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Effects of continuous and released compressive force on osteoclastogenesis *in vitro*

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ABSTRACT

Objective: Compressive force has been found to be catabolic to alveolar bone during orthodontic tooth movement. This study quantified the fusion of mononuclear RAW 264.7 cells (a murine osteoclastic-like cell line) into multinucleated osteoclasts under a hydrostatic pressure-generated mechanical compression-the new model of various magnitudes and durations.

Methods: RAW 264.7 cells were subjected to 0.3, 0.6 or 0.9 g/cm^2 of compressive force by an acrylic cylinder custom-made by laser cutting or no compressive force for 4 days during osteoclastogenic induction. TRAP-positive multinucleated cells were quantified. For the release from force experiment, osteoclastogenesis was induced by 0.6 g/cm^2 mechanical stimuli for 0, 1, 2, 3 or 4 days. Cell viability, TRAP-positive multinucleated cells, *DCSTAMP* and Cathepsin K (*CTSK*) gene expression were evaluated 4 days after release from force.

Results: Compressive force at 0.6 and 0.9 g/cm^2 significantly increase the number of TRAP-positive multinucleated cells ($P < 0.05$). Release from continuous mechanical compression after 4 days significantly elevated the number of TRAP-positive multinucleated cells and *DCSTAMP* and *CTSK* mRNA expression, with no adverse effects on cell viability ($P < 0.05$).

Conclusions: Continuous stimulation with compressive force induced osteoclastogenesis in RAW 264.7 cells by enhancing *DCSTAMP* and *CTSK* expression, which provides new understanding of bone remodeling during orthodontic treatment.

1. Introduction

Osteoclasts are primarily responsible for bone resorption during the bone remodeling process. Osteoclast precursors undergo multiple processes including proliferation, differentiation and fusion into multinucleated mature osteoclasts, which eliminate mineralized bone in response to the application of force.[1] The initiation of osteoclastogenesis is significantly influenced by Receptor activator of nuclear factor-kappa B ligand (RANKL). When RANKL binds to its receptor, receptor activator of nuclear factor-kappa B (RANK), it triggers the activation of various molecules that are crucial for osteoclast maturation and function.[2]

Dendritic cell-specific transmembrane protein (*DCSTAMP*) is a gene closely associated with osteoclasts involved in preosteoclast differentiation and a key factor required for the fusion of mononuclear osteoclasts.[3] The osteoclasts of *DCSTAMP* knockout mice are exclusively mononuclear TRAP-positive cells without evidence of fusion.[4] Despite the fact that DCSTAMP is involved in the modulation of osteoblastic activity, this gene is not expressed in osteoblasts.[5] Cathepsin K (CTSK) is a highly potent lysosomal cysteine protease primarily secreted by mature osteoclasts, which plays a significant role in the degradation of collagen and matrix proteins during bone resorption.[6] Additionally, CTSK can be detected in a variety of bone and non-bone cells.

Mechanical stimulation can enhance the expression of CTSK in both osteoblasts and osteocytes, and may help to maintain bone homeostasis.[7] Furthermore, the upregulation of osteoclast-associated genes such as *RANK*, *DCSTAMP* and *CTSK* in response to compressive stress in RAW264.7 cells led to a rapid increase in the number of osteoclasts *in vitro*.[8]

Thus, compressive force directly affects the regulation of orthodontic tooth movement by triggering the biological cascade that induces

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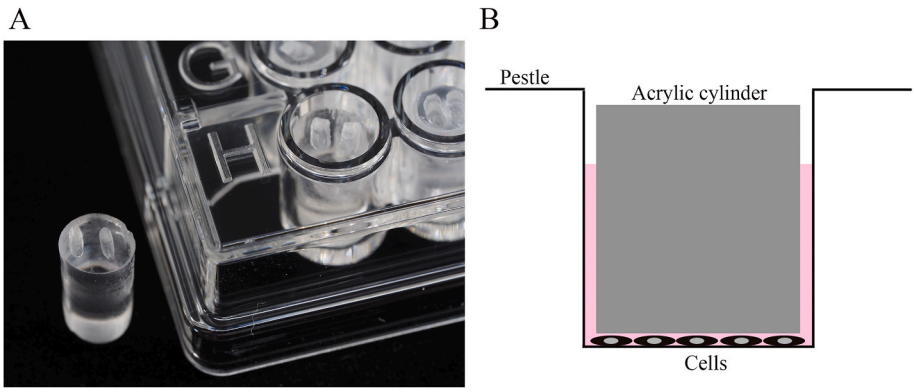

