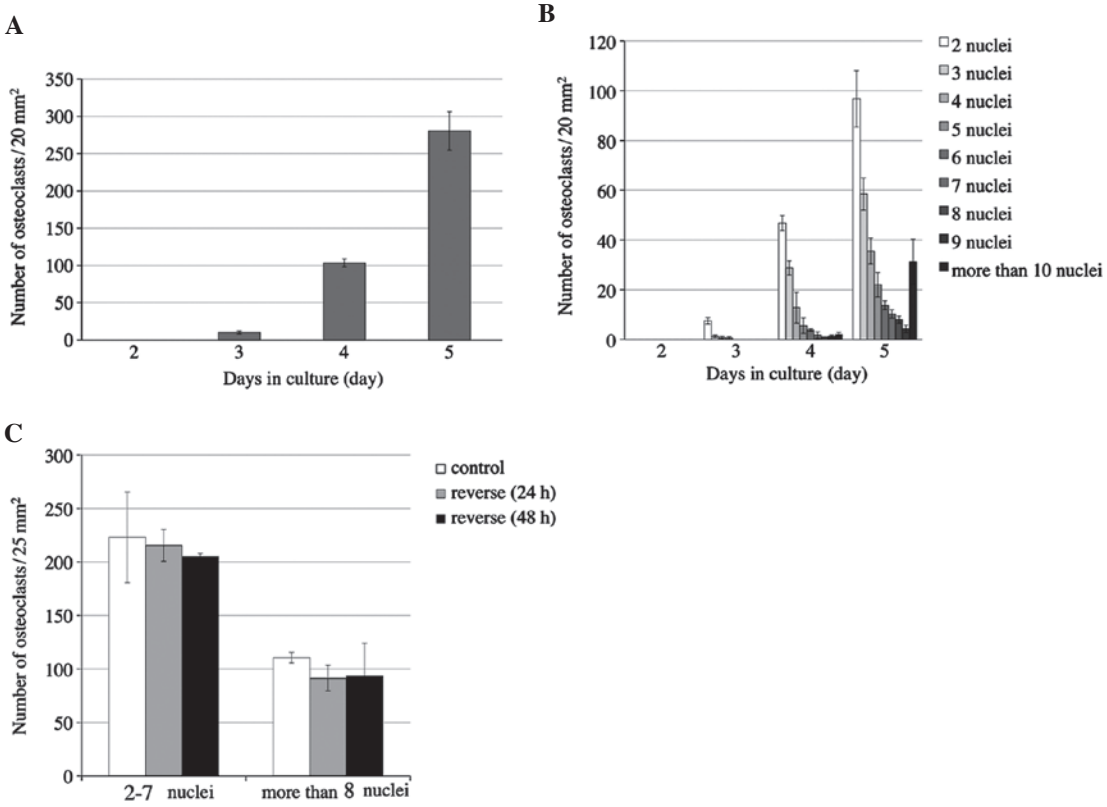


Figure 2. Number of osteoclasts on each culture day up to day five. (A) The number of osteoclasts was assessed daily over five days. (B) The number of osteoclasts with multiple nuclei on days 2-5. (C) Number of small (2-7 nuclei) and large (>8 nuclei) osteoclasts in response to reversal of slips at either 24 or 48 h when compared with control group on day five. The control group was cultured on slips without reversing and was fixed on day five. The number of cells positive for tartrate-resistant acid phosphatase staining with 2-7 nuclei and those with >8 nuclei was counted. Values are expressed as the mean ± standard deviation (n=4).

the following genes were used: NFATc1 (Mm00479445_m1), TRAP (Mm00475698_m1), RANK (Mm00437135_m1), matrix metalloproteinase-9 (MMP-9; Mm00432271_m1), dendritic cell specific trans membrane protein (DC-STAMP; Mm01168058_m1), osteoclast stimulatory trans membrane protein (OC-STAMP; Mm00512445_m1), integrin-av (Mm00434506_m1), integrin-β3 (Mm00443980_m1), cathepsin-K (cath-K; Mm00484036_m1), chloride channel 7 (ClC-7; Mm00442400_m1), adenosine triphosphatase H^+ transporting vacuolar proton pump member I (ATP6i; Mm00469395_g1) and GAPDH (Mm99999915_g1). Real-time PCR was performed using the ABI Prism 7300 sequence detection system (Applied Biosystems). Data obtained for each sample were standardized against the expression of GAPDH and were calculated using the $2^{-\Delta\Delta Ct}$ method (21,22).

