Differential effects of mechanical strain on osteoclastogenesis and osteoclast-related gene expression in RAW264.7 cells

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Abstract. Mechanical strain plays a critical role in the formation, proliferation and maturation of bone cells. However, little is known about the direct effects of different magnitudes of mechanical strain on osteoclast differentiation. The aim of the present study was to investigate how the fusion and activation of osteoclasts can be regulated by mechanical strain magnitude using the RAW264.7 mouse monocyte/macrophage cell line as an osteoclast precursor. Mechanical strain (substrate stretching) was applied via a 4-point bending system when RAW cells were treated with macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (RANK) ligand (RANKL) for an indicated period of time. The numbers of tartrate-resistant acid phosphatase-positive ($TRAP^+$) and apoptotic cells were counted. The expression of TRAP, matrix metalloproteinase-9 (MMP-9), RANK, cathepsin K and carbonic anhydrase II (CAII) was measured by semi-quantitative RT-PCR, and immunocytochemistry staining for RANK was performed. We found that the number of nuclei per osteoclast derived from RAW cells decreased under low magnitude mechanical strain and increased under high magnitude strain within physiological load with an enhanced fusion of $TRAP^+$ osteoclasts, compared to the control with no mechanical strain. The expression of RANK mRNA was downregulated by low magnitude strain and beyond physiological load, while it was upregulated by high magnitude strain within physiological load, correlating with the increased expression of RANK examined by immunocytochemistry, suggesting the mechanical regulation of RANK expression. There was also an increase in the expression of MMP-9 mRNA in the groups subjected to a mechanical strain of 2,000 and 2,500 $\mu\varepsilon$. No significant differences were detected in the expression of TRAP mRNA, cathepsin K and CAII under mechanical strain compared to the control under no strain (0 $\mu\varepsilon$). These findings indicate that low-magnitude strain suppresses osteoclast fusion and activation, while high-magnitude strain within physiological load promotes osteoclast fusion and activation related to a mechanical magnitude-dependent response of RANK expression. These data, therefore, provide a deeper understanding of how different magnitudes of mechanical strains exert their effects on osteoclastogenesis.

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Introduction

Bone is a dynamic tissue that constantly undergoes remodeling in which a coupled process of bone formation and resorption continues throughout life. This remodeling is necessary to maintain the structural integrity of the skeleton under conditions of changing mechanical forces. Over the past few decades, investigators have expounded mechanisms for the adaptive response of bone to mechanical stimuli, including the most well-known theory of mechanostat originally proposed by Frost (1,2). It has been reported that disuse activates remodeling, but inhibits modeling, leading to bone loss, whereas overload inhibits remodeling and activates formation mode modeling, leading to bone gain. Furthermore, strain above 1,500 $\mu\varepsilon$ evokes bone increase (a positive adaptive response) and a strain below 100 $\mu\varepsilon$ causes a loss of bone (a negative adaptive response), while a strain ranging from 100 to 1,500 $\mu\varepsilon$ evokes no response. Bone tissue functionally adjusts its mass and architecture to mechanical stimuli, mainly depending on 2 cell types involved in remodeling, one of which is osteoblasts, engaged in bone formation, and the other is osteoclasts, mainly responsible for bone resorption. Excessive osteoclast bone resorption leads to bone loss resulting in skeletal pathologies, such as rheumatoid arthritis, periodontal disease, postmenopausal osteoporosis, implant osteolysis and tumor-associated bone loss (3). However, the understanding of the cellular mechanisms producing such a mechanically meaningful structure remains poor.

The osteoclast is a macrophage polykaryon developed from the differentiation and fusion of hematopoietic precursors at or near the bone surface in response to the essential tumor necrosis factor (TNF) family-related signal molecule receptor activator of NF-κB (RANK) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (4,5). RANKL directly engages a membrane receptor, RANK, on osteoclast precursors and mature osteoclasts to trigger multiple intracellular signaling cascades that stimulate osteoclast gene expression, development, function and survival (6). Mice deficient in RANKL or RANK have severe osteopetrosis due to osteoclastogenesis (7-10).

