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Compressive Mechanical Force Augments Osteoclastogenesis by Bone Marrow Macrophages Through Activation of c-Fms-Mediated Signaling

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ABSTRACT

Little is known about the effects of mechanical forces on osteoclastogenesis by bone marrow macrophages (BMMs) in the absence of mechanosensitive cells, including osteoblasts and fibroblasts. In this study, we examined the effects of mechanical force on osteoclastogenesis by applying centrifugal force to BMMs using a horizontal microplate rotor. Our findings, as measured by an in vitro model system, show that tumor necrosis factor (TNF)-α is capable of inducing osteoclast differentiation from BMMs and bone resorption in the presence of macrophage-colony stimulating factor (M-CSF) and is further facilitated by receptor activator of nuclear factor-kappaB (NF-κB) ligand (RANKL). Application of force to BMMs accelerated TNF-α-induced osteoclastogenesis; this was inhibited either by anti-TNF-α or anti-TNF-α receptor but not by OPG. TNF-α also increased c-Fms expression at both mRNA and protein levels in BMMs. An anti-c-Fms antibody completely inhibited osteoclast differentiation and bone resorption induced by TNF-α but partially blocked osteoclastogenesis stimulated in combination with RANKL. These results suggest that TNF-α (in the presence of M-CSF) is capable of inducing osteoclastogenesis from BMMs, and that osteoclastogenesis is significantly stimulated by force application through the activation of c-Fms-mediated signaling. Overall, the present study reveals the facilitating effect of mechanical force on osteoclastic differentiation from BMMs without the addition of mechanosensitive cells. J. Cell. Biochem. 111: 1260–1269, 2010.

KEY WORDS: CENTRIFUGAL FORCE; MOUSE BONE MARROW CELLS; TUMOR NECROSIS FACTOR-α; OSTEOCLASTOGENESIS; c-Fms

Orthodontic tooth movement occurs due to alveolar bone resorption on the pressure side and bone formation on the tension side when applying prolonged mechanical force to the tooth. Fibroblasts and osteoblasts that are present in periodontal tissue play important roles in these processes by producing various cytokines, including the receptor activator of nuclear factor-kappaB (NF-κB) ligand (RANKL) [Kanzaki et al., 2001, 2002] and the RANKL decoy receptor osteoprotegerin (OPG) [Kanzaki et al., 2001]. Interaction of RANKL with its receptor (RANK) induces the formation of multinucleated osteoclasts from hematopoietic precursors, stimulating bone resorptive activity [Yamaguchi et al., 2006]. In contrast, OPG inhibits osteoclastogenesis and accelerates osteoclast apoptosis [Kanzaki et al., 2006]. Therefore, it is believed that the balanced expression of RANKL and OPG in periodontal tissue plays a critical role in bone remodeling during orthodontic treatment.

Several studies have shown that the application of mechanical force leads to the expression of tumor necrosis factor (TNF)-α, suggesting a crucial role in inducing bone remodeling during orthodontic treatment [Uematsu et al., 1996; Ogasawara et al., 2004]. A prior report demonstrated reduced orthodontic tooth movement in TNF receptor (TNFR)-deficient mice compared to wild-type mice [Yoshimatsu et al., 2006]. TNF-α has been shown to induce osteoclast differentiation from bone marrow macrophages in vitro [Azuma et al., 2000; Udagawa, 2002; Kim et al., 2005]. It has also

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been suggested that elevated levels of TNF-α are involved in the pathogenesis of chronic inflammatory conditions, such as rheumatoid arthritis [David and Schett, 2000] and periodontal disease [Taubman and Kawai, 2001]. Furthermore, TNF-α induces osteoclast differentiation and bone resorption from osteoclast precursors in the presence of macrophage-colony stimulating factor (M-CSF) independent of the RANK/RANKL pathway [Kobayashi et al., 2000; Kwak et al., 2005]. These findings suggest that TNF-α is an essential osteoclastogenic factor.

Numerous recent investigations have focused on the mechanisms by which cytokine and chemokine expression and biofunctional changes within periodontal tissue are induced by orthodontic mechanical stress. For use in mechanical studies, many investigators have used co-culture systems with periodontal fibroblasts or osteoblasts and monocytes derived from peripheral blood cells [de Vries et al., 2006] or bone marrow cells [Hasegawa et al., 2002; Kook et al., 2009a,b]. Most of these investigations revealed that mechanical stresses such as shear, pressure, or strain sensitively affect osteoclastogenesis in co-cultures through RANKL- and OPG-dependent pathways, which are regulated by mechanosensitive fibroblasts or osteoblasts. However, little information is available on the mechanisms by which mechanical force affects differentiation to osteoclasts in bone marrow cultures without the addition of mechanosensitive cells.

Here, we examined the effects of mechanical force on osteoclast formation and bone resorption by mouse bone marrow-derived macrophages (BMMs) by applying a centrifugal force to these cells using a horizontal microplate rotor.

MATERIALS AND METHODS

MICE, CHEMICALS, AND LABORATORY WARES

Male BALB/c mice (6 weeks old) were purchased from Orient Co. (Seoul, Korea). Recombinant murine RANKL, TNF-α, and M-CSF were purchased from R&D Systems (Minneapolis, MN). Anti-c-Fms antibody was obtained from eBioscience, Inc. (San Diego, CA). Anti-TNFR1 and anti-TNFR-2 antibodies were obtained from Pharmingen (San Diego, CA). Antibodies specific for OPG, RANK, and α-tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise specified, chemicals and laboratory wares were obtained from Sigma Chemical Co. (St. Louis, MO) and SPL Life Sciences (Pochun, South Korea), respectively.

PREPARATION OF BONE MARROW MACROPHAGES

Bone marrow cells were obtained from 6- to 8-week-old BALB/c mice according to the methods described elsewhere [Lee and Lim, 2001]. Animal care and use were approved by the Chonbuk National University Committee on Ethics in the Care and Use of Laboratory Animals (CBU 2010-0007). Briefly, the tibiae and femora were removed from mice killed by cervical dislocation and were washed several times with PBS with antibiotics. The ends of the bones were cut and the marrow cavity was flushed with modified essential medium (MEM) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) and antibiotics. Five milliliters of bone marrow suspension (2×10^6 cells/ml) was divided into 100 mm culture plates in the presence of 50 ng/ml M-CSF. After culturing for 3 days, adherent cells were collected using a scraper and shown by flow cytometric analysis to be negative for CD3 and B220, and positive for F4/80 (data not shown); approximately 95% of these cells were considered to be BMMs.

