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Effects of different magnitudes of mechanical strain on Osteoblasts in vitro

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Abstract

In addition to systemic and local factors, mechanical strain plays a crucial role in bone remodeling during growth, development, and fracture healing, and especially in orthodontic tooth movement. Although many papers have been published on the effects of mechanical stress on osteoblasts or osteoblastic cells, little is known about the effects of different magnitudes of mechanical strain on such cells. In the present study, we investigated how different magnitudes of cyclic tensile strain affected osteoblasts. MC3T3-E1 osteoblastic cells were subjected to 0%, 6%, 12% or 18% elongation for 24 h using a Flexercell Strain Unit, and then the mRNA and protein expressions of osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) were examined. The results showed that cyclic tensile strain induced a magnitude-dependent increase (0%, 6%, 12%, and 18%) in OPG synthesis and a concomitant decrease in RANKL mRNA expression and sRANKL release from the osteoblasts. Furthermore, the induction of OPG mRNA expression by stretching was inhibited by indomethacin or genistein, and the stretch-induced reduction of RANKL mRNA was inhibited by PD098059. These results indicate that different magnitudes of cyclic tensile strain influence the biological behavior of osteoblasts, which profoundly affects bone remodeling.

Keywords: Mechanical strain; Osteoblasts; Osteoprotegerin; Receptor activator of nuclear factor-κB ligand (RANKL)

Mechanical stress is known to be an important factor in the regulation of bone modeling and remodeling [1–3]. Although many investigators have reported a relationship between bone and mechanical stress induced by stretching [4], ultrasound [5] or gravity [6], bone remodeling after the application of mechanical stress is not fully understood.

Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively, so many investigators have studied the interaction between these two cell types and their role in bone remodeling [7]. Recently, osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) have been shown to play very important roles in bone remodeling [7,8]. OPG is a member of the tumor necrosis factor (TNF) receptor family and a soluble decoy receptor against RANKL and soluble RANKL (sRANKL) [9]. It is produced by osteoblasts and other cells, and has been found to be a key factor in the inhibition of osteoclast differentiation and activation [9]. In contrast, RANKL, which is also a member of the TNF family, activates osteoclastogenesis and mature osteoclasts during growth via their receptor activator of nuclear factor-κB (RANK), and sRANKL cleaved from RANKL behaves similarly to RANKL [8,10]. Increased RANKL expression and sRANKL release lead to bone resorption and loss [9]. The regulation of OPG synthesis and RANKL expression causes either activation or inactivation of osteoclasts, which profoundly affects bone remodeling [8].

Recent studies have shown that mechanical strain plays an important role in the regulation of OPG synthesis and RANKL expression. OPG expression in the periodontal ligament cells is specifically up-regulated under intermittent tensile stress, whereas RANKL is barely affected [11]. Using an in vivo experimental model of tooth movement in rat, Kobayashi et al. [12] demonstrated that preexisting osteoclasts disappeared from the bone surface through apoptosis during a rapid force-induced shift from bone

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resorption to bone formation. They also found a marked increase in OPG mRNA expression in the stretched cells on the tensioned distal bone surface, simultaneously with the loss of osteoclasts. Because osteoclast formation is dependent upon RANKL expression, strain-induced reduction in this factor may contribute to the accompanying reduction in osteoclastogenesis. The application of a 2% mechanical strain (10 cycles/min) has been reported to reduce $1,25(OH)_2D_3$-stimulated osteoclast formation by 50% in murine marrow cultures. This was preceded by decreased expression of RANKL [13]. Kusumi et al. [14] reported that the application of cyclic tensile strain to human osteoblasts caused an increase of OPG synthesis and a decrease of sRANKL release and RANKL mRNA expression. The effects of mechanical stress on cells are known to depend on the magnitude [15,16], duration [17], and frequency [17,18] of the stress, but very few reports to date have explored the effects of different magnitudes of mechanical stress on osteoblasts.

