Continuous Compressive Force Induces Differentiation of Osteoclasts with High Levels of Inorganic Dissolution

Osteoclast precursor cells are constitutively differentiated into mature osteoclasts on bone tissues. We previously reported that the continuous stimulation of RAW264.7 precursor cells with compressive force induces the formation of multinucleated giant cells via receptor activator of nuclear factor κB (RANK)-RANK ligand (RANKL) signaling. Here, we examined the bone resorptive function of multinucleated osteoclasts induced by continuous compressive force.

Cells were continuously stimulated with 0.3, 0.6, and 1.1 g/cm² compressive force created by increasing the amount of the culture solution in the presence of RANKL. Actin ring organization was evaluated by fluorescence microscopy. mRNA expression of genes encoding osteoclastic bone resorption-related enzymes was examined by quantitative real-time reverse transcription-polymerase chain reaction. Mineral resorption was evaluated using calcium phosphate-coated plates.

Multinucleated osteoclast-like cells with actin rings were observed for all three magnitudes of compressive force, and the area of actin rings increased as a function of the applied force. Carbonic anhydrase II expression as well as calcium elution from the calcium phosphate plate was markedly higher after stimulation with 0.6 and 1.1 g/cm² force than 0.3 g/cm². Matrix metalloproteinase-9 expression decreased and cathepsin K expression increased slightly by the continuous application of compressive force.

Our study demonstrated that multinucleated osteoclast-like cells induced by the stimulation of RAW264.7 cells with continuous compressive force exhibit high dissolution of the inorganic phase of bone by upregulating carbonic anhydrase II expression and actin ring formation. These findings improve our understanding of the role of mechanical load in bone remodeling.

MeSH Keywords: Bone Resorption • Carbonic Anhydrase II • Cathepsin K • Matrix Metalloproteinase 9 • Osteoclasts • RANK Ligand

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/913674
Background

During osteoclast differentiation, mononuclear osteoclast precursor cells in the monocyte/macrophage lineage fuse with each other in the presence of osteoclast differentiation factors, including receptor activator of nuclear factor κB (RANK) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) derived from marrow stromal cells and osteoblasts [1,2]. Morphologically, mature osteoclasts can become multinucleated giant cells and function in bone resorption; these mature osteoclasts decompose hydroxyapatite and extracellular matrix proteins in the inorganic and organic phase of bone [1,3].

Mechanical load derived from activities of daily living, regular exercise, medical devices for bone fracture healing, or orthodontic tooth movement is an important factor in the bone remodeling process [4–7]. Stimulation with a certain amount of compressive force, tension strain, or fluid shear stress induces osteoblastogenesis via the upregulation of Runx2 and Osterix, which are essential transcription factors for differentiation of mature osteoblasts with osteogenic function, including collagen synthesis and alkaline phosphatase activity, from precursor cells [8–12]. Osteoclastic bone resorption is also affected by mechanical stimulation. Several in vitro studies using osteoclast precursor cells have indicated the impacts of transient or intermittent mechanical stimuli on osteoclastogenesis. Cyclic tension force suppresses the differentiation into mature osteoclasts via the downregulation of cell fusion factors, including dendritic cell-specific transmembrane protein (DCSTAMP) and osteoclast-stimulatory transmembrane protein (OCSTAMP) [13,14]. The short-term application (up to 24 h) of compressive force generated by the superposition of a cover glass on osteoclast precursor cells pre-incubated with culture solution containing RANKL facilitates osteoclastogenesis and resorptive function in multinucleated osteoclast-like cells [15]. The effects of mechanical stimulation on osteoclastic bone resorption differ depending on the type and duration of mechanical loading; however, relatively little is known about osteoclastogenesis in vitro as compared to osteoclastogenesis owing to difficulties in applying mechanical stimuli to monocyte/macrophage lineages.

