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ORIGINAL ARTICLE

Effects of mechanical stress on cytokine production in mandible-derived osteoblasts

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OBJECTIVE: Mechanical stress is known to be an important factor in the regulation of bone remodeling, and mandibular bone is continuously exposed to mechanical stressors such as occlusal force. Therefore, in this study, we investigated the effects of mechanical stress approaching occlusal force, to which mandible-derived osteoblasts (MDOB) are exposed, on cytokine expression and production using an original hydrostatic pressure apparatus.

MATERIALS AND METHODS: The levels of cytokine in MDOB were examined by real-time RT-PCR, ELISA, and western blotting. In addition, mitogen-activated protein kinase inhibitor for ERK1/2, JNK, and p-38 pathways was used to identify the signal transduction pathway.

RESULTS: Hydrostatic pressure increased the expression of IL-6 and TNF-α mRNA in a magnitude- and time-dependent manner and also enhanced IL-6 and TNF-α protein production. Furthermore, hydrostatic pressure changed the RANKL/OPG ratio in favor of RANKL for both mRNA and protein levels. Specific inhibitor of p-38 pathway but not that of the ERK1/2 and JNK pathways suppressed the up-regulation of RANKL production induced by hydrostatic pressure loading.

CONCLUSION: These results suggest that MDOB play a role in cytokine production in response to mechanical stress and that occlusal force may support the maintenance of mandible bone homeostasis by activating bone remodeling through osteoclastogenesis.

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Keywords: mechanical stress; osteoblast; inflammatory cytokine; RANKL; OPG

Introduction

Mechanical stress is known to be an important factor in the regulation of bone remodeling. Unsuitable mechanical conditions such as being subjected to excessive mechanical stress or weightlessness may result in unbalanced bone remodeling (Huiskes *et al*, 2000; Garmeliet *et al*, 2001; Ehrlich and Lanyon, 2002).

It was recently reported that mechanical stress loading affects osteoblasts and periodontal ligament cells to regulate inflammatory cytokine expression (Yamamoto *et al*, 2006; Maeda *et al*, 2007; Koyama *et al*, 2008; Kook *et al*, 2009), receptor activator of nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG) (Tang *et al*, 2006; Kreja *et al*, 2008; Nakajima *et al*, 2008). We have also reported that mechanical stress as hydrostatic pressure induced cytokine production in human periodontal ligament cells (Yamamoto *et al*, 2006, 2011). The changes in the production of these cytokines are thought to play a role in bone remodeling. Skeletal mass homeostasis is maintained by a balance of bone formation and resorption (Boyle *et al*, 2003; Endo and Matsumoto, 2009). Osteoblasts play a crucial function in bone remodeling. They produce bone matrix, such as type I collagen, osteopontin, and osteocalcin (OC), calcificate this bone matrix, and produce RANKL and OPG.

RANKL is a member of the tumor necrosis factor (TNF) ligand family and activates osteoclastogenesis by binding to its receptor RANK on osteoclast progenitors (Takayanagi, 2005a; Wada *et al*, 2006). In contrast, OPG, which is a member of the TNF receptor family, acts as a non-signaling decoy receptor binding to RANKL with higher affinity than RANK and prevents osteoclast differentiation and activation (Khosla, 2001; Takayanagi, 2005a). Inflammatory cytokines such as interleukin-6 (IL-6) and TNF-α are known to activate osteoclastogenesis inducing RANKL expression on osteoblasts, T cells (Takayanagi, 2005b), and periodontal ligament cells (Yamaguchi, 2009).

In the dental region, representative mechanical stress is occlusal force, which reaches approximately 6 MPa (Nagai *et al*, 2001). The mandibula is the part that is most loaded with constant occlusal force. Mammalian bone has two distinct origins. The first is the mesoderm, which gives rise to the axial and appendicular bone. The second is ectodermal, namely the neural crest, which

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gives rise to craniofacial bone (Chung, 2004). And there are two distinct processes of osteogenesis. The first is intramembranous bone formation, during which mesenchymal cells condense and directly differentiate into osteoblasts to deposit bone matrix. The second is endochondral bone formation, during which a cartilage mold is first formed from mesenchymal condensation and then is replaced by bone (Chung, 2004). Thus, in spite of the close resemblance of the end products, osteoblasts and the matrix, bone may have different signaling mechanisms and functions in each part of the processes to produce these differences. The mandibula originates from the neural crest and induces osteogenesis in the intramembranous bone formation mode different from that of other main body bone. Moreover, there is little information available concerning the influence of mechanical stress similar to occlusal force on the mandible osteoblasts. Therefore, we used mandible-derived osteoblasts (MDOB) in this study.

