Hypertonicity down-regulates 1α, 25-dihydroxyvitamin D_3-induced osteoclastogenesis via Runx2 in co-culture system

TIAN YUSHUN, Ph. D.

The Graduate School

Yonsei University

Department of Dentistry

Hypertonicity down-regulates 1α, 25-dihydroxyvitamin D_3-induced osteoclastogenesis via Runx2 in co-culture system

TIAN YUSHUN, Ph. D.

The Graduate School

Yonsei University

Department of Dentistry

Hypertonicity down-regulates 1α, 25-dihydroxyvitamin D_3-induced osteoclastogenesis via Runx2 in co-culture system

Directed by Professor Syng-Ill Lee, D.D.S., Ph.D.

The Doctoral Dissertation
Submitted to the Department of Dental Science and the
Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

TIAN YUSHUN

June 2005

This certifies that the Doctoral Dissertation of TIAN YUSHUN is approved.

Thesis Supervisor: Syng-Ill Lee, D.D.S., Ph.D.

Yun-Jung Yoo, D.D.S., Ph. D.: Thesis Committee Member

Dong Min Shin, D.D.S., Ph. D.: Thesis Committee Member

Kyoo-Sung Cho, D.D.S., Ph. D.: Thesis Committee Member

Seung-Ho Ohk, Ph. D.: Thesis Committee Member

The Graduate School
Yonsei University

June 2005

Acknowledgements

At first, I would like to express my deepest appreciation to Professor Syng-Ill Lee for thoughtful advice and guide from start to the end of my doctoral degree.

I am grateful to Professor Yun-Jung Yoo, Professor Dong Min Shin, Professor Kyoo-Sung Cho, Professor Seung-Ho Ohk. Also, I would like to thank so much Professor Jeong-Taeg Seo, Professor Jeong Heon Cha and Professor Han Seong Jeong, and other professors for the trouble they have taken on my behalf.

I would like to appreciate Hyun-Joo Jeong, she helps me to finish this study. Also, I would like to thank Hae Jo, Min Seuk Kim, Reseach assistant Jeong Hee Hong, Hae Mi Byun, Seong Hye Kim.

I am very grateful to every member of the Department of Oral Biology, who made me encouraged. Thank Research fellow Jae Young Kim, Research fellow Sung Woon Cho, Jong Min Lee, Research fellow Jeong Mi Ahn, Ju Yeon Kim, Du Sik Kim, and Jang-Gi Cho.

Finally, special thanks go to my parents for always making me to cheer up. And thank my Chinese friends Jinglei Cai, Zhengguo Piao, and Zhongmin Che, who are study with me, very much for being great help besides me.

TABLE OF CONTENTS

III. RESULTS

LIST OF FIFURES

Abstract

Hypertonicity down-regulates 1α, 25-dihydroxyvitamin D_3-induced osteoclastogenesis via Runx2 in co-culture system

TIAN YUSHUN

Department of Dentistry

The Graduate School, Yonsei University

(Directed by Prof. Syng Ill Lee, D.D.S., Ph.D.)

Bone integrity is maintained through bone remodeling which is consisted of bone formation and resorption. Osteoblast/stromal cells can express RANKL (receptor activator of NF-κB ligand), M-CSF (macrophage colony-stimulating factor), and OPG (osteoprotegerin). RANKL and M-CSF are essential and sufficient to promote osteoclastogenesis. By contrast, OPG is a secretory protein which binds to RANKL, resulting in blockade of RANKL/RANK axis. When bone is actively resorbed, it might be possible that high osmolality is provided due to the high concentration of Ca^{2+}, PO_4^{2-}, and the degraded organic materials in the extracellular fluid around bone cells. Therefore, we demonstrated the effects of hypertonicity on osteoclastogenesis in osteoblast

(from calvariae)-preosteoclast (bone marrow) co-culture system for the purpose of understanding the hypertonicity on bone metabolism at the molecular level.

Hypertonicity (high osmolar sucrose) down-regulates 1α, 25-dihydroxy-vitamin D_3 (1α, 25$(OH)_2D_3$)-induced osteoclastogenesis in co-culture system. Namely, hypertonicity (sucrose 25, 50 mM) inhibits number of tartrate-resistant acid phoshoatase (TRAP) positive multinucleated cells and pit formation induced by 10 nM 1α, 25$(OH)_2D_3$. In a viability test, sucrose did not show any toxic effect in these concentrations. The mRNA expressions of RANKL, OPG and M-CSF were analyzed by RT-PCR, and RANKL was detected with Western blot and ELISA assay, in order to investigate the mechanism by which hypertonicity inhibits osteoclastogenesis. In hypertonic conditions, expression of RANKL and its mRNA were decreased in dose-dependent manner, while the changes in OPG and M-CSF were not detected significantly. Namely, hypertonicity inhibits osteoclast differentiation by reducing the expression ratio of RANKL/OPG in differentiated osteoblastic cells. However, osteoblast marker genes as well as osteopontin, collagen 1 and osteocalcin expressions were not changed in hypertonic conditions, which indicate calvarial osteoblastic cell specific character was not affected with hypertonicity. Runx2 (Runt-related transcription factor 2)/Cbfa1 (Core-binding factor alpha 1 subunit)/Pebp2αA (Polyomavirus enhancer binding protein 2 alpha A subunit)/AML3 (acute myeloid leukemia 3 protein), which is an essential transcriptional factor that regulates osteoblast differentiation. In this study we clarify whether the Runx2 is linked to 1α, 25$(OH)_2D_3$-induced osteoclastogenesis in hypertonic condition.

Because RANKL gene basic promoter sequence has several binding sites for Runx2 and mutation of these sites abrogates the transcriptional activity of the RANKL promoter. We measured the Runx2 expression by Western blot in the presence or absence of hypertonicity. 1α, $25(OH)_2D_3$ caused the increase of Runx2 expression in osteoblastic cells. Such increase in 1α, $25(OH)_2D_3$-induced Runx2 expression was blunted by hypertonicity, suggesting that hypertonicity could elicit the partial inhibition of osteoclastogenesis due to the supression of Runx2 expression. To confirm this possibility, RNA interference of Runx2 was transfected into the osteoblastic cells. In these transfected cells, 1α, $25(OH)_2D_3$-induced RANKL expression and TRAP-positive cells formation were remarkably decreased.

