Korean J Physiol Pharmacol
Vol 14: 169−176, June, 2010
DOI: 10.4196/kjpp.2010.14.3.169

Hyperosmotic Stimulus Down-regulates 1α, 25-dihydroxyvitamin D_3-induced Osteoclastogenesis by Suppressing the RANKL Expression in a Co-culture System

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The hyperosmotic stimulus is regarded as a mechanical factor for bone remodeling. However, whether the hyperosmotic stimulus affects 1α, 25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$)-induced osteoclastogenesis is not clear. In the present study, the effect of the hyperosmotic stimulus on 1α,25$(OH)_2D_3$-induced osteoclastogenesis was investigated in an osteoblast-preosteoclast co-culture system. Serial doses of sucrose were applied as a mechanical force. These hyperosmotic stimuli significantly evoked a reduced number of 1α,25$(OH)_2D_3$-induced tartrate-resistant acid phosphatase-positive multinucleated cells and 1α,25$(OH)_2D_3$-induced bone-resorbing pit area in a co-culture system. In osteoblastic cells, receptor activator of nuclear factor κB ligand (RANKL) and Runx2 expressions were down-regulated in response to 1α,25$(OH)_2D_3$. Knockdown of Runx2 inhibited 1α,25$(OH)_2D_3$-induced RANKL expression in osteoblastic cells. Finally, the hyperosmotic stimulus induced the overexpression of TonEBP in osteoblastic cells. These results suggest that hyperosmolarity leads to the down-regulation of 1α,25$(OH)_2D_3$-induced osteoclastogenesis, suppressing Runx2 and RANKL expression due to the TonEBP overexpression in osteoblastic cells.

Key Words: Hyperosmotic stimulus, TonEBP, Osteoblast, RANKL, Runx2

INTRODUCTION

Bone is metabolically active tissue that is capable of adapting its structure to the mechanical load and repairing structural damage through the process of remodeling. Bone remodeling is closely coupled with the physiological activities of osteoblasts and osteoclasts to ensure skeletal integrity [1-5]. Recently, it was clearly documented that stromal/osteoblastic cells are essential components of the differentiation of monocytes/macrophages into osteoclasts *in vitro*, through expression of the signal molecules needed to promote osteoclastogenesis: macrophage colony-stimulating factor (M-CSF) and the receptor that activates the nuclear factor kappa B (NF-κB) ligand (RANKL). RANKL, the osteoclast-differentiating factor, binds to RANK on the surface of osteoclast precursor cells [6,7]. In addition, stromal/osteoblastic cells also secrete osteoprotegerin (OPG), which inhibits osteoclastogenesis by binding with RANKL as a decoy receptor [8]. This RANK/RANKL signaling system plays a key role in the development of osteolytic bone lesions such as osteoporosis, cancer-associated osteolytic diseases, and rheumatoid arthritis [9,10].

Physiologically, osteoclast development and activity is the under the control of osteoblast/stromal cells [11], which modulate the RANKL/OPG axis for osteoclastogenesis. Parathyroid hormone, prostaglandins, interleukins, vitamin D_3, corticosteroids, and mechanical load are involved in osteoclastogenesis. Osteoblast/stromal cells translate all these signals into an appropriate RANKL and OPG output to control osteoclast differentiation and bone remodeling. Therefore, osteoblast/stromal cells are regarded as a transducer of cytokine/hormone signal for osteoclasts, where input is the cytokine/hormone/mechanical signal and the output is the RANKL/OPG ratio. In addition, skeletal mass and architecture are adjusted by the critical and competing responsibilities of structural and metabolic factors [12-14]. Metabolic demands on bone tissue are processed through calciotropic hormones, while structural/mechanical stimuli to the skeleton trigger the genetic baseline, and the bone adapts to its loading environment by biomechanical signal transduction.

Received May 12, 2010, Revised May 25, 2010,
Accepted June 7, 2010

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ABBREVIATIONS: TonEBP, tonicity-responsive enhancer binding protein; RANK, receptor activator for nuclear factor κB; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BMMs, bone marrow-derived macrophages; TRAP, tartrate-resistant acid phosphatase.

Recently, much attention has been focused on how mechanical load leads to the biological responses of the bone cells as well as numerous cell types, including endothelial cells chondrocytes, and fibroblasts [15-19]. The load-induced cellular deformation may play a key role in determining the cellular biochemical response that occurs at the whole-cell level [20], and mechanical loading induces global cellular responses such as changes in cell morphology and cytoskeletal structure [21,22]. Therefore, mechanical loading is transduced into biological processes, generating deformation in the bone tissue continuously exposed to mechanical stress including osmotic stress and gravity, pressure in the intramedullary cavity within the cortices, transient pressure waves, shear forces through canaliculi, and even dynamic electric fields as interstitial fluid flows past charged bone crystals. In particular, studies with animal models have demonstrated that mechanical stimulations such as shear stress induce the change in the shape of osteoblasts and an increase in intracellular calcium (Ca^{2+}) response [23] and also regulate bone remodeling [24]. Mechanical loading causes cell membrane deformation such as stretching or folding, which is induced by the hypotonic and hypertonic stress, respectively [25].

As a potent regulator of the bone remodeling, osmotic stimuli result in the modulation of osteoclastic proliferation and/or differentiation, and eventually alters the bone resorption. However, a precise mechanism of these osmotic stimuli remains to be studied. Here, we investigated whether the hyperosmotic stimulus in an osteoblast-preosteoclast co-culture system leads to changes in 1α, 25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$)-induced osteoclastogenesis.