Mitogen-activated protein kinase (MAPK) cascades are one of the most studied and well-established signal transduction systems. Three distinct MAPK cascades have been described in mammalian cells, including the extracellular signal-regulated kinase 1/2 (ERK1/2) cascade, the c-Jun N-terminal kinase (JNK) cascade, and the p-38 cascade. Several studies demonstrated the functional roles of MAPK in regulating cytokine production in osteoblasts (Kim *et al*, 2003; Luo *et al*, 2006; Eda *et al*, 2010). However, the role of MAPK on RANKL production in MDOB-loaded hydrostatic pressure has not yet been estimated. So, we also investigated intracellular transduction pathways for hydrostatic pressure-induced RANKL production in MDOB using MAPK inhibitor.

Mechanical stress is closely related to bone metabolism. In addition, occlusal force is the most representative mechanical stress for mandible bone. Therefore, we presumed that occlusal force is one of the most important regulators for maintaining mandible homeostasis. However, to our knowledge, there has not been any report investigating the effects of mechanical stress similar to occlusal force on cytokine production in mandible osteoblast. In this study, we investigated the effects of mechanical stress approaching occlusal force, to which MDOB are subjected, on cytokine expression and production using an original hydrostatic pressure apparatus.

Materials and methods

Preparation of MDOB

Mouse MDOB were isolated and cultured as in previously described methods (Sodek and Berkman, 1987; Cao *et al*, 2006; Kasama *et al*, 2007). Briefly, the mandibulae were harvested from 4- to 6-week-old male C57BL/6 mice under sterile conditions. After being washed with PBS and having the teeth, condylar process and adhering soft tissue removed, the mandibular bone was minced into small pieces. Subsequently, the bone pieces were treated with 130 U ml^{-1} collagenase at 37°C for 30 min to remove any fibroblastic cells and adipose cells. The bone pieces were washed repeatedly and cultured as explants in culture medium. After 2–4 passages, the cells were used for experiments. The culture medium contained Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 10% FBS and antibiotics (100 U ml^{-1} of penicillin G, 100 μg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin B). To confirm the osteoblastic character of isolated cells, we undertook RT-PCR and alkaline phosphatase (ALP) staining. Animal procedure was approved prior to the study by animal care and use committee of Kyoto Prefectural University of Medicine.

RNA isolation and cDNA synthesis

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), and cDNA was produced using Superscript RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), random primer, and 2.5 mM dNTP mixture (Takara, Otsu, Japan).

RT-PCR

The mRNA expressions of type I collagen, ALP, OC, and aP2 were analyzed. β-Actin was used as an internal control. The primer sequences are shown in Table 1. Reverse transcription polymerase chain reaction (RT-PCR) was performed as described in a previous study (Kita *et al*, 1994). Briefly, a cDNA fragment was amplified by PCR using specific primers and a thermal cycler (Takara). PCR amplification was performed for 35 cycles, each consisting of 1 min at 95°C for denaturation, 1 min at 50°C for annealing, and 1 min at 72°C for extension. Each PCR product was analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide (Bio-Rad Laboratories, Berkeley, CA, USA), and the bands were visualized under a UV light illuminator FAS-III (Toyobo, Osaka, Japan). Distilled water (DW) was used as a negative control for osteoblastic genes, and MC3T3-E1 cells were used as a positive control for osteoblastic genes.

ALP staining

To evaluate the osteoblastic phenotype of isolated cells, we used ALP staining kit (Takara) in accordance with the manufacturer's instructions. Briefly, the cultured cells were washed twice with PBS and fixed with

Table 1 RT-PCR primer sequences

Genes	*Primers*	*Sequences*
β-Actin	Forward	GTGGGGCGCCCCAGGCACCA
	Reverse	CTCCTTAATGTCACGCACGATTTC
Type I collagen	Forward	CCTGGTAAAGATGGTGCC
	Reverse	CACCAGGTTCACCTTTCGCACC
ALP	Forward	GCCCTCTCCAAGACATATA
	Reverse	CCATGATCACGTCGATATCC
Osteocalcin	Forward	CCTCAGTCCCCAGCCCAGATCC
	Reverse	CAGGGCAGAGAGAGGACAGG
aP2	Forward	GCGTGGAATTCGATGAAATCA
	Reverse	CCCGCCATCTAGGGTTATGA

ALP, alkaline phosphatase.

fixation solution for 5 min. Subsequently, having been washed three times with DW, the cells were incubated with ALP staining solution at 37°C for 30 min. After being washed repeatedly with DW, the cells were observed using an optical inverted microscope (Olympus, Tokyo, Japan).