In this study, we investigated how different magnitudes of cyclic tensile strain affected osteoblasts. MC3T3-E1 osteoblastic cells were subjected to 0%, 6%, 12% or 18% elongation using the Flexercell Strain Unit. The effects of different magnitudes of cyclic tensile strain on OPG synthesis, sRANKL release, and expression of mRANKL were then examined. In addition, protein synthesis and signal transduction pathways that mediate cellular responses to mechanical strain were investigated using specific inhibitors.

Materials and methods

MC3T3-E1 cell culture. Mouse osteoblastic MC3T3-E1 cells were obtained from the Center Laboratory for Tissue Engineering, College of Stomatology, Fourth Military Medical University, Xi'an, China. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in α-modified Eagle's minimum essential medium (α-MEM: Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 32 U/ml penicillin G (Meiji Seika, Tokyo, Japan), 250 μg/ml amphotericin B (Nacalai Tesque, Kyoto, Japan), and 60 μl/ml kanamycin (Meiji Seika, Tokyo, Japan). After reaching 90% confluence, the cells were detached by treatment with 10% trypsin-EDTA (Sigma) and cultured for 24 h on six-well, flexible-bottomed plates (type I collagen coated, Flex I; Flexcell International, Mckeesport, PA, USA) at 2×10^5 cells per well, and then the 10% FBS medium was replaced with 1% FBS before strain force was applied to the cells.

Application of strain force. A Flexercell Strain Unit (FX 3000, Flexcell International) was used to generate cyclic tensile strain in the MC3T3-E1 cells. According to the manufacturer's instructions, the cells were subjected to mechanical strain of 6%, 12% or 18% elongation at 6 cycles/min for 24 h. The strain unit consists of a computer-controlled vacuum unit and baseplates to hold the culture dishes. The computer system controls the frequency of deformation and the negative pressure applied to the culture plates. Control cells (0% elongation) were cultured on similar plates and kept in the same incubator without mechanical strain.

Isolation of total RNA. Total cellular RNA was isolated from each culture using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA), according to protocols provided by the manufacturer. To remove contaminating genomic DNA, the RNA samples were treated with RNase-free DNase I (Qiagen) at 37 °C for 30 min.

Reverse transcriptase-polymerase chain reaction. First, 2 μg of total RNA was used as a template to synthesize first-strand complementary DNA (cDNA) with oligo(dT) primer and reverse transcriptase, using the Omniscript RT Kit (Qiagen). Then 1 μl of the cDNA mixture was subjected to polymerase chain reaction (PCR) amplification using specific primers. The primer sequences used in this study are shown in Table 1.

Each PCR was carried out in a 50-μl mixture containing 1 μl cDNA, 5 μl of 10× Qiagen PCR buffer, 10 μl of 5× Q-Solution, 1 μl of each deoxynucleotide triphosphate mix (10 mM), 0.1 μM of each sense and antisense primer, and 0.5 μl *Taq* DNA polymerase (Qiagen). The amplification reaction consisted of initial denaturation at 94 °C for 3 min, followed by three-step cycling: denaturation at 94 °C for 30 s, annealing at a temperature optimized for each primer pair (OPG:59 °C, RANKL:58 °C, and GAPDH:58 °C) for 30 s, and extension at 72 °C for 1 min, all for 30 cycles; then a final extension at 72 °C for 10 min. The amplification products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. The relative intensities of the gel bands were measured using NIH Image software, and the results were normalized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping enzyme. We performed these experiments using samples from at least five different cell preparations, and quantification of mRNA was confirmed using the same cell sample at least in triplicate.

Enzyme-linked immunosorbent assay for the quantitative determination of OPG/sRANKL concentration. Conditioned media harvested from the cultured cells were used as samples. The concentrations of OPG and sRANKL in these supernatants were assayed using OPG and sRANKL enzyme-linked immunosorbent assay (ELISA) kits (OPG: R&D, Minnesota, USA; sRANKL: IDS, Arizona, USA). Measurements were performed in triplicate on samples diluted 1:10 according to the manufacturer's instructions. In brief, 100 μl assay buffer, 50 μl sample, and 50 μl detection antibody were added to a well. After incubation for 16 h at 4 °C, the wells were washed with wash buffer, then 200 μl streptavidin–horseradish peroxidase conjugate was added to each well followed by incubation for 60 min at room temperature. After further washing, substrate was added to all wells and incubated for 20 min, followed by stop solution. The absorption was determined with a microplate reader at 450 nm against 620 nm as a reference. The concentrations of OPG and sRANKL were determined using a standard curve and normalized to the total cell number in each sample.