METHODS

Materials

Routine cell culture media, dispase, TRIzol were obtained from Gibco BRL (Carlsbad, CA, USA). The 1α,25$(OH)_2D_3$, sucrose, tartrate-resistant acid phosphatase (TRAP) staining kit, collagenase, and most of the chemicals were purchased from Sigma-Aldrich (St Louis, MO). ICR mice were purchased from Samtacho (O-san, Kyung-gi-Do, Republic of Korea). Sodium dodecyl sulfate (SDS) was obtained from Bio-Rad Laboratories (Hercules, CA, USA). Anti-Runx2 and anti-RANKL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The TonEBP gene and TonEBP antibodies were gifts by Dr. Sang-do Lee, Chungnam National University College of Medicine. All other chemicals were of the highest grade commercially available.

Preparation of primary osteoblastic cells, bone marrow cells, and hyperosmotic culture medium

Primary osteoblastic cells were prepared from the calvariae of 1-day-old newborn ICR mice using a slightly modified version of a method reported elsewhere [26]. The calvarial osteoblastic cells were isolated by digestion with 0.2% collagenase (Sigma Chemical Co., Ltd., St.Louis, MO, USA) and 0.1% dispase. The isolated cells were cultured for 4 days in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B). The bone marrow cells were flushed out from femurs and tibiae of 4-week-old ICR male mice and cultured overnight in tissue culture dishes in α-MEM containing 10 ng/ml of M-CSF. Osteoblastic cells (1×10^4 cells) and bone marrow cells (1×10^5 cells) were seeded and co-cultured in α-MEM supplemented with 1α,25$(OH)_2D_3$ (10 nM), with or without sucrose addition. After 6 days incubation, the cells were fixed, and stained for TRAP using the Leukocyte Acid Phosphate Assay Kit (Sigma-Aldrich) according to the protocol of the manufacturer. TRAP-positive multinucleated cells (more than three nuclei) were counted as osteoclast. *In vitro* formation assay of osteoclast was repeated four times.

Bone resorption assay using OAAS plates

The osteoblastic cells and bone marrow cells were co-cultured in α-MEM in calcium phosphate apatite-coated osteoclast activity assay substrate (OAAS) plates (Oscotec, Seoul, Republic of Korea). After 4 days incubation, the medium was replaced with fresh medium containing 1α,25$(OH)_2D_3$ and/or sucrose. After culturing, the attached cells were removed from the plate by the addition of a 4% sodium hypochlorite solution. Images of the pit were captured using a digital camera attached to a microscope at ×100 magnification, and the total area of the resorption pits was analyzed using the Image Meta Morph Program (Universal Imaging, Downingtown, PA, USA). The data are presented as means± SEM of four samples.

Assessment of cell viability

Cell viability was determined using a conventional MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay with slight modification [27]. A MTT solution (5 mg/ml in phosphate buffered saline; PBS) was added directly to the assay plates. To dissolve the formazan crystals, dimethyl sulfoxide was then added to assay plates and mixed. The resulting mixture was examined using a MRX spectrophotometer (Dynatech Laboratories, Chantilly, VA, USA) at 570 nm.

Reverse transcription-polymerase chain reaction (RT-PCR)

The expressions of RANKL, OPG, M-CSF, osteoclacin, osteopontin, collagen I and β-actin were evaluated by RT-PCR using total RNA isolated from murine osteoblastic cells. Cells were lysed in TRIzol reagent according to the instructions of the manufacturer (Invitrogen). One microgram of RNA was reverse-transcribed with RT-&GO^{TM} reverse transcriptase (Qbiogene, Carlsbad, CA, USA). The reverse-transcribed cDNA was PCR amplified. Primers used for amplification of murine RANKL, OPG, M-CSF, osteoclacin, osteopontin, collagen I and β-actin gene fragments were RANKL (750 bp) (forward): 5'-ATCAGAAGACAGCACTCACT-3' (reverse): 5'-ATCTAGGACATCCATGCTAATGTTC-3'; OPG (636 bp) (forward): 5'-TGAGTGTGAGGAAGGGCGTTAC-3' (reverse): 5'-TTCCTCGTTCTCTCAATCTC-3'; M-CSF (395 bp) (forward): 5'-CATGACAAGGCCTGCGTCCGA-3' (reverse 1): 5'-AAGCTCTGGCAGGTGCTCCTG-3'(reverse 2): 5'-GCCGCCTCCACCTGTAGAACA-3'; β-actin (366 bp) (forward): 5'-GGACTCCTATGGTGGGTGACGAGG-3' (reverse): 5'-GGGAGAGCATAGCCCTCGTAGAT-3'; Cbfa1 (Runx2) (forward): 5'-AGCCTCTTCAGCCGAGTGACACC-3' (reverse): 5'-CTGGGCCATGGTTGACGAATTTC-3'; Osteopontin (forward): 5'-GAGCGGTGAGTCTAAGGAGT-3' (reverse): 5'-CTAAAT-

GCAAAGTAAGGAAC-3'; Osteocalcin (forward): 5'-AAGCAGGAGGGCAATAAGGT-3' (reverse): 5'-AGCTGCTGTGACATCCCATAC-3'; Collagen 1 (forward): 5'-ACCTTCCTGCGCCTAATGTC-3' (reverse): 5'-TTGGGTTGTTCGTCTGTTTC-3' . The amplification conditions were optimized for each pair of primers as follows: 30 s at 95°C and 30 s at 48°C (RANKL), 58°C (OPG, M-CSF, Runx2, and β-actin), 59°C (osteopontin) or 55°C (osteoclacin, and collagen I), and 1 min at 72°C. For the genes, 30 cycles of amplification were used to generate the probes. Parallel PCR analysis was run for the housekeeping gene β-actin to normalize data for differences in mRNA quantity and integrity. All primers were obtained from BIONEER (Kyung-gi-Do, Republic of Korea). PCR products were separated on 1.2% agarose gels and stained with ethidium bromide.