Hydrostatic pressure apparatus

A specially designed system was devised to apply intermittent hydrostatic pressure to the cells (Hikari Koatsu, Hiroshima, Japan). Petri dishes (35 mm diameter) were placed in a deformable Teflon pouch that was filled with serum-free DMEM. The pouch was placed in a stainless steel pressurization vessel. The pressurization vessel was filled with water to uniformly transmit pressure to the cells through the serum-free culture medium in the packed Teflon pouch in a gas-free environment. The pouch was subjected to hydrostatic pressure with a minimum applied pressure of 0.1 MPa and a maximum pressure of 6 MPa. The temperature was maintained at 37°C using a thermostat placed in the pressure vessel. The advantages of this apparatus are the capacity to separate the pressure medium from the air and to load a three-dimensional force reaching an occlusal force (Yamamoto *et al*, 2006, 2011; Sakao *et al*, 2008).

Loading of hydrostatic pressure

The MDOB were seeded onto Petri dishes (35-mm-diameter dish) at a concentration of 5×10^5 cells per dish. The cells approximately reached confluence following 24 h of culture and were then exposed to intermittent hydrostatic pressure (magnitude: 0.1, 1, 6 MPa; time: 10, 30, 60 min; frequency: 1 Hz). We confirmed that the pH of the DMEM inside the teflon pouch did not change before and after the loading of hydrostatic pressure. Cells in dishes placed in the same apparatus but not exposed to hydrostatic pressure were used as non-pressurized controls.

Microscopic observation

After the loading of hydrostatic pressure, morphologic changes in MDOB were observed using an inverted optical microscope (Olympus) after Giemsa staining.

Cell viability analysis

We performed cell viability test using tetrazolium salt assay. Having loaded a hydrostatic pressure on the MDOB, we added 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) solution (Cell Count Reagent SF; Nacalai Tesque, Kyoto, Japan) to culture medium at a 10% concentration. After 2 h of incubation with WST-8 at 37°C, the supernatant was transferred from a Petri dish to a 96-well plate, and the absorbance of each well was determined using a microplate reader (Emax; Molecular Devices, Sunnyvale, CA, USA) at 450 nm against 650 nm as a reference. The absorbance of control cells was considered to be 100%. The relative viability in the experiment is presented as a percentage of that of the control.

Real-time RT-PCR

We performed real-time RT-PCR as previously described (Oseko *et al*, 2009). Briefly, real-time RT-PCR was carried out using the LightCycler (Roche Diagnostics, Mannheim, Germany) and PCR mixtures. The amplification program was performed as follows: 1 cycle of 95°C for 10 min, followed by 45 cycles consisting of 10 s at 95°C for denaturing and 25 s at 60°C for annealing and extension. The PCR mixtures of 20 μl contained 4 μl of TaqMan Master Mix (Roche Diagnostics), 11.8 μl of RNase-free water, 2 μl cDNA, 0.2 μl UPL probe (Roche Diagnostics), and 2 μl of a specific pair of primers. Primers were designed using Universal ProbeLibrary Assay Design Center (Roche Diagnostics). Glyceraldehyde-3-phosphate (GAPDH) was used as an internal control. The primer sequences are shown in Table 2.

ELISA

The culture supernatant was harvested and the concentration of inflammatory cytokines was measured using ELISA kits obtained from the following sources: IL-6 and TNF-α (e-Bioscience, San Diego, CA, USA). All assays were conducted in accordance with the manufacturer's instructions, and the concentrations of these cytokines were determined using a standard curve. The absorbance of each well was determined using the microplate reader Emax at 450 nm. The determinations were performed in duplicate for each cell culture preparation.

Western blotting analysis

Mandible-derived osteoblasts were lysed in a lysis buffer (Nacalai Tesque). After 30-min incubation on ice, the lysates were centrifuged at 12 000 ***g*** for 20 min at 4°C and heated for 5 min at 96°C before being loaded on a gel. Proteins (20 μg per well) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane (Millipore, Billerica, MA, USA), and probed overnight at 4°C with 1:200 rabbit polyclonal anti-RANKL or 1:200 rabbit polyclonal anti-OPG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and reprobed with 1:2000 mouse monoclonal anti-GAPDH (Santa Cruz Biotechnology).