Taken all together, these findings reveal that hypertonicity around bone cells was supported by active osteoclasts down-regulates 1α, $25(OH)_2D_3$-induced osteoclastogenesis, is via Runx2. Because under the hypertonic condition, 1α, $25(OH)_2D_3$-induced Runx2 expression was suppressed and then inhibited of RANKL synthesis. Therefore, hypertonicity may be a novel candidate for the regulation of bone metabolism through affect bone resorption.

Key words: Hypertonicity, osteoclastogenesis, osteoblast, RANKL, Runx2, 1α, $25(OH)_2D_3$

I. Introduction

Bone morphogenesis and remodeling are strictly regulated by the balance between bone formation and resorption [Teitelbaum, 2000; Gerard *et al.*, 2002]. Osteoclasts, multinucleated giant cells derived from hematopoietic precursors of monocyte/macrophage lineage, which are found in bone marrow, spleen, blood, and other tissues [Tsurukai *et al.*, 2000; Gerard *et al.*, 2002], play a central role in the bone resorption process. The stromal/osteoblastic cells are requisite for differentiation of monocyte/macrophages into osteoclasts *in vitro* coculturing [Takahashi *et al.*, 1988; Udagawa *et al.*, 1990]. It is now clear that these stromal/osteoblastic accessory cells express the two molecules that are essential and sufficient to promote osteoclastogenesis: macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B (NF-κB) (RANK) ligand (RANKL) (also known as OPGL and TRANCE). M-CSF [Udagawa *et al.*, 1990; Yoshida *et al.*, 1990; Tanaka *et al.*, 1993], which is imperative for macrophage maturation, binds to its receptor, c-fms, on early osteoclast precursors, thereby providing signals required for osteoclast survival and proliferation. RANKL [Wong *et al.*, 1997; Jimi *et al.*, 1999], an osteoclast differentiating factor, is a part of the tumor necrosis factor ligand family, binding to its receptor, RANK, which is expressed on the cell surface of osteoclast precursor cells. RANKL is a 317-amino acid peptide [Wong *et al.*, 1997], and two distinct forms of RANKL have been identified, a 45-kDa membrane-associated form (mRANKL) and a 32-kDa soluble form (sRANKL) derived from proteolytic cleavage of the membrane-associated form [Lacey *et*

al., 1998]. The RANK/RANKL signaling system has been shown to play crucial roles in the development of osteolytic bone lesions such as osteoporosis [Suda *et al.*, 1999], cancer-associated osteolytic diseases [Kitazawa S. and Kitazawa R., 2002] and rheumatoid arthritis [Takayanagi *et al.*, 2000]. Stromal/osteoblastic cells also secrete a soluble protein osteoprotegerin (OPG) that inhibits osteoclastogenesis by binding and neutralizing RANKL [Simonet *et al.*, 1997; Takayanagi *et al.*, 2000]. Animals lacking OPG have accelerated osteoclastogenesis and develop severe osteoporosis [Simonet *et al.*, 1997].

Bone is a mineralized tissue that is composed of an organic matrix. Type I collagen constitutes 95% of the organic matrix, and the remaining 5% is composed of proteoglycans and noncollagenous proteins such as osteopontin and osteocalcin [Liu *et al.*, 2001]. For the resorption process, osteoclasts secrete protons and a large variety of proteolytic enzymes into resorption lacunae to dissolve minerals and degrade collagenous matrix and noncollagenous proteins such as osteonectin and some phosphoproteins [Baron *et al.*, 1985; Vaes *et al.*, 1988; Blair *et al.*, 1989; Väänänen *et al.*, 1990]. Mainly, proteolytic enzymes of osteoclasts, interstitial collagenase, lysosomal enzymes and cysteine proteases are together to digest organic matrix [Salo *et al.*, 1996]. Bone resorption leads to the localized degradation of fully mineralized bone matrix, including removal of both inorganic and organic matrix components [Salo *et al.*, 1996]. The degredation of inorganic matrix precedes the degradation of organic matrix, which mostly takes place in extracellular resorption lacunae [Jones SJ, Boyde A., 1987]. During osteoclastic bone resorption, crystal hydroxyapatite is dissolved into free Ca^{2+}/PO_4^{2-} ions in the resorptive hemivacuole where it can

reach concentrations as high as 40 mM [Silver *et al.*, 1988; Xu *et al.*, 2005]. High extracellular Ca^{2+} results in the inhibition of bone resorption [Zaidi *et al.*, 1999a; Zaidi *et al.*, 1999b] and induction of osteoclast apoptosis [Lorget *et al.*, 2000]. On the other hand, when bone is actively resorbed, it might be possible that high osmolality is provided due to the high concentration of Ca^{2+}/PO_4^{2-} and the degraded organic materials (mainly collagen) in the extracellular fluid around bone cells. Under these high osmotic conditions, the function of bone cells will be changed through the cell shrinkage. Namely, when osteoclast was activated, there are two pathways in arrest osteoclastogenesis, one is regulated by high concentration of Ca^{2+}, which induces osteoclast apoptosis, and the other one is regulated by hypertonicity, which affect bone cells metabolism. In these two pathways, we are interested in the latter one, because the way how the degraded organic materials from the resorbed bone affect the bone cells (osteoblast and osteoclast) in terms of high osmolality is yet not examined. Here, we hypothesized that the hypertonicity in bone could be one of the regulating factors for bone remodeling. In the our study, we will use sucrose to make hypertonic condition and apply 1α, 25-dihydroxyvitamin D_3 (1α, 25$(OH)_2D_3$) to stimulate osteoclast formation. Understanding of hypertonicity in bone metabolism is necessary at the molecular level of osteoblast and osteoclast. Therefore, we hypothesized that hypertonicity will might concern 1α,25$(OH)_2D_3$-induced osteoclastogenesis and bone metabolism with respect to RANKL, OPG, and other genes on the osteoblast. To demonstrate whether hypertonicity affect the osteoclastogenesis, we applied hypertonicity to an osteoblast/stromal cell and preosteoclast co-culture system.