APPLICATION OF MECHANICAL FORCE

BMMs (1×10^5 cells/ml) were divided into 96-well plates (100 μl/well; 3.1×10^4 cells/cm^2) or 6-well culture plates (2 ml/well; 2×10^4 cells/cm^2) and cultured in α-MEM supplemented with 50 ng/ml M-CSF and various concentrations of TNF-α (0–40 ng/ml) or in combination of RANKL (0–100 ng/ml). After 12 h of incubation, mechanical force was applied at 10–200 g/cm^2 for 1 h by centrifuging the culture plates using a horizontal microplate rotor (Universal 32 R, Hettich, Germany) as described elsewhere [Redlich et al., 1998]. The applied force calculation was based on the following equation [Redlich et al., 2004a,b]: $P = (m \times r \times rpm^2 \times \pi^2)/(A \times 9.8 \times 900)$, where P is the pressure on cells (kg/cm^2), m the mass of the medium (g), r the radius (0.12 m), rpm the revolutions/min, and A the area of contact between the medium and cells (cm^2).

OSTEOCLASTIC DIFFERENTIATION AND TRAP STAINING

To examine the effect of compressive mechanical force on osteoclasts formation from BMMs, the cells were subjected to centrifugal force and cultured for 7 days with 50 ng/ml M-CSF in the presence of TNF-α or in combination with RANKL. Culture media were replaced totally with fresh media on days 2 and 5 after application of force. The control and force-exposed cultures were fixed in 4% PBS-buffered *para*-formaldehyde 7 days after force exposure and stained with tartrate-resistant acid phosphatase (TRAP) using the Sigma–Aldrich kit according to the manufacturer's instructions. TRAP-positive mononuclear cells were counted using optic microscopy; cells containing three or more nuclei were considered to be multinucleated osteoclasts. In some experiments, BMMs subjected to centrifugal force were incubated with osteoclast-inducing factors in combination with antibodies specific for c-Fms, TNF-α, or TNFR and subjected to TRAP staining or pit formation assays after 7 days of incubation.

MEASUREMENT OF DNA SYNTHESIS AND CELL VIABILITY

The control and force-applied BMMs were cultured in 96-multiwell plates for 36 h, followed by treatment with 0.5 μCi/well of [methyl-^{3}H] thymidine deoxyribose (TdR; Amersham Pharmacia Biotech, Inc., Piscataway, NJ). At 12 h after TdR treatment, the cells were collected using a cell harvester (Inotech, Inc., Switzerland) and the level of DNA synthesis was determined by measuring tritium-specific radioactivity using a liquid scintillation counter (Packard Instrument Co., Downers Grove, IL).

In addition, cell viability was determined using water-soluble tetrazolium salt (WST)-8 reagent. Briefly, the control and force-exposed cultures were treated with WST-8 reagent 48 h after force application. Following incubation for an additional 4 h, the WST-8-specific absorbance was measured at 450 nm using a microplate reader (Packard Instrument Co.).

BONE RESORPTION ASSAY

BMMs (1×10^5 cells/ml) were suspended in α-MEM containing various concentrations of M-CSF, TNF-α, RANKL, or anti-c-Fms antibody, and then divided across a 24-well plate coated with calcium-phosphate nanocrystal (OAAS-24; Osteoclast Activity Assay Substrate, Oscotec, Inc., Choongnam, South Korea) at a density of 2×10^4 cells/cm^2. After 12 h of incubation, they were subjected to centrifugal force as described above and further cultured in the same medium containing the osteoclastogenic regulatory factors. Seven days after force exposure, the cells were removed from the plates by treatment with 5% sodium hypochlorite, and pit formation was observed under an optic microscope. The resorbed area was also measured by image analyzer and expressed as percentage (%) of control value.

RNA PREPARATION AND POLYMERASE CHAIN REACTION (PCR)

Total RNA was extracted from the control and force-exposed BMMs at various times according to the manufacturer's instructions (SV Total RNA Isolation System, Promega, Madison, WI). Reverse transcription and PCR amplification were performed using an Access RT-PCR System (Promega) according to the manufacturer's protocol. The primer sequences were as follows: RANKL (−233 bp), 5′-TGG TTC CCA TAA AGT GAG TC-3′ (forward), 5′-AGG GTA TGA GAA CTT GGG AT-3′ (reverse); c-Fms (−753 bp), 5′-AAC AAG TTC TAC AAA CTG GTG AAG G-3′ (forward), 5′-GAA GCC TGT AGT CTA AGC ATC TGT C-3′ (reverse). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control and was amplified with the primers 5′-AAC CTG CCA AAT ATG ATG AC-3′ (forward) and 5′-ATA CCA GGA AAT GAG CTT GA-3′ (reverse). PCR was performed for 30–35 cycles of 94°C for 30 s, 50–60°C for 30 s, and 72°C for 1 min in a DNA thermal cycler (model PTC-100, Waltham, MA). PCR products were analyzed on 1.5–2% agarose gels and visualized using ethidium bromide staining. Band intensity was calculated using a gel imaging system (model F1-F2 Fuses type T2A, Bio-Rad, Segrate, Italy).

Quantitative real-time PCR amplification was also performed with gene-specific primers using a 7500 real-time PCR System (Applied Biosystems). The primer sequences used for c-Fms and β-actin are described elsewhere [Yao et al., 2006]. Relative c-Fms expression was obtained from relative standard curves run in triplicate after dividing each value by the actin value.

WESTERN BLOT ANALYSIS

BMM suspensions (1×10^5 cells/ml) containing various concentrations of M-CSF, TNF-α, and/or RANKL were divided into six-well plates with 2 ml per well. These cells were exposed to force as described above. Twenty-four hours after the application of force, protein lysates were prepared from the control and force-applied BMMS using a NP-40 lysis buffer (30 mM Tris–Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 1% NP-40). Protein content was quantified using the Bradford method [1976]. Equal amounts of protein extracts were separated by 12% SDS–PAGE and blotted onto polyvinyl difluoride membranes. The blots were probed with primary antibodies overnight at 4°C prior to incubation with secondary antibody in a blocking buffer for 1 h. The blots were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Inc.) and exposed on X-ray film (Eastman-Kodak, Rochester, NY).

STATISTICAL ANALYSIS

Unless otherwise indicated, all data are expressed as mean ± standard deviation (SD). One-way ANOVA was used for multiple comparisons using SPSS version 17.0 software. A P-value <0.05 was considered statistically significant.