Table 2 Real-time RT-PCR primer sequences

Genes	*Primers*	*Sequences*
GAPDH	Left	tgtccgtcgtggatctgac
	Right	cctgcttcaccaccttcttg
IL-6	Left	gctaccaaactggatataatcagga
	Right	ccaggtagctatggtactccagaa
TNF-α	Left	tcttctcattcctgcttgtgg
	Right	ggtctgggccatagaactga
RANKL	Left	agccatttgcacacctcac
	Right	cgtggtaccaagaggacagagt
OPG	Left	gtttcccgaggaccacaat
	Right	ccattcaatgatgtccaggag

RANKL, receptor activator of nuclear factor kB ligand; OPG, osteoprotegerin.

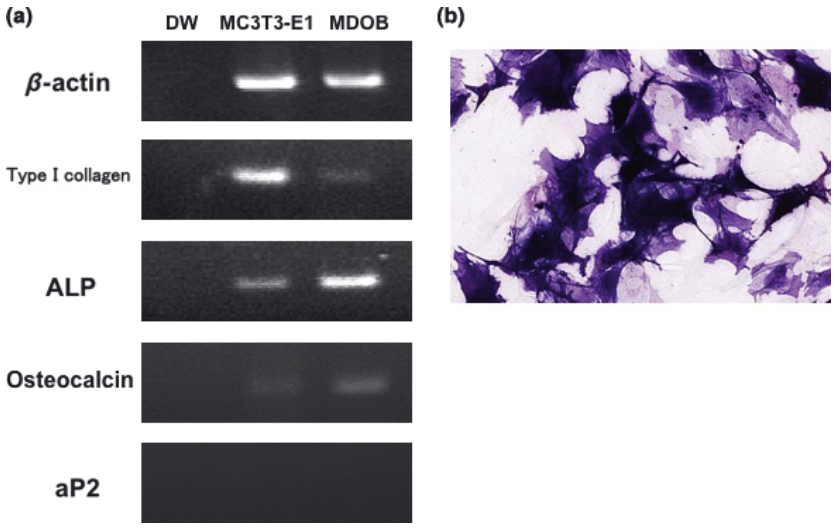

Figure 1 Characterization of osteoblasts isolated from mandible bone. (**a**) The mandible-derived osteoblasts (MDOB) were positive for type I collagen, alkaline phosphatase (ALP), and osteocalcin, but negative for aP2 mRNA expression. MC3T3-E1 was a positive control for osteoblast-specific genes, and distilled water (DW) was a negative control for osteoblast-specific genes. (**b**) Almost all MDOB (>95%) were positive for ALP staining under microscope observation

After 1-h incubation at room temperature with 1:5000 anti-rabbit or 1:5000 anti-mouse HRP-labeled secondary antibody (GE Healthcare, Buckinghamshire, UK), blots were enhanced using the ECL Plus Western Blotting Detection System (GE Healthcare) and visualized using Molecular Imager (Bio-Rad Laboratories). The intensity of each protein band was analyzed using Image Quant 5.1 (GE Healthcare) and standardized with the intensity of the actin band to evaluate the amount of cytokine produced.

MAPK inhibition
To investigate the role of MAPK signal pathway on hydrostatic pressure-induced RANKL production, MDOB were pretreated with each MAPK inhibitors ERK1/2 pathway inhibitor, PD98059 (Cayman Chemical, Ann Arbor, MI, USA), JNK pathway inhibitor, SP600125 (Enzo Life Science, Farmingdale, NY, USA), or p-38 pathway inhibitor, SB202190 (Cayman Chemical), for 10 μM 1 h prior to the application of hydrostatic pressure. MDOB were then loaded to hydrostatic pressure, and lysate samples of MDOB were collected for western blot analysis.

Statistical analysis
Data are expressed as mean ± standard deviation (s.d.) or ± standard error (s.e.). Statistical significance was analyzed using Student's *t*-test and ANOVA with Tukey–Kramer correction for multiple comparisons. *P* values <0.05 were considered significant.