Skeletal integrity is regulated by a variety of hormones such as parathyroid hormone (PTH), prostagrandin E_2 (PGE_2), interlukin-1 (IL-1), interlukin-6 (IL-6), estrogen, 1α, 25$(OH)_2D_3$, and local factors like fluid movement, located Ca ion [Suda *et al.*, 1992; Yasuda *et al.*, 1998; David *et al.*, 1998; Suda *et al.*, 1999]. 1α, 25$(OH)_2D_3$, a physiologically active form of vitamin D_3, plays multiple roles in the control of bone resorption and bone formation. Cellular levels of 1α,25$(OH)_2D_3$ present as a function of the stage of osteoblast maturation which can positively or negatively influence expression of a gene or protein [Owen *et al.*, 1991; Shalhoub *et al.*, 1992; Gerstenfeld *et al.*, 1996; Lian *et al.*, 1997]. It regulates bone metabolism by enhancing expression of many genes necessary to accommodate the events that govern osteoclast maturation and osteoblast lineage commitment and differentiation [Drissi *et al.*, 2002]. For instance, when osteoblasts/stromal cells are stimulated by 1α,25$(OH)_2D_3$, RANKL is expressed and induces the differentiation of osteoclast progenitors by binding to the receptor activator of NF-κB (RANK) [Jimi et al., 1999]. Receptor activator of NF-κB ligand (RANKL)-RANK signaling is essential for osteoclastogenesis and plays important role. Interestingly, RANKL genes contain functional Runx2 binding sites and mutation of these sites abrogates the transcriptional activity of the RANKL promoter [O'Brien *et al.*, 1998] [Kitazawa *et al.*, 1999]. Runx2 (runt-related transcription factor 2)/ Cbfa1 (core-binding factor α1)/ AML-3 (acute myeloid leukemia 3 protein)/ Pebp2αA (Polyomavirus enhancer binding protein 2 alpha A subunit), is essencial for osteoblast differentiation [Komori *et al.*, 1997; Otto *et al.*, 1997; Ducy P., 2000; Karsenty *et al.*, 2001]. Runx2 plays important roles in multiple processed of

endochondral ossification, including chondrocyte maturation, vascular invasion into the cartilage, osteoclast differentiation, and osteoblast differentiation [Komori T., 2002]. Runx2 expression is regulated by 1α, 25$(OH)_2D_3$, because its proximal promoter sequences contain a functional VDRE (VD_3-responsive element) that binds a VDR/retinoid X receptor heterodimer [Drissi *et al.*, 2002]. Members of the Runx2 family of transcription factors function by binding to the consensus sequence AACCACA, known as osteoblast-specific element 2 (OSE2) [Ducy, P. and Karsenty, G., 1995]. This AACCACA sequence or highly related sequences are found in the promoter regions of many genes such as osteocalcin [Ducy *et al.*, 1997], bone sialoprotein [Benson *et al.*, 1999], osteopontin [Tezuka *et al.*, 1996; Sato *et al.*, 1998], type 1 collagen [Porte *et al.*, 1999], collagenase 3 (MMP-13) [Jimenez *et al.*, 1999], OPG [Thirunavukkarasu *et al.*, 2000], and RANKL [O'Brien *et al.*, 1998; Kitazawa *et al.*, 1999].

Komori and coworkers [Komori *et al.*, 1997] reported that osteoclastogenesis was markedly retarded in Runx2-deficient ($Runx2^{-/-}$) mice. These results suggested that the maturational arrest of osteoblasts caused by disruption of the Runx2 gene might be related to the insufficient osteoclastogenesis in Runx2-deficient mice. However, hematopoietic cells from Runx2-deficient mice are capable of forming osteoclasts *in vitro* when cultured with wild-type stromal/osteoblastic cells indicating that the osteoclast deficit results from a lack of support from stromal/osteoblastic cells. In agreement with this evidence, RANKL expression is reduced in tibiae and femurs from $Runx2^{-/-}$ embryos, and 1α, 25$(OH)_2D_3$ can not induce RANKL mRNA in cells from these embryos [Gao *et al.*, 1998]. On the other hand, according to Enomoto, in Runx2-deficient

mice, osteoclasts were absent, in which OPG and M-CSF were normally expressed, but RANKL expression was severely diminished [Enomoto *et al.*, 2003]. Thus, it is likely, at least in part, that Runx2 is involved in osteoclastogenesis by regulating the expression of RANKL. In addition to explaining the osteoblast-specific expression of RANKL, the requirement of Runx2 for RANKL gene expression may constitute the molecular mechanism of the linkage between osteoblastogenesis and osteoclatogenesis [Porte *et al.*, 1999]. And, they [Kitazawa *et al.*, 1999] [Kabe *et al.*, 2005] found inverted TATA- and CAAT-boxes and binding sites for Runx2 as a characteristic structure of the mouse RANKL/TRANCE/OPGL/ODF gene basic promoter. More extensive studies on the regulation of RANKL by Runx2 will provide insight into the molecular mechanism involved in the classical hypothesis proposed by Rodan and Martin [Rodan GA, Martin TJ, 1981] concerning the interaction between osteoblasts and osteoclasts during bone remodeling. Runx2 through its effects on osteoblast lineage commitment and function could also directly or indirectly regulate the bone resorption process [Kannan *et al.*, 2000]. As previously reported [Komori T., 2002; Kitazawa *et al.*, 2003], one vitamin D_3 responsive element (VDRE) is located further upstream in the region of the mouse RANKL gene. Others also observed that $1\alpha,25(OH)_2D_3$ accelerated *in vitro* osteoclastogenesis by upregulating RANKL gene [Kitazawa R., Kitazawa S., 2002; Kitazawa *et al.*, 2003]. Furthermore, identified RANKL as one of the target genes of vitamin D, and shown that the vitamin D receptor (VDR) [Farach-Carson *et al.*, 1998; Javed *et al.*, 1999] formed VDR-RXRα(retinoid X receptor α)(human) or VDR-RXRβ (mouse) heterodimers to

bind to the VDRE. Therefore, RANKL promoters is well preserved and composed of inverted TATA- and CAAT-boxes and a consensus binding sites of Runx2 flanked by VDRE, indicating the importance of these structures for the regulation of the gene. More interestingly, in Runx2-transgenic adult mice, express bone formation marker genes, osteocalcin (a marker of mature osteoblast) and ALP (alkaline phosphate), are unaffected; however, that of osteoclastic related genes as well as OPG, RANKL, and MMP-13 are increased [Geoffroy *et al.*, 2002]. In addition, osteocyte number is reduced in transgenic animals irrespective of bone turnover status. Matrix synthesis level per osteoblast is unchanged in transgenic animals, and matrix mineralization is reduced. On the other hand, Runx2 inhibits the late stage of osteoblast maturation, restricting Runx2's positive function to the early differentiation stage in the process of osteoblast development [Liu *et al.*, 2001; Viereck *et al.*, 2002; Toshihisa K., 2003]. Furthermore, the regulation of RANKL expression by Runx2 seems to be dependent on the maturational stage of osteoblast lineage cells [Komori T., 2002].

