ARTICLE
Journal of Cellular Biochemistry 106:1010–1019 (2009)

Journal of Cellular Biochemistry

Mechanical Force Inhibits Osteoclastogenic Potential of Human Periodontal Ligament Fibroblasts Through OPG Production and ERK-Mediated Signaling

Sung-Ho Kook,[1,2] Young-Ok Son,[3] Jung-Min Hwang,[2,4] Eun-Mi Kim,[2,4] Choon-Bong Lee,[2,5] Young-Mi Jeon,[2,5] Jong-Ghee Kim,[2,5] and Jeong-Chae Lee[2,4,5*]

[1] *Division of Biological Sciences, Chonbuk National University, Jeonju 561-756, South Korea*

[2] *Institute of Oral Biosciences and 21 Century Education Center for Advanced Public Dental Health (BK 21 Program), Chonbuk National University, Jeonju 561-756, South Korea*

[3] *Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky 40536-0001*

[4] *Department of Bioactive Material Sciences and Research Center of Bioactive Materials, Chonbuk National University, Jeonju 561-756, South Korea*

[5] *Department of Orthodontics, School of Dentistry, Chonbuk National University, Jeonju 561-756, South Korea*

ABSTRACT

Periodontal ligament and gingival fibroblasts play important roles in bone remodeling. Periodontal ligament fibroblasts stimulate bone remodeling while gingival fibroblasts protect abnormal bone resorption. However, few studies had examined the differences in stimulation of osteoclast formation between the two fibroblast populations. The precise effect of mechanical forces on osteoclastogenesis of these populations is also unknown. This study revealed that more osteoclast-like cells were induced in the co-cultures of bone marrow cells with periodontal ligament than gingival fibroblasts, and this was considerably increased when anti-osteoprotegerin (OPG) antibody was added to the co-cultures. mRNA levels of receptor activator of nuclear factor-kappaB ligand (RANKL) were increased in both populations when they were cultured with dexamethasone and vitamin D_3. Centrifugal forces inhibited osteoclastogenesis of both populations, and this was likely related to the force-induced OPG up-regulation. Inhibition of extracellular signal-regulated kinase (ERK) signaling by a pharmacological inhibitor (10 μM PD98059) or by siERK transfection suppressed the force-induced OPG up-regulation along with the augmentation of osteoclast-like cells that were decreased by the force. These results suggest that periodontal ligament fibroblasts are naturally better at osteoclast induction than gingival fibroblasts, and that centrifugal force inhibited osteoclastogenesis of the periodontal fibroblasts through OPG production and ERK activation. J. Cell. Biochem. 106: 1010–1019, 2009.

KEY WORDS: CENTRIFUGAL FORCE; PERIODONTAL LIGAMENT FIBROBLASTS; GINGIVAL FIBROBLASTS; OSTEOPROTEGERIN; OSTEOCLASTOGENESIS; EXTRACELLULAR SIGNAL-REGULATED KINASE

In human periodontal tissue anatomy, gingiva is located at the most occlusal area of the tooth while periodontal ligament is a band of dense connective tissue located between the tooth and the alveolar bone [Sodek and Limeback, 1979; Hassel and Stanek, 1983; Berkovitz, 1990]. Periodontal ligament fibroblasts play an essential role in maintaining the integrity and dimension of the periodontal ligament, and allow it to respond to extracellular matrix damage and mechanical forces [Lekic and McCulloch, 1996]. Although gingival and periodontal ligament fibroblasts are heterogeneous cell populations, they share common functions of alveolar bone remodeling and tooth movement.

Many studies have suggested that periodontal ligament fibroblasts can induce bone degradation by stimulating the formation of osteoclast-like cells [Kanzaki et al., 2001, 2002; Nishijima et al., 2006] through the production of various active cytokines and enzymes which are essential for osteoclastic differentiation [Wada et al., 2004; Yamaguchi et al., 2006]. Periodontal ligament fibroblasts express receptor activator of nuclear factor-kappaB

Grant sponsor: Korea Research Foundation Grant; Grant number: KRF-2006-005-J03102.

*Correspondence to: Prof. Jeong-Chae Lee, Department of Orthodontics and Institute of Oral Biosciences, Research Center of Bioactive Materials, Chonbuk National University, Jeonju 561-756, South Korea.
E-mail: leejc88@chonbuk.ac.kr

Received 30 January 2008; Accepted 7 January 2009 • DOI 10.1002/jcb.22086 • 2009 Wiley-Liss, Inc.
Published online 10 February 2009 in Wiley InterScience (www.interscience.wiley.com).

(NF-κB) ligand (RANKL), which is required for osteoclast maturation through the interactions of osteoclast precursors and osteoblastic cells [Hasegawa et al., 2002; Kanzaki et al., 2002]. Accumulating evidence suggests that periodontal ligament fibroblasts induce osteoclasts in co-culture with peripheral blood mononuclear cells [de Vries et al., 2006] or bone marrow cells [Wada et al., 2001; Hasegawa et al., 2002] through a RANKL-dependent mechanism. This clearly supports the positive role of periodontal ligament fibroblasts in osteoclastogenesis. Periodontal ligament fibroblasts also secrete osteoprotegerin (OPG) [Wada et al., 2001], which is a soluble member of the tumor necrosis factor (TNF) receptor superfamily and is involved in the regulation of bone density [Simonet et al., 1997]. Importantly, OPG inhibits osteoclastogenesis and accelerates osteoclast apoptosis by acting as a decoy receptor for RANKL [Hofbauer et al., 2001; Kanzaki et al., 2006]. These findings suggest that periodontal ligament fibroblasts regulate partially osteoclastogenesis through RANKL/OPG-dependent ways during alveolar bone remodeling by orthodontic force.

Gingival fibroblasts also play regulatory roles in mechanically induced-tooth movement. However, the cellular responses of gingival fibroblasts to mechanical stresses differ from those of periodontal ligament fibroblasts and are primarily anti-osteoclastic in nature. The interleukin (IL)-1α-stimulated OPG expression in gingival fibroblasts was higher than expression in periodontal ligament fibroblasts [Hormdee et al., 2005]. Lipopolysaccharide (LPS)-stimulated gingival fibroblasts inhibit monocyte differentiation into osteoclasts by production of OPG [Nagasawa et al., 2002]. Further, gingival fibroblasts are more effective at inhibiting osteoclast formation than periodontal ligament fibroblasts, and this behavior is closely associated with relatively high circulating OPG levels [de Vries et al., 2006]. These findings suggest that periodontal ligament fibroblasts positively regulate bone remodeling, whereas gingival fibroblasts protect from abnormal bone resorption. However, there is little information regarding RANKL and OPG expression in these fibroblasts in response to mechanical forces. The mechanisms by which mechanical forces affect osteoclast formation by either periodontal or gingival fibroblasts and osteoclastogenesis of the fibroblast populations are unclear.

In the present study, we compared the abilities of the two fibroblast populations in stimulating osteoclastic differentiation of monocytes derived from mouse bone marrow. We also investigated the effects of mechanical forces on the production of RANKL and OPG and on the formation of osteoclast-like cells by applying centrifugal forces to these fibroblast populations. In addition, we attempted to characterize the role of extracellular signal-regulated kinase (ERK) on OPG/RANKL expression and osteoclastogenesis in both the control- and force-exposed periodontal fibroblasts.