RESULTS

TNF-α INDUCES OSTEOCLAST FORMATION FROM BMMs IN A DOSE-DEPENDENT MANNER

To understand the nature of TNF-α in stimulating osteoclast differentiation from BMMs, cells were cultured with various concentrations (0–40 ng/ml) of TNF-α for 7 days in the presence of 10 ng/ml M-CSF. The number of TRAP-positive cells (Fig. 1A) and TRAP-positive MNCs (Fig. 1B) was increased by TNF-α in a dose-dependent manner. There was a limitation on stimulation by TNF-α; 40 ng/ml TNF-α was the maximum concentration capable of

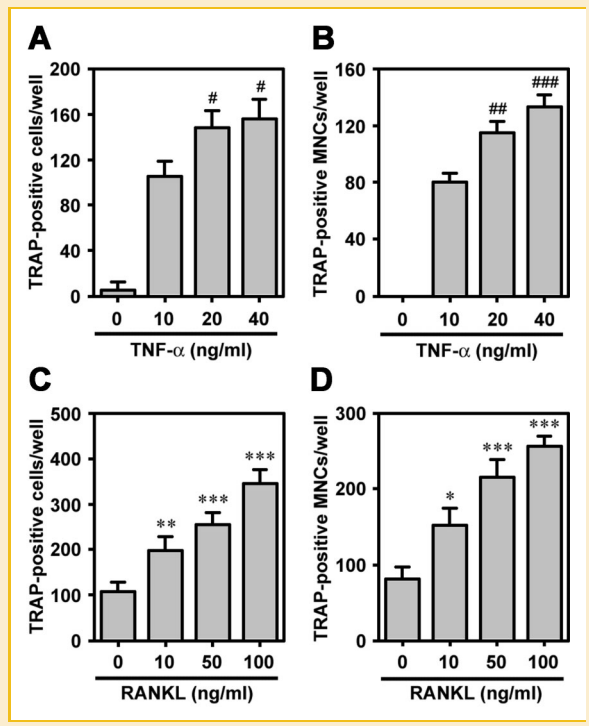

Fig. 1. TNF-α induces osteoclastic differentiation of BMMs in a dose-dependent manner and this further accelerated by RANKL. BMMs were cultured in 96-multiwell plates for 7 days in the presence of 50 ng/ml M-CSF and increasing doses (0–40 ng/ml) of TNF-α (A,B) or with 50 ng/ml M-CSF and 10 ng/ml TNF-α in the presence of the indicated concentrations (0–100 ng/ml) of RANKL (C,D). TRAP-positive cells (A,C) and TRAP-positive MNCs (B,D) containing three or more nuclei were counted. $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ versus 10 ng/ml TNF-α-treated cells. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ versus the M-CSF and TNF-α treatment group.

inducing osteoclastogenesis from BMMs (data not shown). TNF-α-induced osteoclast differentiation from BMMs was dramatically accelerated by the addition of RANKL in a dose-dependent manner (Fig. 1C,D). TNF-α-stimulated bone resorption, as measured by an in vitro model system, was also facilitated by RANKL (data not shown).

CENTRIFUGAL FORCE AFFECTS OSTEOCLASTOGENESIS, PROLIFERATION, AND VIABILITY IN BMMs DEPENDING ON THE MAGNITUDE OF THE APPLIED FORCE

We next determined the optimal force magnitude for stimulation of osteoclast differentiation from BMMs. The number of TRAP-stained cells varied depending on the force applied (0–200 g/cm^2); the most