Inhibitors. Protein synthesis and signal transduction pathways that mediated cellular responses to tensile stress were investigated using specific inhibitors: cycloheximide (10 μM) to inhibit de novo protein synthesis, indomethacin (10 μM) to inhibit cyclooxygenase (COX), genistein (20 μM) to inhibit tyrosine kinase, and PD098059 (10 μM) for specific inhibition of extracellular signal-related kinase (ERK). All inhibitors were obtained from Sigma (St. Louis, MO, USA) and the concentrations used were the effective doses previously reported [19,20]. After the MC3T3-E1 cells had been pre-incubated in the presence of each inhibitor for 30 min to permit

Table 1
Primer sequences used in semi-quantitative RT-PCR

OPG (product 578 bp)	Forward 5′-TCCTGGCACCTACCTAAAACAGCA-3′ Reverse 5′-CTACACTCTCGGCATTCACTTTGG-3′
RANKL (product 223 bp)	Forward 5′- ATGATGGAAGGCTCATGGTTG-3′ Reverse 5′-TGTTGGCGTACAGGTAATAGAA-3′
GAPDH (product 983 bp)	Forward 5′-GGTCGGTGTGAACGGATTTGG-3′ Reverse 5′-ATGTAGGCCATGAGGTCCACC-3′

these compounds to penetrate the cells and block their respective pathways, cyclic tensile strain at 18% elongation, 6 cycles/min was applied in culture for 24 h. Total RNA was extracted from the cells, and the expression levels of OPG and RANKL mRNA were determined by Reverse transcriptase-polymerase chain reaction (RT-PCR).

Statistical analysis. Values were calculated as means ± standard deviation (SD). Some data were subjected to multiple measurement analyses of variance (ANOVA), and Student's t test was used to analyze differences between the cultures tested. A P value of less than 0.05 was accepted as significant.

Results

Initially, the MC3T3-E1 cells were stretched to 6%, 12% or 18% elongation at a frequency of 6 cycles/min, each cycle consisting of 5 s strain and 5 s relaxation, for 24 h. No damaged, dead or apoptotic cells were observed microscopically in the stretch-loaded cultures (Fig. 1). The orientation of cells grown on stretched culture dishes differed from the uniform random distribution observed in non-stretched cultures (Fig. 1). Cells from stretched and non-stretched cultures were harvested and extracted to quantify mRNA expression by RT-PCR.

Fig. 2 shows how different magnitudes of strain affected OPG mRNA expression level in the MC3T3-E1 cells. The expression of OPG mRNA was increased significantly and in a magnitude-dependent manner by mechanical strain with 6%, 12% or 18% elongation in comparison with the control (0% elongation) cultures.

The elevated expression of OPG mRNA in the stretched cells was further confirmed by ELISA for quantifying OPG protein in conditioned medium from these cells. The amount of OPG protein in the stretched cultures also increased significantly in a magnitude-dependent manner compared to that in the control (Table 2).