MATERIALS AND METHODS

CHEMICALS AND LABORATORY WARES

Unless specified otherwise, all chemicals and laboratory wares were obtained from Sigma Chemical Co. (St. Louis, MO) and SPL Life Sciences (Pochun, South Korea), respectively. ERK inhibitor, PD98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one, was purchased from TOCRIS (MI).

FIBROBLAST CULTURE

Gingival and periodontal ligament fibroblasts were obtained from healthy male individuals aged 20–30 years who had molar extraction procedures. Fibroblasts were cultured according to methods described elsewhere with slight modifications [Howard et al., 1998]. Written informed consent for tissue use was obtained from all donors. This study was approved by the Review Board of Chonbuk National University Hospital. Briefly, single cell suspensions of gingival and periodontal ligament fibroblasts were incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and antibiotics (100 IU/ml penicillin G and 100 μg/ml streptomycin) in 100-mm culture dishes. The cultures were switched to a fresh batch of medium every 3 days. After the cells reached confluence, they were sub-cultured for several passages and spread onto 6-well or 96-well flat-bottomed plates before the application of mechanical force. All the experiments were performed at passages 4–7.

APPLICATION OF MECHANICAL FORCE

Culture plates with gingival and periodontal ligament fibroblasts were centrifuged at approximately 50 g/cm^2 for 60 min using a horizontal microplate rotor (Universal 32 R, Hettich, Germany) as described previously [Redlich et al., 1998]. The applied force calculation was based on this equation [Redlich et al., 2004a,b]: $P = (m \times r \times rpm^2 \times \pi^2)/(A \times 9.8 \times 900)$, where P = kg pressure per cm^2 of cells, m = mass of the medium (g), r = radius (0.12 m), rpm = revolution per min, and A = area of contact between the medium and cells (cm^2).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from the control or force-exposed gingival and periodontal ligament fibroblasts 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 are as follows: OPG (−253 bp), 5′-GAG TGT CTA TAC TGC AGC CC-3′ (forward) and 5′-TGT TTT CTA CAG GGT GCT TT-3′ (reverse), and RANKL (−233 bp), 5′-TGG TTC CCA TAA AGT GAG TC-3′ (forward) and 5′-AGG GTA TGA GAA CTT GGG AT-3′ (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control and 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 at 94°C for 30 s, at 50–55°C for 30 s, and at 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 by ethidium bromide staining. Band intensity was calculated using a gel imaging system (model; F1-F2 Fuses type T2A, BIO-RAD, Segrate, Italy).

MEASUREMENT OF OPG AND RANKL PROTEIN LEVELS

When gingival and periodontal ligament fibroblasts had reached approximately 95% confluence in 96-multiwell plates, they were replaced with new culture media containing 100 nM dexamethasone and/or 10 nM vitamin D_3 (1α,25(OH)$_2$D$_3$). After 4 days of incubation,

conditioned media were prepared from the cultures and analyzed for the OPG and RANKL levels using ELISA. ELISA for the detection of human OPG (BI-20402, Biomedica, Vienna, Austria) and human RANKL (BI-20422H, Biomedica) was performed according to the manufacturer's instructions.

WESTERN BLOT ANALYSIS

Gingival and periodontal ligament fibroblasts were cultured in the presence of 100 nM dexamethasone and 10 nM vitamin D_3 in 6-well culture plates and exposed to force for 60 min. After 24 h of the force, cell lysates from the control and force-applied fibroblasts were made in a NP-40 lysis buffer (30 mM Tris–Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, and 1% NP-40). Protein contents were quantified using the Bradford method (1976). Equal amounts of protein extracts were separated by 12% SDS–PAGE and blotted onto poly vinyl 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., Buckinghamshire, UK) and exposed to X-ray film (Eastman-Kodak, Rochester, NY). The polyclonal antibodies specific to RANKL (SC-7628) and OPG (SC-8468) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

OSTEOCLASTOGENESIS AND TRAP STAINING

Single cell suspensions (1×10^5 cells/ml) of gingival and periodontal ligament fibroblasts were divided into each well in a 96-multiwell plate at a cell density of 2×10^4 cells/well. When the cells were confluent, mouse bone marrow cells were plated on top of the cultured fibroblasts at 10^5 cells per well and cultured in RPMI-1640 medium containing 100 nM dexamethasone and 10 nM vitamin D_3 ($1\alpha,25(OH)_2D_3$) with and without various concentrations of anti-OPG antibody (0.5–5 μg/ml). After 2 days of co-incubation, the cells were subjected to centrifugal forces as described above and then further incubated for an additional 5 days. The control-/force-exposed cultures were fixed in 4% PBS-buffered *para*-formaldehyde and stained with tartrate-resistant acid phosphatase (TRAP) using the Sigma–Aldrich kit, according to the manufacturer's instructions. In this study, bone marrow cells were obtained from the femurs and tibiae of 6 weeks old BALB/c mice according to the methods described elsewhere [Lee and Lim, 2001]. This experiment was approved by the Chonbuk National University Committee on Ethics in the Care and Use of Laboratory Animals.

BONE RESORPTION ASSAY

Single cell suspensions (1×10^5 cells/ml) of the two fibroblasts were divided into each well in a 24-well bone coated plate (OAAS™; Osteoclast Activity Assay Substrate, Oscotec Inc., Choongnam, Korea) at a cell density of 5×10^4 cells/well. After the fibroblast cells reached confluence, mouse bone marrow cells were plated on top of the cultured fibroblasts at 3×10^5 cells per well and co-cultured in RPMI-1640 medium as described above. After 7 days of co-culture, the cells were removed from the plates by treating 5% sodium hypochlorite, and the pit formation was observed under optic microscope.

siRNA TRANSFECTION

RNA interference of ERK was performed using 25-bp Stealth™ siRNA duplex oligoribonucleotides (Invitrogen, USA). The coding strands for ERK siRNA were 5′-AAA GGU UAA CAU CCG GUC CAG CAG G-3′ and 5′-UGU CGA ACU UGA AUG GUG CUU CGG C-3′ for ERK1 and ERK2, respectively. A non-related control siRNA that targeted the green fluorescent protein (GFP) sequence 5′-CCA CTA CCT GAG CAC CCA GTT-3′ was used as a control. For transfection, periodontal ligament fibroblasts were seeded in 96-multiwell plates and transfected with siRNA duplexes using Lipofectamine 2000 (Invitrogen) according to the manufacturer's introduction. At 36 h thereafter, the cellular levels of ERK and p-ERK were determined by Western blot analysis. The antibodies specific to ERK (SC-94) and p-ERK (SC-7383) were obtained from Santa Cruz Biotechnology. In addition, the transfected cells were co-cultured with mouse bone marrow cells in the presence of 50 ng/ml M-CSF. After 2 days of co-culture, these cells were exposed to centrifugal force for 1 h, and were adjusted to TRAP staining after 5 days of exposure to the force.

STATISTICAL ANALYSIS

Unless specified otherwise, all the data were expressed as the mean ± standard deviation (SD). A one-way ANOVA was used for multiple comparisons using SPSS version 16.0 software. A P value <0.05 was considered statistically significant.