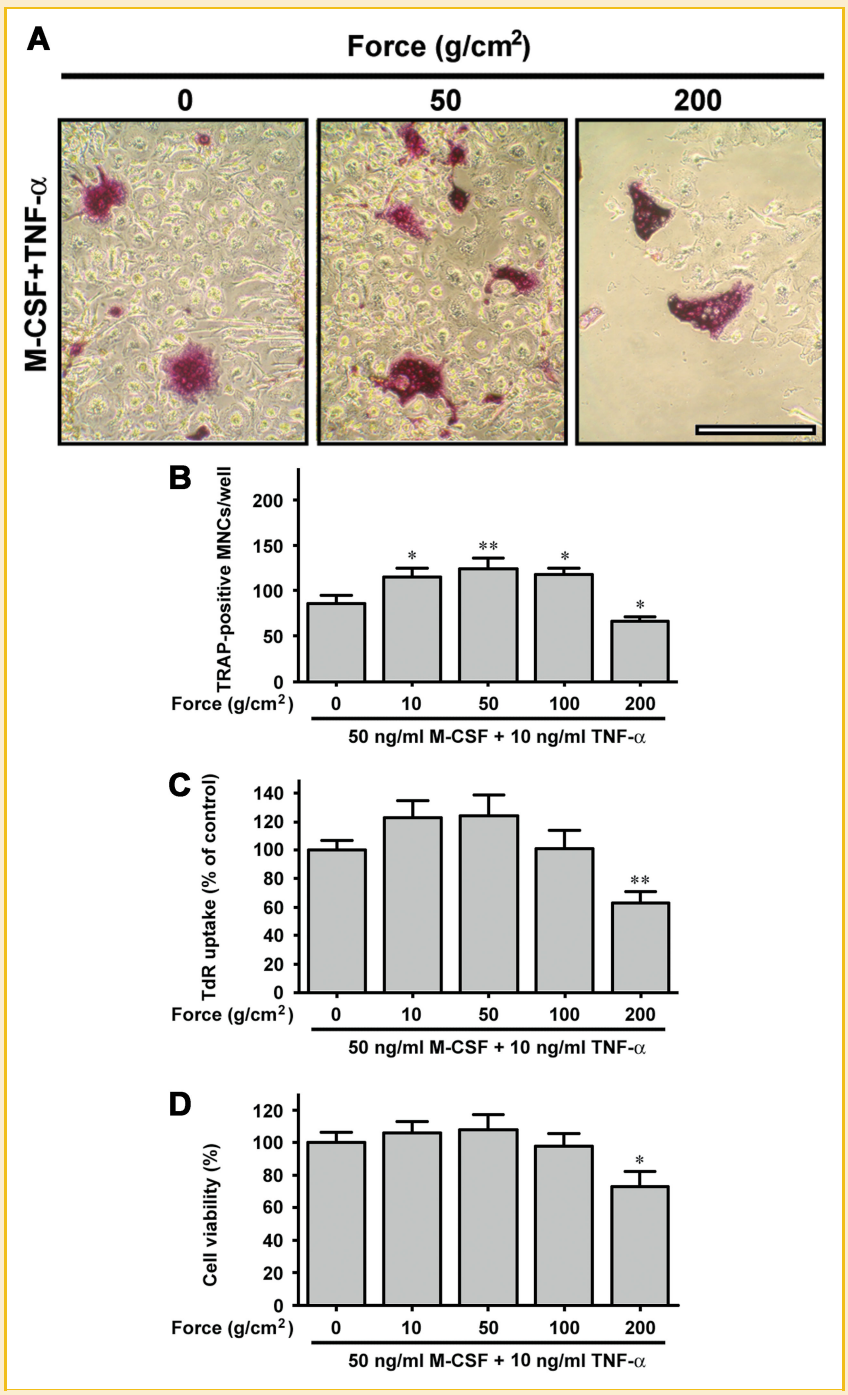

Fig. 2. Centrifugal force of more than 100 g/cm^2 reduces the number of TRAP-positive MNCs, TdR incorporation, and viability of BMMs. BMMs were exposed to the indicated magnitudes of centrifugal force (0–200 g/cm^2) for 1 h in the presence of 50 ng/ml M-CSF and 10 ng/ml TNF-α. The control and force-exposed BMMs were subjected to TRAP staining (A), and the number of TRAP-positive MNCs was counted 7 days after force exposure (B). Bar = 50 μm. In addition, the levels of DNA synthesis (C) and cell viability (D) were determined 48 h after application of centrifugal force. $^*P < 0.05$ and $^{**}P < 0.01$ versus the unloaded control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

prominent increase was observed in cultures subjected to a force of 50 g/cm^2 for 1 h, whereas a force of more than 100 g/cm^2 significantly reduced TRAP-positive MNCs compared to control levels (Fig. 2A,B). Centrifugal force at a magnitude of 200 g/cm^2 for 1 h significantly reduced proliferation (Fig. 2C) as well as viability of BMMs (Fig. 2D). These findings suggest that approximately 50 g/cm^2 and 1 h are the optimum model force and time for osteoclast differentiation of BMMs, and that subjecting to a force of more than 100 g/cm^2 reduces the rate of DNA synthesis and viability of these cells.

CENTRIFUGAL FORCE STIMULATES OSTEOCLAST DIFFERENTIATION BY BMMs

We subsequently examined whether TNF-α stimulates osteoclast formation independent of RANKL and how compressive mechanical force affects osteoclastogenesis. When BMMs were cultured in the presence of M-CSF and TNF-α for 7 days, many cells stained with TRAP; the number of stained cells further increased in cultures subjected to centrifugal force at a magnitude of 50 g/cm^2 (Fig. 3A).

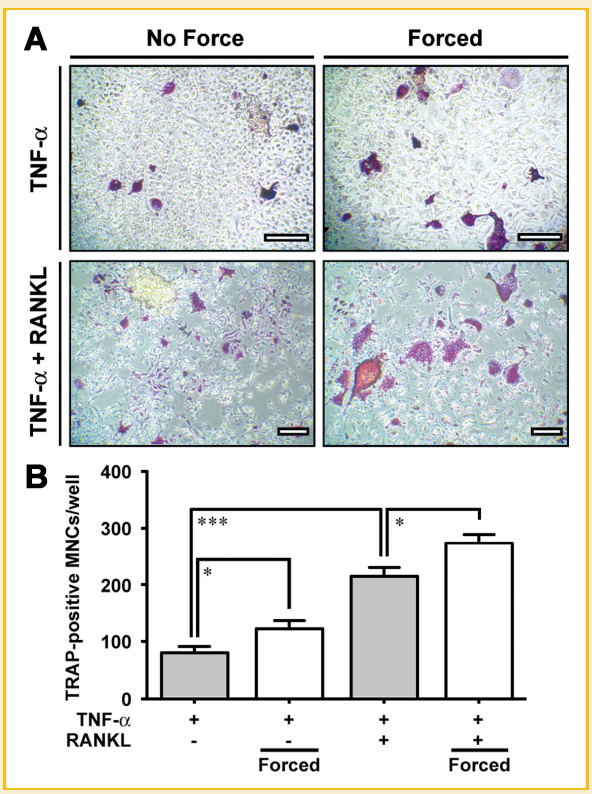

Fig. 3. Centrifugal force facilitates osteoclast differentiation in BMMs. BMMs were suspended in α-MEM containing 50 ng/ml M-CSF, 10 ng/ml TNF-α, and/or 50 ng/ml RANKL, and then spread onto 96-well plates at 2×10^4 cells per well. After 12 h of incubation, the cells were exposed to centrifugal force (50 g/cm^2) for 1 h and cultured for 7 days. A: Cells were fixed and subjected to TRAP staining; representative data from triplicate experiments are shown. Bar = 50 μm. B: The number of TRAP-positive MNCs with three or more nuclei was counted 7 days after force exposure and is expressed as numbers of cells per well of 96-multiwell plates. $^*P < 0.05$ and $^{***}P < 0.001$ versus the experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

The numbers of TRAP-positive MNCs were significantly higher in force-applied cultures than untreated controls (Fig. 3B). Combined treatment of BMMs with RANKL dramatically increased TNF-α-induced osteoclast formation, which was also facilitated by applying centrifugal force (Fig. 3). However, TNF-α did not stimulate the formation of TRAP-positive MNCs in cultures incubated without M-CSF (data not shown). These results suggest that TNF-α induces osteoclast differentiation from BMMs in the presence of M-CSF, and that this induction is augmented by compressive mechanical force.

OPG DOES NOT INHIBITS OSTEOCLAST DIFFERENTIATION INDUCED BY TNF-α

In order to understand the mechanism by which centrifugal force stimulates osteoclastogenesis from BMMs, the control and force-exposed cells were cultured with 200 ng/ml OPG for 7 days in the presence of 50 ng/ml M-CSF and 10 ng/ml TNF-α, or in combination with 50 ng/ml RANKL. TNF-α-stimulated TRAP-positive staining of BMMs was not inhibited by adding OPG in both control and force-applied cultures (Fig. 4A). In contrast, OPG significantly suppressed osteoclast differentiation by the cells incubated in the medium combined with M-CSF, TNF-α, and RANKL (Fig. 4B). When BMMs subjected to centrifugal force for 1 h were incubated for 7 days in the presence of anti-TNF-α antibody or a mixture of anti-TNFR1 and TNFR2 antibodies, the TNF-α-induced increase in TRAP-positive staining and its amplification following application of force were completely blocked (Fig. 4C). These results indicate that TNF-α-induced osteoclastogenesis and its facilitation by centrifugal force in BMMs are independent of RANKL-mediated signaling.