The constitutive differentiation of osteoclast precursor cells into mature osteoclasts occurs on bone tissue [1]; therefore, osteoclastic bone resorption occurs as well as mature osteoclasts and osteoblasts are continuously exposed to mechanical stimuli. In orthodontic treatments, continuous mechanical force induces alveolar bone remodeling within the physiological range during orthodontic tooth movement [6]. Recently, we investigated the effects of compressive force on osteoclastogenesis in vitro using the RAW264.7 mouse monocyte/macrophage lineage; cells were continuously exposed to compressive force created by increasing the amount of the culture solution (over 4 days) for RANKL-induced osteoclast differentiation [16]. Continuous stimulation with compressive force induced the fusion of cells by the up-regulation of the two cell fusion factors described above via RANK-RANKL signaling. As a result, multinucleated cells positive for tartrate-resistant acid phosphatase (TRAP), a marker of mature osteoclasts, increased depending on the magnitude of the compressive force that was exerted on the cells [16]. To the best of our knowledge, this is the first report of the continuous effects of the direct stimulation of osteoclast precursor cells by compressive force on osteoclastogenesis. In the present study, we hypothesized that resorptive function might be enhanced in osteoclast-like multinucleated cells induced by continuous compressive force; therefore, we examined the expression of bone resorption-related enzymes as well as actin ring organization, which are typical characteristics of mature osteoclasts that efficiently degrade bone matrix. Moreover, we also conducted a pit assay using calcium phosphate-coated plate to determine bone resorption activity in cells.

Material and Methods

Materials

The RAW264.7 cells obtained from Dainippon Pharmaceutical (Osaka, Japan) and maintained in our laboratory [16] were used as osteoclast precursor cells in this study. Penicillin/streptomycin, bovine serum albumin, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT, USA). Soluble RANKL, α-minimal essential medium (α-MEM), phenol red-free α-MEM/F-12, phosphate-buffered saline (PBS), paraformaldehyde, sucrose, and sodium chloride were purchased from Wako Pure Chemical (Osaka, Japan). NucleoSpin RNA, the RNA PCR Kit (PrimeScript), and SYBR Premix Ex Taq solution were purchased from Thermo Fisher Scientific (Rockford, IL, USA). The TRAP staining kit was purchased from Cosmo Bio (Sapporo, Japan). The mouse anti-carbonic anhydrase II (sc-48351), mouse anti-matrix metalloproteinase-9 (sc-393859), mouse anti-cathepsin K (sc-48353), and mouse anti-β-tubulin (sc-5274) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Biotin-conjugated goat anti-mouse antibodies were obtained from Abcam (Cambridge, UK). The western ECL substrate kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Osteoclast cultures and continuous stimulation with compressive force

Cells were stimulated with compressive force, as described previously [16]. Briefly, cells were seeded on a 96-well plate at a density of 1×10^4/cm^2 in α-MEM containing 10% FBS and 1% (v/v) antibiotic and maintained overnight at 37°C.
in a humidified atmosphere of 95% air and 5% CO₂. After discarding the medium, 0, 100, or 250 µL of α-MEM containing only antibiotics in addition to 100 µL of differentiation medium (α-MEM supplemented with 50 ng/mL soluble RANKL, 10% FBS, and 1% antibiotics) were added to each well. When the total volume of medium in the well was 100, 200, or 350 µL, cells that settled on the bottom of the culture plate were exposed to approximately 0.3, 0.6, or 1.1 g/cm² compressive force, respectively, for the same total amounts of RANKL and FBS (Figure 1A). A previous study reported that RANKL is indispensable for differentiation of RAW264.7 cells into TRAP-positive osteoclast-like cells, whereas M-CSF is not required for osteoclastogenesis in in vitro study using this cell line [17]. The medium was replaced every other day. TRAP staining was conducted as previously described [16,18,19]; then, the formation of osteoclast-like cells was observed by light microscopy.

Actin ring observation

Cells were seeded onto glass coverslips placed at the bottom of culture plates, and then were stimulated with compressive force generated by the aforementioned procedure for 4 days. Cells were fixed and permeabilized by the same method as in the previous study [18] using 4% (v/v) paraformaldehyde/2% sucrose and 0.1% Triton X-100, respectively. Actin filaments and nuclei of cells were labeled with two fluorescent dyes, 66 nM Alexa Fluor 488-phallolidin for actin and DAPI for nucleus, after blocking nonspecific binding of the fluorescent dyes by 1% bovine serum albumin. Pictures of actin filaments and nuclei were separately obtained through fluorescence microscope BZ 9000 (Keyence, Osaka, Japan) and merged on a conventional personal computer.

Quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA including mRNA was eluted from RAW264.7 cells harvested after stimulating with compressive force for 3 or 4 days using NucleoSpin RNA. cDNA synthesis was carried out with 1 µg RNA in 20 µL of a solution containing random primers, dNTP mixture, and reverse transcriptase, which were bundled with PrimeScript; 2 µL aliquots of the cDNA solution were subjected to quantitative real-time RT-PCR. Twenty-five microliters of the reaction solution contained 1×RPR buffer, 1.5 mM dNTP mixture, 1×SYBR green I, 15 mM NgCl₂, 0.25 unit of Ex Taq polymerase (these components were bundled with SYBR Premix Ex Taq solution), and 20 µM forward and reverse primers. Primers were designed against mouse sequences as follows: carbonic anhydrase II (forward: CATTACTGCTACGACCGAGCA, reverse: GAGGCCAGTTGTCCACCCATC), matrix metalloproteinase-9 (forward: GCCCGTAACTCAGACGCAGA, reverse: TTGGAGGCGTACACGCGAAG), cathepsin K (forward: CAGCAGACTGACTGACGACA, reverse: GACGCCAGTGATTGTTAC), matrix metalloproteinase-9 (forward: AAATGGTGAGGCGTGGTGTGG, reverse: TGAAGGGGTCTCG TGATGG) [18,19]. The reaction conditions such as temperature for denaturation, annealing, and extension were the same as those for the previous study [18,19]. Results were analyzed using Smart Cycler software (Cepheid, Sunnyvale, CA, USA). The specificity of the PCR products was verified by a melting curve analysis. The expression levels of carbonic anhydrase II, matrix metalloproteinase-9, and cathepsin K were normalized to those of GAPDH.

Western blotting

Supernatant containing intracellular protein was prepared by the same method described in previous reports [18,19]. Protein (20 µg) was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After blocking of nonspecific binding using 5% skim milk solution, the membranes were incubated with primary antibodies against carbonic anhydrase II, matrix metalloproteinase-9, cathepsin K, and β-tubulin. Then, biotin-conjugated secondary antibodies were added and incubated. Membranes were labeled with streptavidin-horseradish peroxidase and visualized using ECL chemiluminescence. Western blot intensities were quantified using digital image analysis software (Quantity One; Bio-Rad Laboratories).

Mineral resorption activity and pit formation assay

RAW264.7 cells were placed on a fluoresceinated calcium phosphate-coated plate (Bone Resorption Assay Kit 48). Then, cells were continuously stimulated with compressive force generated by increasing the volume of phenol red-free α-MEM/F-12. After 4 days of culture, the amount of calcium eluted from the calcium phosphate plate into the culture medium was evaluated based on fluorescence intensity (wavelength of 485 nm for excitation and of 535 nm for emission). For the observation of the resorption pit, the plates were removed with 5% NaClO. The pit area was observed by light microscopy.

Statistical analysis

Mean differences were tested by ANOVA and Tukey’s multiple comparison tests. Results with P < 0.05 were considered statistically significant.

Results

Effect of continuous compressive force on actin ring organization

We first investigated actin ring organization in cells stimulated with continuous compressive force in the presence of RANKL.
Figure 1. Schema of compressive force generated by increasing the volume of culture medium and effect of compressive force on actin ring organization and TRAP staining. White arrow indicates compressive force (A). Cells were continuously stimulated with 0.3, 0.6, or 1.1 g/cm² compressive force in the presence of 5 ng of RANKL for 4 days. Actin labeled with fluorescently tagged phalloidin (green) and nuclei labeled with DAPI (blue) were observed with a fluorescence microscope (B). The cells were stimulated with 0.3, 0.6, or 1.1 g/cm² compressive force for 4 days, and then stained with TRAP and observed by light microscopy (C). Scale bar=100 μm.
Multinucleated osteoclast-like cells with actin rings were observed for all three magnitudes of compressive force, and the area of actin rings increased as a function of the applied force (Figure 1B). TRAP staining also revealed that the area of each osteoclast-like cell increased as a function of applied compressive force (Figure 1C).