Results

Identification of isolated cells
To investigate the characterization of MDOB, the mRNA expressions of the osteoblast-specific genes, type I collagen, ALP, and OC, and the adipocyte-specific gene, aP2, were analyzed by RT-PCR. The MDOB were positive for type I collagen, ALP, and OC, but negative for aP2 mRNA expression (Figure 1a). Moreover, we performed ALP staining to evaluate the osteoblastic phenotype for MDOB. Almost all MDOB (>95%) were positive for ALP staining under the microscope (Figure 1b).

Morphology and cell viability of the MDOB
Exposure to hydrostatic pressure ranging from 1 to 6 MPa caused no changes in the morphology of MDOB (data not shown). The viability of MDOB after exposure to hydrostatic pressure of 6 MPa was 93.3 ± 8.6% as determined by tetrazolium salt assay (Figure 2).

Expression of inflammatory cytokine mRNA in MDOB
The levels of IL-6 and TNF-α mRNA expressions were determined by real-time RT-PCR after loading hydrostatic pressure onto MDOB. As shown in Figure 3a, the level of IL-6 and TNF-α mRNA in MDOB was increased by applying a hydrostatic pressure to the cells in a time-dependent manner. The levels of IL-6 and TNF-α mRNA in MDOB were increased in a magnitude-dependent manner; for example, the IL-6 mRNA level was elevated 1.6-, 2.9-, and 3.7-fold for 0.1, 1, and 6 MPa, respectively (Figure 3b).

Concentration of inflammatory cytokines in the culture supernatant of MDOB
The amounts of IL-6 and TNF-α in the culture supernatant of MDOB were measured using specific

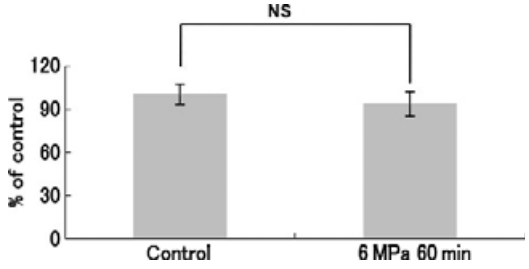

Figure 2 Cell viability of mandible-derived osteoblasts (MDOB) after exposure to hydrostatic pressure; MDOB were not affected in terms of cell viability ($n = 4$). N.S., not significant

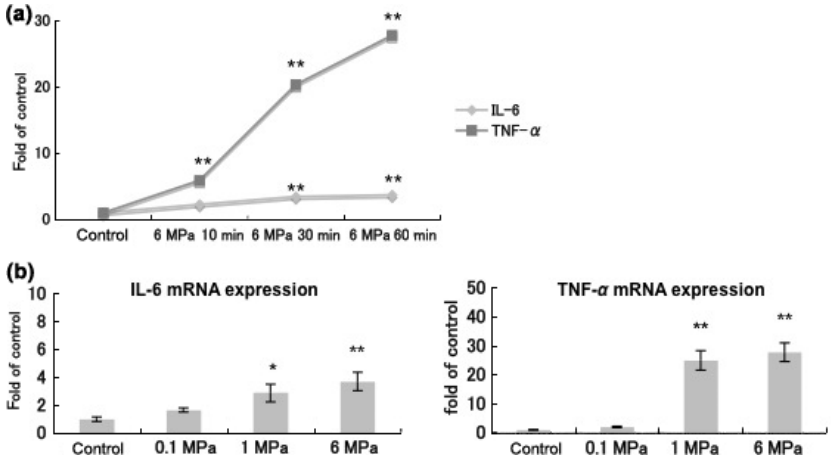

Figure 3 Effects of hydrostatic pressure on the expression of inflammatory cytokines in mandible-derived osteoblasts (MDOB). The mRNA levels of inflammatory cytokines were analyzed using real-time PCR. The results of real-time PCR are shown as a fold ratio between the expression level of the control and that of the experiments (experiments/control). (**a**) MDOB were exposed to a pressure of 6 MPa for various durations of time. (**b**) MDOB were exposed to various pressures for 60 min. $*P < 0.05$ and $**P < 0.01$ *vs* the control. ($n = 7$, values are mean ± s.e.)

ELISA kit. The amounts of IL-6 and TNF-α in the conditioned medium from control MDOB were 481.0 and 16.3 pg ml^{-1}, respectively. As the amount of IL-6 increased to 689.9 and 798.6 pg ml^{-1} after exposure to 1 and 6 MPa, respectively, the hydrostatic pressure augmented the amount of IL-6 production by MDOB significantly in a magnitude-dependent manner. Furthermore, TNF-α production was also augmented by hydrostatic pressure in a magnitude-dependent manner (Figure 4).