Bone tissue is sensitive to mechanical strain. In a previous study, in the establishment of a mechanobiology model of bone and functional adaptation, the ulna was subjected to peak strains of 2,000 and 3,000 $\mu\varepsilon$, suggesting a dose-dependent adaptation of bone to mechanical stimuli (11). However, to date, little is known about the mechanisms invovled in the dose-response relationship between mechanical stimuli and osteoclasts *in vitro*. Therefore, in the present study, the cells were stretched with cyclic predominantly uniaxial strain by substrate movement along a given axis in order to mimic the mechanical stimuli within or beyond physiological load. Strain magnitudes ranging from 0 to 5,000 $\mu\varepsilon$ were applied over a period of 3 days at a constant cycle number and frequency to explore the effect of mechanical strain magnitude on osteoclast fusion and activation.

Materials and methods

Cell culture. The RAW264.7 murine monocyte/macrophage cell line (obtained from the School of Basic Medicine of the Peking Union Medical College, Beijing, China) was used as an osteoclast precursor, that has been shown to differentiate into osteoclast-like cells in the presence of M-CSF and soluble RANKL (PeproTech Inc., Rocky Hill, NJ, USA) over a period of 4-5 days (12-14). The cells were cultured in α-minimal essential medium (α-MEM; Gibco BRL, Rockville, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco), 1% (v/v) penicillin-streptomycin solution (Gibco), 10 mM HEPES at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. For treatment with mechanical stretching, the cells were seeded onto 34.8-mm cell culture plates at a density of $5x10^6$ cells/cm^2. After overnight incubation, the cells were maintained in α-MEM containing 10% FBS, 1% (v/v) penicillin-streptomycin solution, 10 mM HEPES, M-CSF (40 ng/ml) and RANKL (40 ng/ml) for 7 days. The medium was changed every 3 days.

Application of mechanical strain to cultured cells. A 4-point bending system (invented by the Institute of Medical Equipment, Academy of Military Medical Science, Tianjin, China), composed of a cell culture unit, loading unit and circuit controller, was used to apply uniaxial and homogeneous mechanical strain, as described previously (Fig. 1) (15). Strain magnitudes (substrate stretching) ranged from 1,000 to 5,000 $\mu\varepsilon$, with a strain frequency fixed at 0.5 Hz. After treatment with RANKL and M-CSF for 3 days, the cells were divided into 6 groups (0, 1,000, 1,500, 2,000, 2,500 and 5,000 $\mu\varepsilon$) at random, and subjected to substrate stretching for 3 days for 1 h per day, keeping the original culture condition unchanged (16). After 3 days of substrate stretching, the cells were harvested for the following experiments. The control culture was grown under the same condition without mechanical strain.

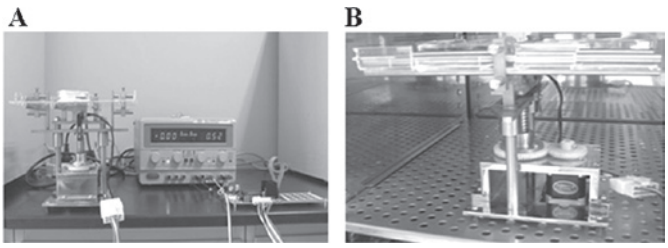

Figure 1. The principle of the mechanical strain (substrate streching) device composed of a cell culture unit, loading unit and circuit controller, supplying uniaxial and homogeneous mechanical strain. (A) Mechanical strain (substrate streching) system. (B) Cell culture unit and loading unit of the mechanical strain (substrate streching) device running in the sterile incubator.

Morphological observation and tartrate-resistant acid phosphatase (TRAP) staining. After substrate stretching, the cells from each group were washed twice with phosphate-buffered saline (PBS) and stained with TRAP, using a leukocyte acid phosphatase kit (Institute of Hematology and Blood Diseases Hospital Chinese Academy of Medical Sciences, Tianjin, China). The $TRAP^+$ multinucleated cells (≥5 nuclei) in 3 representative fields were manually enumerated as osteoclasts under a microscope.