CENTRIFUGAL FORCE AUGMENTS c-Fms EXPRESSION INDUCED BY TNF-α ALONE AND IN COMBINATION WITH RANKL

It has been reported that antibody of c-Fms, a specific M-CSF receptor, prevents osteoclastogenesis and bone resorption induced by TNF-α [Kitaura et al., 2008]. Therefore, we explored whether c-Fms expression in BMMs is affected by TNF-α. As shown in Figure 5A, the mRNA level of c-Fms was increased by TNF-α in a dose-dependent manner. The data from real-time PCR also indicated that centrifugal force amplified c-Fms expression significantly, as force at a magnitude of 50 g/cm^2 for 1 h increased the expression level to 2.4-fold ($P < 0.01$) compared to the control (Fig. 5B). This was similar to the results obtained from reverse transcriptase PCR using the same mRNA extract (Fig. 5C). Western blot analysis also revealed a force-dependent increase in c-Fms protein levels in BMMs (Fig. 5D).

We next compared the levels of RANK and c-Fms at the mRNA and protein levels between the control and the force-applied BMMs. Treating the cells with the combined media containing M-CSF, TNF-α, and RANKL increased the mRNA levels of both RANK and c-Fms, while the level of c-Fms mRNA was only significantly augmented in cells subjected to centrifugal force (Fig. 6A,B). These results were quite similar to immunoblot data showing that the protein bands specific for c-Fms, but not for RANK, were amplified after application of force (Fig. 6C,D). These results suggest that RANK expression is increased in response to RANKL, while c-Fms expression is stimulated by TNF-α and is further increased in response to centrifugal force in a RANKL-independent manner. This

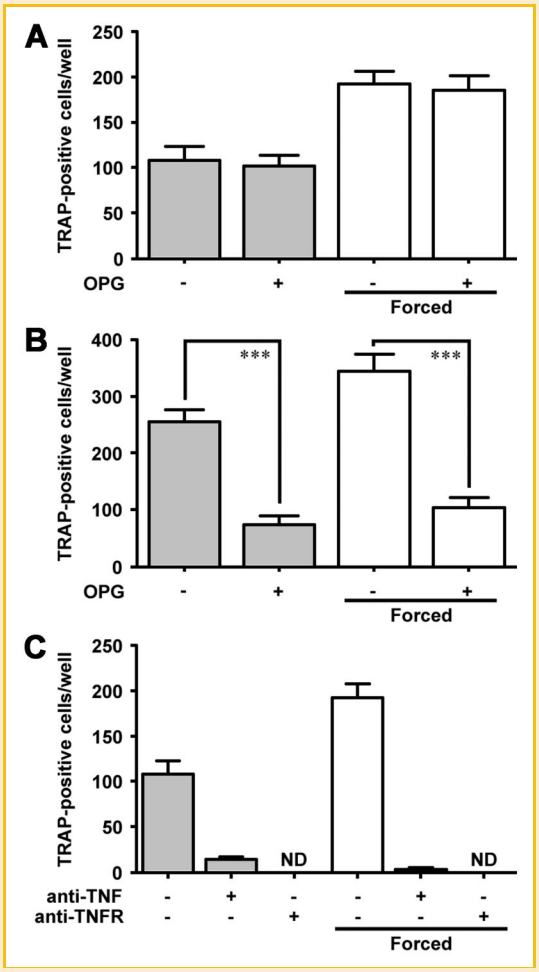

Fig. 4. TNF-α-stimulated osteoclast differentiation from BMMs is not inhibited by OPG treatment. BMMs cultured in 96-well plates were exposed to centrifugal force (50 g/cm^2) for 1 h and further cultured with and without 200 ng/ml OPG for 7 days in the presence of 50 ng/ml M-CSF and 10 ng/ml TNF-α (A) or in combination with 50 ng/ml RANKL (B). These cells were fixed and processed for TRAP staining 7 days after force exposure, and the number of TRAP-positive cells was counted. $^{***}P < 0.001$ versus the experiments. C: BMMs were exposed to force (50 g/cm^2) for 1 h and grown for an additional 7 days with 50 ng/ml M-CSF and 10 ng/ml TNF-α in the presence of either anti-TNF-α antibody (10 μg/ml) or a mixture containing anti-TNFR1 (10 μg/ml) and ani-TNFR2 (10 μg/ml) antibodies. These cells were then stained with TRAP, and TRAP-positive cells were counted as osteoclasts. ND, not detected.

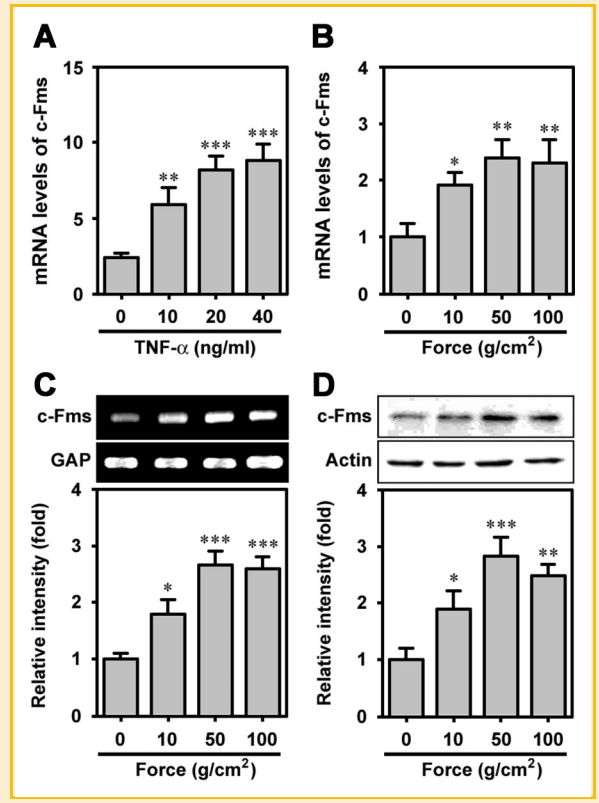

Fig. 5. Centrifugal force augments c-Fms expression induced by TNF-α in BMMs. A: BMMs suspended in α-MEM containing 50 ng/ml M-CSF were spread onto six-well plates, then exposed to increasing concentrations (0–40 ng/ml) of TNF-α. The expression level of c-Fms was determined after 12 h of culture by real-time PCR using c-Fms and β-actin-specific primers. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus M-CSF treatment alone. BMMs were also exposed to the indicated magnitudes of centrifugal force in the presence of 50 ng/ml M-CSF and 10 ng/ml TNF-α. The mRNA level of c-Fms was evaluated 12 h after force exposure by real-time PCR (B) or reverse transcriptase PCR (C). In experiment (C), data from triplicate experiments were quantified by densitometry after normalizing bands to GAPDH. D: BMMs were exposed the force in the presence of M-CSF and TNF-α for 1 h; 48 h later, the cells were analyzed by Western blotting. A representative result from triplicate experiments is shown. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the cells not exposed to force.

is supported by the finding that RANKL induced the RANK mRNA expression (this was significantly increased in co-cultures with TNF-α), whereas RANKL did not affect c-Fms mRNA expression in cultures treated with M-CSF and TNF-α (Fig. 7A,B).