RANKL and OPG level in MDOB

To investigate the effect of hydrostatic pressure on the expression and production of RANKL and OPG, we performed real-time RT-PCR and western blotting. Not only RANKL but also OPG expression level was increased after applying a hydrostatic pressure, in terms of the mRNA level (Figure 5a). However, RANKL production level was increased (band intensity: 19316–25327) and OPG production level was not changed (band intensity: 10406–10835) in terms of the protein level (Figure 5c). RANKL/OPG mRNA and protein ratio increased significantly by 2.0-fold (6 MPa, 60 min) and 1.3-fold (6 MPa, 60 min) after loading of hydrostatic pressure (Figure 5b,d), respectively. These results indicate that hydrostatic pressure changes the RANKL/OPG ratio in favor of RANKL at the mRNA and protein levels.

Effect of MAPK inhibitor on hydrostatic pressure-induced RANKL expression in MDOB

We used MAPK inhibitor for JNK, ERK1/2, and p-38 to identify the signal transduction pathway on RANKL production in MDOB. As shown in Figure 6, RANKL production was increased significantly by hydrostatic pressure loading (6 MPa, 60 min) (1.33-fold), but up-regulation of RANKL production exposed with hydrostatic pressure was significantly suppressed by the addition of SB203580 (1.06-fold).

Discussion

In this study, we have shown that hydrostatic pressure affects the cytokine expression and production regulating bone remodeling in osteoblasts. Although several studies have suggested that mechanical stress induces cytokine production in osteoblasts, this is the first study to investigate the effects of hydrostatic pressure on the cytokine production in MDOB. Our results showed that hydrostatic pressure induced inflammatory cytokine production in a time- and magnitude-dependent manner and changed the RANKL/OPG ratio in favor of RANKL, suggesting that occlusal force could support the activation of bone remodeling via osteoclastogenesis.

It was reported that physiological occlusion condition is approximately 6 MPa with a 0.9- to 1.7-Hz interval (Morimoto *et al*, 1984; Nagai *et al*, 2001). Therefore,

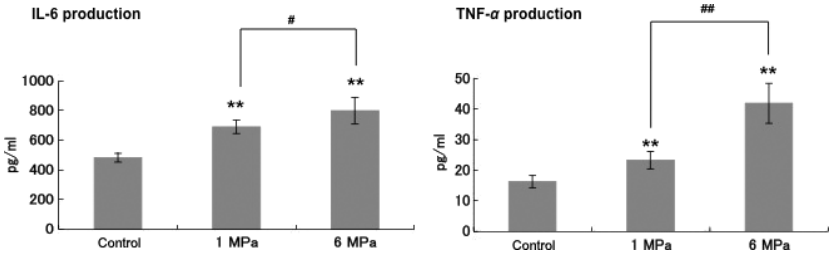

Figure 4 Effect of hydrostatic pressure on the production of inflammatory cytokines in mandible-derived osteoblasts. Supernatant fluids were collected 24 h after exposure to the indicated magnitude of hydrostatic pressure for 60 min. IL-1β, IL-6, and TNF-α production amounts were measured using an ELISA kit. $*P < 0.05$ and $**P < 0.01$ *vs* the control. $\#P < 0.05$ and $\#\#P < 0.01$ indicate significant differences between experiments. ($n = 4$, values are mean ± s.d.)