RESULTS

PERIODONTAL LIGAMENT FIBROBLASTS ARE BETTER AT INDUCING OSTEOCLASTOGENESIS THAN GINGIVAL FIBROBLASTS

This study firstly examined the activities of gingival and periodontal ligament fibroblasts in inducing osteoclastogenesis. Both fibroblasts revealed TRAP-positive cells after 7 days of co-culture (Fig. 1A). However, the number of TRAP-positive cells differed dramatically according to the type of fibroblasts and the conditions studied. Namely, more TRAP-positive cells were induced in the co-culture with periodontal ligament fibroblasts than gingival fibroblasts. TRAP-positive cells were also detectable only when co-cultured cells were incubated in the presence of both 100 nM dexamethasone and 10 nM $1,25\text{-}(OH)_2D_3$, while the positive cells was dramatically increased in periodontal ligament fibroblasts after treatment with anti-OPG antibody.

A bone resorption assay was subsequently carried out for understanding whether the osteoclast-like cells induced by the co-cultures are active (Fig. 1B). As shown in the figure, a pit formation was detectable in the co-cultures with periodontal ligament fibroblasts. The number and the size of pits formed were further augmented when the co-cultures were treated with anti-OPG antibody. However, there was no a pit in the co-cultures with gingival fibroblasts even when these cells were incubated at the same conditions (data not shown).

TREATING WITH ANTI-OPG ANTIBODY INCREASES THE FORMATION OF TRAP-POSITIVE CELLS IN A DOSE-DEPENDENT MANNER

Figure 1 shows that periodontal ligament fibroblasts produced a greater number of osteoclast-like cells than gingival fibroblasts, and this was augmented by the addition of anti-OPG antibody. To

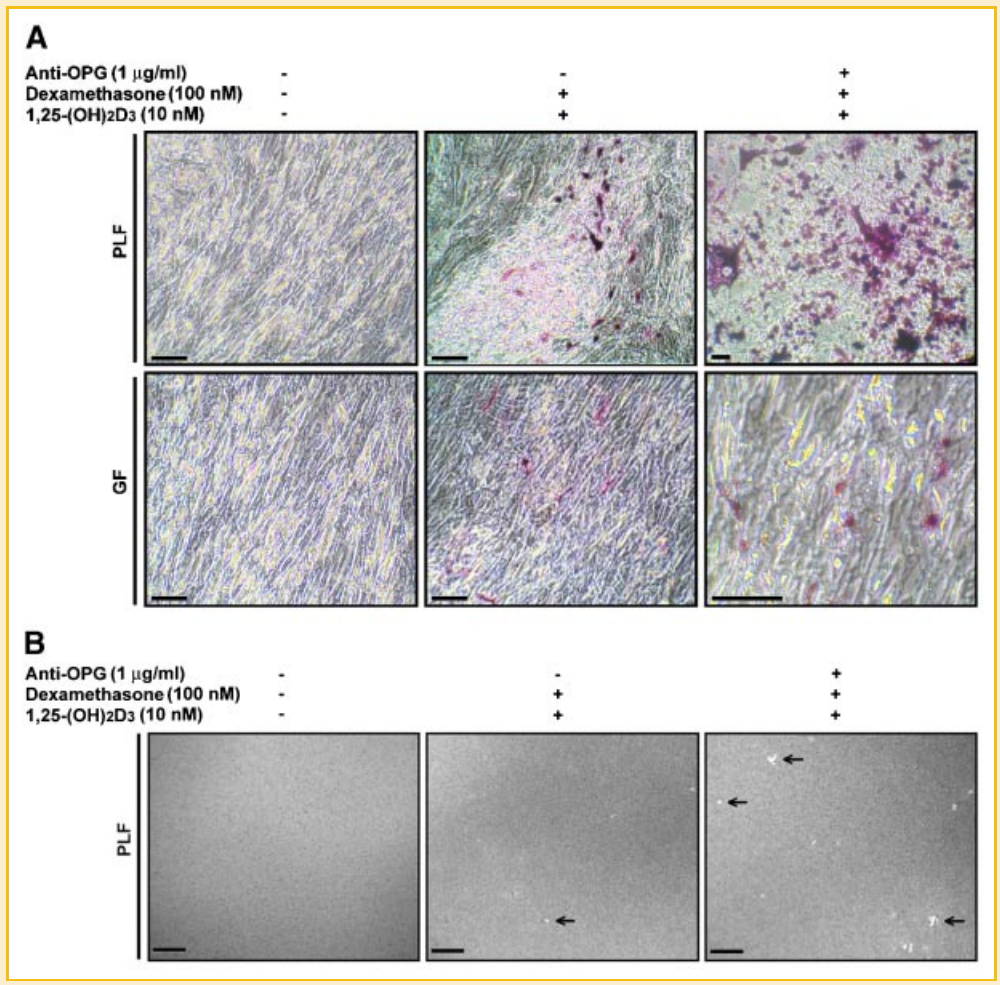

Fig. 1. Periodontal ligament fibroblasts were better at inducing osteoclastogenesis and bone resorption than gingival fibroblasts. Gingival fibroblasts (GF) and periodontal ligament fibroblasts (PLF) were co-cultured with mouse bone marrow cells in the presence and absence of 100 nM dexamethasone, 10 nM 1,25-$(OH)_2D_3$, and/or 1 μg/ml anti-OPG antibody. These cells were adjusted to TRAP staining (A) and bone resorption assay (B) after 7 days of co-culture, and a representative data from triplicate experiments was shown. In order to visualize pits formed on bone coated-24 well plate, the plate was treated 5% sodium hypochlorite for 20 min. Arrows indicate pit formed on the plate. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

investigate the roles of OPG in osteoclastogenesis induced by the co-cultures with gingival or periodontal ligament fibroblasts, these were exposed to various anti-OPG antibody concentrations (0.5–5 μg/ml) in the presence of dexamethasone and vitamin D. The number of TRAP-positive cells in both the co-cultures was significantly increased by adding OPG neutralizing antibody in a dose-dependent manner (Fig. 2A). Greater quantities of TRAP-positive cells were noted by periodontal ligament fibroblasts rather than in gingival fibroblasts. However, there were limited effects of anti-OPG antibody on the augmentation of TRAP-positive cells. There were no additional increases in osteoclast-like cells in the co-cultures with 5 μg/ml OPG antibody as compared to those with 1 μg/ml of the antibody. When the number of TRAP-positive multinucleated cells (more than 3) was counted after 7 days of the co-culture, they were only detectable in the periodontal ligament fibroblast co-cultures (Fig. 2B). The number of TRAP-positive multinucleated cells increased approximately 10-fold with the addition of 1 μg/ml of anti-OPG antibody.

