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Mechanical stress directly suppresses osteoclast differentiation in RAW264.7 cells

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Abstract. Although it is known that mechanical stress to osteoblast and periodontal ligament cells suppresses osteoclast differentiation, little is known about the direct effect of mechanical stress on osteoclast differentiation. In this study, we examined the role of mechanical stress on osteoclast differentiation using murine pre-osteoclastic RAW264.7 cells treated with receptor activator of nuclear factor-κB ligand (RANKL). RAW cells were cultured with RANKL, and mechanical stress was applied for a given period. We counted the number of osteoclast cells which were tartrate-resistant acid phosphatase (TRAP)-positive and multinucleated (2 nuclei or more), and measured mRNA by RT-PCR. There was a decrease in the number of osteoclasts under mechanical stress compared with the number under no mechanical stress. The number of nuclei per osteoclast also decreased compared to the number of nuclei per osteoclast cultured with the application of mechanical stress. As the cells were cultured for a period of 1-7 days and/or for different periods of mechanical stress application, osteoclast differentiation decreased with mechanical stress and increased after removing mechanical stress. Expression of mRNA for the osteoclast-specific genes, TRAP, matrix metalloproteinase-9, cathepsin-K and calcitonin receptor, decreased with mechanical stress and was associated with the number of osteoclasts. Inducible nitric oxide synthase mRNA which inhibits osteoclast differentiation, increased with mechanical stress. In spite of the decrease in osteoclast number with mechanical stress, nuclear factor of activated T cell cytoplasmic 1 (NFATc1) and NFATc2 mRNA expression increased with mechanical stress. These findings indicate that mechanical stress directly suppresses osteoclast differentiation and increases NFATc1 and NFATc2 suggesting delayed differentiation.

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Introduction

The amount of bone remodeling is controlled by the balance between bone formation and bone resorption (1). Many osteopenic diseases, including osteoporosis (2), rheumatoid arthritis, Paget disease and lytic bone metastases of malignancies are characterized by progressive bone resorption by osteoclasts, which are multinucleated giant cells that originate from hematopoietic cells (3). Through previous studies, the close relationship between the immune and skeletal systems has gradually become clear (4,5). Most important is the receptor activator of nuclear factor-κB (RANK) ligand (RANKL) (6-8), which is one of the tumor necrosis factor (TNF) family members. It is expressed as a membrane-bound protein in osteoblasts and stromal cells and promotes the differentiation of osteoclast precursor cells into osteoclasts. Gene-targeted mice deficient in RANKL expression exhibit severe osteopetrosis with complete absence of osteoclast formation. These findings indicate that RANKL is an essential factor for osteoclast differentiation. In addition, osteoclast differentiation is regulated by RANKL and the macrophage colony-stimulating factor (M-CSF) (9). M-CSF (also known as CSF-1) has also been identified as an essential factor for osteoclastogenesis. The main role of M-CSF is that it acts as a survival factor of osteoclast precursor cells and induces the expression of RANK which is a receptor of RANKL. The RAW264.7 cells used in this study are able to differentiate into osteoclasts with stimulation by RANKL without M-CSF.

A known key regulator of osteoblast and osteoclast activity in bone is mechanical stress. The skeleton is able to continually adapt to mechanical stress by adding new bone to withstand increased amounts of loading, and by removing bone in response to unloading or disuse (10). To date, experiments have been conducted with various methods to elucidate the relationship between mechanical stress and bone metabolism. *In vitro* it is known that application of mechanical stress is the method used to deform the culture matrix (strain) (11,12), to give flow to culture medium (fluid flow) (13), to add pressure (hydrostatic pressure) (14), and to directly stimulate the cell (perturbation) (10,15,16). But. *in vivo,* evaluating each method accurately is difficult.

Mechanical stress induces activation and differentiation of osteoblasts (17,18). On the other hand, it seems that mechanical stress inhibits osteoclast differentiation in co-cultures between osteoblasts and osteoclasts (19). However, few studies have reported about the direct effect of mechanical stress on osteoclast differentiation. In this work, we investigated osteoclast differentiation in RAW cells, murine macrophage-like osteoclast precursors, under mechanical stress using the Flexcell tension system.