The mechanisms translating mechanical stress into a signal that activates increased OPG gene expression are as yet unidentified. To investigate whether the up-regulation of OPG mRNA by stretching was dependent on de novo protein synthesis, we added cycloheximide to the MC3T3-E1cell cultures. Cycloheximide did not reduce the up-regulation of the mRNA level (Fig. 3). In contrast, indomethacin suppressed the strain-induced OPG mRNA expression, suggesting that the effect was mediated by cyclooxygenase or prostaglandin synthesis (Fig. 3). Genistein also significantly suppressed stretch-induced OPG mRNA expression, suggesting that the effect was also dependent on protein tyrosine phosphorylation (Fig. 3). In contrast, PD098059 showed no inhibitory effects on the up-regulation of OPG mRNA (Fig. 3), indicating that the effect is

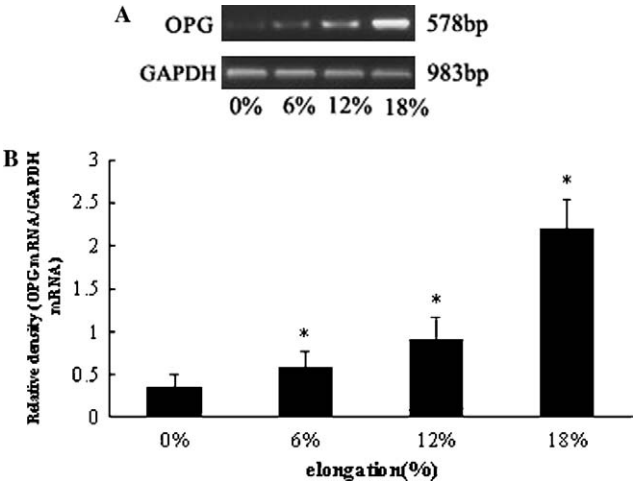

Fig. 2. Effects of percentage elongation on OPG mRNA expression with the application of cyclic tensile strain in mouse osteoblastic MC3T3-E1 cells. The cells were seeded at a density of 2×10^5/well on Flex I culture plates and cultured in α-MEM supplemented with 10% FBS for 24 h. Then they were loaded with (+) or without (−) cyclic tensile strain at the indicated percentage elongation at 6 cycles/min using a Flexercell strain unit for 24 h. Total RNA was extracted from the cells; the expression levels of OPG mRNA were determined by RT-PCR as described in Materials and methods. (A) Agarose gel electrophoresis of the products of PCR using specific primers for OPG or GAPDH. (B) Relative density of OPG mRNA to GAPDH mRNA. The results shown are means ± SD of five independent experiments. *Significant difference from other stress culture ($P < 0.05$): ANOVA, Student's t test.

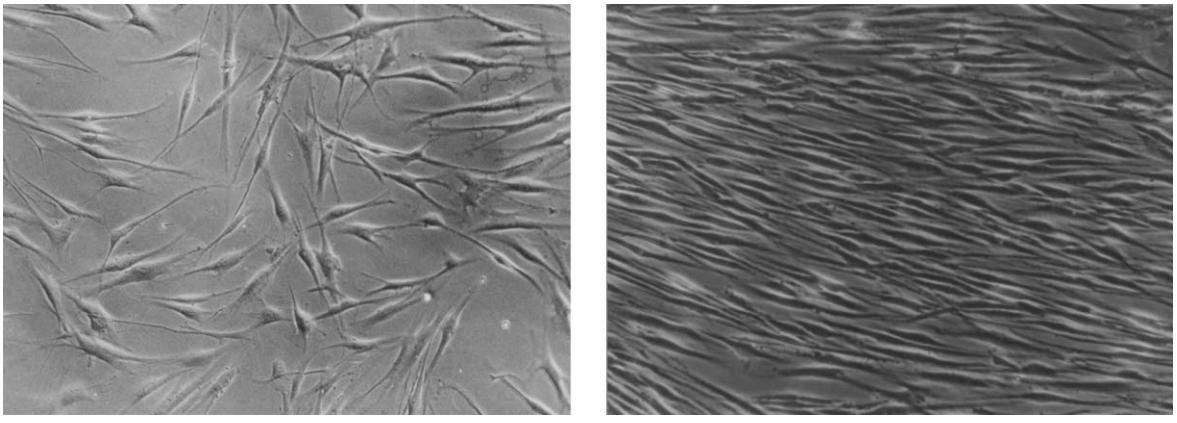

Fig. 1. MC3T3-E1 cells of stretched and non-stretched cultures. The cells were seeded at a density of 2.0×10^5/well on Flex I culture plates, cultured in α-MEM supplemented with 10% fetal bovine serum (FBS) for 24 h, and then loaded with (+) (right) or without (−) (left) cyclic tensile strain with 6%, 12% or 18% elongation at 6 cycles/min for 24 h using a Flexercell strain unit. During application of the mechanical stress, the cells were cultured in α-MEM supplemented with 1% FBS. Magnification × 100.