ANTI-c-Fms ANTIBODY INHIBITS OSTEOCLAST DIFFERENTIATION AND BONE RESORPTION IN BMMs

To examine the possible mechanism by which TNF-α stimulates osteoclastogenesis in BMMs, control and force-exposed cells were cultured with osteoclastogenic factors in the presence and absence of anti-c-Fms antibody for 7 days. Treating the cells with anti-c-Fms antibody blocked osteoclast differentiation from BMMs (Fig. 8A) and reduced the number of TRAP-positive cells in a dose-dependent manner (Fig. 8B). This inhibitory activity of anti-c-Fms antibody was more prominent in cultures incubated with TNF-α alone than in combination with RANKL, where the complete inhibition of TNF-α-induced osteoclasts by anti-c-Fms antibody was achieved at a dose of 10 ng/ml. Force-mediated osteoclast differentiation was also sensitively inhibited by adding anti-c-Fms antibody (data not shown). These findings suggest that the anti-c-Fms antibody completely prevents TNF-α-induced osteoclastogenesis both in the control and force-exposed BMMs, while partially inhibiting osteoclastogenesis induced by RANKL.

Figure 9A reveals that the anti-c-Fms antibody prevents the force-mediated increase of pit formation induced by M-CSF and TNF-α. A significant increase in bone resorption occurred even when these cells were subjected to force at a magnitude of 10 g/cm^2

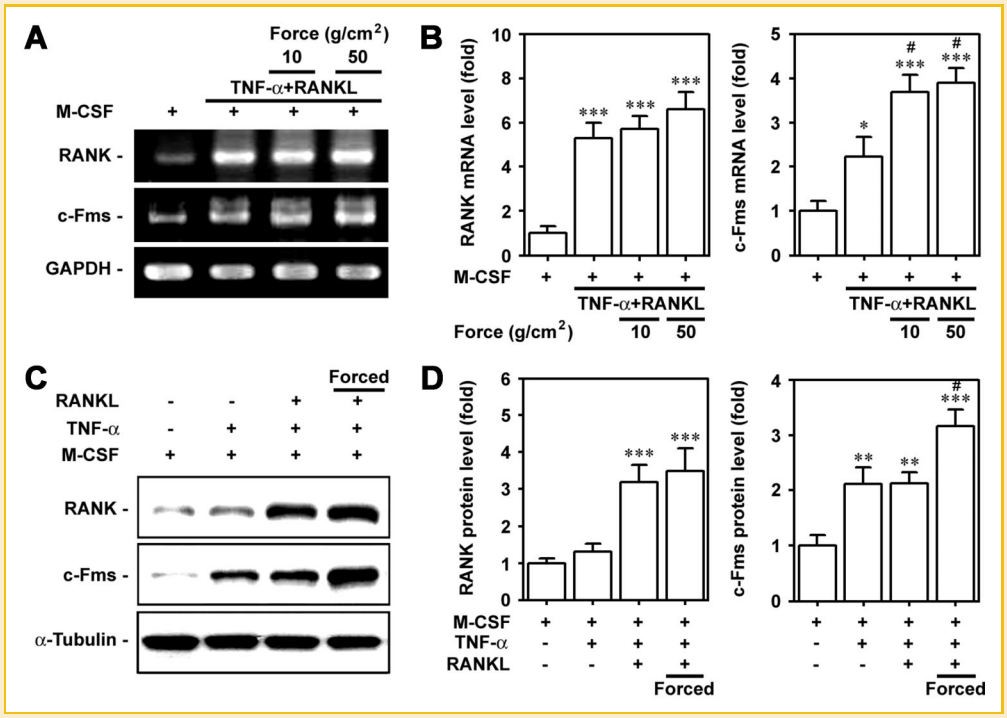

Fig. 6. c-Fms is also stimulated by centrifugal force in cultures incubated in the presence of RANKL. BMMs were incubated with 50 ng/ml M-CSF and 10 ng/ml TNF-α in the presence and absence of 50 ng/ml RANKL, then exposed to centrifugal force at a magnitude of 10 or 50 g/cm^2. A: After 12 h, mRNA levels of RANK and c-Fms were determined by reverse transcriptase PCR. B: The ratio of RANK and c-Fms from triplicate experiments was quantified by densitometry after normalizing bands to GAPDH. $^{***}P < 0.001$ versus M-CSF treatment alone. $^{\#}P < 0.05$ versus the cultures with M-CSF, TNF-α, and RANKL but without force. C: BMMs were also exposed to 50 g/cm^2 centrifugal force for 1 h in the presence of 50 ng/ml M-CSF, 10 ng/ml TNF-α, and/or 50 ng/ml RANKL. The cellular levels of RANK and c-Fms proteins were determined by Western blot analysis 48 h after application of force. D: The levels of RANK and c-Fms proteins from triplicate experiments were quantified by densitometry after normalizing the bands to α-tubulin. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus M-CSF treatment alone. $^{\#}P < 0.05$ versus the cultures with M-CSF, TNF-α, and RANKL but without force.

($P < 0.05$); this was maintained up to 100 g/cm^2 (Fig. 9B). The anti-c-Fms antibody (at a dose of 5 ng/ml) reduced bone resorption to approximately 45% in the force-exposed cells compared to controls, and almost complete inhibition of bone resorption by the antibody was found at dose of 10 ng/ml (Fig. 9C). Similarly, anti-c-Fms antibody suppressed dose-dependently bone resorption stimulated by TNF-α in non-forced BMMs (data not shown).