Materials and methods

Cell culture. We used the murine monocyte/macrophage cell line RAW264.7 (Riken Cell Bank no. RCB0535, Japan) as osteoclast precursors. RAW cells differentiate into osteoclast-like cells in the presence of RANKL. The cells were grown in αMEM (Invitrogen, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 66.7 μg/ml kanamycin-sulfate and 284 μM L-ascorbic acid 2-phosphate at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 (20). The cells were seeded onto 100-mm standard dishes (Falcon™; Becton Dickinson Labware, Franklin Lakes, NJ, USA). After overnight culture, the cells were used for analysis.

Tartrate-resistant acid phosphatase (TRAP) staining. The cells cultured for a given period were washed with PBS and fixed with 10% neutral formalin. They were then washed with distilled water and stained with Fast Red Violet LB Salt (Sigma Aldrich, St. Louis, MO, USA) (18). After washing, TRAP-positive cells with more than two nuclei were considered to be osteoclast-like cells. The number of osteoclasts was counted under a light microscope.

Pit assay. RAW cells were cultured together with mammoth dentine slices (Hokudo, Sapporo, Japan) ~10 mm in diameter and 1 mm in thickness. The mammoth dentine slices were collected after 6 days in culture, stabilized, and observed with SEM (21).

Purification of GST-RANKL. Glutathione S-transferase (GST)-RANKL was purified as described previously (22). Briefly, a fragment of murine RANKL cDNA was cloned into the pGEX vector (GE Bioscience, Amersham Place, UK) and expressed in BL21 pLysS bacteria (Merck, Darmstadt, Germany) induced with 1 mM isopropylthio-ß-D-galactoside-mediated (IPTG) (Wako, Osaka, Japan) for 3 h at 30˚C. Bacteria were lysed, and soluble proteins were recovered using Glutathione-Sepharose™ 4B beads (GE Bioscience) followed by dialysis against PBS. The protein purity was assessed by SDS-PAGE with Coomassie Brilliant Blue by comparison with BSA and was shown to be equivalent. The bioactivity of GST-RANKL (RANKL) was verified by osteoclastogenesis and TRAP expression with RAW cells before experimental use.

Concentrations of RANKL and culture periods. RAW cells were cultured in a 48-well plate ($1x10^4$ cells/well) and were added to different concentrations (0, 20, 50, 100 and 200 ng/ml) of RANKL. The medium was changed every 2 days containing the same dose of RANKL. After the 6th day, the cells were fixed and stained with TRAP, and the number of TRAP-positive multinucleated osteoclasts was counted.

After RAW cells were cultured using the same procedure with RANKL (200 ng/ml) for the indicated days (2, 4, 5, 6 and 7), the number of osteoclasts was counted.

Flexcell tension system and cell culture. The FX-3000™ Flexercell Strain Unit (Flexcell International, Hillsbough, NC, USA) was used for application of mechanical stretch to osteoclasts (23). RAW cells ($9x10^4$/well) treated with RANKL (100 ng/ml) were cultured on a Flexcell 6-well culture plate with a type I collagen-coated and flexible-bottomed well. The experimental regimens used in this study delivered 10% elongation at 30 cycles per min (0.5 Hz). Mechanical stress was applied for a period of 48 h of 3-4 days (MS3-4) or 5-6 days (MS5-6), or for a period of 96 h of 3-6 days (MS3-6). The cells were fixed at the end of the 6th day, and TRAP-positive multinucleated cells were counted after TRAP staining. The number of large osteoclasts with >8 nuclei as well as the maximum number of nuclei in each respective well were investigated. The control culture was grown under the same condition without mechanical stress.