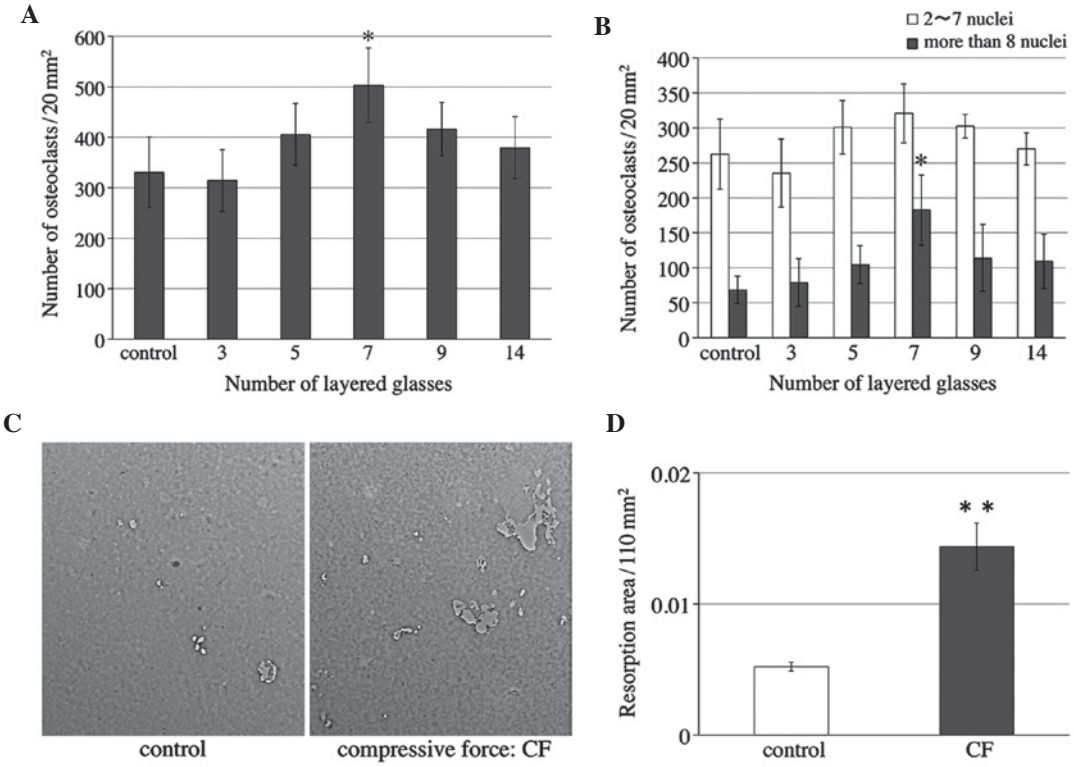


Figure 3. Effects of various compressive forces for 24 h on osteoclastogenesis. RAW264.7 cells ($1.0x10^4$ cells/well) on slips were pre-cultured with culture medium containing 50 ng/ml receptor activator of nuclear factor kappa-B ligand in 24-well culture plates for three days. Cells on slips were reversed onto the collagen gel layer after four days. Compressive force was applied with 3, 5, 7, 9 and 14 layered slips for 24 h from days four to five, while the control group was not loaded. (A) Total numbers of osteoclasts. (B) Number of small (2-7 nuclei) and large (>8 nuclei) osteoclasts. Values are expressed as the mean ± standard deviation (n=4). *P<0.05 vs. control. (C) Pit assay. RAW264.7 cells on Osteologic™ discs were pre-cultured in 24-well culture plates for three days. Cells on the discs were reversed onto collagen gel layers after four days. Compressive force was applied via 3-14 layered slips for 24 h from days four to five, while the control group was not subjected to loading. Light micrographs of Osteologic™ discs (magnification, x100). Resorption areas of the discs in the control and CF groups are shown. (D) The resorption area in C was quantified using Image J software. Values are expressed as the mean ± standard deviation (n=3). **P<0.01 vs. control. CF, compressive force.