Enzyme-linked immunosorbent assay (ELISA)

A Quantikine® M murine Mouse RANK Ligand kit (R&D Systems, Minneapolis, MN, USA) was used to analyze the RANKL protein according to the instructions of the manufacturer. The intensity of the color of each well in assay plates was determined using a microplate reader at 450 nm. The method used to detect the OPG was the same as that used for the RANKL assay.

Western blot analysis

Osteoblastic cells were harvested and lysed using RIPA buffer containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1% NP-40, and protease and phosphatase inhibitors. An equal amount of protein was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a nitrocellulose membrane. The following primary antibodies and dilutions were used: RANKL polyclonal antibody (pAb) (1 : 1,000), Runx2 pAb (1 : 1,000), TonEBP pAb (1 : 2,000), and actin monoclonal Ab (1 : 2,000). The immune complexes were detected using an enhanced

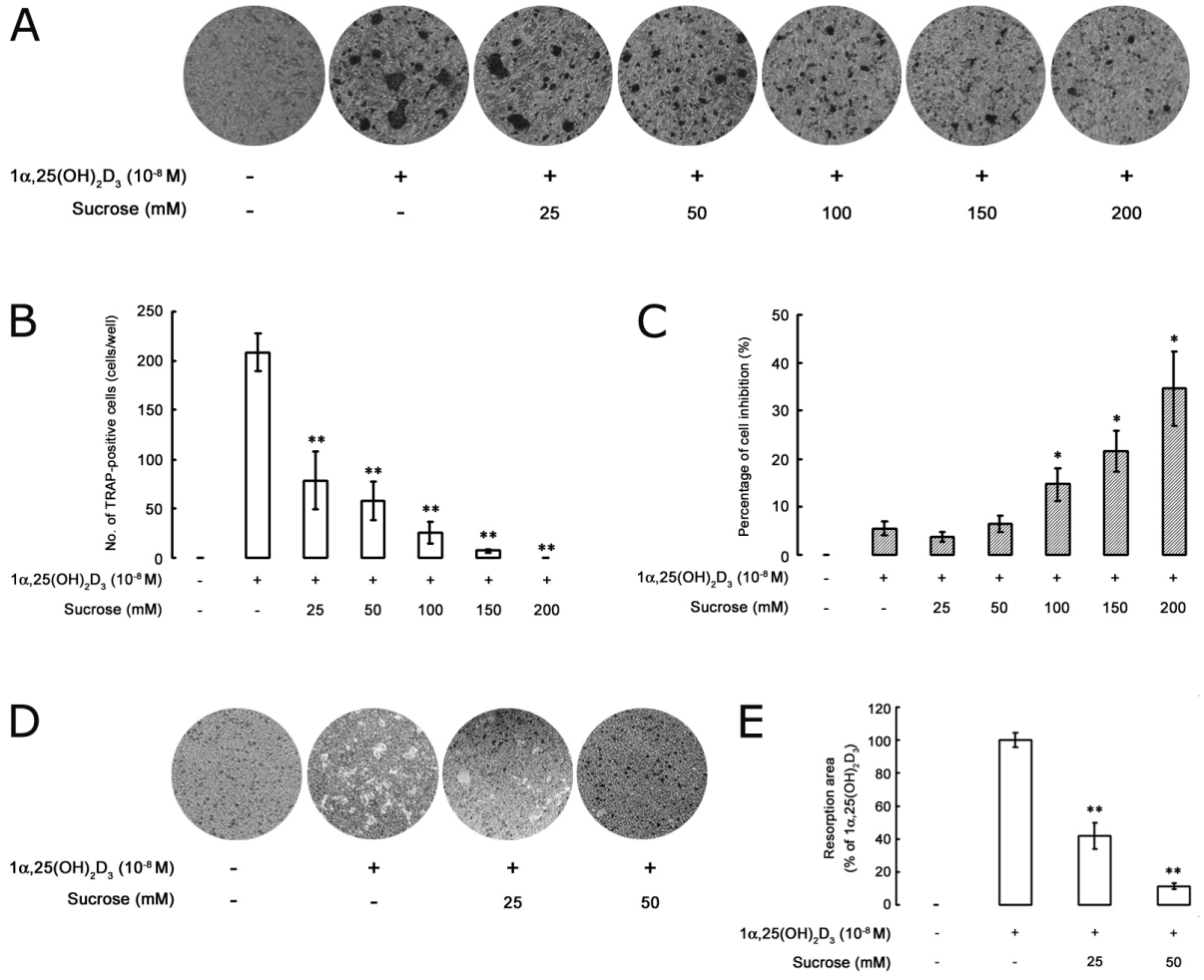

Fig. 1. Effect of hyperosmotic stimulus of sucrose on 1α,25(OH)$_2$D$_3$-induced osteoclast differentiation in a co-culture system. Mouse BMMs were co-cultured with calvarial osteoblastic cells in the presence of 1α,25(OH)$_2$D$_3$ and the indicated concentration of sucrose. (A) Preosteoclast cells underwent differentiation into TRAP-positive multinuclear cells in the presence of 1α,25(OH)$_2$D$_3$, and different concentrations of sucrose. (B) The number of TRAP-positive multinuclear cells was counted. (C) Cell viability was determined by a MTT assay. Results are presented as the mean values±SEM (n=4; *$p<0.05$, **$p<0.001$ versus 1α,25(OH)$_2$D$_3$ only). (D) Osteoclasts had bone resorbing activity on calcium phosphate apatite-coated plates. (E) Total areas of formation of pit resorption. Osteoclast differentiation was significantly inhibited as the increase in sucrose concentration. Bar indicates mean±SEM (n=5; *$p<0.05$, **$p<0.001$ versus 1α,25(OH)$_2$D$_3$ only).

chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Alington Heights, IL, USA).