RT-PCR. RAW cells were cultured in a Flexcell culture plate ($9x10^4$ cells/well, 100 ng/ml of RANKL) and mechanically stressed (48 h of 5-6 days). Total RNA was extracted using Trizol according to the manufacturer's instructions. RT-PCR analysis was performed as described previously (24). The following primers were used: RANK, *c-fms*, TRAP, matrix metalloproteinase-9 (MMP-9), cathepsin-K (Cath-K), calcitonin receptor (CTR), nuclear factor of activated T cell cytoplasmic 1 (NFATc1), NFATc2, NFATc3, inducible nitric oxide synthase (iNOS) and interferon-ß (IFN-ß). The sequences of the used primers are as shown in Table I. PCR products were separated on a 1% agarose gel and obtained with ethidium bromide. As an internal control for RNA quantity, the same cDNA was amplified using primers specific for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA.

Results

The number of TRAP-positive multinucleated osteoclasts varies depending on RANKL concentration and culture days. The number of osteoclasts increased depending on RANKL concentration, and 100 ng/ml of RANKL sufficiently induced osteoclasts when RAW cells were cultured with different concentrations (Fig. 1A). The cells induced by the same concentration of RANKL used, formed resorption pits on a dentine slice (Fig. 1B), and the cells were regarded as osteoclasts.

The number of TRAP-positive multinucleated osteoclasts was counted after culturing with RANKL (100 ng/ml) for 7 days. Many osteoclasts were observed on the 4th day, and the number continued to increase until the 6th day (Fig. 1C).

The number of TRAP-positive multinucleated osteoclasts decreases with mechanical stress during osteoclast differentiation. To examine the effect of mechanical stress on osteoclast differentiation, the number of osteoclasts was

The sequences of the used primers.

Gene		Primer sequence (5'-3')
RANK	Forward	CCAGGGGACAACGGAATCA
	Reverse	GGCCGGTCCGTGTACTCATC
IFN-ß	Forward	CTCCAGCTCCAAGAAAGGACG
	Reverse	GAAGTTTCTGGTAAGTCTTCG
c-fms	Forward	GCGATGTGTGAGCAATGGCAGT
	Reverse	AGACCGTTTTGCGTAAGACCTG
iNOS	Forward	ACGGAGAAGCTTAGATCTGGAGCAGAAGTG
	Reverse	CTGCAGGTTGGACCACTGGATCCTGCCGAT
TRAP	Forward	AAATCACTCTTTAAGACCAG
	Reverse	TTATTGAATAGCAGTGACAG
NFATc1	Forward	CAACGCCCTGACCACCGATAG
	Reverse	GGCTGCCTTCCGTCTCATAGT
MMP-9	Forward	CTGTCCAGACCAAGGGTACAGCCT
	Reverse	GTGGTATAGTGGGACACATAGTGG
NFATc2	Forward	GGGCCATGTGAGCAGGAGGAGA
	Reverse	GCGTTTCGGAGCTTCAGGATGC
Cath-K	Forward	CCTCTCTTGGTGTCCATACA
	Reverse	ATCTCTCTGTACCCTCTGCA
NFATc3	Forward	CTTTCAGTTCCTTCACCCTTTACCT
	Reverse	TGCCAATATCAGTTTCTCCTTTTC
CTR	Forward	ACCGACGAGCAACGCCTACGC
	Reverse	GCCTTCACAGCCTTCAGGTAC
G3PDH	Forward	CGGAGTCAACGGATTTGGTCGTAT
	Reverse	AGCCTTCTCCATGGTGGTGAAGAC