RANKL AND OPG EXPRESSION IN GINGIVAL AND PERIODONTAL LIGAMENT FIBROBLASTS ARE AFFECTED DIFFERENTLY BY COMBINATION OF DEXAMETHASONE AND VITAMIN D

A balanced expression of RANKL and OPG is the most critical event to the regulation of osteoclastogenesis [Hofbauer and Heufelder, 2001]. In order to examine the ability of gingival and periodontal ligament fibroblasts on the expression of RANKL and OPG in the presence of dexamethasone and/or vitamin D, the mRNA levels specific for RANKL and OPG were analyzed by RT-PCR. RANKL and OPG mRNA expression were unchanged in periodontal ligament fibroblasts treated with either dexamethasone or vitamin D (Fig. 3A). In combination of both components, RANKL expression was increased significantly ($P < 0.001$) in periodontal ligament fibroblasts while OPG expression was decreased ($P < 0.05$; Fig. 3B). In gingival fibroblasts, RANKL mRNA levels were detectable only in the culture with a control medium, while the levels were increased when treated with dexamethasone, and further augmented by the combination of dexamethasone and vitamin D. In contrast, OPG

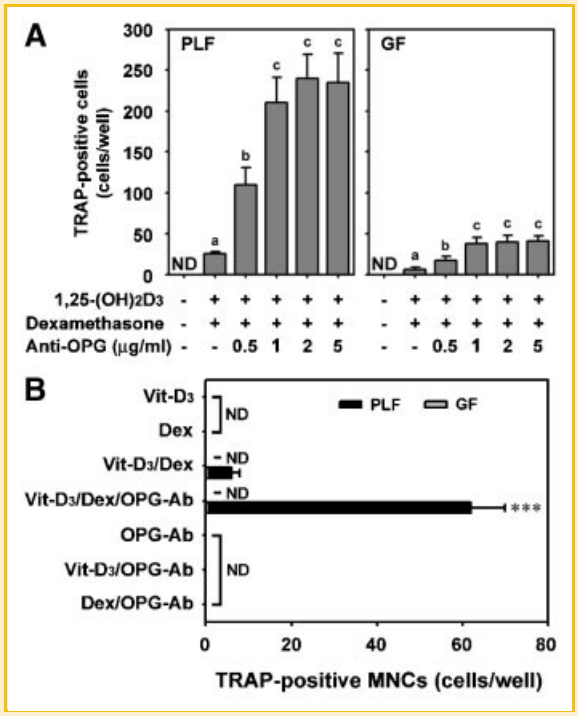

Fig. 2. Treatment with OPG neutralizing antibody increased the formation of TRAP-positive cells in the two fibroblast populations. Co-cultures with gingival or periodontal ligament fibroblasts were incubated in the presence of 100 nM dexamethasone, 10 nM 1,25-$(OH)_2D_3$, and various concentrations of anti-OPG antibody (0.5–5 μg/ml). The number of TRAP-positive cells (A) and TRAP-positive multinucleated cells (MNCs) (B) were counted after 7 days of co-culture from triplicate experiments and expressed as numbers of cells per well of 96-multiwell plates. ND, not detected; Vit-D_3, 1,25-$(OH)_2D_3$; Dex, dexamethasone. $^{a-c}$ represent significant differences ($P < 0.05$) between the experiments. $^{***}P < 0.001$ versus the co-culture with Vit-D_3/Dex.

mRNA levels were unchanged in gingival fibroblasts regardless of the presence of dexamethasone and vitamin D.

The levels of OPG and RANKL production by the fibroblasts were also determined by ELISA using the conditioned medium prepared from the culture supernatants (Fig. 3C). Consistent with the data from RT-PCR, the levels of RANKL secreted were increased in both the fibroblasts only when they were incubated in combined medium with dexamethasone and vitamin D (Fig. 3C, upper panel). Unexpectedly however, there were not any changes in OPG levels presented both in the culture supernatants prepared from gingival and periodontal ligament fibroblasts (Fig. 3C, below panel).

CENTRIFUGAL FORCE INHIBITS OSTEOCLASTOGENESIS IN BOTH THE CO-CULTURES WITH GINGIVAL AND PERIODONTAL LIGAMENT FIBROBLASTS, WHICH IS CLOSELY ASSOCIATED WITH THE FORCE-INDUCED UP-REGULATION OF OPG

A previous study identified that mechanical forces augment OPG expression with attendant inhibition of osteoclastogenesis in gingival fibroblasts [Kook et al., in press]. In addition, previous studies and our current findings indicated that periodontal ligament fibroblasts had greater osteoclastogenic potential than gingival fibroblasts. Therefore, we examined the precise effects of centrifugal

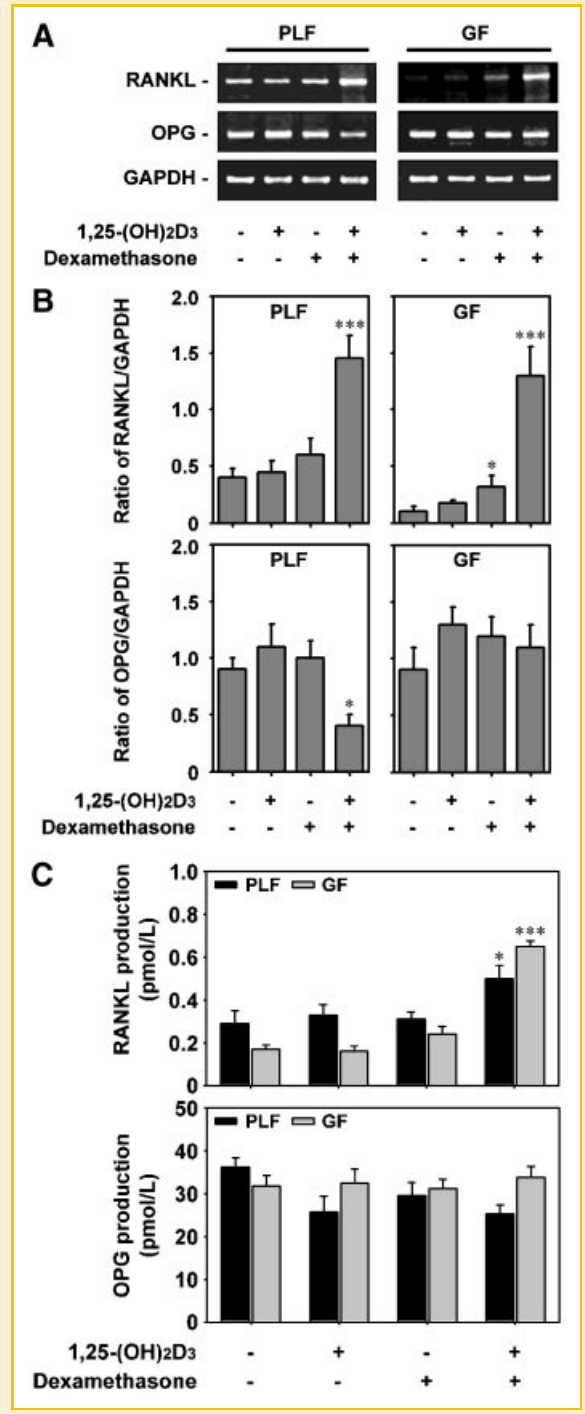

Fig. 3. RANKL and OPG induction in gingival and periodontal ligament fibroblasts differed according to the combination of dexamethasone and 1,25-$(OH)_2D_3$. A: These fibroblasts were cultured with the control medium or the medium containing 100 nM dexamethasone and/or 10 nM 1,25-$(OH)_2D_3$, and the mRNA levels of RANKL and OPG were analyzed by RT-PCR after 4 h of culture. A representative result from triplicate experiments was shown. B: The data from triplicate experiments was quantified by densitometry after normalizing the bands to GAPDH. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus the cultures without both the components. C: Conditioned media were prepared from gingival and periodontal ligament fibroblasts cultured in the presence of 100 nM dexamethasone and/or 10 nM 1,25-$(OH)_2D_3$ for 4 days. The levels of RANKL and OPG secreted were then analyzed by ELISA. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus the cultures without both the components.

force on the formation of TRAP-positive cells by gingival and periodontal ligament fibroblasts and compared their osteoclastogenic potentials. Formation of TRAP-positive cells and its acceleration by combining anti-OPG antibody were significantly reduced by exposing the co-cultures to centrifugal force (Fig. 4).