Fig. 1. Acrylic cylinders were laser-cut and placed into 96-well plates to apply compressive force. A: Photo of an acrylic cylinder; B: illustration of the hydrostatic pressure-generated compressive model.

osteoclastogenesis. *In vitro* studies have demonstrated the induction of osteoclast differentiation in response to generation of various types of compressive force, varied cellular responses have been observed in these models.[8–12] Weight loading is simple for mechanical loading model but physical cell damage and nutrient restriction from direct contact between weight-generating materials and cells are limitations. In order to prevent direct contact and assure the constant force distribution on the cell culture, an air-pump compressor to create air pressure is suggested. Anyway, physiologic leakage of fluid from cells undergo compressive force cannot be occurred in this method.[13–15] Additionally, applying the same force characteristics in different mechanical loading models can result in varied cellular functions.[16]

Different types of mechanical forces or loading regimens may lead to varied responses in osteoclast formation and function. A single study found that increasing the number of coverslips covering cells cultured on collagen gel at 0.3 g/cm^2 increased osteoclastogenesis.[8] It's probable that the tightly sealed environment under the coverslip has less level of both nutrients and oxygen. Another study exert force by increasing the amount of culture medium to osteoclast precursor cells.[9] It is possible that the increased of nutrients in the environments. In this study, to address the limitations of existing models of compressive force, we developed a novel hydrostatic pressure-generated compressive model based on pestles and cylinders. We examined the effects of both compressive stress and release from compressive stress on osteoclastogenesis in murine macrophage RAW 264.7 cells. *DCSTAMP* gene expression was analyzed to detect osteoclasts that have undergone cell-cell fusion. Expression of the *CTSK* and *DCSTAMP* genes and the numbers of TRAP-positive multinucleated cells (MNC), the typical characteristics of mature osteoclasts, were evaluated to identify the optimal magnitude and duration of compressive stress in this model.

2. Materials and methods

2.1. Cell culture and application of continuous compressive stress

RAW 264.7 cells (TIB-71TM; American Type Culture Collection, Manassas, VA, USA) were cultured in α-Minimal Essential Medium (α-MEM; Gibco BRL, Rockville, MD, USA) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% fungizone. The cells were incubated at 37 °C in humidified atmosphere with 5% CO_2. To induce osteoclast differentiation, cells were seeded into 96-well plates at 2.0 × 10^3 cells/well in media containing 50 ng/mL mouse recombinant RANKL and cultured overnight. The media was replaced every other day. Cells with a confluency level of 70–80% were exposed to a continuous compressive force at three different levels: 0.3 g/cm^2, 0.6 g/cm^2, and 0.9 g/cm^2 (total given force was 0.1027, 0.2058, 0.2780 gm, using a different weight of acrylic mass and calculated by formula P═F/A) or left without any applied force as a control and then harvested after a period of 4 days. Acrylic mass was created by laser-cutting. The diameter of acrylic mass is nearly the diameter of a 96-well plate (Fig. 1).

2.2. Osteoclastogenesis after release of compressive force

RAW 264.7 cells were cultured in 96-well plates with α-MEM supplemented with 50 ng/mL of mouse recombinant RANKL. These cells were then subjected to a compressive force of 0.6 g/cm^2; no force was used as a control. After loading for 1, 2, 3 or 4 days, the acrylic cylinders were removed to release the compressive force. The cells were harvested until day 4 and then each group was immediately quantified cell viability, the numbers of TRAP-positive multinucleated cells (MNC ≥3 nuclei), and mRNA expression.

2.3. Cell viability assay

PrestoBlue® Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA) was mixed with fresh culture medium at a ratio of 1:10. The resulting mixture was then incubated with cells for 1 h at 37 °C. After incubation, the absorbance values were determined at 600 nm to assess cell viability.

2.4. TRAP staining assay

Cells were subjected to staining using a TRAP staining kit (Takara Bio, USA) following the manufacturer's guidelines. TRAP-positive multinucleated cells (MNC) with three or more nuclei were then counted under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 10× objective. The counting process was performed by two individuals.

Table 1
Primers used for quantitative real-time polymerase chain reaction (qPCR).