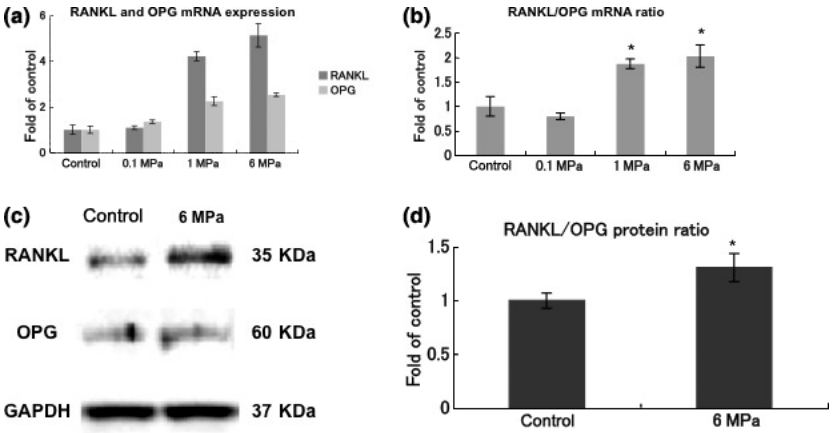

Figure 5 Effects of hydrostatic pressure on the expression of RANKL and osteoprotegerin (OPG) in mandible-derived osteoblasts. The mRNA levels of RANKL and OPG were analyzed using real-time PCR. The results of RANKL/OPG mRNA ratio are shown as a fold ratio between the expression level of the control and that with 6 MPa for 60 min (experiments/control). (**a**) RANKL and OPG mRNA, (**b**) RANKL/OPG mRNA ratio. The protein levels of RANKL and OPG were investigated using western blot analysis. (**c**) Exhibition of RANKL, OPG, and GAPDH protein bands and (**d**) RANKL/OPG protein ratio. RANKL/OPG protein ratio is shown as a fold ratio between the expression level of the control and that with 6 MPa for 60 min (experiment/control). $*P < 0.05$ *vs* the control. ($n = 5$, values are mean ± s.d.)

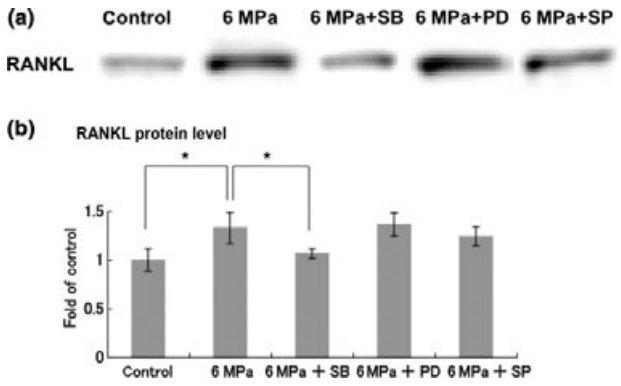

Figure 6 Effects of mitogen-activated protein kinase (MAPK) inhibitor on hydrostatic pressure-induced RANKL production. The RANKL production was investigated using Western blot analysis. (**a**) Exhibition of RANKL protein band and (**b**) RANKL protein level ratio. RANKL level ratio was shown as a fold ratio between the level of the control and that of the experiments (6 MPa, 60 min or 6 MPa, 60 min + each MAPK inhibitor/control). $*P < 0.05$ indicates significant differences between experiments. ($n = 5$, values are mean ± s.d.)

our model resembles the natural environment *in vivo*. In fact, exposure to hydrostatic pressure ranging from 1 to 6 MPa caused no changes in the morphology and viability of MDOB in this study. Parkkinen *et al* (1995) and Yamamoto *et al* (2011) also reported that 5- to 6-MPa hydrostatic pressure did not change the morphology of the cells.

Under physiological conditions, bone is resorbed periodically by osteoclasts, while new bone is formed by osteoblasts. The bone remodeling process is initiated by osteoclast activation and old bone resorption occurs (Sims and Gooi, 2008). Subsequently, osteoblasts are activated and new bone formation proceeds (Boyce and Xing, 2008). Osteoclast activation is induced by the presence of RANKL, which is expressed in osteoblast membrane, and macrophage colony-stimulating factor (Takayanagi, 2005a). RANKL binds to its receptor RANK on osteoclast progenitors and stimulates their differentiation and activity. Otherwise, OPG acts as a non-signaling decoy receptor binding to RANKL and prevents the activation of RANK, which results in a decrease in osteoclast recruitment. This RANK/RANKL/OPG axis controls the balance between bone formation and resorption (Khosla, 2001; Sims and Gooi, 2008). The expression of RANKL in osteoblasts is enhanced by 1α,25-dihydroxyvitamin D_3 (Suda *et al*, 2003), parathormone (PTH) (Suda *et al*, 2003), prostaglandin E_2 (PGE_2) (Takayanagi, 2005b), and inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Takayanagi, 2005b; Cochran, 2008). In addition, recent studies have shown that TNF-α directly stimulates the differentiation of osteoclast progenitors to osteoclasts, not dependent on RANKL/RANK interaction (Kobayashi *et al*, 2000; Kim *et al*, 2009).