In order to examine the mechanism by which centrifugal force inhibited osteoclastogenesis, mRNA levels of RANKL and OPG in fibroblasts were determined by RT-PCR at 4 h after applying centrifugal force to these cells for various times (30–90 min). RANKL mRNA levels in periodontal ligament fibroblasts were increased by exposing the cells to centrifugal force in a force-dependent manner (Fig. 5A,B). In contrast, OPG mRNA level was increased transparently from the exposure to centrifugal force for 30 min, and this increase was also seen similarly at the cells subjected to the force for 90 min (Fig. 5A,C). Greater OPG mRNA levels were detected in gingival fibroblasts than in periodontal ligament fibroblasts in the force-exposed cells (data not shown). The stimulatory effects of centrifugal force on RANKL and OPG expression were also examined by Western blot analysis (Fig. 5D). The cellular levels of membrane-bound RANKL protein (approximately 40 kDa) were increased considerably by applying centrifugal force to these cells, and the increase was more prominent in gingival rather than periodontal ligament fibroblasts (Fig. 5E). Soluble RANKL protein (approximately 26 kDa) was enhanced, but not in a significant level, in the force-exposed periodontal ligament fibroblasts as compared to the unforced control. However, the cellular level of soluble RANKL was significantly ($P < 0.05$) reduced in the gingival fibroblasts after the force. Similar to the results from RT-PCR, the cellular levels of OPG protein (approximately 60 kDa) were markedly increased after the force for 60 min in the fibroblasts, and more OPG protein levels were observed in gingival fibroblasts than in periodontal ligament fibroblasts regardless of the application of force.

We further examined the roles of anti-OPG antibody on the centrifugal force-mediated inhibition of osteoclastogenesis. The numbers of TRAP-positive cells (Fig. 6A) as well as TRAP-positive multinucleated cells (Fig. 6B) were almost completely inhibited when the co-cultures with periodontal ligament fibroblasts were exposed to centrifugal forces for 60 min. Treating the co-cultures with anti-OPG antibody significantly inhibited the force-induced decrease in osteoclast-like cells in a dose-dependent manner (Fig. 6A–C). The force-mediated decreases in TRAP-positive cells in co-culture with gingival fibroblasts were also inhibited by treatment with anti-OPG antibody, similar to periodontal ligament fibroblasts, though not with the same magnitude (data not shown).

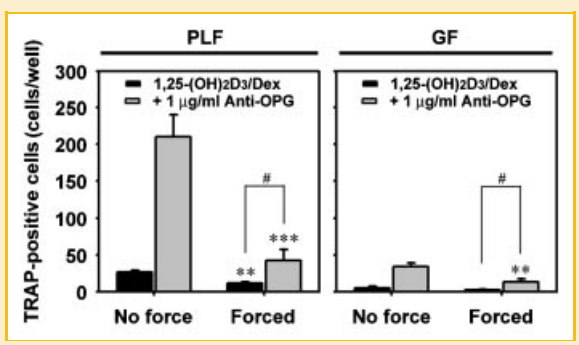

Fig. 4. Centrifugal force inhibited osteoclastogenesis in both the co-cultures with gingival and periodontal ligament fibroblasts. Co-cultures of bone marrow cells with gingival and periodontal ligament fibroblasts were incubated in the combination of 100 nM dexamethasone, 10 nM 1,25-$(OH)_2D_3$, and/or 1 μg/ml anti-OPG antibody. After 2 days of culture, these cells were exposed to centrifugal force for 1 h, and were adjusted to TRAP staining after 5 days of exposure to the force. The number of TRAP-positive cells was calculated from triplicate experiments and expressed as described above. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the unforced control cultures. $^{\#}P < 0.05$ versus the experiments.

CENTRIFUGAL FORCE INHIBITS OSTEOCLASTOGENESIS THROUGH ACTIVATION OF ERK-MEDIATED SIGNALING

A preliminary study revealed that application of centrifugal forces to gingival fibroblasts induced the rapid and transient activation of ERK, such that p-ERK levels reached maximum levels as compared with unforced control levels between 30 min and 1 h after application of the force [Jeon et al., 2009]. In addition, numerous studies have suggested that ERK activation is required for signal transduction involved in mechanotransduction in cells [Iqbal and Zaidi, 2005; Liedert et al., 2006; Kook et al., 2008]. This led us to postulate that ERK-mediated pathways have important roles on the force-induced inhibition of osteoclastogenesis in the co-cultures with periodontal fibroblasts.

Figure 7A shows that the force-mediated decrease of TRAP-positive cells was prevented significantly by adding anti-OPG antibody in a dose-dependent manner, which was further prominent by combined treatment with 10 μM PD98059 in the co-cultures with periodontal ligament fibroblasts. Addition of the ERK inhibitor to periodontal ligament fibroblasts reduced OPG mRNA levels that had been increased by the application of centrifugal forces to the basal levels (Fig. 7B,C). In contrast, RANKL mRNA levels, which had also increased after force application, were not affected by the ERK inhibitor.

To verify the involvement of ERK in the force-mediated inhibition of osteoclastogenesis, siRNAs directed against ERK1/2 were transfected into periodontal ligament fibroblasts. As shown in Figure 8A, transfecting the cells with ERK1/2-specific siRNAs, but not with GFP siRNA, inhibited significantly ($P < 0.01$) the force-mediated increase of ERK expression and its phosphorylation. Consistent with the previous data (Fig. 7), ERK inhibition by siRNA transfection transparently increased the number of TRAP positive cells that had decreased by centrifugal force (Fig. 8B). These results strongly suggest that ERK-mediated signaling is closely related to the force-induced inhibition of osteoclastogenesis in the co-cultures with periodontal ligament fibroblasts.