Effects of continuous compressive force on carbonic anhydrase II, matrix metalloproteinase-9, and cathepsin K expression

We next examined the effects of continuous compressive force on the mRNA expression of genes encoding carbonic anhydrase II, matrix metalloproteinase-9, and cathepsin K, which are osteoclastic bone resorption-related enzymes, by real-time PCR. Figure 2 shows that carbonic anhydrase II expression was significantly higher, whereas matrix metalloproteinase-9 expression was significantly lower in cells stimulated with 0.6 and/or 1.1 g/cm² than in cells stimulated with 0.3 g/cm² on days 3 and 4 of culture (Figure 2A, 2B). The effect of compressive force on cathepsin K expression was observed in cells only on day 4; cathepsin K expression was slightly higher in cells stimulated with 0.6 and 1.1 g/cm² than in cells stimulated with 0.3 g/cm², but the difference was only significant for the comparison between 0.3 g/cm² and 1.1 g/cm² (Figure 2C). The degree of change induced by compressive force was markedly higher for carbonic anhydrase II than for cathepsin K and matrix metalloproteinase-9; carbonic anhydrase II expression changed by 1.78–2.28-fold, cathepsin K expression changed by 0.98–1.35-fold, and matrix metalloproteinase-9 expression changed by 0.62–0.91-fold in cells stimulated with 0.6 and 1.1 g/cm² as compared to 0.3 g/cm². Subsequently, we

![Figure 2](image-url)

**Figure 2.** Effect of compressive force on mRNA levels of osteoclastic bone resorption-related enzymes in RAW264.7 cells. The mRNA expression levels of carbonic anhydrase II (A), matrix metalloproteinase-9 (B), and cathepsin K (C) were determined by real-time RT-PCR on days 3 and 4 of culture. Bars indicate the means ± standard deviation of 4 independent experiments.

* P<0.05, ** P<0.01, *** P<0.001 (vs. 0.3 g/cm²), ## P<0.01 (0.6 vs. 1.1 g/cm²).
examined the effects of compressive force on the protein expression of carbonic anhydrase II, matrix metalloproteinase-9, and cathepsin K by western blotting. On day 4 of culture, the protein expression of carbonic anhydrase II and cathepsin K was increased, whereas that of matrix metalloproteinase-9 was decreased, by a compressive force of 1.1 g/cm² compared to that of 0.3 g/cm² (Figure 3). Measurement of western blot intensities revealed that the degree of compressive force-induced change was markedly higher for carbonic anhydrase II (7.0-fold) than for cathepsin K (2.0-fold) and matrix metalloproteinase-9 (0.9-fold) in cells stimulated with 1.1 g/cm² as compared to 0.3 g/cm².

**Effect of continuous compressive force on mineral resorption activity**

To further examine whether actin ring formation and the elevated expression of carbonic anhydrase II expression affected bone resorptive function, cells on calcium phosphate plates were stimulated with continuous compressive force. After 4 days of culture, restorative pits of various sizes were observed for all three magnitudes of compressive force (Figure 4A). Fluorescence intensity in the culture medium was significantly higher for cells stimulated with 0.6 or 1.1 g/cm² compressive force than for cells stimulated with 0.3 g/cm² compressive force and was significantly higher for cells stimulated with 1.1 g/cm² compressive force than for cells stimulated with 0.6 g/cm² compressive force (Figure 4B), suggesting that the amount of calcium eluted from the calcium phosphate plate increased as a function of the applied compressive force.

The microenvironment established by the acidic conditions of the actin ring is also important for dissolving the inorganic and organic components of bone. Carbonic anhydrase II converts CO₂ into H⁺ and HCO₃⁻; then, vacuolar H⁺-ATPase transports H⁺ to the resorptive zone [22,23]. Increasing H⁺ induces the mobilization of mineralized components of bone; subsequently, the demineralized organic matrix is degraded by proteinases [21]. In the present study, carbonic anhydrase II expression was markedly elevated by compressive force. Moreover, calcium elution from the calcium phosphate plate increased after the application of compressive force for 4 days. These results suggested that continuous compressive force facilitates the dissolution of the inorganic component of bone via the upregulation of carbonic anhydrase II expression in mature osteoclasts.