Gene	Forward (5′-3′)	Reverse (5′-3′)	Accession number
CTSK	CAGCAGAACGGAGGCATTGA	CCTTTGCCGTGGCGTTATAC	NM_007802.4
DC-STAMP	CTAGCTGGCTGGACTTCATCC	TCATGCTGTCTAGGAGACCTC	NM_029422.4
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT	XM_003819132.3

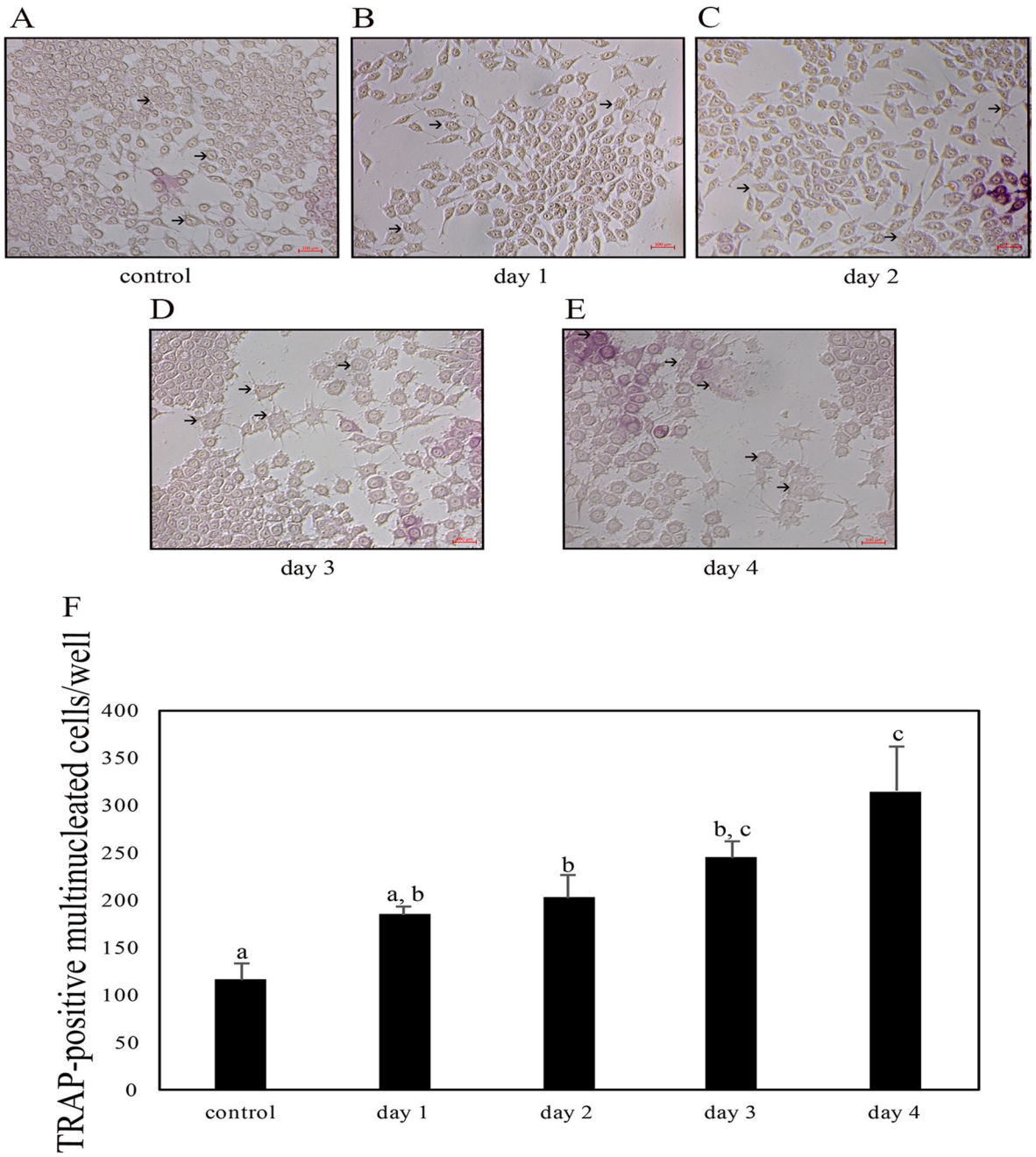

Fig. 2. Numbers of TRAP-positive MNC in RAW 264.7 cells released from continuous compressive force. Representative images of TRAP-staining of (A) control RAW 264.7 cells not exposed to compressive force and cells released from compressive force on (B) day 1, (C) day 2, (D) day 3, and (E) day 4. The cells were harvested 4 days after release from compressive force. Black arrows indicate TRAP-positive MNCs (magnification = 40×, bar = 20 μm). F: The numbers of TRAP-positive MNCs (≥3 nuclei) were significantly higher in cells exposed to compressive force for 3 or 4 days and then released. Data are representative of three independent experiments. All values are mean ± SD. Significant differences between groups are indicated by different letters (a, b and c; $P < 0.05$, $n = 3$, Tukey's HSD test).