Table 2
OPG concentration in conditioned medium induced by tensile stress in mouse osteoblastic MC3T3-E1 cells

Tensile stress	0%	6%	12%	18%
OPG concentration (pM)	$9.38 \pm 1.01^{**}$	$11.48 \pm 0.95^{**}$	$15.02 \pm 0.85^{**}$	$19.02 \pm 0.99^{**}$

OPG, osteoprotegerin; values are means ± SD ($n = 6$).
** A significant difference ($P < 0.01$) in cells when compared with other groups of cells.

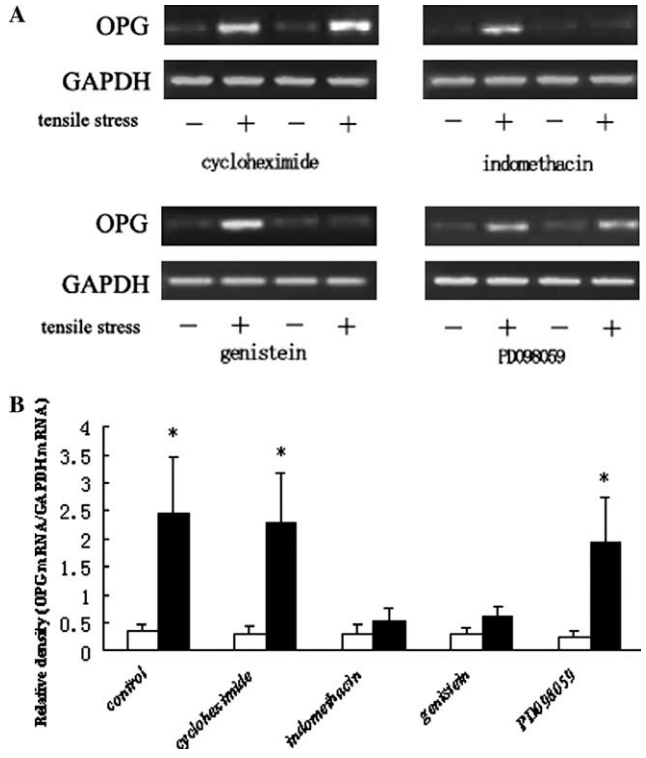

Fig. 3. Effects of inhibitors on induction of OPG mRNA expression by tensile stress in MC3T3-E1 cells. The cells were seeded at a density of 2×10^5/well on Flex I culture plates and cultured in α-MEM supplemented with 10% FBS for 24 h. They were then cultured in a medium containing cycloheximide (10 μM), indomethacin (10 μM), genistein (20 μM), PD098059 (10 μM) or vehicle (control) for 30 min. The cells were cultured with (+) or without (−) loading with tensile stress at 18% elongation at 6 cycles/min for 24 h. Total RNA was extracted from the cells, and expression levels of OPG mRNA were determined by RT-PCR as described in Materials and methods. (A) Agarose gel electrophoresis of the products of PCR using specific primers for OPG or GAPDH. (B) Relative density of the OPG mRNA to GAPDH mRNA. The results shown are means ± SD of five independent experiments. *Significant difference from stress (+) culture ($P < 0.05$): Student's t test.