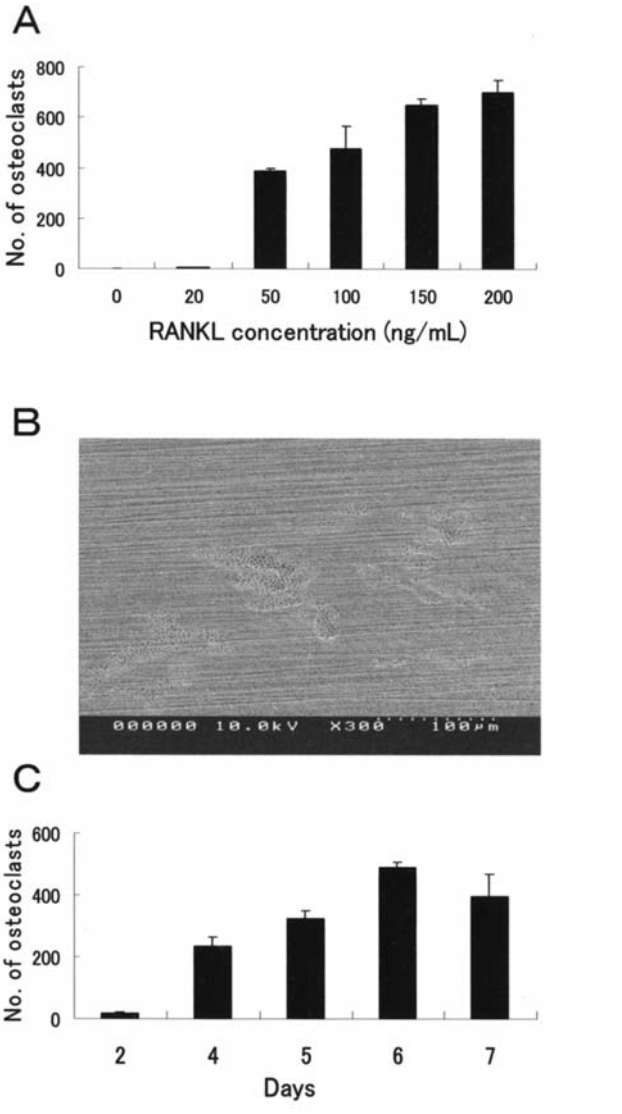

Figure 1. The number of TRAP-positive multinucleated osteoclasts varied depending on the concentration of RANKL and the period of culture days. (A) RAW cells ($1x10^4$/well) were cultured in 48-well plates with various concentrations of RANKL. After 6 days in culture, TRAP (+) multinucleated cells were counted. (B) SEM picture of resorption pits by RANKL (200 ng/ml)-induced osteoclasts on a dentine slice. Magnification, x300. (C) RAW cells ($9x10^4$/well) were counted in 6-well plates with RANKL (100 ng/ml). After various days in culture, osteoclasts were counted. Results are shown as the mean ± SD (n=6).

observed. Mechanical stress clearly decreased the development of osteoclasts in RAW cells (Fig. 2A). The mechanical stress-induced suppression was at least 50%, compared with controls plated on similar Flexcell plates, cultured in the same incubator, but not exposed to direct mechanical stress. The number of large osteoclasts with >8 nuclei was significantly decreased by mechanical stress (Fig. 2B). The ratio of the number of large osteoclasts per counted osteoclasts was 16% in the control and 7% in the mechanical stress groups (data not shown). Upon comparing the maximum number of nuclei in osteoclasts per well, we observed the largest osteoclasts having >40 nuclei in the control group, compared to nearly 14 nuclei in the mechanical stress groups (Fig. 2C).

Mechanical stress suppresses and delays osteoclast differentiation. To verify the time-dependent frequency of osteoclast differentiation, the medium with RANKL (100 ng/ml) was replaced every 2 days, and mechanical stress for a conditional period (for 48 h of 3-4 and 5-6 days, and for 96 h of 3-6 days) was applied. We observed that the control without mechanical stress on the Flexcell 6-well culture plate during 1-7 days showed a similar time-dependent frequency to that in Fig. 1C. The number of osteoclasts in MS3-4, MS5-6 and MS3-6 was smaller than that of the control on the 6th day (Fig. 3). In MS3-4, applying mechanical stress for 48 h, the number of osteoclasts slowly increased after removing the mechanical stress until the 6th day, but suddenly increased on the 7th day and reached a similar number as in the control on the 6th day. In MS5-6, applying mechanical stress for 48 h, the osteoclasts suddenly increased on the 7th day upon the removal of mechanical stress and reached a similar number as the control on the 6th day. In MS3-6, applying mechanical stress for 96 h, the osteoclasts suddenly increased on the 7th day reaching a similar number as in MS3-4 and MS5-6. But the number of osteoclasts were fewer than that in MS3-4 and MS5-6 for 48 h during observation and did not recovered to a similar number as that in the control on the 6th day.