From these reports, we found Runx2 is related osteoclastogenesis, probably. So, in this report, we will to identify transcription factor Runx2 and osteoclast differentiation relationship with siRNA of Runx2 technique.

We expect that hypertonicity regulates $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis via Runx2.

Ⅱ. Materials and Methods

1. Materials

Routine cell culture media was obtained from GIBCO/BRL (Grand Island, NY). The Tartrate-Resistant Acid Phosphatase Staining Kit was purchased from the Sigma Chemical Co., Ltd. (St. Louis, MO). Trizol was purchased from Invitrogen Corp. (Carlsbad, CA), and the ICR mice were from Samtacho Co., Ltd. (O-san, Kyung-gi-Do, Korea). Sucrose (α-D-Glucopyranosyl β-D-fructo-furanoside; Saccharose; Cane sugar) and 1α, 25-dihydroxyvitamin D_3 [1α, $25(OH)_2D_3$] were also purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). All other chemicals were of reagent grade. The osmolalities of all media and solutions were measured with a FISKE ONE-TEN Osmometer (FISKE ASSOCIATES, USA).

2. Preparation of primary osteoblastic cells

Primary osteoblastic cells were prepared from the calvariae of 1-day-old newborn ICR mice (Samtak Inc., O-san, Kyung-gi-Do, Korea) by a previously reported method [Takahashi *et al.*, 1988; Choi, B. K. *et al.*, 2001] with a slight modification. The calvariae removed from 10 mice were subjected to digestion four times at 20-min intervals using 0.2% collagenase (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and 0.1% dispase (Gibco/BRL, Life Technologies, Grand Island, NY, USA) in a shaking water bath at 37℃. The primary osteoblastic cells isolated in the first digestion were discarded, and those of the second to fourth digestions were collected and cultured to

confluence in an α-minimun essential medium (α-MEM) (Gibco/BRL, Life Technologies) containing 10% fetal bovine serum (Gibco/BRL, Life Technologies) and antibioticin-antimycoticin (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotricin B) (Gibco/BRL, Life Technologies) in 10-cm culture dish at 37℃ with 5% CO_2.

3. Histochemical staining of alkaline phosphatase (ALP)

ALP staining was performed by a standard protocol. In brief, cultured cells were rinsed in PBS (pH 7.4), fixed in 2% paraformaldehyde at 20-30min, rinsed with PBS, and then overlaid with 5 ml reation solution, which containing 0.5 mg of Naphthol AS-BI phosphate (sigma) plus N-N' dimethylformamide (sigma) and 3 mg of fast blue RR solt (sigma) in 0.1 M Tris-HCl (pH 8.3-8.5), followed by incubation at room temperature for 2 hours in the dark.

4. RT-PCR (Reverse Transcription-Polymerase Chain Reaction)

RT-PCR was performed by a previously reported method [Choi, B. K. *et al.*, 2001; Yun *et al.*, 2004]. Primary osteoblastic cells were seeded in 35-mm culture dishes (1×10^4 cells/dish) and grown to 4 days in α-MEM containing 10% fetal bovine serum. After 4 days the cells were treated with each reagent and incubated at 37℃ with 5% CO_2 for 4 days, and then the cells were collected and total RNA was extracted from the primary osteoblastic cells using a TRIzol reagent (Invitrogen Corp. Carlsbad, CA) according to the manufacturer's instructions. The concentration of the RNA obtained was determined by measuring the absorbance at 260 and 280 nm. RT-PCR for RANKL, OPG, M-CSF, osteocalcin, osteopotin, collagen 1 and β-actin mRNA was carried out

with a commercial two-step RNA-PCR kit (ELPisbio). That mRNAs (1 μg) isolated from each sample were used as templates for the cDNA synthesis. Each reaction tube contained 1 μg of mRNA, 1 μl Anchored primer $(dT)_{25}V$ or N8 random octamers (40 μM), 8 μl DEPC treated sterile water, 2 μl DTT (100 mM), and 8 μl RT-&GO^{TM}MasterMix 2.5×C. Polymerase chain reactions were performed in a thermal Cycler 96 PCR machine (BIO-RAD, MyCyclear^{TM}Thermal CyCler, U.S.A) using 2 μg of cDNA, 5 μl Taq-&GO^{TM}MasterMix 5×C, 2 μl of each forward and reverse oligonucleotice primer (*Table 1*) and sterile water in a 25 μl volume. The cycling conditions were 5 min at 95℃ (reverse trascription) and 10 min at 72℃ (reverse transcriptase inactivation) and 30 cycles of 95℃ 30 s, T_a (annealing temperature) 30 s, 72℃ 1 min. After amplification, 15 μl of each PCR products was analyzed by 1.2% agarose gel electrophoresis in TAE buffer. The gels were stained with ethidium bromide (10 μg/ml) and photographed on top of a 280 nm UV light box. The quantity and base pair size of the PCR generated DNA fragments were estimated relative to DNA ladder standards. β-actin used for standardization control.

Table 1. Synthetic oligonucleotide primers used for reverse transcription-polymerase chain reaction (RT-PCR)

Target gene	**Primer sequence**	**T_a** (annealing temperature)
RANKL (750 bp)	(forward): 5'-ATCAGAAGACAGCACTCACT-3' (reverse): 5'-ATCTAGGACATCCATGCTAATGTTC-3'	48℃