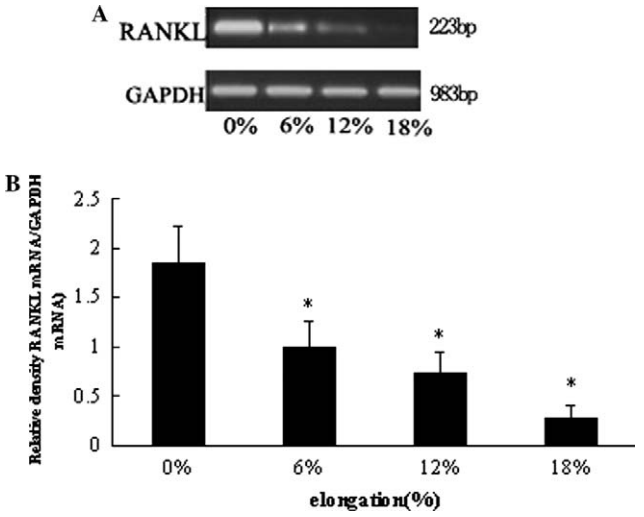

Fig. 4. Expression of receptor activator of nuclear factor-κB ligand (RANKL) mRNA with the application of tensile stress in mouse osteoblastic MC3T3-E1 cells as assessed by RT-PCR. The cells were seeded at a density of 2×10^5/well, cultured for 24 h, and then loaded with (+) or without (−) tensile stress for the indicated percentage elongation at 6 cycles/min using a Flexercell strain unit for 24 h. Total RNA was extracted from the cells, and the expression levels of RANKL mRNA were determined by RT-PCR. (A) Agarose gel electrophoresis of the products of PCR using specific primers for RANKL or GAPDH. (B) Relative density of RANKL mRNA to GAPDH mRNA. The results shown are means ± SD of five independent experiments. *Significant difference from other stress culture ($P < 0.05$): ANOVA, Student's t test.

not mediated by signaling molecules related to extracellular signal-regulated kinase (ERK).

Fig. 4 shows how different magnitudes of strain affect the RANKL mRNA expression level in MC3T3-E1 cells. Mechanical strain at 6%, 12% or 18% elongation caused a significant magnitude-dependent decrease in RANKL mRNA expression compared to that in control (0% elongation) cultures (Fig. 4). These results were confirmed by ELISA for quantifying RANKL protein in the conditioned medium from these cells (Table 3). We further examined whether the aforementioned inhibitors abrogated the strain-induced decrease in expression of RANKL mRNA. The effect was inhibited by PD098059 but not by indomethacin, genistein or cycloheximide, suggesting that it depended on the ERK-MAPK pathway (Fig. 5).

Discussion

It is well known that mechanical stress is a fundamental physiological factor for regulating structure and function in bones. Mechanical strain also plays a crucial role in orthodontic tooth movement [21]. To date, many investigators have studied the responses of bone cells to mechanical stresses such as stretch [15,16,22–27], fluid flow [28], four-point bending [29], and hydrostatic pressure [17].

It is generally accepted that the magnitude of applied strain strongly influences cell responses on the tissue level. Frost [30] has described a window of mechanical usage, which is defined by an upper boundary (1500 μstrain) called the minimum effective strain, above which bone undergoes modeling and changes its structure in order to reduce the local strain. Osteoblasts are located on the surface of cancellous and cortical bone on the yet

Table 3
RANKL concentration in conditioned medium induced by tensile stress in mouse osteoblastic MC3T3-E1 cells

Tensile stress	0%	6%	12%	18%
RANKL concentration (pM)	$9.00 \pm 0.70^{**}$	$7.53 \pm 0.47^{**}$	$5.46 \pm 0.46^{**}$	$3.32 \pm 0.37^{**}$

RANKL, receptor activator of nuclear factor-κB ligand; values are means ± SD ($n = 6$).
** A significant difference ($P < 0.01$) in cells when compared with other groups of cells.