Statistical analysis. Values are expressed as the mean ± standard deviation. Statistical analysis was performed using Microsoft Excel for Mac 2011, version 14.4.8 (Redmont, WA, USA). Comparisons between two groups were analyzed using the two-tailed unpaired Student's *t*-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Ostoclastogenesis increases with culturing time. In order to determine at what time-point osteoclast differentiation and fusion of RAW264.7 cells were activated, the number of osteoclasts and the number of nuclei per cell were monitored for five days (Fig. 2). RAW264.7 cells were cultured with 50 ng/ml RANKL in 24-well culture plates for five days to induce osteoclastonenesis. No osteoclasts were identified on day two, while the number of osteoclasts was slightly increased on days three and four, and was markedly increased on day five (Fig. 2A). Osteoclasts contained 2-4 nuclei on day three and began to appear with >5 nuclei on day four. On day five, the number of multinuclear cells further increased, and cells with >6 nuclei were increasingly present (Fig. 2B). Only upon osteoclastic differentiation, RQW264.7 cells begin to adhere to the cover slips. Day five was therefore determined to be the ideal time-point for reversing the cover slips in order to apply compressive forces on the cells.

Reversed culture without application of compressive forces does not affect osteoclastogenesis. The present study investigated the effects of reversing the slips on osteoclastogenesis on sliced silicone tubes for 24 and 48 h (Fig. 1B). Controls were cultured on slips without reversing and were fixed on day 5. The number of TRAP-positive cells with 2-7 nuclei and those with >8 nuclei was counted. The number of osteoclasts among the reversed cells showed no significant difference when compared with that in the control group at either 24 h or 48 h (Fig. 2C).

A compressive force exerted by loading with seven cover slips for 24 h accelerates osteoclastogenesis. Various intensities of compressive force were applied to osteoclasts for 24 h after 4-5 days of culture. RAW264.7 cells on slips were cultured as described above. Cells were reversed onto the collagen gel layer in other plates on the 4th day. Cells were then loaded with layers of 3-14 slips after being reversed. The number of osteoclasts was significantly increased when the cells were compressed with seven slips (Fig. 3A), and the number of osteoclasts with >8 nuclei was significantly elevated (Fig. 3B).

Bone resorption activity of osteoclasts subjected to compressive forces with 7 slips. The present study then examined the bone resorption activity of osteoclasts when the cells were subjected to a compressive force of seven slips. RAW264.7 cells were cultured on Osteologic™ discs as described above. Light