OPG (636 bp)	(forward): 5'-TGAGTGTGAGGAAGGGCGTTAC-3' (reverse): 5'-TTCCTCGTTCTCTCAATCTC-3'	58℃
M-CSF (395 bp)	(forward): 5'-CATGACAAGGCCTGCGTCCGA-3' (reverse 1): 5'-AAGCTCTGGCAGGTGCTCCTG-3' (reverse 2): 5'-GCCGCCTCCACCTGTAGAACA-3'	58℃
β-actin (366 bp)	(forward): 5'-GGACTCCTATGGTGGGTGACGAGG-3' (reverse): 5'-GGGAGAGCATAGCCCTCGTAGAT-3'	58℃
Cbfa1 (Runx2)	(forward): 5'-AGCCTCTTCAGCCGAGTGACACC-3' (reverse): 5'-CTGGGCCATGGTTGACGAATTTC-3'	58℃
Osteopontin	(forward): 5'-GAGCGGTGAGTCTAAGGAGT-3' (reverse): 5'-CTAAATGCAAAGTAAGGAAC-3'	59℃
Osteocalcin	(forward): 5'-AAGCAGGAGGGCAATAAGGT-3' (reverse): 5'-AGCTGCTGTGACATCCCATAC-3'	55℃
Collagen 1	(forward): 5'-ACCTTCCTGCGCCTAATGTC-3' (reverse): 5'-TTGGGTTGTTCGTCTGTTTC-3'	55℃

5. *In vitro* osteoclast formation assay

The prepared calvarial osteoblastic cells were detached from the culture dish by treating them with trypsin-EDTA (Gibco/BRL, Life Technologies) and collected by centrifugation. The bone marrow cells were collected from the femurs and tibiae of 4-week-old ICR male mice. The ends of the femurs and tibiae were removed and the marrow cavity was flushed by slowly injecting media in at one end using a 25-gauge needle. The collected bone marrow cells were washed and treated with 10 mM Tris-HCl, 0.83% ammonium chloride to remove the red blood cells. After 2 days of incubation at 37°C on cell culture dishes, nonadherent cells (termed BMM precursors) were removed from culture for use in osteoclastogenic cultures [Quinn *et al.*, 2002]. Primary osteoblastic cells (1×10^4 cells/well) were co-cultured with bone marrow cells (1×10^5

cells/well) in α-MEM containing 10% FBS in 48-well plates (Corning Inc., Corning, NY). The culture volume was made up to 400 μl per well with α-MEM supplemented with 10% FBS, in the presence of 1α, 25$(OH)_2D_3$ (10 nM), without or with sucrose. All cultures were maintained at 37℃ in a humidified atmosphere containing 5% CO_2. After incubation for 4 days, the cells were fixed and stained for Tartrate-Resistant Acid Phosphatase (TRAP, an osteoclast marker enzyme [Minkin C., 1982]), using an acid phosphatase kit (Sigma Chemical Co.). TRAP-positive multinucleated cells showing more that three nuclei were counted as such. *In vitro* formation assay of osteoclast was repeated four times.

6. Assessment of cell viability

Cell viability was determined by the conventional MTT assay [Mosmann T., 1983; Choi, B. K. *et al.*, 2001] with slight modification. The number of viable cells was determined based on the reduction of MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) dye (Sigma Chemical Co., St. Louis, MO, USA) by mitochondria dehydrogenase in live cells to form blue formazan crystals. Primary osteoblastic cells (1×10^4 cells/well) were seeded in 48-well plates and grown in α-MEM containing 10% fetal bovine serum to 4 days. The cells were then treated with 10 nM 1α, 25-$(OH)_2D_3$ and various concentrations of sucrose for an additional 4 days. In addition, after the primary osteoblastic cells (1×10^4 cells/well) had been co-culture with bone marrow cells (1×10^5 cells/well) in α-MEM containing 10% fetal bovine serum in 48-well plates for 4 days, 1α, 25-$(OH)_2D_3$ and various concentrations of sucrose were added to each well and the cells cultured for an additional 4 days. After 4 days of culture, 40

μl of MTT solution (5 mg/ml) was added to each well (160 μl media) and the cells incubated for 4 hours at 37℃. The supernatant was discarded and 100 μl of demethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals at room temperature for 20 minutes and was diluted with suitable volumn of demethyl sulfoxide in 96-well plates. The optical density of the formazan solution was measured at 570 nm.

7. Pit formation assay

Osteoblastic cells obtained from the calvariae of newborn ICR mise and bone marrow cells obtained from the tibiae and femur of 4 months male ICR mise were co-cultured in α-MEM in calcium phosphate apatite-coated 24-well OAAS (Oscotec, Korea) plate and used according to the method [Jimi et al., 1999; Choi YN et al., 2001] with a slight modification. Briefly, the osteoblastic cells and bone marrow cells were resuspended in complete α-MEM medium and plated into OAAS plate at 2×10^4 cells/0.8 ml/well calvarial osteoblastic cells and 2×10^5 cells/0.8 ml/well bone marrow cells, respectively. The cells were cultured for 4 days at 37℃ in a humidified 5% CO_2 atmosphere. Then the cells treated with 1α, $25(OH)_2D_3$ (10 nM) and sucrose by different concentration, respectively. Cultures were maintained for 4 days. The medium in each well was replaced with respective fresh completive medium and 1α, $25(OH)_2D_3$ (10 nM) and sucrose. The experiments were performed four times. After termination of culture, attached cells were removed from the plate by addition with 4% sodium hypochlorite solution (Sigma). Images of pit were gathered with a digital camera attached to a microscope at × 100 magnification, and total

areas of resorption pits were analyzed by Image Meta Morph Program. The data were presented as mean ± SEM of four times sample.

8. ELISA (Enzyme Linked Immuno-Solbent Assay)

Quantikine® M murine Mouse RANK Ligand kit (R&D systems Inc., Minneapolis, IN) was used to analyze RANKL protein following the manufacturer's instructions. Briefly, mRANKL standard was diluted in Calibrator Diluent RD-12 solution to make final concentration of 0, 31.2, 62.5, 125, 250, 500, 1000, and 2000 pg/ml. Assay Diluent RD1W and standards (50 µl) were added to each well and incubated for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed. Each well was aspirated and washed, repeating the process four times for a total of five washes. mRANKL conjugate (100 µl) was added to each well and incubated for 2 hours at room temperature. Washing was repeated as described above. Substrate solution (100 µl) was added to each well and incubated for 30 minutes at room temperature in dark room. Stop Solution (100 µl) was added to each well and mixed by gentle tapping. Then the enzyme reaction yields a blue product that turns yellow. The intensity of the color of each well was determined within 30 minutes, using a microplate reader at 450 nm.