Transfection of small interfering RNA (siRNA)

Runx2 and TonEBP synthesis were inhibited using a RNA interference technique involving the generation of siRNA. In brief, siRNA for Runx2 and TonEBP were annealed with T4 DNA ligase to form a double-stranded DNA fragment, and inserted into the psiSTRIKE^{TM} vector (Promega, Madison, WI, USA) by following the manufacture's procedure. Osteoblastic cells were transfected with 4 μg/ml Runx2/or TonEBP siRNA using Lipofectamine^{TM} 2000 (Invitrogen) and were selected with 0.2 mg/ml G418. The sequence of siRNA for Runx2 [5'-ACCGCTCTGGCGTTTAAATGGTTTCAAGA GAACCATTTAAACGCCAGAGCTTTTTC-3' (sense), 5'-TGCAGAAAAAGCTCTGGCGTTTAAATGGTTCTCTTG AAACATTTAAACGCCAGAG-3' (anti-sense)] or TonEBP [5'-ACCGAGTTCCTGAGATCTTAAATTCAAGAG ATTGATCTCAGGAACTCTTTTTC-3' (sense), 5'-TGCAGAAAAAGAGTTCCTGAATCTTAAATCTCTTGAATTT AAGATCTCAGGAACT-3' (anti-sense)] (Bioneer Oligo Synthesis) were generated by the Promega siDESIGN Center (Promega).

Statistical analysis

Unless otherwise stated, all experiments were reproduced at least three times. The results are expressed as mean±SE. The statistical significance between comparable groups was determined using the Kruskal-Wallis and Bonferroni's test. In statistical tests, the p-value<0.05 was considered significant.

RESULTS

Inhibition of 1α,25(OH)$_2$D$_3$-induced osteoclast formation and activity by hyperosmotic stress in a co-culture system

To provide hyperosmotic stress, 25, 50, 100, 150, and 200 mM (306, 326, 368, 410, and 460 mOsm) of sucrose were added to the co-culture medium, and cells were then incubated at 37°C for 4 days. When 10 nM 1α,25(OH)$_2$D$_3$ was added to the co-culture system, 209±19 TRAP-positive multinucleated cells were formed, whereas no TRAP-positive cells were detected in the media alone. However, the 1α,25(OH)$_2$D$_3$-induced osteoclast formation was reduced in response to the addition of sucrose to the co-culture medium (Fig. 1A, 1B). The addition of 25 mM or 50 mM sucrose lowered the number of multinucleated osteoclast to 78±29 and 58±20 cells per well, respectively. The addition of sucrose (25 and 50 mM) did not show any toxic effect when added up to 50 mM (Fig. 1C), indicating the inhibitory effect of the addition of sucrose on osteoclast formation was caused by a change in osteoclastogenesis, not by its direct toxic effect on the cells. To evaluate osteoclastic activity, osteoblastic cells and bone marrow cells were co-cultured in α-MEM on OAAS plates coated with carbonated calcium phosphate instead of dentin slice [28], and the resorption lacuna were then measured for bone resorbing activity. No resorption pits were observed on the OAAS plates in the absence of 1α,25(OH)$_2$D$_3$, while treating with 10 nM 1α,25(OH)$_2$D$_3$ produced numerous resorption pits and multiple lacunae. Additionally, the hyperosmotic stimulus caused by the addition of sucrose to the co-culture medium resulted in a decrease of the 1α,25(OH)$_2$D$_3$-induced resorption pits and area (42±8% of control at 25 mM sucorose, 11±2% of control at 50 mM sucrose; Fig. 1D, 1E). These findings indicate that hyperosmotic stimulus (25 and 50 mM sucrose addition to co-culture medium) inhibits osteoclast differentiation and osteoclast activity without causing toxic damage to the cell.

Effects of hyperosmotic stimulus on expression of genes for osteoclast differentiation factor and osteoblast marker genes in mouse calvarial osteoblasts

Fig. 2A shows the expression levels of the genes for osteoclast differentiation factor such as RANKL, OPG, and M-CSF from osteoblastic cells in the presence of 1α,25(OH)$_2$D$_3$. Fig. 2B displays the quantified results. The expression of mouse RANKL mRNA was remarkably increased in the calvarial osteoblastic cells treated with 1α,25(OH)$_2$D$_3$ alone. However, hyperosmotic stimulus caused by the addition of sucrose to culture medium resulted in a dose-dependent decrease in RANKL mRNA expression without changing the OPG and M-CSF mRNA expression levels in the presence of 1α,25(OH)$_2$D$_3$. The effects of hyperosmotic stimulus to culture medium on the 1α,25(OH)$_2$D$_3$-induced RANKL and OPG protein expressions in osteoblastic cells were also quantitatively examined by ELISA using anti-RANKL and anti-OPG antibodies. As shown in Fig. 2C, the 1α,25(OH)$_2$D$_3$-induced RANKL protein level was lowered with increasing sucrose concentrations. Although the OPG levels in osteoblastic cells were slightly elevated by the addition of sucrose, this level of upregulation was not statistically significant (Fig. 2D). Consequently, hyperosmotic stimulus as a result of the addition of sucrose to the culture medium inhibited the 1α,25(OH)$_2$D$_3$-induced RANKL mRNA and soluble RANKL protein, leading to the down-regulation of osteoclastogenesis in a dose-dependent manner.