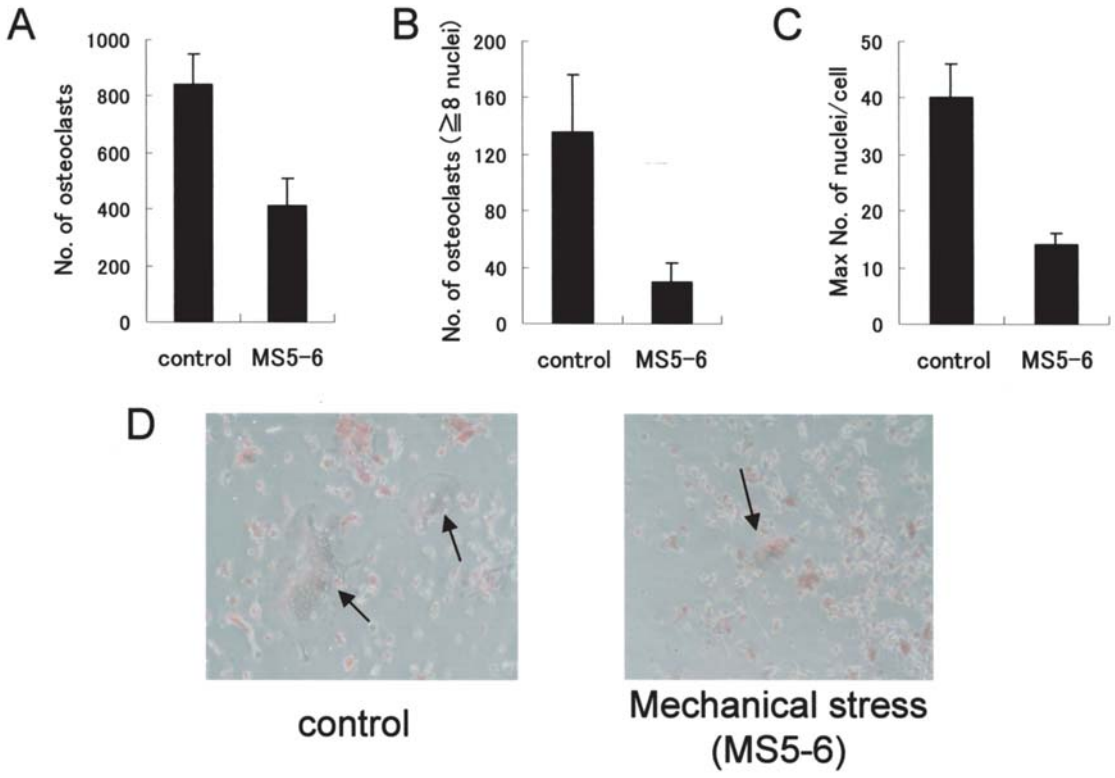

Figure 2. The number of osteoclasts decreased with mechanical stress during osteoclast differentiation. RAW cells ($9x10^4$/well) were cultured on Flexcell 6-well plates with RANKL (100 ng/ml) for 6 days. Mechanical stress was applied for 48 h for 5-6 days (MS5-6), but not to the control. (A) The number of osteoclasts after 6 days in culture, (B) the number of osteoclasts with 8 nuclei or more, and (C) the maximum number of nuclei per cell in all osteoclasts. (D) Microscopic images of osteoclasts in the control and osteoclasts in MS5-6. Magnification, x100. Results are shown as the mean ± SD (n=6).