There have been many studies confirming the results of the present study. Koyama *et al* (2008) subjected human osteosarcoma cell line Saos-2 cells to various magnitudes of compressive forces and found that Saos-2 up-regulated inflammatory cytokine expression, such as IL-6 and TNF-α, in a time- and magnitude-dependent manner. Sakao *et al* (2009) also reported that osteoblasts derived from human osteophytes augmented IL-6 and IL-8 production after loading with hydrostatic pressure of 1–50 MPa in a magnitude-dependent manner. Nakao *et al* (2007) exposed human periodontal ligament cells to compressive forces and found strong up-regulation of RANKL. An increase in RANKL expression and production was found after loading fluid flow to murine osteoblasts (Mehrotra *et al*, 2006). Moreover, Kreja *et al* (2008) loaded various kinds of mechanical stress to osteoblasts derived from human femur or tibia and found that intermittent force enhanced the RANKL mRNA expression level.

On the other hand, there are some reports that contrast with our results. In the human osteoblastic cell

line MG-63, OPG production was up-regulated and RANKL expression was not changed after exposure of a cyclic strain (Saunders *et al*, 2005). Tang *et al* (2006) exposed mouse osteoblastic cell line MC3T3-E1 cells to a tensile strain, which up-regulated OPG production and down-regulated sRANKL production. These studies demonstrated that mechanical stress could depress osteoclastogenesis and inhibit bone resorption. These contradictory results may be due to differences in mechanical conditions (strength, time, and quality) and types of cells.

It is well known that MAPK signaling plays a role in cytokine production in osteoblast. However, there is little information available concerning the signaling pathway on RANKL production when osteoblasts were loaded with hydrostatic pressure. Thus, we assessed the MAPK signaling pathway on RANKL production using MAPK inhibitor. In this study, RANKL up-regulation was significantly suppressed when SB203580 was added, suggesting that p-38 cascade is involved in RANKL augmentation by hydrostatic pressure in MDOB. Previously, it was reported that p-38 is involved in osteoblast proliferation and differentiation (Zhao *et al*, 2007). Moreover, it was reported that soluble RANKL production from human osteoblast is attenuated in the presence of p-38 MAPK inhibitor in cyclic tensile stress model (Kusumi *et al*, 2005). These finding could support our results.

The bone remodeling cycle is altered by stimulation, such as mechanical stress. Our results indicate that mechanical stress induces osteoclastogenesis. In the bone remodeling process, osteoclastogenesis and bone resorption occur initially and subsequently bone formation proceeds. In this study, we investigated the effect of mechanical stress on the cytokine production in MDOB only for a short time. Therefore, it seems that our results may represent the initial stage of the bone remodeling cycle. On the other hand, in experiments with a model loaded with long-term mechanical stress (Zhu *et al*, 2008; Liu *et al*, 2009), osteogenic marker up-regulation was observed in osteoblasts loaded with mechanical stress compared with that for non-loaded osteoblasts. In future, it will be necessary to examine the effect of long-term mechanical stress loading on the cytokine production in MDOB.

Periostin, a novel secreted disulfide-linked 90-kDa protein, is highly expressed in osteoblasts and periodontal ligament cells and supports cell adhesion (Horiuchi *et al*, 1999; Afanador *et al*, 2005). Rios *et al* (2008) reported that mechanical stress loading maintains the integrity of periodontium through the up-regulation of periostin expression in periodontal ligament cells. Moreover, it was reported that periostin is one of the local contributing factors in bone and periodontal tissue remodeling following mechanical stress during tooth movement (Wilde *et al*, 2003). Thus, periostin plays a role in the relationship between mechanical stress and bone remodeling. As periostin may play essential role in mandible bone remodeling in response to occlusal force, it is necessary to investigate the periostin for the analysis of molecular mechanism in mandible bone remodeling in future.

In conclusion, we demonstrated that hydrostatic pressure augmented the production of inflammatory cytokines in a magnitude- and time-dependent manner and changed the RANKL/OPG ratio in favor of RANKL in MDOB. Moreover, RANKL is up-regulated by hydrostatic pressure via p-38 pathway in MDOB. These results suggest that MDOB play a role in cytokine production in response to mechanical stress and that occlusal force may support the maintenance of mandible bone homeostasis by activating bone remodeling through osteoclastogenesis *in vivo*.

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Author contributions

Kenta Yamamoto, Toshiro Yamamoto, Narisato Kanamura, and Masakazu Kita designed the study. Kenta Yamamoto performed the experiments and analyzed the data. Masakazu Kita and Kenta Yamamoto drafted the paper. Hiroaki Ichioka, Yuki Akamatsu, and Fumishige Oseko assisted the experiments. Osam Mazda and Jiro Imanishi guided the technical procedure

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