DISCUSSION

Osteoblasts present in the alveolar bone surface and play essential roles in a force-induced tooth movement by stimulating osteoclastogenesis. This study revealed that periodontal ligament fibroblasts were better at inducing TRAP-positive cells than gingival fibroblasts in co-cultures with bone marrow cells, as consistent with a previous report [de Vries et al., 2006]. Our present study demonstrates that

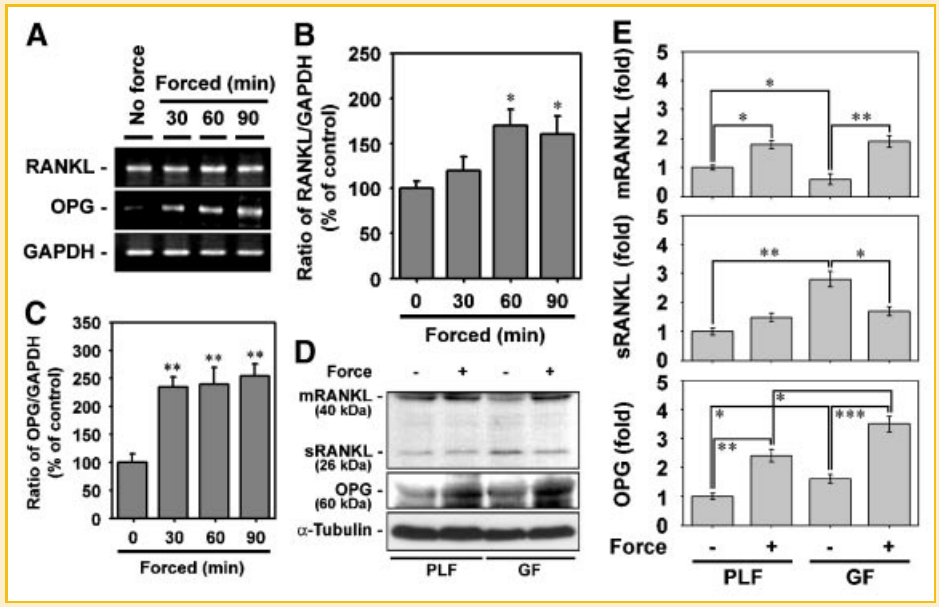

Fig. 5. Centrifugal force up-regulated the expression of RANKL and OPG in periodontal fibroblast populations. A: Periodontal ligament fibroblasts were incubated in the medium supplemented with 100 nM dexamethasone and 10 nM 1,25-$(OH)_2D_3$, and then exposed to centrifugal force for the indicated times at a magnitude of approximate 50 g/cm^2. After 4 h of the force, levels of RANKL and OPG mRNA expression were determined by RT-PCR. The ratio of RANKL (B) and OPG (C) from triplicate experiments was quantified by densitometry after normalizing the bands to GAPDH. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the unforced control cultures. D: Gingival and periodontal ligament fibroblasts that were incubated in the presence of dexamethasone and 1,25-$(OH)_2D_3$ were exposed to centrifugal force for 1 h. The cellular levels of membrane-bound (mRANKL)/soluble RANKL (sRANKL) and OPG proteins were determined by Western blot analysis at 24 h after application of force. E: The levels of mRANKL/sRANKL and OPG proteins from triplicate experiments were quantified by densitometry after normalizing the bands to tubulin. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the experiments.

periodontal ligament fibroblasts, unlike gingival fibroblasts, induce large numbers of TRAP-positive multinucleated cells in co-culture with mouse bone marrow cells. In addition, centrifugal force stimulated OPG production in theses cells and inhibited the formation of osteoclast-like cells, which was closely associated with ERK-mediated pathways.

It has been recognized that gingival and periodontal ligament fibroblasts are functionally different but may have dual effects. They are capable of inducing the formation of osteoclast-like cells and also inhibiting it according to the conditions examined [Wada et al., 2001; de Vries et al., 2006]. However, gingival fibroblasts are primarily different from periodontal ligament fibroblasts with regard to their osteoclastogenic potential. Gingival fibroblasts induce fewer TRAP-positive cells in response to IL-1 stimulation than periodontal ligament fibroblasts [Wada et al., 2001]. Treatment of gingival fibroblasts with LPS also decreases their osteoclastogenic potential through OPG production [Nagasawa et al., 2002]. Further, gingival fibroblasts are superior at inhibiting the formation of osteoclast-like cells than periodontal ligament fibroblasts [de Vries et al., 2006]. These findings suggest that gingival fibroblasts in our study responded to mechanical forces in an anti-inflammatory and anti-osteoclastic manner, although they partially induced osteoclastogenesis in a co-culture system. Periodontal ligament fibroblasts may therefore play positive roles in the formation of osteoclasts which are required for bone remodeling [Nishijima et al., 2006] while gingival fibroblasts are essential in the maintenance of gingival tissue and tissue protection from oral pathogens [Nagasawa et al., 2002; Hormdee et al., 2005]. Overall, alveolar bone remodeling and tooth movement induced by extracellular stimuli is likely due to periodontal ligament rather than gingival fibroblasts.

This study also revealed that TRAP-positive cells in co-cultures of bone marrow cells with fibroblasts were only generated in the presence of dexamethasone and vitamin D. The combination of both dexamethasone and vitamin D is critical in the formation of osteoclast-like cells in a co-culture system with periodontal ligament fibroblasts. As such, many studies have shown that both compounds are essential for the proper development of active osteoclasts in co-cultures with osteoblastic cells [Kondo et al., 2008] as well as with periodontal fibroblasts [Wada et al., 2001; Hasegawa et al., 2002; de Vries et al., 2006]. Both compounds have also been shown to up-regulate osteoclast-related genes in the co-cultures with periodontal fibroblasts and increase cell size and the number of multinucleated cells [de Vries et al., 2006].

The difference in osteoclast-inducing potentials between gingival and periodontal ligament fibroblasts appears unrelated to their RANKL producing ability, at least in the co-culture system used in this study. This is likely because that the combined treatment with dexamethasone and vitamin D caused significant increases in mRNA and protein levels of RANKL in both fibroblasts populations. In regard to that the number of TRAP-positive cells was extremely increased when the co-cultures with periodontal ligament fibroblasts rather than gingival fibroblasts were incubated in combination with anti-OPG antibody, it is considered that there is a different sensitivity between these cells to respond to OPG. It can be also suggested that unlike periodontal ligament fibroblasts, gingival fibroblasts secrete other anti-osteoclastogenic factors in addition to