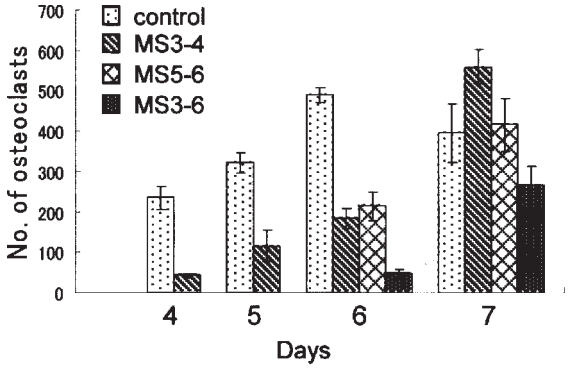

Figure 3. Mechanical stress suppressed and delayed osteoclast differentiation. RAW cells ($9x10^4$/well) were cultured in Flexcell 6-well plates with RANKL (100 ng/ml). Mechanical stress was applied to the cells for 3-4 (MS3-4), 5-6 (MS5-6) or 3-6 (MS3-6) days. After the indicated days, the number of osteoclasts was counted. Results are shown as the mean ± SD (n=6).

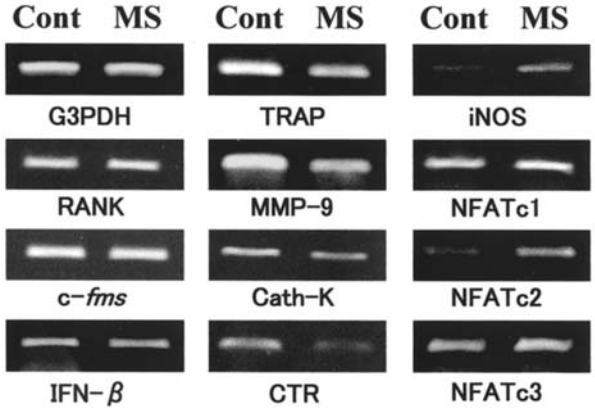

Figure 4. Mechanical stress was related to the expression of osteoclast differentiation genes. RAW cells ($9x10^4$/well) were cultured in Flexcell 6-well plates with RANKL (100 ng/ml) with or without mechanical stress for 5-6 days (MS5-6). After day 6, the total RNA was extracted, and RT-PCR analysis was performed using the primers indicated in Table I. Cont, control; MS, mechanical stress.

Mechanical stress is related to the expression of osteoclast differentiation genes. Mechanical stress was applied for only 5-6 days with RANKL in RAW cells, and RNA was collected on the 6th day. We examined the expression of mRNA in RT-PCR analysis for osteoclast-specific genes (TRAP, MMP-9, Cath-K and CTR) which were differentiation markers from mature osteoclasts after forming. The expression of mRNA of these genes with mechanical stress was decreased correlating with the decrease in osteoclasts (Fig. 4). The expression of iNOS mRNA increased with mechanical stress. The expression of NFAT family mRNA which is a necessary factor in osteoclast differentiation increased with mechanical stress and particularly NFATc2 mRNA exhibited a distinct increase when compared to the control. NFATc1 and NFATc3 mRNA also increased with mechanical stress. The expression of RANK, *c-fms* and IFN-ß mRNA was not affected by mechanical stress.

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Since Wolff's law was reported, many researchers have performed *in vivo* and *in vitro* studies to clarify the relationship between bone tissue and mechanical stress (10,25). No ideal methods have yet been established to provide cells with mechanical stress efficiently since, *in vitro*, it is difficult to reproduce the physiological conditions under which various factors operate. Conversely, *in vivo*, it is difficult to direct stimulation only to specific cells. In this study, the advantage of the Flexcell tension system was the easy control of the time and force of stimulation.

The effect of mechanical stress on osteoclasts remains unsolved, although many investigators have reported on its effect on osteoblasts and osteocytes. In one study on mechanical stress to osteoblasts, it was reported that osteoblast proliferation was promoted by straining the culture matrix in a monolayer. The effect of mechanical stress on differentiation factors such as alkaline phosphatase and osteocalcin is not clear, but as for intracellular signaling in osteoblasts there are some reports regarding the activation of phospholipase A2, increase of cAMP, increase of intracellular Ca^{2+} density, and promotion of *c-fos* expression (17,18).