Immunocytochemistry. The cells were washed 3 times with PBS after substrate stretching and then fixed in 4% (v/v) paraformaldehyde. Permeabilization was performed with 0.2% (v/v) Triton X-100 (Sigma Aldrich Chemical Co., St. Louis, MO, USA), then the cells were incubated in 3% H_2O_2 to quench the endogenous peroxidase activity. After washing with PBS, the samples were incubated with anti-RANK polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; working dilution, 1:200) at 4˚C overnight using the peroxidase-conjugated mouse IgG SABC kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China). After the peroxidase detection, cells were washed with PBS 4 times using the DAB Chromogenic kit (Wuhan Boster Biological Technology, Ltd.). Seal slide with balsam neutral and average optical density of RANK was calculated. The control samples were incubated in the same way with 0.01 M PBS alone, instead of primary antibody.

Apoptosis of osteoclasts. The cells from each group were washed twice with PBS and then fixed in 4% (v/v) paraformaldehyde. The fixed cells were stained with 1 μg/ml DAPI for 20 min at room temperature. After washing with PBS, the nuclear morphology of the cells was observed by fluorescence microscopy. Triplicate samples were prepared for each group and cells with condensed nuclei were counted in a selected field of each sample.

RT-PCR. The cells from each group were harvested as described above followed by RNA extraction using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized with 3 μg of total RNA, oligo(dT) primer and

Table I. Nucleotide sequences of the used primers.

Gene	GeneBank™ accession no.	Product size (bp)	Temperature cycling (cycle no.)	Forward (F) and reverse (R) primer sequences (5'-3')
TRAP	NM_007388	465	94˚C, 25 sec; 57˚C, 30 sec; 68˚C, 35 sec (32)	ACACAGTGATGCTGTGTGGCAACTC (F) CCAGAGGCTTCCACATATATGATGG (R)
MMP-9	NM_013599	354	94˚C, 25 sec; 64˚C, 30 sec; 68˚C, 35 sec (32)	CGAGTGGACGCGACCGTAGTTGG (F) CAGGCTTAGAGCCACGACCATACAG (R)
RANK	NM_009399	351	94˚C, 25 sec; 57˚C, 30 sec; 68˚C, 35 sec (32)	ACCTCCAGTCAGCAAGAAGT (F) TCACAGCCCTCAGAATCCAC (R)
CAII	NM_009801	407	94˚C, 25 sec; 54˚C, 30 sec; 68˚C, 35 sec (32)	CTTCAGGACAATGCAGTGC (F) ATCCAGGTCACACATTCCAGC (R)
Cath K	NM_007802	364	94˚C, 25 sec; 56˚C, 30 sec; 68˚C, 35 sec (32)	CTGAAGATGCTTTCCCATATGTGGG (F) GCAGGCGTTGTTCTTATTCCGAG (R)
β-actin	NM_007393	306	94˚C, 25 sec; 55˚C, 30 sec; 68˚C, 35 sec (32)	GAAGAGCTATGAGCTGCCTG (F) CACAGAGTACTTGCGCTCAG (R)

TRAP, tartrate-resistant acid phosphatase; MMP-9, matrix metalloproteinase-9; RANK, receptor activator of nuclear factor-κB; CAII, carbonic anhydrase II; Cath K, cathepsin K.

the TIANScript RT kit (Tiangen Biotech, Beijing, China). The following primers were used: RANK, TRAP, matrix metalloproteinase-9 (MMP-9), cathepsin K and carbonic anhydrase II (CAII). PCRs were conducted by initial denaturation at 94˚C for 3 min 30 sec, with 1 μl of reverse transcriptase product added. The sequences of the used primers are shown in Table I. PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, photographed using Gel-Doc (Bio-Rad) and quantified by density determination using Quantity One image analysis software (Bio-Rad). The results were normalized to β-actin signals determined in parallel for each sample, and the data expressed as a ratio of the target gene to β-actin. All amplicons were of the expected size (Table I), and products were directly sequenced to confirm identities by comparison with sequences published using computation performed at NCBI and the BLAST network service.