9. Western blot analysis

Western blot analysis followed by a previously reported method [Kim *et al.*, 2003]. Protein extracts were prepared from osteoblastic cells as follows. The cells that cultured in 60-mm culture dish were washed with ice-cold 1×PBS and then lysed by adding RIPA (radio-immuno precipitation assay) buffer [Tris-

HCl (10 mM; pH 7.8), 150 mM of NaCl, 1 mM of EDTA, and 1% NP-40], 2 mM of Na_3VO_4, 10 mM of NaF, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 10 μg/ml of PMSF. The lysates were clarified by centrifugation at 14,000 rpm for 20 minutes. An equal amount of protein was subjected 10% sodium deoxyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Hercules, CA) and electrotransferred to a nitrocellulose membrane. After transfer, the blot was washed with 1×PBS (pH 7.4) for 5 minutes at room terperature and then incubated in blocking buffer (1× PBS containing 0.1% Tween 20 with 5% nonfat dry milk) for 1 hour at room temperature. The blot was washed three times for 10 minutes each with 1× PBS. Primary antibody of Runx2 or RANKL (Santa Cruz Biotech, Santa Cruz, CA) was added to the blocking buffer at 1:5000 with gentle agitation overnight at 4℃. The blot was washed three times with 1×PBS and incubated with the horseradish peroxidase-conjugated rabbit anti-mouse antibody (Santa Cruz Biotech, Santa Cruz, CA) for 1 hour at room temperature. After three washes, the signal was detected by ECL Chemiluminescence Plus reagents (Amersham, Alington Heights, IL, USA) following the procedure recommended by the supplier.

10. RNA interference

10.1. Cloning of samll interfering RNA

siSTRIKE^{TM}U6 hairpin cloning systems kit (Promega, Madison, USA) were used to synthesize 19-nucleotied single-stranded RNA. Briefly, two hairpin oligonucleotides are annealed to form a double-stranded DNA fragment for inserting it into the psiSTRIKE^{TM} vector. For assistance with hairpin oligonucleotide design visit the siRNA Designer at:

www.promega.com/techserv/tools.

The primers used were: for Runx2 (56 bp), 5'-ACCGCTCTGGCGTTTAA ATGGTTTCAAGAGAACCATTTAAACGCCAGAGCTTTTTC-3' (sense), 5'-TGCAGAAAAAGCTCTGGCGTTTAAATGGTTCTCTTGAAACCATTTAA ACGCCAGAG-3' (anti-sense) (Bioneer Oligo Synthesis; Korea);

For cloning the psiSTRIKE™ vectors inserting hairpin oligonucleotides, the annealing reaction was performed at 90℃ for 3 minutes followed by incubating at 37℃ for 15 minutes using 2 μg/μl each oligonucleotide.

10.2. Ligation of small interfering RNA into the psiSTRIKE™ vectors

The annealed hairpin oligonucleotides were diluted at the final concentration 4 μg/ml. And for ligating hairpin insert to the psiSTRIKE™ vectors. psiSTRIKE™ vectors and diluted hairpin oligonucleotides were incubated at room temperature for 1 hour using T4 DNA ligase.

10.3. Transformation of *E.coli* with psiSTRIKE™ vectors

To obtain a reasonable number of the ligation of fragments with a hairpin, performed transformation reaction using the *ECOS-1* min Competent cells (Yeastern Biontech. Taipei, Taiwan). Recombinant plasmid DNA was isolated.

10.4. Confirmation of recombinant plasmid DNA

The recombinant plasmid DNA was digested with restriction enzyme *Pst*I yielded 3655bp and 958bp. This construct was confirmed by electrophoresis on 1.5% agarose gels in TAE buffer. The gels were stained with ethidium bromide (10 μg/ml) and photographed on top of a 280 nm UV light box.

11. Transfection experiments

The calvarial osteoblastic cells that were isolated from 1-day-old newborn ICR mice were seeded at 60-70% confluence in culture dishes. Cells were resuspended in α-MEM containing 10% fetal bovine serum (FBS) and antibioticin-antimycoticin. The day before transfection, osteoblastic cells changed the α-MEM antibiotics-free medium. Transfection of recombinant DNA (1 μg/ml) (final concentration) was carried out using lipofectamine™2000 (Invitrogen Corp., Carlsbad, CA) for 24 hours following the manufacturer's recommendations. After 24 hours the cells changed with α-MEM containing 10% FBS and antibioticin-antimycoticin medium and were cultured for 1 day at 37℃ in a humidified 5% CO_2 atmosphere. Then add 1% of GENETICIN® (G418-neomycin) (100 mg/ml) (Invitrogen Corp.) to selet transfected cells to use. The contrast groups were added psiSTRIKE™ vectors and were selected with G418-neomycin followed same procedure.

12. Data analysis and statistics

Unless stated otherwise, all experiments were reproduced at least three times. The results are expressed as the mean ± SEM. The statistical significances of the data were analyzed by the Student *t* test (two-tailed). A *p* value of less than 0.05 was considered to be statistically significant.

III. Results

1. Confirmation of osteoblastic cells

To investigate whether osteoblastic cells, which were prepared from new born mice calvariae according to “Methods”, were performed an alkaline phosphatase staining (Figure 1A) and RT-PCR (Figure 1B). From Figure 1A, we found many alkaline phosphatase-positive spindle-shaped cells, but NIH3T3 cell (fibroblast cell line) for negative control wasn’t expressed any one stained cell. And, from Figure 1B, we also found that these cells expressed osteoblast marker genes as well as collagen 1, osteopontin, and osteocalcin (late stage marker gene). From these results, we could distinguish the cells from mice calvarea are osteoblastic and already differentiated cells.