The effect of hyperosmotic stimulus on the differentiation of osteoblasts was investigated since insufficient osteoclastogenesis may occur due to the immaturation of preosteoblast cells in a co-culture system. The expression levels of the osteoblast marker genes such as collagen I, osteopontin, and osteocalcin were examined by RT-PCR in the presence of hyperosmotic stimulus (Fig. 2E). The hyperosmotic stimulus as a result of sucrose addition did not cause any distinct changes in the osteocalcin, osteopontin, and collagen I mRNA, which are expressed in the matured osteoblasts. These results indicate that hyperosmotic stimulus does not affect osteoblast differentiation.

Effect of hyperosmotic stimulus on 1α,25(OH)$_2$D$_3$-induced Runx2 expression in calvarial osteoblastic cells

With respect to the expression of RANKL at the gene level, Runx2 is involved in osteoclastogenesis at the upstream level of RANKL protein, since the basic promoter sequence of the RANKL gene contains the binding sites for Runx2 [30]. Therefore, we examined that the effect of hyperosmotic stimulus on 1α,25(OH)$_2$D$_3$-induced Runx2 expression in osteoblastic cells. The level of expression of Runx2 in osteoblastaic cells was dramatically elevated in response to 10 nM 1α,25(OH)$_2$D$_3$ compared to that of the control (Fig. 3A).

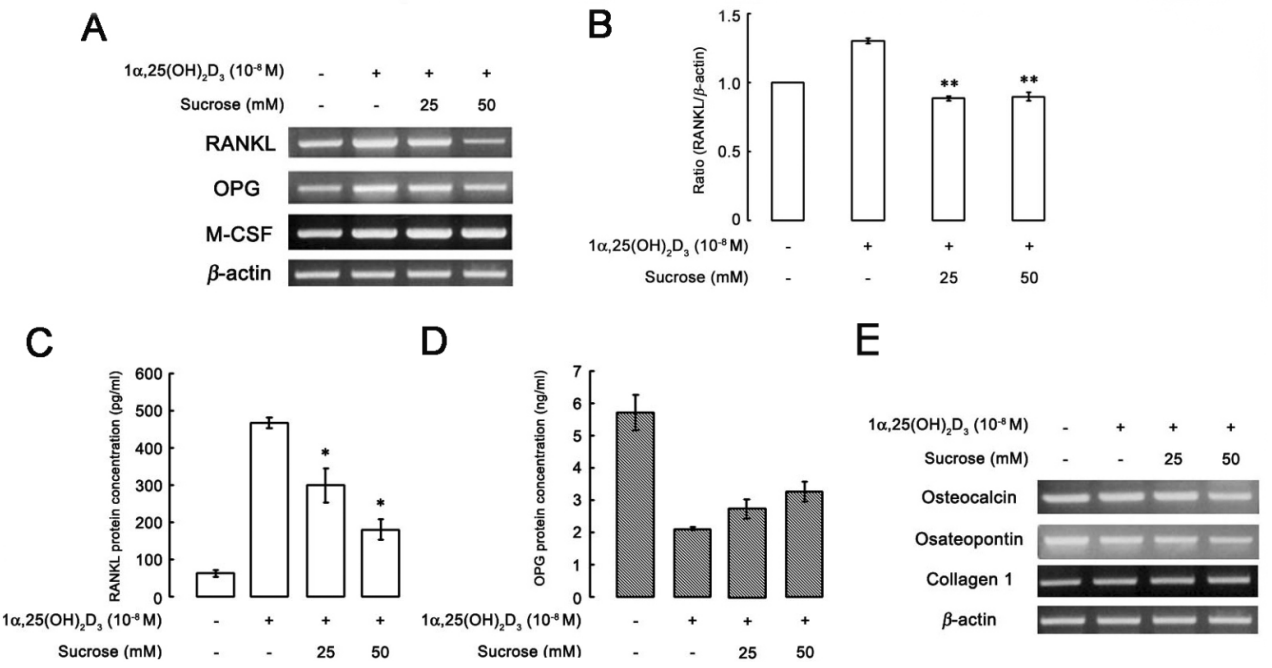

Fig. 2. Effect of hyperosmotic stimulus on the genes of osteoblast. Osteoblastic cells were cultured with $1\alpha,25(OH)_2D_3$ and the indicated sucrose concentration. (A) Total RNA was extracted, and then mRNA expression was analyzed by RT-PCR. (B) Bar graph shows the quantitative analysis of RANKL/β-actin mRNA. (C, D) For quantitative analysis, sRANKL and OPG productions were determined by ELISA. The bar graph shows the concentration level of RANKL and OPG protein. (E) Osteoblast marker genes were amplified by RT-PCR using RNA the primary osteoblastic cells that were exposed to the hyperosmotic stimulus. Bar represents the mean±SEM (n=4; *$p<0.05$ versus $1\alpha,25(OH)_2D_3$ only).