Statistical analysis. Data are presented as the means ± SE, typically from 2 to 3 independent trials, each with 3 replicates. Statistical comparisons between the treatment groups were performed using one-way analysis of variance. For simultaneous comparisons between multiple treatments, significant differences were determined using Bonferroni's post hoc analysis of variance test; $p<0.05$ denoted a statistically significant difference.

Results

Osteoclasts change morphologically and the number of TRAP-positive multinucleated osteoclasts vary depending on the magnitude of mechanical strain. Osteoclasts in culture with M-CSF and RANKL were observed with representative typical multinuclei. After substrate stretching, the number of TRAP$^+$ multinucleated osteoclasts significantly decreased in the groups subjected to a strain of 1,000 and 1,500 $\mu\varepsilon$ as compared to the control, whereas there was no significant difference in the groups subjected to a strain of 2,000 and 2,500 $\mu\varepsilon$ compared to the control (Figs. 2A and B, and 3A).

Expression of RANK increases under high mechanical strain within physiological load. RANK is the sole signaling receptor for RANKL in the process of inducing the development and activation of osteoclasts. In immunocytochemistry staining for RANK, the groups subjected to a strain of 2,000 and 2,500 $\mu\varepsilon$ showed stronger positive staining than the control after substrate stretching (Fig. 3B).

Osteoclast apoptosis is induced by substrate stretching of low magnitude. The number of apoptotic osteoclasts increased with the mechanical strain of 1,000 and 1,500 $\mu\varepsilon$ compared to the control, whereas no significant stimulation was observed in the groups subjected to a strain of 2,000, 2,500 and 5,000 $\mu\varepsilon$ (Fig. 4A and B).

Expression of RANK and MMP-9 genes differs depending on different magnitudes of mechanical strain. The expression of RANK mRNA increased in the groups subjected to a strain of 2,000 and 2,500 $\mu\varepsilon$, while it decreased in the groups subjected to a strain of 1,000, 1,500 and 5,000 $\mu\varepsilon$. The expression of MMP9 mRNA increased in the groups subjected to a strain of 2,000 and 2,500 $\mu\varepsilon$, while there were no significant differences in the groups subjected to a strain of 1,000, 1,500 and 5,000 $\mu\varepsilon$. There were no significant differences in the expression of TRAP, cathepsin K and CAII mRNA under mechanical strain compared to the control (Fig. 5).

Discussion

Since the pioneering work of Frost, it has become generally accepted that bone tissue maintains its structure throughout

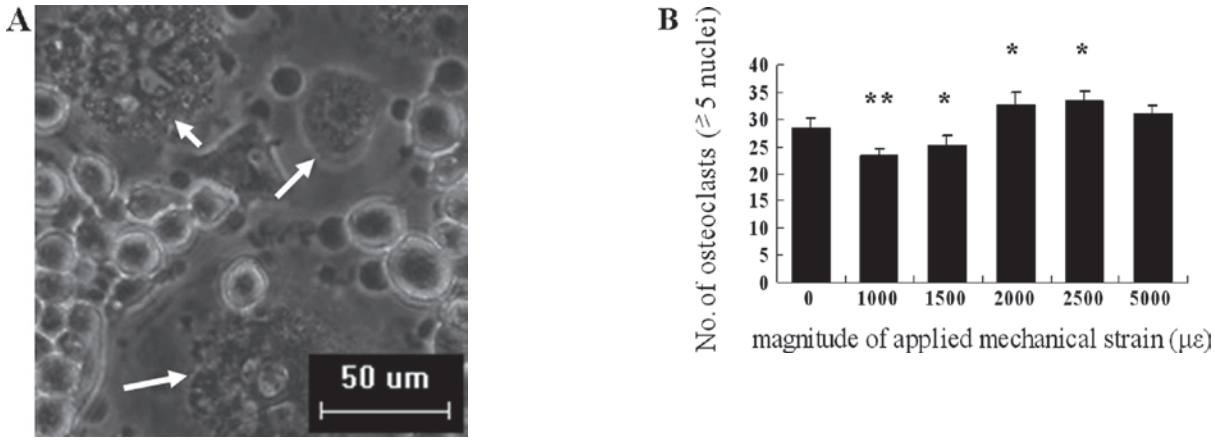

Figure 2. RAW264.7 cells ($5x10^6/cm^2$) were cultured in 34.8-mm tissue culture plates with macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). Mechanical strain of different magnitudes was applied for 1 h/day for 3 days and at day 4, but not to the control, keeping the culture condition unchanged. (A) Typical multinucleated cells without substate streching. (B) The number of neclei/cell varied depending on the magnitude of mechanical strain, osteoclasts with ≥5 nuclei were counted at day 7. Results are presented as the means ± SD (n=6). $^{*}p<0.05$, $^{**}p<0.01$.