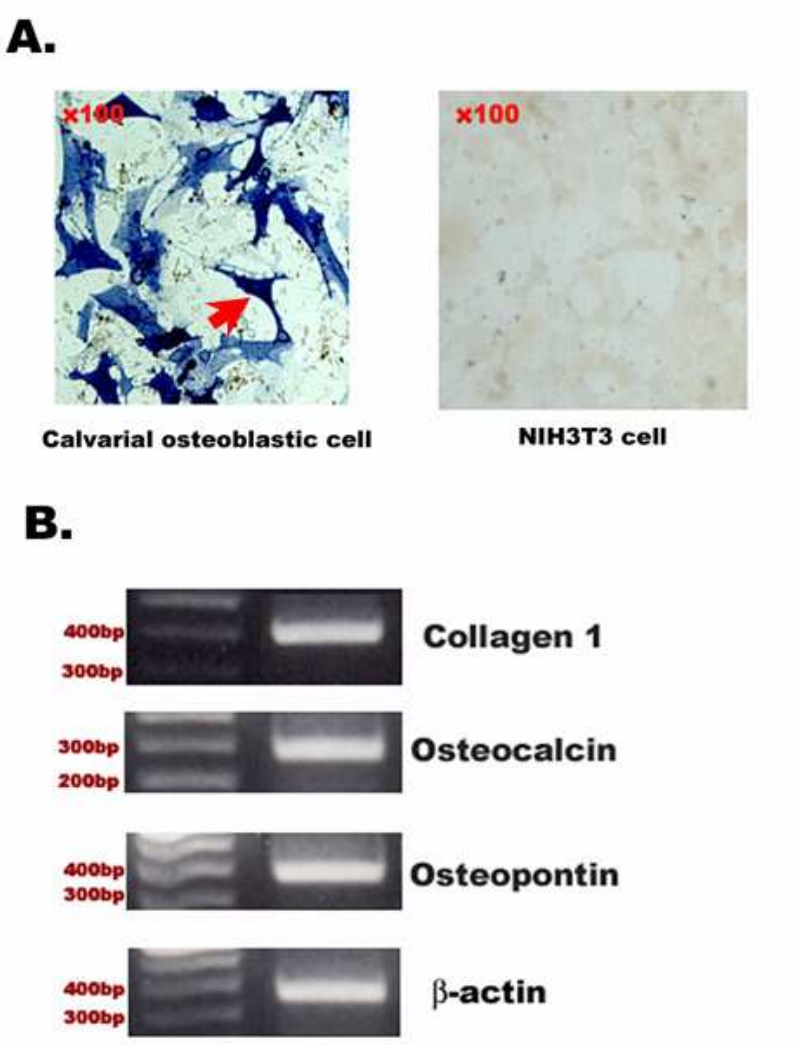
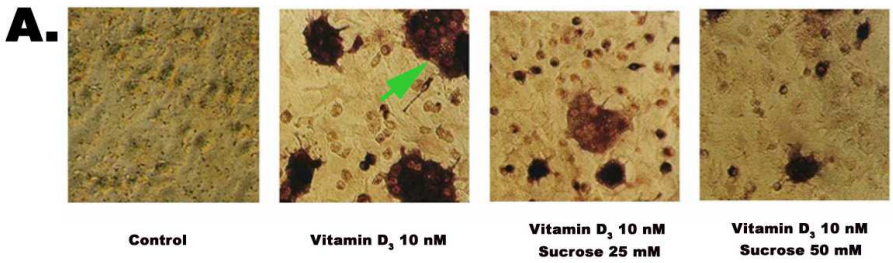
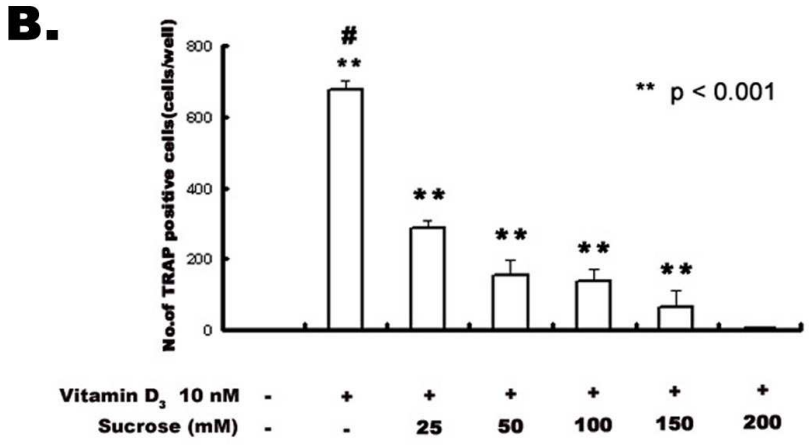


Figure 1. Confirmation of osteoblastic cells. Primary osteoblastic cells prepared from 1-day-old ICR mise calvariae according to as mentioned above "Methods" and cultured for 4 days in α-MEM medium. (A), cells were performed with alkaline phosphatase (ALP) staining (x 100). The red arrow indicated ALP stained cell; (B), the total RNA was extracted with TRIzol reagent and confirmed the osteoblast marker genes by RT-PCR.

2. Inhibition of 1α, 25(OH)$_2$D$_3$-induced osteoclastogenesis by hypertonicity (high osmolar sucrose) in co-culture system

Osteoclastogenesis was induced by 1α, 25(OH)$_2$D$_3$ in osteoblastic cells and bone marrow cells co-culture system. To clarify the role of hypertonicity on bone metabolism, 25, 50, 100, 150, and 200 mM of sucrose were added for hypertonic conditions to co-culture system and incubated at 37℃ for 4 days to investigate osteoclast differentiation. When 10 nM of 1α, 25(OH)$_2$D$_3$ was added to the co-culture system, TRAP positive multinucleated cells were formed, whereas no TRAP positive cells were detected in media only. In the presence of sucrose, 1α, 25(OH)$_2$D$_3$-induded osteoclast differentiation was reduced (Figure 2A). In detail, the addition of 25, 50, 100, 150, and 200 mM of sucrose reduced the number of TRAP positive multinucleated cells up to about 70% in 50 mM sucrose (Figure 2B). However, it might be possible that sucrose causes cell damage directly without interrupting the normal maturation of osteoclasts. To rule out this possibility, we used a viability test. As shown in Figure 2C, sucrose did not show toxic effect when treated at up to 50 mM. These results suggest that the effect of sucrose (25 mM: +20 mOsm; 50 mM: +40 mOsm) on osteoclast formation was caused by hypertonicity, not by sucrose direct toxic effect upon the cells.