In osteoclast differentiation from RAW cells with RANKL, the number of osteoclasts significantly decreased with mechanical stress. We next examined the relationship between the number of osteoclasts and that of nuclei. The number of nuclei in osteoclasts *in vivo* can range widely from several to hundreds (26). Commonly, 90% of all osteoclasts have ≤10 nuclei, but *in vitro* we observed osteoclasts having >10. The results of this study indicated that mechanical stress suppressed the differentiation from RAW cells to mononuclear osteoclasts because the number of nuclei in the cell and the number of large osteoclasts (≥8 nuclei) decreased as well as the number of osteoclasts. Furthermore, these findings indicated that the fusion of mononuclear osteoclasts was also suppressed.

The number of forming osteoclasts fluctuated with the time and period when mechanical stress was applied. By comparison with the control, the number of osteoclasts decreased on the 6th day of culture in the groups under applied mechanical stress (MS3-4, MS5-6 and MS3-6) (Fig. 3). In MS3-4 and MS3-6, with initial mechanical loading on the 3rd day, osteoclasts were fewer than those in MS5-6 with initial mechanical loading on the 5th day. This result suggested that osteoclast differentiation in MS5-6 advanced similar to that of the control because the time of initial observation of osteoclasts was on the 3rd and/or 4th culture day according to the control lapse. But the number of osteoclasts in each mechanical stress group suddenly increased after the 6th day when mechanical stress was removed. This may indicate that mechanical stress did completely inhibit osteoclast differentiation, but rather suppressed osteoclast differentiation.

In RT-PCR analysis (Fig. 4), osteoclast-specific gene expression (TRAP, MMP-9, Cath-K, CTR) decreased when mechanical stress was applied, correlating with the decrease in the number of osteoclasts (27). It is well known that the expression of iNOS inhibits osteoclast differentiation and decreases bone resorption (28). In this study, the increase in iNOS expression with mechanical stress induced suppression of osteoclast differentiation. Of the NFAT family, NFATc1 is a key transcription factor for osteoclast differentiation and is regulated by RANKL (29-32). NFATc2 is present in the cell before RANKL stimulation and induces NFATc1 activation (33,34). In this study, NFATc1 and NFATc2 mRNA expression increased when mechanical stress was applied, and the number of osteoclasts decreased in relation. This result suggested that, first, at the time of extraction of RNA on the 6th day, NFATc1 was present because of suppressed osteoclast differentiation in the mechanical stress group, although in the control group osteoclast differentiation advanced sufficiently and NFATc1 was consumed. Second, mechanical stress increased the expression of NFATc1 mRNA but signal transduction to osteoclast differentiation stopped downstream of NFATc1 although NFATc1 transcription functioned properly. The role of NFATc3 in osteoclasts is unknown (32). Our data showed that the expression of NFATc3 mRNA increased slightly with mechanical stress, and we reasoned that NFATc3 was related to other NFATs. The expression of RANK and c-*fms* mRNA in RAW cells was not affected by RANKL and mechanical stress. Furthermore, the expression of INF-ß with negative feedback function at the time of osteoclast differentiation also was not affected by mechanical stress in mRNA levels (5,28).

In summary, since there has been no study regarding the direct application of mechanical stress during osteoclast differentiation *in vitro*, our aim was to undertake an initial investigation. We found that the effect of mechanical stress on RANKL-induced osteoclast differentiation in RAW cells directly suppressed osteoclast differentiation during the period of mechanical stress application, and promoted osteoclast formation after removing the mechanical stress. However, as previously mentioned, because the experimental system which applied the mechanical stress to the cell does not accurately reflect an *in vivo* reaction, further study of the reliability and validity of the system *in vivo* and the pursuit of target genes influenced by mechanical stress is necessary.

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