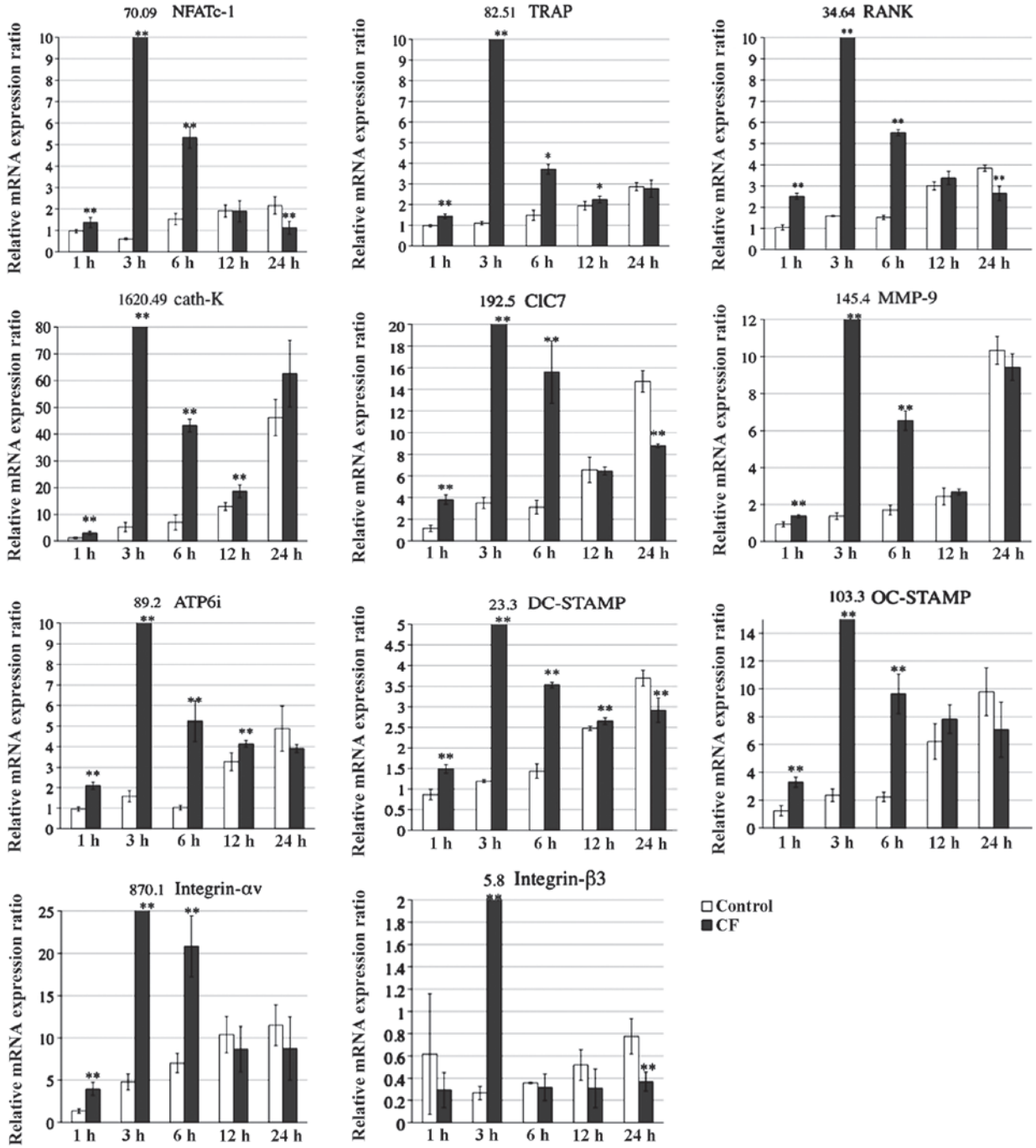


Figure 4. Effects of compressive force on mRNA levels of osteoclast-associated genes. Force was applied to cells for 1-24 h. mRNA levels of osteoclast-associated genes were evaluated by reverse transcription quantitative polymerase chain reaction. The control group, which was cultured for the same duration without compressive force, was defined as the standard. Values are expressed as the mean ± standard deviation (n=4). **P<0.01; *P<0.05 vs. control at the same time-point. CF, compressive force.

micrographs of the Osteologic™ discs of the control and compressed (CF) groups were captured (Fig. 3C). The areas in which the calcium phosphate on the discs was completely resorbed were measured. Quantification of the results showed that the resorption areas in the compressed group were significantly larger when compared with those in the control group (Fig. 3D).

Expression of osteoclast-associated mRNA. The present study examined the effects of compressive forces on mRNA levels of osteoclast-specific genes associated with differentiation, fusion and adherence using RT-qPCR analysis (Fig. 4). The analysis showed that the respective mRNA levels of osteoclast-associated genes, including NFATc-1, TRAP, RANK, cath-K, ClC7, MMP-9, ATP6i, DC-STAMP, OC-STAMP as well as Integrin-αv and -β3 (4), peaked at 3 h in the CF group, whereas the mRNA levels peaked at 24 h in the control group.

Discussion

The present study was the first, to the best of our knowledge, to directly apply compressive forces to osteoclast-precursor cells

using the abovementioned method. It was shown that application of the optimal compressive force to RAW264.7 cells led to an upregulation of the expression of various osteoclast-associated genes and promoted osteoclast differentiation and fusion. Under normal culturing conditions and stimulation with RANKL, osteoclastogenesis advanced rapidly on days four and five. A previous study demonstrated that small osteoclasts with 2-7 nuclei appeared on the third day (22). Previous studies also showed that formation of osteoclasts continued to increase up to the sixth day (22,23) and cells began to undergo apoptosis from the seventh day (3). Therefore, the present study applied compressive forces to the cells after 4-5 days of culture under osteoclastic induction conditions.