2.5. RNA extraction and real-time polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using theInnuPREP DNA/RNA mini kits (Analytic-Jena, Jena, Germany), following the manufacturer's protocol. Each experiment was repeated at least three times. The extract total RNA was then converted into cDNA using Superscript III First Strand Synthesis System (Invitrogen). For cDNA synthesis, 0.5 μg of total RNA was utilized and employed for qRT-PCR analysis. *The gene expression levels of DCSTAMP and CTSK* were assessed, with glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* serving as the reference gene. The primer sequences used are listed in Table 1. The relative gene expression was analyzed using the$2^{-\Delta\Delta Cq}$ method.

3. Statistical analysis

The data are reported as mean standard deviation (SD), with each experiment independently performed at least three times. One-way ANOVA was applied to analyze the variances between the means, with either the Games-Howell test or the Tukey multiple comparisons test followed. The P-value was considered significant when it was less than 0.05 (P 0.05).

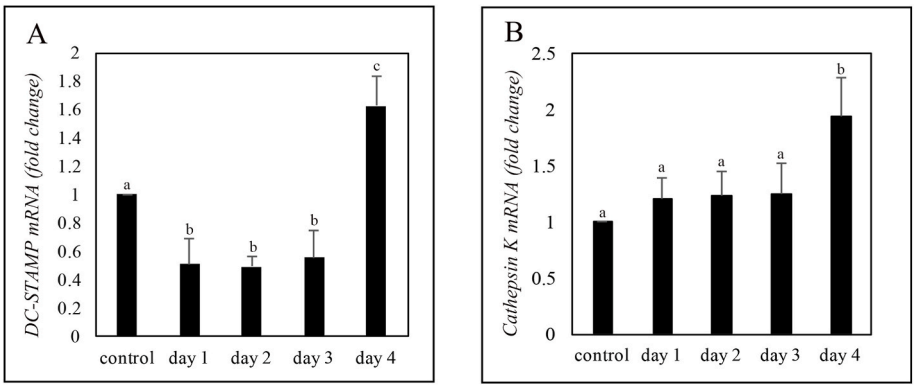

Fig. 3. *DCSTAMP* and *CTSK* mRNA expression in RAW 264.7 cells released from continuous compressive force. Cells were released from compressive force on (B) day 1, (C) day 2, (D) day 3, or (E) day 4 and harvested 4 days after release from compressive force. Control cells were not exposed to compressive force. All data are mean standard deviation of triplicate experiments. Significant differences between groups are indicated by different letters (a, b and c; $P < 0.05$, $n = 3$, Games-Howell test).

4. Results

4.1. Continuous compressive force stimulates the differentiation of RAW 264.7 cells into osteoclasts

Significant elevations of the numbers of TRAP-positive MNC were observed after stimulation with compressive force of 0.3, 0.6 and 0.9 g/cm^2, in compared to the control group of unstimulated cells. Cells stimulated with a compressive force of 0.3 g/cm2 exhibited a significantly lower number of TRAP-positive multinucleated cells (MNC) compared to cells stimulated with 0.6 or 0.9 g/cm2. However, no significant difference in the number of TRAP-positive MNC was observed between cells stimulated with 0.6 and 0.9 g/cm2 of continuous compressive force.

4.2. Released compressive force affects osteoclast differentiation and expression of osteoclastogenic genes

Released compressive force by removal of acrylic cylinders on day 1 and 2 led to significantly lower numbers of TRAP-positive MNC compared to cells released on day 4. No significant differences were observed between cells released from force on days 1, 2 and 3; the number of osteoclasts was lower for cells released on day 3 than day 4, though this difference was not significant. On days 2, 3, and 4, the removal of compressive force resulted in increased numbers of TRAP-positive multinucleated cells (MNC) compared to control cells that were not subjected to any stress (as shown in Fig. 2). There were no significant differences in cell viability between cells released from force on days 1, 2, 3, or 4, and the control cells that were not exposed to compressive force.

DCSTAMP mRNA expression was significantly upregulated in cells released from compressive force on day 4 (Fig. 3A). *DCSTAMP* expression was significantly lower in cells released from force on day 1, 2 or 3 than control cells that were not exposed to compressive force. Cells released from compressive force on day 4 expressed significantly higher levels of *CTSK* compared to control cells (as shown in Fig. 3B). There was no significant difference in *CTSK* mRNA expression between cells released from force on days 1, 2 or 3 and control cells that were not exposed to compressive force.