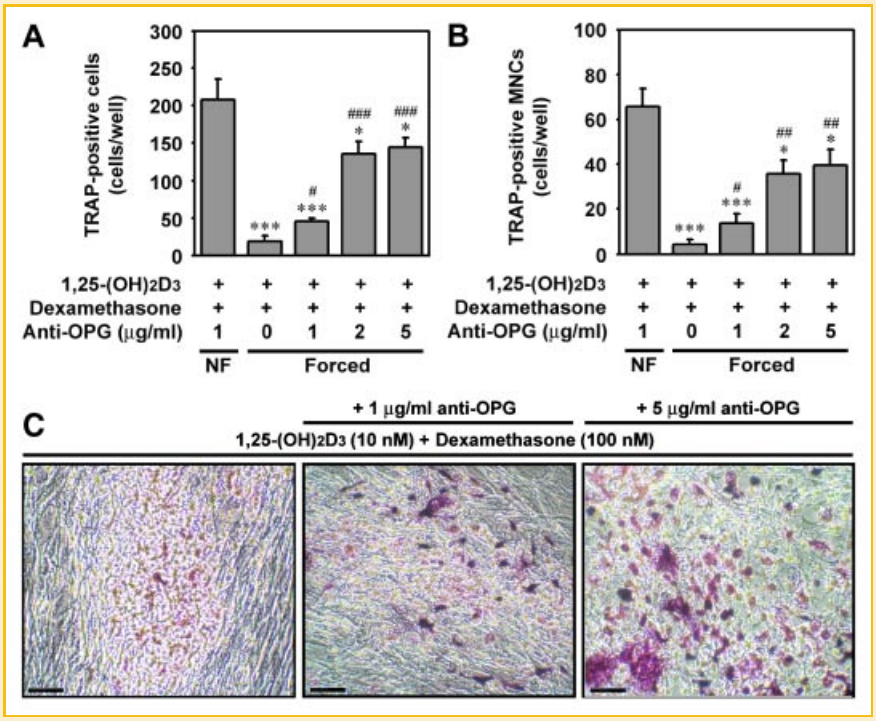

Fig. 6. Treatment with OPG neutralizing antibody increased osteoclastogenesis that was inhibited by centrifugal force. Co-cultures with periodontal ligament fibroblasts in the combination of dexamethasone, 1,25-$(OH)_2D_3$, and/or various concentrations of anti-OPG antibody were exposed to centrifugal force for 1 h. At 5 days culture after the force, the cells were processed for TRAP staining, and the number of TRAP-positive cells (A) and TRAP-positive multinucleated cells (B) were counted from the data obtained from triplicate assays as mentioned above. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus the unforced co-cultures. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the force-exposed co-cultures without anti-OPG-antibody. NF, no force. C: Co-cultures with periodontal ligament fibroblasts were also incubated in the indicated doses of dexamethasone, 1,25-$(OH)_2D_3$ and/or anti-OPG antibody. After 2 days culture, the cells were exposed to centrifugal force for 1 h and adjusted to TRAP staining after 5 days of the force. Bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

OPG. Collectively, it is likely that OPG rather than RANKL is closely related to the different capacities of the fibroblasts on osteoclastogenic induction. Similarly, the force-induced suppression of osteoclastogenesis was involved in OPG expression, as evidenced by the force-induced decrease of osteoclastogenesis and its inhibition by OPG antibody, and the relatively high ratio of OPG to RANKL in forced-exposed cells. This strongly suggests the involvement of OPG in the force-mediated anti-osteoclastogenesis.

Mechanically induced tooth movements are likely achieved by the induction of specific genes that control cellular responses to

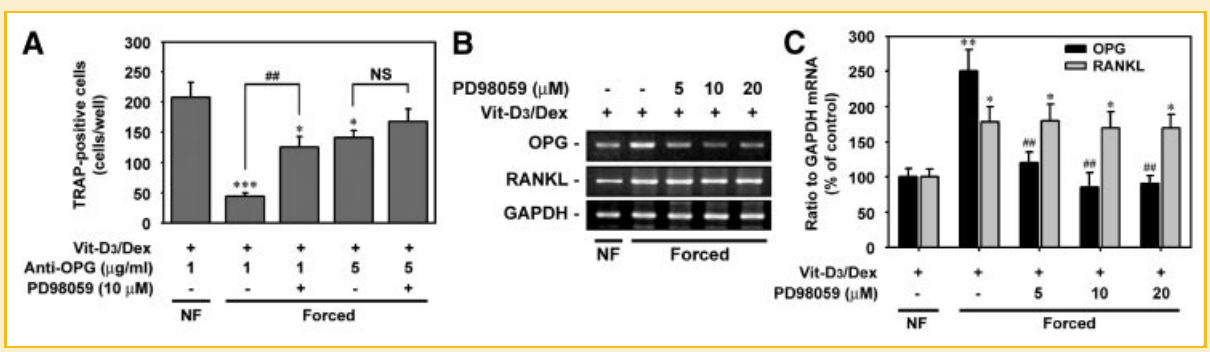

Fig. 7. ERK inhibitor augmented the numbers of TRAP-positive cells that were increased by the addition of anti-OPG antibody while it inhibited the force-induced up-regulation of OPG. A: Co-cultures with periodontal ligament fibroblasts were incubated in the medium containing 100 nM dexamethasone, 10 nM 1,25-$(OH)_2D_3$, and the indicated doses of anti-OPG antibody with and without 10 μM PD98059. After 2 days culture, they were subjected to centrifugal force for 1 h, and processed for TRAP staining after 5 days of the force. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus the unforced co-culture. $^{\#\#}P < 0.01$ versus the experiments. NS, not significant. B: Periodontal ligament fibroblasts were cultured with and without 100 nM dexamethasone, 10 nM 1,25-$(OH)_2D_3$, and the indicated doses of PD98059, and then exposed to centrifugal force for 1 h. After 4 h of the force, they were analyzed by RT-PCR and (C) the data from triplicate experiments were quantified by densitometry after normalizing the bands to GAPDH. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the unforced cultures. $^{\#\#}P < 0.01$ versus the force-exposed cells without PD98059.

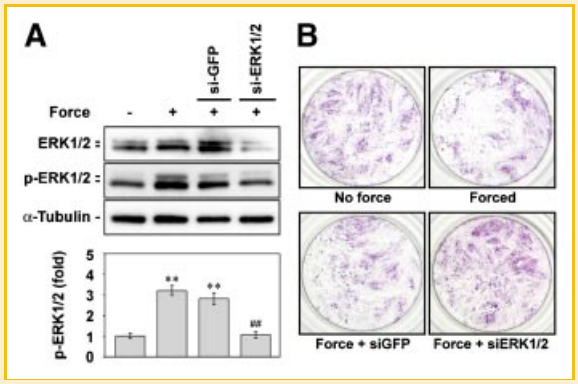

Fig. 8. ERK-directed siRNA transfection enhanced osteoclastogenesis that was inhibited by centrifugal force. siRNAs directed against ERK1/2 or green fluorescent protein were transfected into periodontal ligament fibroblasts. A: After 36 h of transfection, ERK1/2 proteins and its phosphorylated levels were determined by Immunoblotting. B: siRNA-transfected cells were co-cultured with mouse bone marrow cells in the presence of 50 ng/ml M-CSF. After 2 days of incubation, they were exposed to centrifugal force for 1 h and adjusted to TRAP staining at 5 days after the force. A representative data from triplicate experiments was shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mechanical force. The expression of these genes is mediated by various bone-active signals, where members of the mitogen-activated protein kinase (MAPK) family play critical roles [Peverali et al., 2001; Kletsas et al., 2002]. This study revealed that ERK-mediated signaling is related to the force-induced suppression of osteoclastogenesis. ERK inhibition significantly accelerated the neutralizing effects of anti-OPG antibody and directly inhibited the force-mediated increases in OPG mRNA levels to the basal level. In addition, a direct blockage of ERK expression by its siRNA transfection increased the number of TRAP-positive cells that were diminished by centrifugal force. This suggests that ERK-mediated signaling regulates the mechanotransduction to centrifugal forces in periodontal fibroblasts and acts as the upstream effector of OPG gene.

In summary, this study provides in vitro evidence that periodontal ligament fibroblasts are superior in the induction of osteoclast-like cells than gingival fibroblasts. This may be secondary to the differences in the expression of and/or cellular response to OPG between the two groups. This study also shows that centrifugal force inhibits the formation of osteoclast-like cells through production of OPG, where ERK-mediated signaling is closely involved. Although these are in vitro findings and were obtained under a condition of force, the current findings could elucidate better the cellular roles of periodontal fibroblast populations in osteoclastogenesis as well as in alveolar bone remodeling by mechanical stress. Further, this study suggests that gingival fibroblasts may secrete other factors to inhibit osteoclastogenesis in addition to OPG.

ACKNOWLEDGMENTS

This work was supported by Korea Research Foundation Grant endowed to RCBM in Chonbuk National University (KRF-2006-005-J03102).