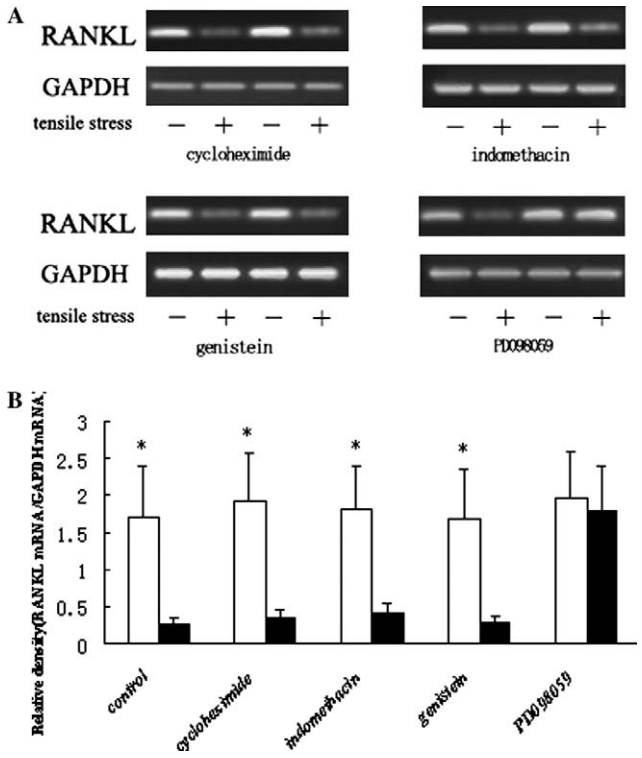

Fig. 5. Expression of receptor activator of nuclear factor-κB ligand (RANKL) mRNA with the application of tensile stress in MC3T3-E1 cells as assessed by RT-PCR. The cells were seeded at a density of 2×10^5/well and cultured for 24 h. They were then cultured in a medium containing cycloheximide (10 μM), indomethacin (10 μM), genistein (20 μM), PD098059 (10 μM) or vehicle (control) for 30 min. The cells were cultured with (+) or without (−) loading with tensile stress at 18% elongation at 6 cycles/min for 24 h. Total RNA was extracted from the cells, and expression levels of RANKL mRNA were determined by RT-PCR. (A) Agarose gel electrophoresis of the products of PCR using specific primers for RANKL or GAPDH. (B) Relative density of RANKL mRNA to GAPDH mRNA. The results shown are means ± SD of five independent experiments. *Significant difference from stress (+) culture ($P < 0.05$): Student's t test.

unmineralized cell matrix. Therefore, deformation of the substrate to which these cells adhere would be an appropriate signal for stimulating them. This situation was simulated in our experiment by culturing osteoblasts as a monolayer on flexible cell substrate surfaces. We used an in vitro cell strain system that allowed mechanical stimulus to be quantified at different magnitudes of stress, using cyclic strains of 6%, 12% or 18% elongation. Physiological strains recorded in human long bones during strenuous activity are of the order of 2000–4000 μstrain [31], and 1500 μstrain is the minimum effective strain under which bones will undergo modeling [30]. It has been reported that strains of the order of 1–3% elongation (10,000–30,000 μstrain) are needed to obtain a cellular response in vitro [32]. It is difficult to compare the magnitudes of strains in vitro with those in vivo because the characteristics of the strains differ.

The interaction between osteoblasts and osteoclasts is known to be responsible for bone formation. Recent studies have shown that OPG synthesis, and RANKL and RANK expression, which are related to the differentiation and activation of bone cells, play important roles in bone remodeling. Increased osteoblast expression of RANKL stimulates osteoclast recruitment and activity, and increases bone resorption. Expression of OPG by osteoblasts antagonizes the effects of RANKL and decreases bone resorption. The application of a 20% tensile stretch has been reported to result in the induction of OPG mRNA and protein in periodontal ligament cells [11]. As in the present report, this cited study indicates that stretching induced an increase of OPG synthesis, though osteoblasts and bone cells were not examined. A marked increase in OPG mRNA expression has been observed by in situ hybridization in stretched cells on the tensioned mandibular bone surface, with loss of osteoclasts, when a slight tensional force was exerted using a dental appliance [12].This report also indicated that stretched cells showed an increase in OPG synthesis. Recently, Kusumi et al. [14] reported that 7%, 0.25-Hz cyclic tensile strain, which is thought to place considerable stress on osteoblasts, induced an increase in OPG synthesis. These reports seem to support our experimental results showing up-regulation of OPG synthesis on the application of mechanical strain to osteoblasts. In the present study, however, the significant increase observed in OPG synthesis was magnitude-dependent.