In a pilot study, compressive force was applied using layered glass cover slips on osteoclasts cultured on a 24-well culture plate. However, cell counts markedly decreased due to the hindered supply of culture medium to the cells. Therefore, a collagen gel (Cellmatrix) was used, against which cells were pressed in subsequent experiments. The technical manual for collagen gel noted that the gel was developed for tissue culture, allowing it to be used for the culture of various cells. Osteoclastogenesis was not promoted on the collagen gel, and cells merely underwent cell division to form a mass of mononucleated cells (results not shown). It was also observed that RAW264.7 cells differentiated and fused on rigid surfaces. The impaired osteoclast differentiation observed under these non-adherent conditions was due to the absence of RANK-dependent signaling by osteoclast precursors (24). When osteoclasts were cultured on the bottom of a 24-well culture plate subjected to compressive forces exerted by the collagen gel layer, it was difficult to determine the cell number under the light microscope and to measure mRNA expression in these osteoclasts.

The present study then cultured cells on slips and reversed them onto the collagen gel layer in order to compress the osteoclasts. Innutrition and cell death during application of compression was thereby avoided, and the collagen gel layer was able to act as a cushion material when compressive forces were applied to the osteoclasts. Studies have shown that osteoclast precursors and osteoclasts are present on alveolar bone and adjacent to compressed PDL (13,25), which suggested that compressive forces from orthodontic treatment are transmitted to osteoclasts on the alveolar bone via the PDL. Application of compressive forces to osteoclasts on slips via the collagen gel therefore represents an *in vitro* model of the application of orthodontic forces onto osteoclasts on the alveolar bone surface via the PDL *in vivo*.

As a control experiment, the present study examined the effects of reversing cover slips on osteoclast differentiation when compared with osteoclasts on the same slips without reversal. Differences in the numbers of osteoclasts with or without reversing were not detected in the absence of compressive forces.

The present study examined the effects of various weights (layers of 3-14 cover slips) on the number of osteoclasts. The number of osteoclasts and multinuclear cells was greatest when seven slips were used, which were therefore considered to exert the optimal compressive force. Furthermore, the optimal compressive force may have accelerated the fusion of osteoclasts. Application of this compressive force also accelerated osteoclastogenesis of RAW264.7 cells as compared with that in the control group. Bone resorption areas of osteoclasts on Osteologic™ discs were significantly elevated when the optimal compressive force was applied.

There are several types of tooth movement in orthodontic treatment, including tipping, bodily movement and rotation. The optimal orthodontic forces are different for each type of tooth movement and area of the root surface. Schwartz and Austria (26) recommended that the forces of orthodontic treatment should not exceed the capillary bed blood pressure (~20-26 g/cm^2 of the root surface) for the most favorable outcomes. This is not in accordance with the results of the present study, in which a force of ~300 mg/cm^2 exerted by eight cover slips was found to be optimal for accelerating osteoclastogenesis. Each tooth is attached to and separated from the adjacent alveolar bone by heavy collagen fibers known as Sharpey's fibers in the PDL (25). The optimal orthodontic force in teeth may therefore be diminished by Sharpey's fibers in the PDL.

In the present study, mRNA expression of osteoclast-associated genes, including NFATc-1, TRAP, RANK, cath-K, ClC7, MMP-9, ATP6i, DC-STAMP, OC-STAMP as well as Integrin-αv and -β3, was shown to be upregulated and accelerated by compression at the optimal force when compared with that in the controls. In the compressed group, the expression of the respective mRNAs peaked at 3 h, whereas in the control group mRNA expression increased slowly over 24 h. Among the genes assessed, NFATc1 is thought be an essential transcriptional factor for autoamplification. These results indicated that the optimal compressive force activates osteoclastogenesis and upregulates osteoclast-associated mRNA expression.

Numerous studies have suggested that applying compressive forces to osteoblasts and PDL cells promotes osteoclastogenesis. The present study succeeded in applying compressive forces to osteoclasts and found that the optimal compressive force accelerated osteoclastogenesis. These results provided the basis for an alternative approach to the consideration of optimal force, which focuses on osteoclast precursors and osteoclasts.

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