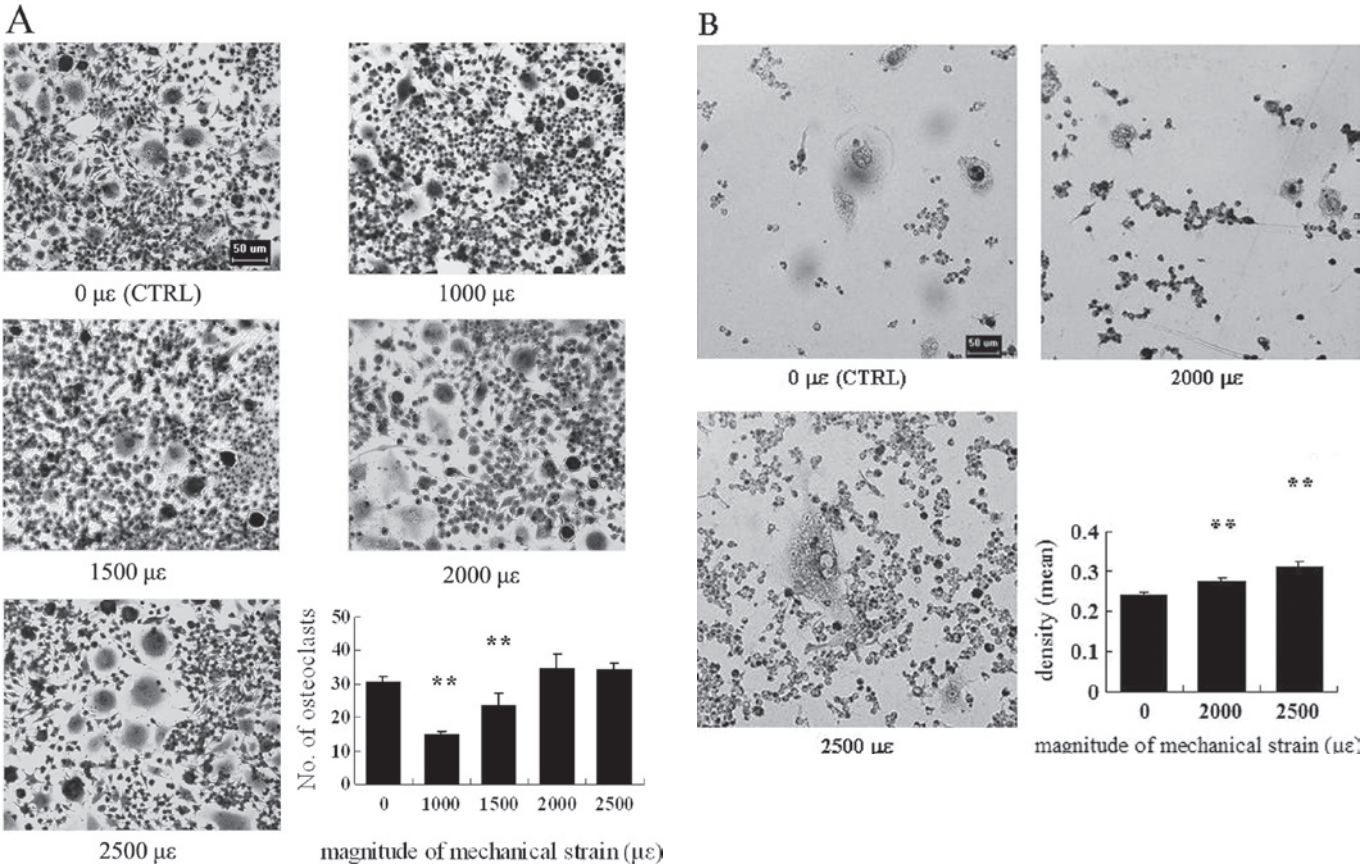

Figure 3. The number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts and receptor activator of nuclear factor-κB (RANK) expression varied depending on the magnitude of mechanical strain. RAW cells ($5x10^6/cm^2$) were cultured in 34.8-mm tissue culture plates for 3 days, followed by the cells that were subjected to substrate mechanical stretching at different magnitudes for another 3 days, keeping the culture condition unchanged. After 6 days in culture, immunocytochemistry was performed. (A) $TRAP^+$ multinucleated cells were counted in 3 representative fields. (B) Optical density (mean) of RANK was calculated. Results are presented as the means ± SD (n=6). $^{**}p<0.01$.

life by the coupled activities of bone-forming osteoblasts and bone-resorbing osteoclasts. The skeleton is able to continually adapt to the mechanical environment by adding new bone to withstand increased amounts of loading, and by removing bone in response to unloading or disuse. Furthermore, most adult skeletal diseases are due to excess osteoclastic activity. Studies have shown that mechanical strain inhibits osteoclast differentiation indirectly by osteoblasts and stromal cells (17,18). Other reports have shown that compressive mechanical stress promoted osteoclast formation through RANKL expression in