DISCUSSION

Osteoclasts are derived from hematopoietic precursors of the monocyte/macrophage lineage in response to osteoclastogenic cytokines, including RANKL, M-CSF, and TNF-α. Among these factors, RANKL is considered the most essential factor for osteoclast formation and bone resorption. The finding that RANKL-deficient mice develop osteopetrosis and entirely lack osteoclasts clearly supports the requirement of RANKL in osteoclastogenesis [Kong et al., 1999; Saidenberg-Kermanac'h et al., 2004]. It was also reported that a minimum level of RANKL was needed for TNF-α-induced osteoclast differentiation [Lam et al., 2000]. However, there is considerable evidence that TNF-α induces differentiation of osteoclasts from M-CSF-derived macrophages in a RANKL-independent manner. In fact, TNF-α at high doses directly stimulates the formation of mature osteoclasts from hematopoietic precursors in the absence of osteoblasts or stromal cells [Azuma et al., 2000; Kobayashi et al., 2000]. It also exerts osteoclastogenic effects by stimulating bone marrow stromal cells and their derivative osteoblasts [Cenci et al., 2000]. Kwak et al. [2005] reported that TNF-α is capable of inducing osteoclast differentiation from human blood mononuclear cells independent of RANKL. This is in agreement with the results of the present study, which demonstrated the potential of TNF-α to induce osteoclast differentiation from BMMs without RANKL. This is also supported by the current findings showing that OPG treatment did not inhibit TNF-α-induced osteoclast differentiation but did block RANKL-induced osteoclastogenesis. Although the role of TNF-α in regulating osteoclastogenesis has been studied extremely, this study suggests an important role for this factor in osteoclast induction from BMMs.

Mechanical stress generates responses from mechanosensitive cells; this is mainly due to bone cells, although other many types of cells (including fibroblasts and epithelial cells) are also responsible to mechanical stress [Iqbal and Zaidi, 2005; Liedert et al., 2006; Rubin et al., 2006]. Numerous approaches have been used in understanding the mechanisms by which osteoclast differentiation and bone remodeling are modulated by mechanical force. The cellular responses to mechanical force differ according to the mode of force applied and the types of cells studied. Klein-Nulend et al. [1990] reported that exposure of fetal mouse cartilaginous bone cells to intermittent compressive force decreased mineral resorption and

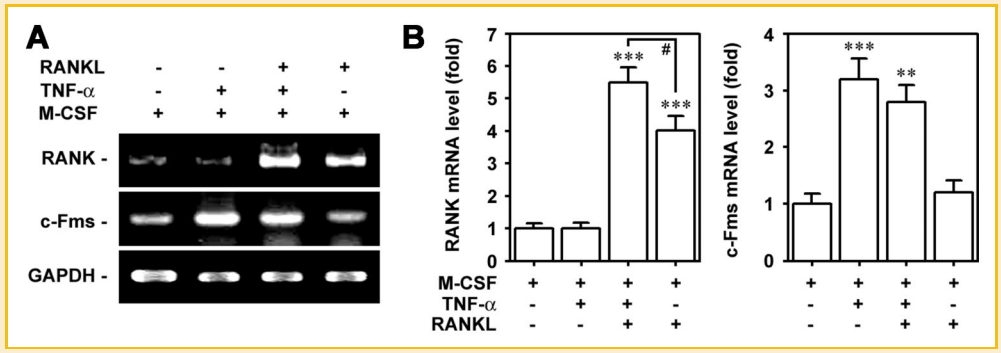

Fig. 7. TNF-α augments RANK expression induced by RANKL in BMMs. BMMs suspended in α-MEM containing 50 ng/ml M-CSF were incubated in six-well plates in the presence or absence of 10 ng/ml TNF-α and 50 ng/ml RANKL. A: After 12 h, mRNA levels specific for RANK or c-Fms were determined by reverse transcriptase PCR. B: The levels of RANK and c-Fms were quantified from triplicate experiments by densitometry after normalizing bands to the GAPDH. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus M-CSF treatment alone. $^{\#}P < 0.05$ versus the experiments.

the number of TRAP-positive cells. Application of cyclic pressure to bone marrow cells (10–40 kPa at 1.0 Hz) significantly lowered osteoclast differentiation and bone resorption by reducing the production of cytokines involved in osteoclast formation [Nagatomi et al., 2002]. Mechanical strain also inhibited the expression of RANKL by murine stromal cells [Rubin et al., 2000]. Conversely, compressive mechanical force promoted osteoclast formation through RANKL expression on synovial cells derived from rat knee joints [Ichimiya et al., 2007]. A recent report also showed that pressure loading to mesenchymal stem cells during early osteodifferentiation promoted osteoclastogenesis through up-regulation of the RANKL/OPG ratio [Liu et al., 2009]. In the present study, centrifugal force increased osteoclast differentiation and bone resorption induced both by TNF-α alone and in combination with RANKL in BMMs. TNF-α-induced osteoclast differentiation and its facilitation by force exposure were completely blocked only by adding anti-TNF or anti-TNFR antibodies. Furthermore, centrifugal force itself stimulated the production of TNF-α in BMMs (data not shown). These results strongly suggest that TNF-α induces osteoclast differentiation from BMMs through a RANKL-independent pathway, and that the mechanism by which the force facilitates osteoclastogenesis is closely related to a TNF-α-mediated pathway.

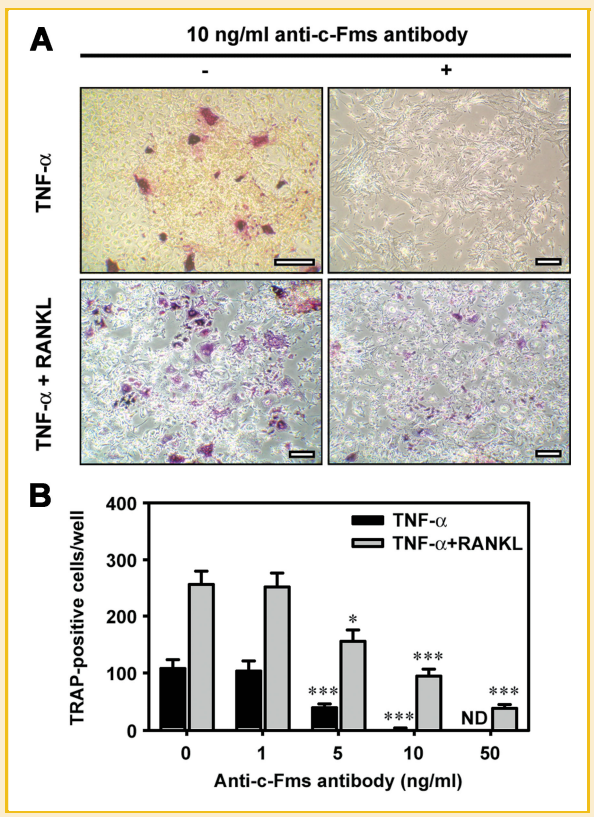

Fig. 8. Anti-c-Fms antibody inhibits osteoclast differentiation in BMMs. BMMs were cultured in 96-well plates for 7 days with 50 ng/ml M-CSF and 10 ng/ml TNF-α or in combination with 50 ng/ml RANKL in the presence of the indicated doses of anti-c-Fms antibody. A: Cells were fixed and subjected to TRAP staining; representative data from triplicate experiments are shown. Bar = 50 μm. B: TRAP-positive cells were also counted at the same time from triplicate experiments and expressed as the number of cells per well. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus the control values. ND, not detected.