5. Discussion

Several *in vitro* applications of mechanical stimuli have been proposed to simulate orthodontic force; however, these models have several limitations.[17–21] To address this issue, we fabricated laser-cut acrylic cylinders that precisely fit into 96 wells to apply hydrostatic pressure-generated compressive force loading, without directly contacting the cells. The culture media is displaced by the pestles, which mimics fluid leakage from tissues under physiological conditions. The pestle and cylinder models exert a one-dimensional compressive force on the cells, without introducing tension, bending, or shear forces.[16] As shown in Fig. 1, exposure to mechanical compression for 1–4 days did not have any significant effect on cell viability, which suggests that the culture media in the pestle and cylinder model provides an adequate supply of nutrients and oxygen to the cells. In addition to responding to changes in oxygen tension, hematopoietic stem cells can also respond to mechanical stimuli via molecules or structures involved in cellular mechanosensors.[22] However, the mechanisms underlying cellular sensing remain unclear and the response of RAW 264.7 cells to mechanical stimuli remains to be elucidated.

In our pestle and cylinder model, stimulation of RAW 264.7 cells with 0.6 g/cm^2 continuous compressive force resulted in a significant increase in the number of TRAP-positive multinucleated cells (MNC). Stimulation with 0.9 g/cm^2 compressive force did not lead to any further increase in the number of TRAP-positive multinucleated cells (MNC). Therefore, 0.6 g/cm^2 compressive force was defined as the optimal magnitude of compressive force and was selected for the force release experiments.

Exposure to 4 days of compressive force prior to release from the force led to higher numbers of TRAP-positive MNC compared to precursor cells that were released from compressive force on days 1, 2 or 3. This finding was consistent with our observation of the highest levels of *DCSTAMP* expression in RAW 264.7 cells occurring after 4 days of stimulation with compressive force. Moreover, early release from compressive force decreased osteoclast formation. These results indicate that exposure to a longer duration of compressive stress rises the cell's number undergoing osteoclastogenesis, indicating that the cell-cell fusion process is dependent on time. In their study, Ikeda et al. (2016) examined the impact of releasing RAW 264.7 cells from optimal compressive force on the expression of the nuclear factor of activated T cell cytoplasmic 1 (NFATc1) gene., and found *NFATc1* mRNA expression was downregulated in the first 6 h after force cessation.[23] Following stimulation of RANKL, *NFATc1* plays a significant role as a regulator of *DCSTAMP* expression.[3] Further study of *NFATc1* expression using the pestle and cylinder model may help to explain the mechanisms that inhibit *DCSTAMP* expression in RAW 264.7 cells released from compressive force.

Released compressive force also influencd the expression of *CTSK*. Secretion of the enzyme CTSK indicates that osteoclasts are mature, well differentiated and have the capability to resorb bone.[24] In this study, TRAP-positive cells with three or more nuclei were defined as mature osteoclasts. The decrease in level of *CTSK* expression on each day after release from compressive force was similar to the reduce in the number of TRAP-positive multinucleated cells (MNC). This data further suggests

that released optimum compressive stress suffer the maturation and function of osteoclasts.

The duration of force activation is a crucial factor in orthodontic tooth movement and root resorption. A rest period in force loading allows for healing and repair of the cementum to occur.[25,26] However, there is limited knowledge of the variations in the rate of tooth movement and root resorption when different types of force are applied. The limitation of this study is that a variety of factors affect cellular responses, and the responses in vivo to mechanical stimulation are more complicated compared to the responses observed *in vitro*. Consequently, conducting longer-term in vivo studies becomes necessary to delve deeper into the underlying mechanisms that govern cellular responses to mechanical stimulation. Applying constant compressive force during orthodontic treatment is crucial. We suspect the inhibition of osteoclast activity that occurs after the release of the optimum compressive force contributes to the reduction of tooth movement in the bone. Thus, the pestle and cylinder model presented in this study provides provide a foundation for conducting further research on the effects of continuous exposure and release from compressive force on osteoclast activity.

6. Conclusions

Application of continuous compressive force using a modified acrylic mass has no adverse effect on cell viability. In this novel pestle and cylinder model, 0.6 g/cm^2 represents the optimum continuous compressive force for osteoclastogenesis in RAW 264.7 cells. Compressive force increases the number of TRAP-positive multinucleated cells (MNC) over time, with at least 4 days' exposure to continuous compressive force required to promote differentiation into mature osteoclasts.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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