**Discussion**

Our results indicated that decomposition of the inorganic phase of bone was promoted in osteoclast-like multinucleated cells induced by continuous compressive force during RANKL-induced osteoclastogenesis. The formation of an actin ring that surrounds and isolates the microenvironment of osteoclasts is considered a hallmark of the degradative capacity of osteoclasts because this closed ring contributes to the maintenance of effector molecules, such as hydrogen chloride and proteinases [20,21]. We previously observed TRAP-positive multinucleated cells with a large area when increasing force was applied to RAW264.7 cells in the presence RANKL [16]. In the present study, actin ring organization was observed in the multinucleated giant cells formed by the continuous stimulation of RAW264.7 cells with compressive force following the same methods and conditions used in our previous study. These results suggested that the multinucleated giant cells could create a resorptive microenvironment.

Cathepsin K and matrix metalloproteinase-9 produced by mature osteoclasts efficiently cleave type I collagen, which is abundant in the organic phase of bone [21,24,25]. In the present study, matrix metalloproteinase-9 expression decreased, whereas cathepsin K expression increased slightly by the continuous application of compressive force. This variation in the effect of continuous compressive force on proteinase expression in osteoclasts might be involved in the difference between pathologic bone resorption and physiological bone remodeling. Rheumatoid arthritis and periodontitis lead to bone destruction in articular tissue or alveolar bone. Previous studies have indicated that inflammatory cytokines, which are elevated in rheumatoid arthritis and periodontitis, or lipopolysaccharides, the cell wall component of gram-negative rods (including periodontal pathogens, such as *Porphyromonas gingivalis*), strongly induce the expression of cathepsin K and matrix metalloproteinase-9 as well as carbonic anhydrase II [26–30]. Mitogen-activated protein kinases
(MAPKs), including extracellular signal-regulated kinase (ERK), p38, and Jun-N-terminal kinase, play important roles in the protease expression of osteoclasts [31]. Two previous studies using a specific inhibitor of each kinase revealed that the effects of these kinases on the expression of matrix metalloproteinase-9 and cathepsin K varied by the type of stimulation [32,33]. Kim and Lee [32] reported that upregulation of matrix metalloproteinase-9 expression was attenuated only by ERK inhibitor in tumor necrosis factor-α-stimulated osteoclasts. In contrast, Matsumoto et al. [33] revealed that RANKL-induced cathepsin K expression was inhibited by p38 but not ERK inhibitor. The effects of mechanical stress on the expression of matrix metalloproteinase-9 and cathepsin K may also depend on MAPK signals; however, the effects of mechanical stimulation on protease expression in osteoclasts remain controversial. Hayakawa et al. [15] previously revealed that the expression of both cathepsin K and matrix metalloproteinase-9 in RAW264.7 cells pre-incubated with RANKL was increased by stimulation with compressive force (~0.3 g/cm²). Xu et al. [34] reported that the effects of mechanical strain on the expression of matrix metalloproteinase-9 and cathepsin K differed, with mechanical strains of 2000 and 2500 με inducing matrix metalloproteinase-9 expression, while cathepsin K expression was not affected by mechanical strain of this magnitude. Mechanical stimulation from physical activity, exercise, and adaptive stress contributes to bone tissue homeostasis [4,5]. Our present results and these previous findings suggest that decomposition of the organic phase of bone might be less aggressive in physiological bone remodeling than in pathologic bone resorption. Further studies are required to clarify the differences in intracellular signal transductions that are involved in the expression of proteases in osteoclasts between inflammatory cytokines and mechanical stimuli.

**Conclusions**

Our study demonstrated that the stimulation of RAW264.7 cells with continuous compressive force in the presence of RANKL induces the formation of polynuclear giant cells with actin rings and carbonic anhydrase expression. Moreover, these osteoclast-like cells have a high capacity for decalcification, suggesting that continuous compressive force contributes to the promotion of bone resorption in bone remodeling.
Conflicts of interest.

None.

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