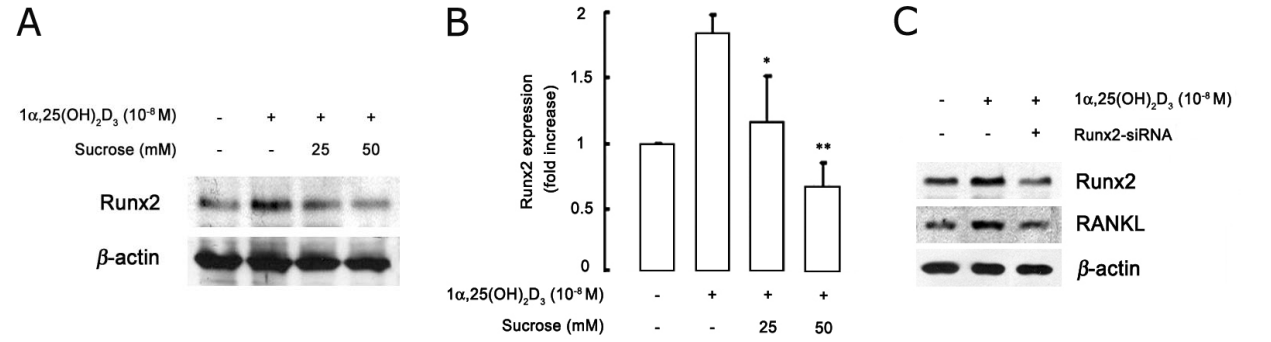

Fig. 3. Effects of hyperosmotic stimulus in $1\alpha,25(OH)_2D_3$-induced Runx2 expression. (A) Western blot analysis of Runx2 expression in osteoblastic cells stimulated with 1α, $25(OH)_2D_3$ in the presence of indicated sucrose concentration. (B) Densitometric analysis of Runx2 and β-actin expression was used to normalize Runx2 data. (C) Western blot analysis of the levels of $1\alpha,25(OH)_2D_3$-induced RANKL and Runx2 expression in osteoblastic cells transfected with Runx2 siRNA and selected with G418-neomycine. Bar represents the mean±SEM (n=6; *$p<0.05$, **$p<0.001$ versus $1\alpha,25(OH)_2D_3$ only).

However, the hyperosmotic stimulus (25 and 50 mM sucrose addition) blunted the increase in Runx2 expression in a dose-dependent manner (Fig. 3A, 3B). These down- regulations of RANKL and Runx2 protein suggest that hyperosmotic exposure to osteoblastic cells inhibits RANKL expression via Runx2, eventually interupting osteoclastogenesis in the co-culture system. RANKL expression was examined after disrupting the Runx2 production using the siRNA method. As expected, an upregulated level of $1\alpha,25(OH)_2D_3$-induced RANKL expression in mouse calvarial osteoblastic cells was abolished to the control level by the siRNA-mediated knockdown of Runx2 (Fig. 3C), indicating that hyperosmotic stimulus to osteoblastic cells is able to decrease $1\alpha,25(OH)_2D_3$-induced RANKL expression via the inhibition of Runx2, which in turn leads to the down-regulation of $1\alpha,25(OH)_2D_3$-induced osteoclast differentiation.

TonEBP expression and its modulation in response to hyperosmotic stimulus

With respect to hyperosmotic stimulus, it has been demonstrated that TonEBP is responsible for transcriptional ac-