Several studies have shown that orthodontic force induces the expression of TNF-α, and that TNF-α plays an important role in mechanically induced osteoclastogenesis and bone resorption. In contrast, administration of anti-c-Fms antibody has been shown to block osteoclastogenesis at the pressure side during orthodontic tooth movement [Kitaura et al., 2008, 2009]. c-Fms is the receptor of M-CSF and has been shown to increase the viability of osteoclast precursors and stimulate osteoclast maturation [Teitelbaum, 2000]. Results from the present study reveal that TNF-α stimulates c-Fms expression and that this is augmented by centrifugal force. Anti-c-Fms antibody completely inhibited osteoclast differentiation induced by M-CSF and TNF-α but partially blocked osteoclastogenesis stimulated by combination with RANKL. This result is consistent with previous findings that anti-c-Fms antibody inhibits RANKL-induced osteoclastogenesis in a significant level but completely blocks pathological osteoclast formation and bone resorption induced by the direct injection of TNF-α [Kitaura et al., 2005]. These results suggest that TNF-α-induced osteoclastogenesis is more easily inhibited by anti-c-Fms antibody than is RANKL-induced osteoclast differentiation. It is also important to consider that TNF-α induces M-CSF gene expression in stromal cells and increases

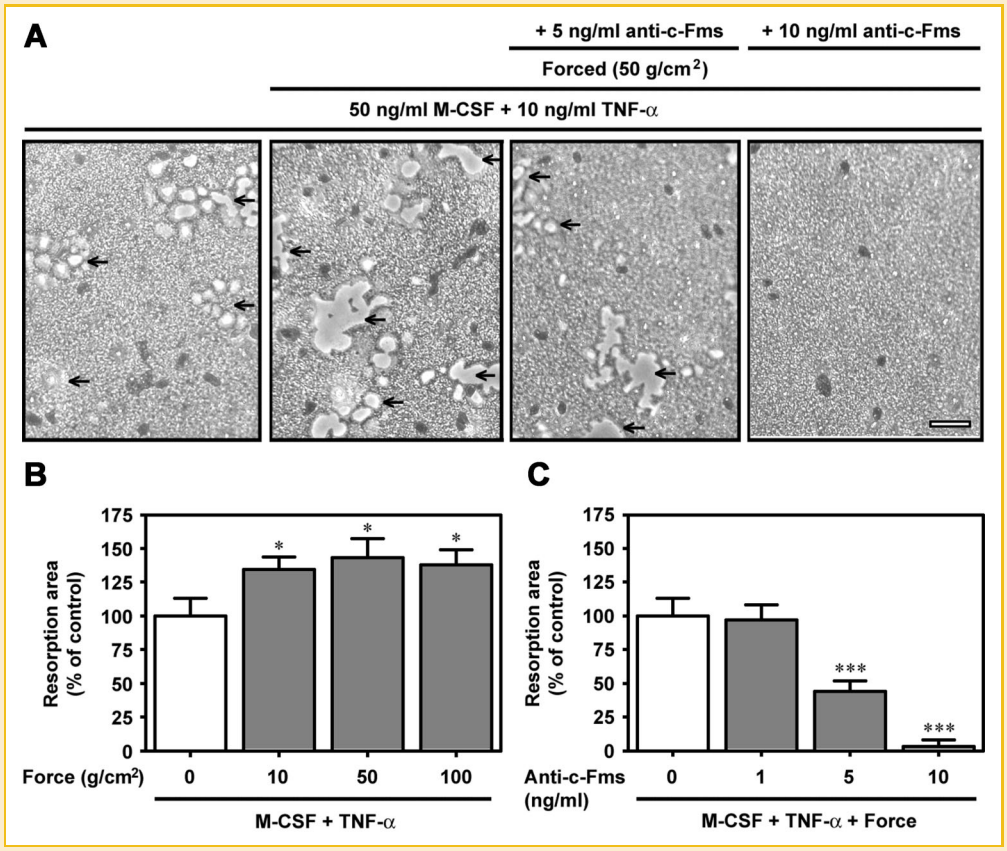

Fig. 9. Anti-c-Fms antibody prevents the force-mediated increase of TNF-α-induced pit formation in BMMs in a dose-dependent manner. A: BMMs were subjected to centrifugal force (50 g/cm^2) for 1 h in bone coated 24-well plates and cultured for 7 days in the presence of 50 ng/ml M-CSF and 10 ng/ml TNF-α with the indicated concentrations of anti-c-Fms antibody. Pit formation on the plate was observed under optic microscopy. Bar = 10 μm. B: BMMs were also exposed to various centrifugal forces (0–100 g/cm^2) in the presence of 50 ng/ml M-CSF and 10 ng/ml TNF-α, and 7 days later, the resorbed area was quantified from triplicate experiments and expressed as % of control value. C: In addition, BMMs were subjected to 50 g/cm^2 centrifugal force for 1 h in the presence of M-CSF and TNF-α with increasing doses (0–10 ng/ml) of anti-c-Fms antibody, and pit area was measured 7 days after force application. $^*P < 0.05$ and $^{***}P < 0.001$ versus the control values.

the number of monocyte/macrophages [Kitaura et al., 2005]. Collectively, we suggest that TNF-α directly stimulates c-Fms-mediated signaling pathway and partially activates the action of RANKL in stimulating osteoclastogenesis and bone resorption and that these are accelerated by compressive force.

In summary, the present study evaluated the effect of TNF-α on osteoclast differentiation from BMMs. The results support the ability of TNF-α to induce osteoclastogenesis from BMMs. This study also revealed that centrifugal force accelerated the TNF-α-induced osteoclast differentiation from BMMs; this was closely associated with up-regulation of c-Fms expression and TNF-α production by the cells. In addition, anti-c-Fms antibody completely inhibited TNF-α-mediated osteoclastogenesis and its facilitation by application of force. Importantly, however, TNF-α did not induce the formation of MNCs from BMMs in the absence of M-CSF. The present findings, in combination with previous reports, strongly suggest that TNF-α, in the presence of M-CSF, plays a critical role in inducing osteoclast formation and bone resorption at the compression side that occurs during orthodontic tooth movement. Furthermore, the current findings demonstrate strongly the facilitating effect of mechanical force on osteoclastic differentiation from BMMs even at the absence of mechanosensitive cells. More detailed experiments are needed to clarify the mechanisms by which mechanical force stimulates c-Fms expression and facilitates osteoclastogenesis from BMMs.

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