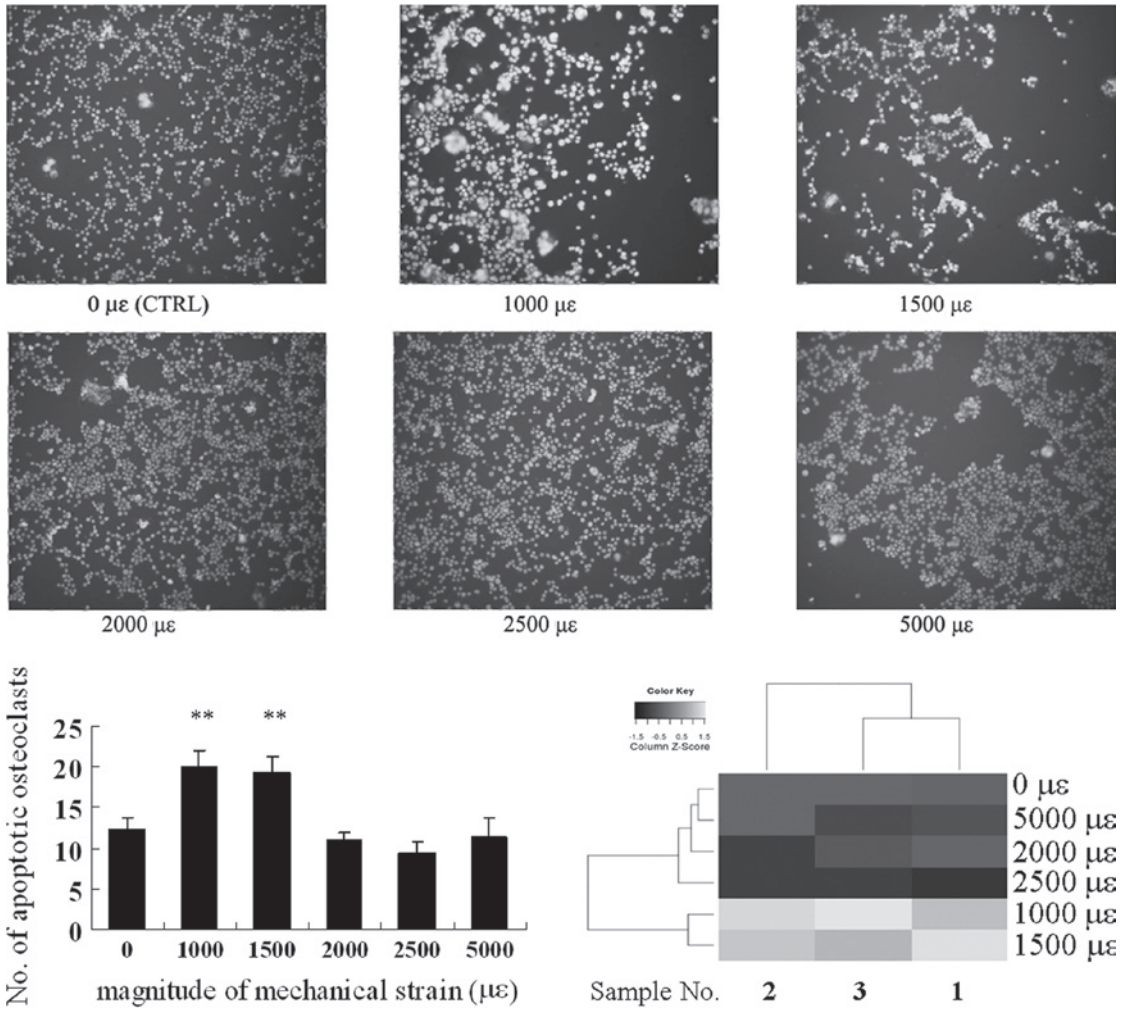

Figure 4. The number of apoptotic osteoclasts varied depending on the magnitude of mechanical strain. After 7 days in culture with different magnitudes of mechanical strain, apoptotic cells were counted with DAPI staining in a representative field of each sample. Heatmap shows the relationships between data categories and diverse trends of cell survival between the groups of different mechanical strain magnitudes. Results are presented as the means ± SD (n=6). **p<0.01. CTRL, control.

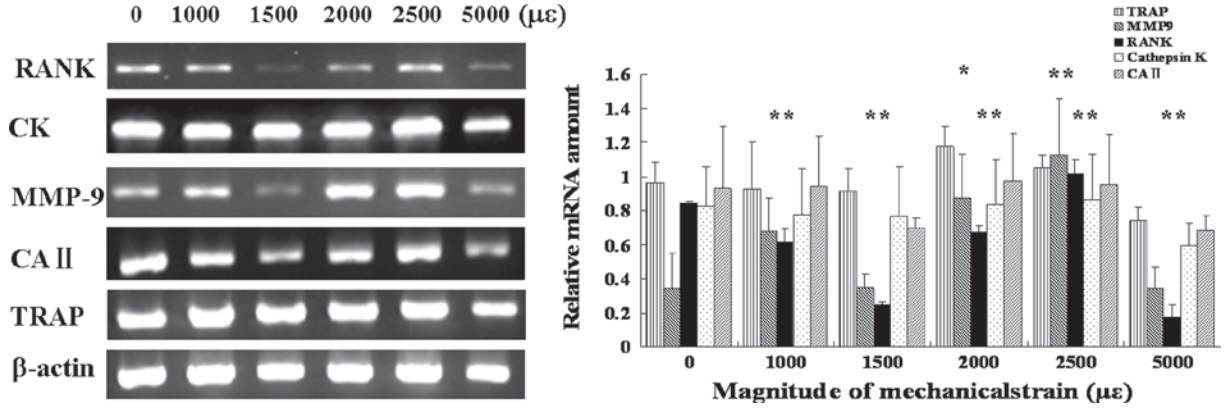

Figure 5. Mechanical strain was related to the expression of osteoclast differentiation genes. RAW cells ($5x10^6/cm^2$) were cultured in 34.8-mm tissue culture plates with receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), and subjected to different magnitudes of mechanical strain (substrate stretching) at day 4. After 3 days of culture with or without mechanical stimulation, total RNA was extracted, and RT-PCR analysis was performed using the primers shown in Table I. The mRNA amount of the target gene/β-actin was calculated. Results represent the means ± SD (n=6). *p<0.05, **p<0.01.

synovial cells (19). Ceratin studies have shown that mechanical strain also directly suppresses osteoclast differentiation (13,20,21); others have proven that mechanical stimuli enhance osteoclast differentiation and activities (22-24). However, little is known about the direct effects of different magnitudes of mechanical strain on osteoclast differentiation.