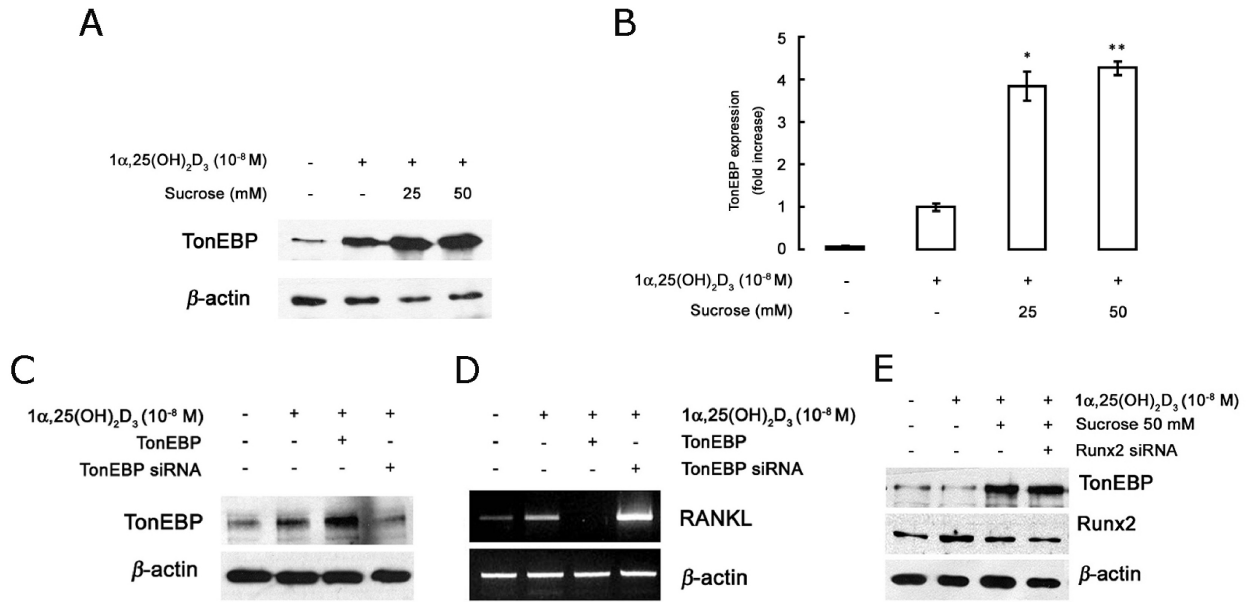

Fig. 4. Changes in the expression of TonEBP and Runx2 by hyperosmotic stimulus. Expression of TonEBP by hyperosmotic stress inhibits $1\alpha,25(OH)_2D_3$-induced RANKL expression in osteoblastic cells. (A) Western blot analysis of TonEBP expression in osteoblastic cells treated with $1\alpha,25(OH)_2D_3$ and 25 and 50 mM sucrose addition to the culture medium for 2 days. (B) TonEBP was stimulated by hyperosmotic stress caused by the addition of sucrose. (C) Western blot analysis of TonEBP expression levels in osteoblastic cells transfected with TonEBP (overexpression) or TonEBP siRNA. (D) The levels of RANKL expression were analyzed by RT-PCR using osteoblastic cells transfected with TonEBP siRNA and TonEBP in the presence or absence of $1\alpha,25(OH)_2D_3$. (E) The down-regulation in Runx2 expression by the stimulation of TonEBP (in response to the addition of 50 mM sucrose to the culture medium) in osteoblastic cells and TonEBP expression in osteoblastic cells transfected with Runx2 siRNA. Bar represents the mean±SEM (n=3; *$p<0.05$, **$p<0.005$ versus $1\alpha,25(OH)_2D_3$ only).

tivation of the genes encoding the regulation of a compatible osmolyte transporter under hyperosmotic conditions in kidney cells [29]. Appropriately, an experimented investigated whether TonEBP could be expressed in osteoblastic cells in response to hyperosmotic stimulus, and assessed how TonEBP affects $1\alpha,25(OH)_2D_3$-induced osteoclast differentiation. Hyperosmotic stimulation caused by the addition of sucrose to the culture medium surprisingly led to the enhancement of TonEBP expression (3.8～4.3-fold at 25 and 50 mM sucrose addition) in osteoblastic cells (Fig. 4A, 4B). To clarify how TonEBP works in the process of $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis, TonEBP overexpression and silence of TonEBP expression into calvarial osteoblastic cells were checked, and also applied in BMMs in the presence of $1\alpha,25(OH)_2D_3$. As shown in Fig. 4C, the overexpression of TonEBP in osteoblastic cells led to enhancement of the TonEBP protein, while silencing of TonEBP resulted in attenuation. When TonEBP was overexpressed in the calvarial osteoblastic cells, the $1\alpha,25(OH)_2D_3$-induced RANKL was suppressed. On the other hand, silence of TonEBP in osteoblastic cells caused an increase in $1\alpha,25(OH)_2D_3$-induced RANKL expression (Fig. 4D). From these results, it could be speculated that the TonEBP gene may be capable of down-regulation the RANKL expression by suppressing the Runx2 at the upstream level, which in turn leads to inhibition of $1\alpha,25(OH)_2D_3$-induced osteoclast differentiation.

When osteoblastic cells were cultured in presence of $1\alpha,25(OH)_2D_3$ plus 50 mM sucrose, the level of TonEBP expression was dramatically higher than that of expression in presence of $1\alpha,25(OH)_2D_3$ only, whereas $1\alpha,25(OH)_2D_3$-induced Runx2 expression was reduced to the level of the unstimulated condition (no hyperosmotic stimulus; Fig. 4E). However, this enhancement of TonEBP expression caused by hyperosmotic stimulus (addition of 50 mM sucrose) was maintained even though osteoblastic cells were transfected with the Runx2 siRNA (Fig. 4E). These results demonstrate that TonEBP down-regulates Runx2 expression at the downstream level. To further confirm this, the effect of TonEBP and Runx2 on osteoclast differentiation was checked in a co-culture system. As shown in Fig. 1, the number of $1\alpha,25(OH)_2D_3$-induced TRAP-positive multinucleated cells (169±29 cells) was remarkably decreased up to 48±8 cells under hyperosmotic stimulus of 50 mM sucrose to the co-culture medium. In addition, the number of $1\alpha,25(OH)_2D_3$-induced TRAP-positive multinucleated cells was significantly reduced when calvarial osteoblastic cells transfected with both Runx2 siRNA and the overexpression of TonEBP were co-cultured with BMMs in the presence of $1\alpha,25(OH)_2D_3$. Unlike the case of knockdown of Runx2 by siRNA (61±6 cells), knockdown of TonEBP in osteoblastic cells (140±26 cells) did not significantly alter the number of $1\alpha,25(OH)_2D_3$-induced TRAP-positive multinucleated cells in co-culture system.

DISCUSSION

In the present work, a role of hyperosmotic stimulus in regulating osteoclastogenesis, particularly $1\alpha,25(OH)_2D_3$-induced osteoclast differentiation in a co-culture system, was investigated. Initially, the addition of sucrose to the culture medium inhibited $1\alpha,25(OH)_2D_3$-induced osteoclast formation in the co-culture system, indicating that hyperosmotic stimulus can negatively regulate $1\alpha,25(OH)_2D_3$- in-

duced osteoclastogenesis (Fig. 1A, 1B). Furthermore, hyperosmotic stimulus up to 50 mM sucrose did not change the normal cellular viability of the cells in this co-culture system (Fig. 1C), and the osteoclasts stimulated by the 1 α,25$(OH)_2D_3$ function well on calcium phosphate apatite-coated plates in the co-culture system, inducing the pit formation as shown in Fig. 1D. These results support that hyperosmotic stimulus induces the alteration of physiological function of the osteoblastic cells, which, in turn, results in the inhibition of 1α,25$(OH)_2D_3$-induced osteoclast formation in co-culture system.