In contrast, RANKL has been shown to activate osteoclasts via RANK. Uniform equibiaxial mechanical strain of 0.25% has been reported to reduce RANKL mRNA expression in murine bone marrow cells [33]. Again, Kusumi et al. [14] reported that 7%, 0.25-Hz cyclic tensile strain decreases RANKL mRNA expression in osteoblasts. It has recently been found that the inhibiting effect of dynamic strain on RANKL and RANK expression in fibrochondrocytes was magnitude-dependent, with higher magnitudes being more effective [34]. Tsuji et al. [11] demonstrated that although periodontal ligament cells expressed RANKL mRNA, its expression level did not change when the cells were loaded by intermittent stretching with 20% elongation. In the present study, the application of different magnitudes of cyclic tensile strain (0%, 6%, 12% or

18%) induced a magnitude-dependent decrease of sRANKL release and RANKL mRNA expression in osteoblasts.

What is the mechanism by which tensile stress controls expression of OPG in MC3T3-E1 cells? Indomethacin suppressed the effect, suggesting that it was mediated by cyclooxygenase or prostaglandin synthesis. There are two COX isoforms, COX-1 and COX-2, which are variably expressed in different cell types. We were unable to determine which isoform of COX is up-regulated by cyclic stretching in MC3T3-E1 cells. Several previous reports have indicated that COX-2 is a key molecule in the response of periodontal ligament cells to various mechanical stresses [35]. It is proposed that the induction of OPG mRNA by stretching in MC3T3-E1 cells could in part involve an intracellular tyrosine kinase cascade, because genistein partially blocked the effects. We obtained the same results that Tsuji et al. [11] found in periodontal ligament cells: indomethacin and genistein suppressed strain-induced OPG mRNA expression. However, Kusumi et al. [14] reported that the biological responses of OPG synthesis in osteoblasts to the application of cyclic tensile strain are regulated via the p38 MAPK pathway.

Inhibition of RANKL mRNA expression by the application of cyclic tensile strain was abrogated by pretreatment with PD098059 (Fig. 5). This experiment indicated that the ERK-MAPK pathway is involved in sRANKL release. Some investigators have reported findings on the relationship between mechanical stress and MAPK activation in osteoblastic cells [36,37]. It has been reported that the application of cyclic strain (peak 3400 $\mu\varepsilon = 0.34\%$, 1 Hz) for 10 min induced ERK1/2 activation in ROS 17/2.8 rat osteosarcoma cells [38,39]. Nitric oxide and prostaglandins induced by this cyclic strain have been reported to induce proliferation and differentiation of osteoblasts via the ERK1/2 pathway [38]. It has also been reported that the application of *g*-loading with a centrifugal force of 12–27*g* (= 0.012–0.027%) induced ERK1/2 activation but not p38 MAPK or JNK activation in mouse MC3T3-E1 osteoblasts [40]. Recently, however, Kusumi et al. [14] reported that the biological responses of RANKL synthesis to the application of cyclic tensile strain in osteoblasts are regulated via the p38 MAPK pathway. The reason for such differences might lie in the amount of stretch used in the experiments. Other reasons might be differences between cells or differences in strain methods.

In summary, the application of different magnitudes of cyclic tensile strain (0%, 6%, 12% or 18%) induced a magnitude-dependent increase of OPG synthesis and a decrease of sRANKL release and RANKL mRNA expression in cultured osteoblasts. Furthermore, we have provided evidence that indomethacin and genistein suppressed the strain-induced OPG mRNA expression, while the stretch-induced RANKL mRNA reduction was inhibited by PD098059. This study has yielded important evidence on bone remodeling by cyclic tensile strain, and the findings strongly suggest that osteoblasts subjected to different magnitudes of mechanical strain might modulate and regulate bone metabolism.

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