The amount of nuclei per osteoclast decreased significantly with a mechanical strain of 1,000 and 1,500 $\mu\varepsilon$, while the number of osteoclasts with 5 or more nuclei increased when the loading magnitude was tuned to 2,000 and 2,500 $\mu\varepsilon$ compared to the control with no strain (0 $\mu\varepsilon$) (Fig. 2). When TRAP staining was performed, the number of osteoclasts was found to be downregulated by low mechanical strain (Fig. 3A). It is well known that RANK signaling is essential for osteoclast differentiation, activation and survival. In immunocytochemistry staining of osteoclasts for RANK, the groups subjected to a strain of 2,000 and 2,500 $\mu\varepsilon$ showed stronger positive staining than the control after 3 days of substrate stretching (Fig. 3B). These findings indicate that a mechanical strain of low magnitude within physiological load inhibits osteoclast differentiation, but promotes the fusion of mononuclear osteoclasts related to high magnitude strain. A strain of 5,000 $\mu\varepsilon$ regarded as high magnitude beyond physiological load had little effect (data not shown).

In RT-PCR analysis (Fig. 5), RANK mRNA expression decreased with the mechanical strain of 1,000, 1,500 and 5,000 $\mu\varepsilon$, while both RANK and MMP-9 increased with the mechanical strain of 2,000 and 2,500 $\mu\varepsilon$ compared to the control. RANK mediated the ability of precursor cells to undergo differentiation. The selective inhibition of RANK with RANK:Fc or RANK receptor inhibitor has been shown to block osteoclast maturation and function *in vivo* or *in vitro* (25-27). MMP-9 has been proven to be indispensable for the migration of osteoclasts through collagen, both in the periosteum and developing marrow cavity of primitive long bones (28,29). MMP-9 antisense oligonucleotides exert an inhibitory effect on osteoclastic bone resorption by suppressing cell migration (30). Bone resorption is specifically reduced by the chemical inhibition of MMP-9 (31,32). The results of this study suggest the involvement of both RANK and MMP-9 expression depending on the mechanical strain magnitude, thus indicating that low-magnitude strain suppresses osteoclast differentiation, while high-magnitude strain within physiological load stimulates osteoclast fusion and activation.

We examined the apoptotic osteoclasts under mechanical strain. Compared to the control, apoptotic osteoclasts increased with the mechanical strain of 1,000 and 1,500 $\mu\varepsilon$, whereas there was no significant stimulation was observed with a strain of 2,000, 2,500 and 5,000 $\mu\varepsilon$ (Fig. 4), suggesting that apoptosis occurs when a mechanical strain of low magnitude is applied. A heatmap produced by the heatmap function from R intuitively indicated the diverse trends of cell survival between the groups of different mechanical strain magnitudes as well.

Currently, mechanical exercise appears to be a concern for clinicians as a co-ordinated treatment. Little is known about how different magnitudes of mechanical strain exert an effect on the differentiation and fusion of osteoclasts. The data from our study provide a further understanding of the diverse regulation by different magnitudes of mechanical strain, leading to the development of therapeutics optimized for diseases related to bone loss. We found that mechanical strain turned out to be inhibitory towards RAW264.7 cell differentiation at a low magnitude, but stimulatory at a high magnitude within physiological load. The osteoclasts morphologically changed depending on the different magnitudes mechanical strain. The expression of RANK and related genes also changed depending on different magnitutes of strain. TRAP is often used as one of the macrophage/osteoclast lineage markers (33,34). However, the results of the present study on mRNA expression are in disagreement with those from the study of Fujisaki *et al*, who observed that the expression of CAII and cathepsin K induced by RANKL was increased according to the maturity and differentiation of the RAW264.7 cells (35). No significant differences were observed in the expression of TRAP, cathepsin K and CAII mRNA under mechanical strain compared to the control. Therefore, RANKL/osteoprotegerin-dependent signal transduction pathways are possibly more active during late differentiation than TRAP-dependent pathways. However, further study of other co-stimulators and/or mechanisms unknown during various stages of osteoclast development differentially regulated by mechanical strain is warranted.

Acknowledgements

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