Of the physiological processes linked to osteoclastogenesis in osteoblastic cell, we first examined the changes in RANKL, M-CSF, and OPG level, which are the key molecules in the osteoblast to modulate osteoclastogenesis. As shown in Fig. 2, hyperosmotic stimulus of sucrose to the cultured osteoblastic cells lowered the expression level of the RANKL mRNA without any significant changes in the expression of M-CSF and OPG mRNA. Evidently, the hyperosmotic stimulus to the osteoblastic cells inhibited 1α,25$(OH)_2D_3$-induced osteoclastogenesis via the down-regulation of RANKL. To date, the ability of a hyperosmotic stimulus to down-regulate 1α,25$(OH)_2D_3$-induced RANKL expression in osteoblastic cells has not been reported, except studies showing the effects of osmotic loading on cartilage [31] and intervertebral disc [32]. Few studies have examined the influence of increasing extracellular glucose on osteoblast growth, differenciation, gene expression as well as intracellular Ca^{2+} change in diabetes [33]. Intriguingly, the osmotic stress caused by hyperglycemia in diabetic condition elevates the activator protein 1 (AP-1) level and the activity in osteoblasts [34], suggesting that an osmotic stimulus could be a primary inducer of the genetic modulation. Such an osmotic effect may be specific to osteoblasts since a small addition (4.5～16.5 mM) of either glucose or mannitol can enhance the expression of c-jun and collagen I of osteoblast, whereas osteocalcin expression decreases [34]. Our results also demonstrate that hyperosmotic stimulus by the addition of 25～50 mM sucrose is sufficient to suppress the 1α,25$(OH)_2D_3$-induced osteoclast formation and pit formation in a co-culture system, down-regulating the RANKL expression in osteoblastic cells (Fig. 2). Moreover, we confirmed that the osteoblastic cells normally mature by expressing the mRNA of the marker genes (osteocalcin, osteopontin and collagen 1) when exposed to the hyperosmotic stimulus.

Based on the facts that the *RANKL* gene contains functional Runx2-binding sites, and a mutation of these sites abrogates the transcriptional activity of the RANKL promoter [30], we examined whether the Runx2 intervenes in the upstream of RANKL. In this study, 1α,25$(OH)_2D_3$-induced Runx2 and RANKL expression in osteoblastic cells were lowered as a result of hyperosmotic stimulus of sucrose (Fig. 2 and 3), implying that the decrease in Runx2 expression by hyperosmotic stimulus causes the down-regulation of 1α,25$(OH)_2D_3$-induced RANKL expression in osteoblastic cells. Additional studies using the osteoblastic cells transfected with siRNA of Runx2 demonstrated that 1α,25$(OH)_2D_3$ treatment showed neither enhancement of RANKL protein nor decrease in the number of osteoclasts in a co-culture system, implicating Runx2 as a crucial factor in modulating the RANKL level downstream. Although it is still controversial whether Runx2 regulates RANKL expression in terms of the interrelationship between RANKL and Runx2 for cell signaling, it is likely that Runx2 is an essential factor in osteoblast differentiation, since the lack of osteoclasts in $Runx2^{-/-}$ mice suggests a critical role of Runx2 in osteoclastogenesis [35,36]. Overall, Runx2 could be essential to osteoclast formation in conjunction with RANKL expression, even though it has been reported that Runx2 is not responsible for the RANKL expression [37,38].

The next question is how hyperosmotic stimulus leads to the decrease in 1α,25$(OH)_2D_3$-induced Runx2 expression in osteoblastic cells, thus down-regulating the differentiation of osteoclasts. Basically, most cells are sensitive to physical forces and respond to osmotic stress by activating various mechanisms. These processes might involve the coordinated organization of the cytoskeleton and the activation of transporters in the plasma membrane that rapidly mediates mobilization of osmotically-active solutes [39]. These changes in the intracellular ion concentration, particularly those that function as secondary messengers, might influence other cellular responses, such as gene expression and cellular metabolism, including the reorganization of the cytoskeleton. Of these possible contributors to the hyperosmotic effect of the osteoblastic cells, we employed TonEBP, which is a transcriptional factor for the cellular accumulation of compatible osmolytes under the circumstances of hyperosmotic stimulus, because it is reasonable to assume that TonEBP is involved in processes affecting tissue hydration and osmotic environment, as well as a variety of physiologic functions. Our results demonstrate that a hyperosmotic stimulus of 50 mM sucrose enhances the expression of TonEBP, following the suppression of the Runx2, which, in turn, down-regulates 1α,25$(OH)_2D_3$-induced RANKL expression as expected (Fig. 4A, 4B). Additionally, we confirmed that the 1α,25$(OH)_2D_3$-induced RANKL protein level was suppressed when TonEBP was overexpressed in the calvarial osteoblastic cells, whereas silencing of TonEBP caused an increase in 1α,25$(OH)_2D_3$-induced RANKL expression (Fig. 4C, 4D). From the above results, it can be speculated that the activation of TonEBP gene by the hyperosmotic stimulus suppresses Runx2 expression, possibly following the downstream suppression of RANKL expression.

Our findings suggest that hyperosmotic stimulus leads to the down-regulation of 1α, 25$(OH)_2D_3$-induced osteoclastogenesis, suppressing Runx2 and RANKL expression due to the activation of TonEBP expression. Thus, hyperosmotic stress could play a pivotal role as a regulator that modulates osteoclastogenesis and bone resorption.

ACKNOWLEDGEMENTS

This work was supported by the Research Fund from Yonsei University College of Dentistry for 2007 to Syng-Ill Lee.

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