REFERENCES

Berkovitz BK. 1990. The structure of the periodontal ligament: An update. Eur J Orthod 12:51–76.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

de Vries TJ, Schoenmaker T, Wattanaroonwong N, van den Hoonaard M, Nieuwenhuijse A, Beertsen W, Everts V. 2006. Gingival fibroblasts are better at inhibiting osteoclast formation than periodontal ligament fibroblasts. J Cell Biochem 98:370–382.

Hasegawa T, Yoshimura Y, Kikuiri T, Yawaka Y, Takeyama S, Mstsumoto A, Oguchi H, Shirakawa T. 2002. Expression of receptor activator of NF-kappaB ligand and osteoprotegerin in culture of human periodontal ligament cells. J Periodontal Res 37:405–411.

Hassel TM, Stanek EJ. 1983. Evidence that healthy human gingival contains functionally heterogeneous fibroblast subpopulations. Arch Oral Biol 28:617–625.

Hofbauer LC, Heufelder AE. 2001. The role of osteoprotegerin and osteoprotegerin ligand in the pathogenesis and treatment of metabolic bone diseases. J Clin Endocrinolo Metab 85:2355–2363.

Hofbauer LC, Neubauer A, Heufelder AE. 2001. Receptor activator of nuclear factor-kappaB ligand and osteoprotegerin. Cancer 92:460–470.

Hormdee D, Nagasawa T, Kiji M, Yashiro R, Kobayashi H, Koshy G, Noguchi K, Nitta H, Ishikawa I. 2005. Protein kinase-A-dependent osteoprotegerin production on interleukin-1 stimulation in human gingival fibroblasts is distinct from periodontal ligament fibroblasts. Clin Exp Immunol 142:490–497.

Howard PS, Kucich U, Taliwal R, Korostoff JM. 1998. Mechanical forces alter extracellular matrix synthesis by human periodontal ligament fibroblasts. J Periodontal Res 33:500–508.

Iqbal J, Zaidi M. 2005. Molecular regulation of mechanotransduction. Biochem Biophys Res Communi 328:751–755.

Jeon YM, Kook SH, Son YO, Kim EM, Park SS, Kim JG, Lee JC. 2009. Role of MAPK in mechanical force-induced up-regulation of type I collagen and osteopontin in human gingival fibroblasts. Mol Cell Biochem 320:45–52.

Kanzaki H, Chiba M, Shimizu Y, Mitani H. 2001. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. J Dent Res 80:887–891.

Kanzaki H, Chiba M, Shimizu Y, Mitani H. 2002. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. J Bone Miner Res 17:210–220.

Kanzaki H, Chiba M, Sato A, Miyagawa A, Arai K, Nukatsuka S, Mitani H. 2006. Cyclical tensile force on periodontal ligament cells inhibits osteoclastogenesis through OPG induction. J Dent Res 85:457–462.

Kletsas D, Basdra E, Papavassiliou AG. 2002. Effect of protein kinase inhibitors on the stretch-elicited c-Fos and c-Jun up-regulation in human PDL osteoblast-like cells. J Cell Physiol 190:313–321.

Kondo T, Kitazawa R, Yamaguchi A, Kitazawa S. 2008. Dexamethasone promotes osteoclastogenesis by inhibiting osteoprotegerin through multiple levels. J Cell Biochem 103:335–345.

Kook SH, Son YO, Choi KC, Lee HJ, Chung WT, Hwang IH, Lee JC. 2008. Cyclic mechanical stress suppresses myogenic differentiation of adult bovine satellite cells through activation of extracellular signal-regulated kinase. Mol Cell Biochem 309:133–141.

Kook SH, Son YO, Choe Y, Kim JH, Jeon YM, Heo JS, Kim JG, Lee JC. in press. Mechanical force augments the anti-osteoclastogenic potential of human gingival fibroblasts in vitro. J Periodontal Res.

Lee JC, Lim KT. 2001. Inhibitory effects of the ethanol extract of *Ulmus davidiana* on apoptosis induced by glucose-glucose oxidase and cytokine

production in cultured mouse primary immune cells. J Biochem Mol Biol 34:463–471.

Lekic P, McCulloch CA. 1996. Periodontal ligament cell population: The central role of fibroblasts in creating a unique tissue. Anat Rec 245:327–341.

Liedert A, Kaspar D, Blakytny R, Claes L, Ignatius A. 2006. Signal transduction pathways involved in mechanotransduction in bone cells. Biochem Biophys Res Communi 349:1–5.

Nagasawa T, Kobayashi H, Kiji M, Aramaki M, Mahanonda R, Kojima T, Murakami Y, Saito M, Morotome Y, Ishikawa I. 2002. LPS-stimulated human gingival fibroblasts inhibit the differentiation of monocytes into osteoclasts through the production of osteoprotegerin. Clin Exp Immunol 130:338–344.

Nishijima Y, Yamaguchi M, Kojima T, Aihara N, Nakajima R, Kasai K. 2006. Levels of RANKL and OPG in gingival crevicular fluid during orthodontic tooth movement and effect of compression force on releases from periodontal ligament cells in vitro. Orthod Craniofacial Res 9:63–70.

Peverali FA, Basdra EK, Papavassiliou AG. 2001. Stretch-mediated activation of selective MAPK subtypes and potentiation of AP-1 binding in human osteoblastic cells. Mol Med 7:68–78.

Redlich M, Palmon A, Zaks B, Geremi E, Rayzman S, Shoshan S. 1998. The effect of centrifugal force on the transcription levels of collagen type I and collagnase in cultured canine gingival fibroblasts. Arch Oral Biol 43:313–316.

Redlich M, Roos H, Reichenberg E, Zaks B, Mussig D, Baumert U, Golan I, Palmon A. 2004a. Expression of tropoelastin in human periodontal ligament fibroblasts after simulation of orthodontic force. Arch Oral Biol 49:119–124.

Redlich M, Roos H, Reichenberg E, Zaks B, Grosskop A, Bar Kana I, Pitaru S, Palmon A. 2004b. The effect of centrifugal force on mRNA levels of collagenase, collagen type-I, tissue inhibitors of metalloproteinases and **β**-actin in cultured human periodontal ligament fibroblasts. J Periodontal Res 39:27–32.

Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang M-S, Lüthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ. 1997. Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell 89:309–319.

Sodek J, Limeback HF. 1979. Comparison of the rates of synthesis, conversion and maturation of type I and type III collagens in rat periodontal tissues. J Biol Chem 254:10496–10502.

Wada N, Maeda H, Tanabe K, Tsuda E, Yano K, Nakamuta H, Akamine A. 2001. Periodontal ligament cells secrete the factor that inhibits osteoclastic differentiation and function: The factor is osteoprotegerin/osteoclastogenesis inhibitory factor. J Periodontal Res 36:56–63.

Wada N, Maeda H, Yoshimine Y, Akamine A. 2004. Lipopolysaccharide stimulates expression of osteoprotegerin and receptor activator of NF-kappaB ligand in periodontal ligament fibroblasts through the induction of interleukin-1beta and tumor necrosis factor-alpha. Bone 35:629–635.

Yamaguchi M, Aihara N, Kojima T, Kasai K. 2006. RANKL increase in compressed periodontal ligament cells from root resorption. J Dent Res 85:751–756.