A.
Control
Vitamin D3 10 nM
Vitamin D3 10 nM
Sucrose 25 mM
Vitamin D3 10 nM
Sucrose 50 mM


B.
No.of TRAP positive cells(cells/well)
800
600
400
200
0
#
**
** p < 0.001
Vitamin D3 10 nM - + + + + + +
Sucrose (mM) - - 25 50 100 150 200

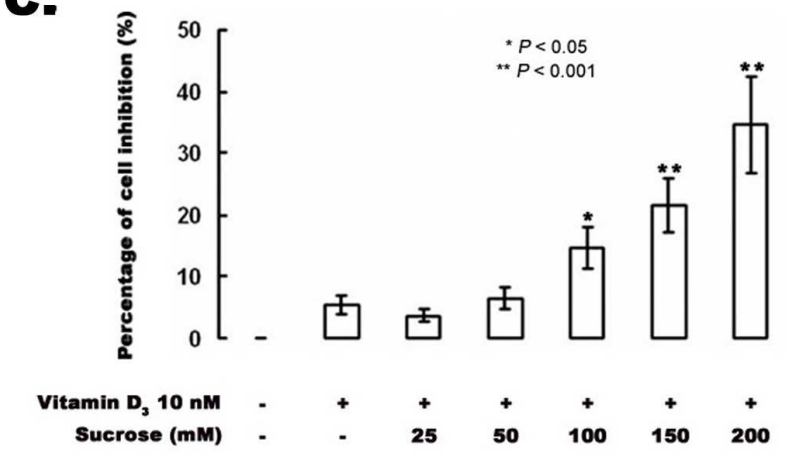

C.
Percentage of cell inhibition (%)
50
40
30
20
10
0
* P < 0.05
** P < 0.001
Vitamin D3 10 nM - + + + + + +
Sucrose (mM) - - 25 50 100 150 200

Figure 2. Hypertonicity (high osmolar sucrose) inhibited 1α, 25(OH)$_2$D$_3$-induced osteoclast differenttiation in co-culture system. (A) In the presence of sucrose, 10 nM 1α, 25(OH)$_2$D$_3$-induced osteoclast differentiation was reduced (x 200). The green arrow indicated osteoclast cells. (B) TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. (C) MTT test results. 100 mM of sucrose showed remarkable high cell inhibition. $^{\#}$**, significantly different ($p < 0.001$) in compared to control group; *, significantly different ($p < 0.05$) in compared to 10 nM 1α, 25(OH)$_2$D$_3$ group; **; significantly different ($p < 0.001$) in compared to 10 nM 1α, 25(OH)$_2$D$_3$ group. The statistical significance of differences between the groups was determined using the two-tailed Student *t* test. Each data was shown in mean ± SEM of four cultures.

3. Effects of hypertonicity on resorption pit formation

Osteoblastic cells and bone marrow cells were co-cultured in α-MEM on OAAS (osteoclast activity assay substrate) plates and measured resorption lacuna. There was no resorption pit on OAAS plates in the absence of 1α, $25(OH)_2D_3$. On the other hand, 10 nM 1α, $25(OH)_2D_3$ induced a number of bone resorption pits and we could find multiple lacunae, which was used as a positive control. But in the experimental group, resorption pits were distinctly decreased as the function of hypertonicity up to 50 mM sucrose (Figure 3). As show in Figure 3, total resorption area was also decreased by the addition of sucrose.

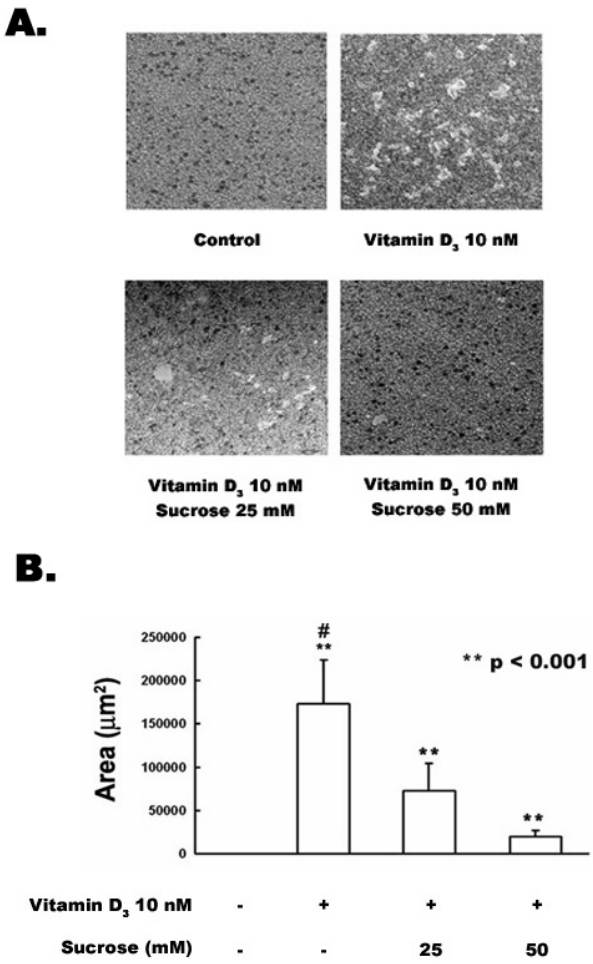


Figure 3. Hypertonicity inhibited 1α, 25(OH)$_2$D$_3$-induced pit formation. (A) The resorbed lacunae on the OAAS (osteoclast activity assay substrate) plates were photographed with microscope (x 100). (B) Total resorption area per well was measured by Image Meta Morph Program. $*^{\#}*$, significantly different ($p <$ 0.001) in compared to control group; **; significantly different ($p < 0.001$) in compared to 10 nM 1α, 25(OH)$_2$D$_3$ group. The data were presented as mean ± SEM of four times sample.

4. The expressions of osteoblast maker genes were not changed by hypertonicity in osteoblastic cells

We invesgated that the effect of hypertonicity in osteoblasts specific character. If the maturational arrest of osteoblasts in hypertonic conditions, it will be able to affect the insufficient osteoclastogenesis. We examined the expressions of osteoblast marker genes as well as collagen 1, osteopontin, and osteocalcin by RT-PCR (Figure 4). Figure 4 indicated that osteoblasts specific character was not changed by hypertonicity.

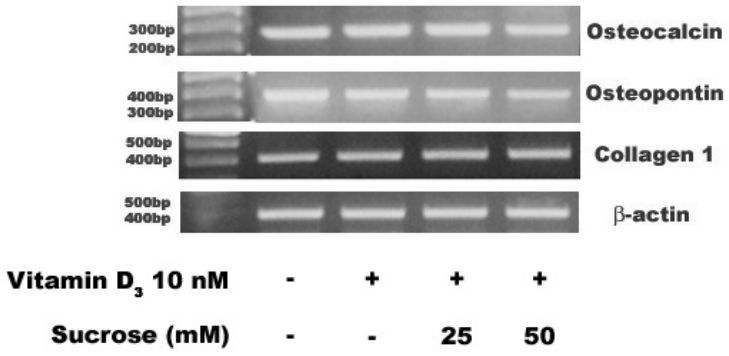
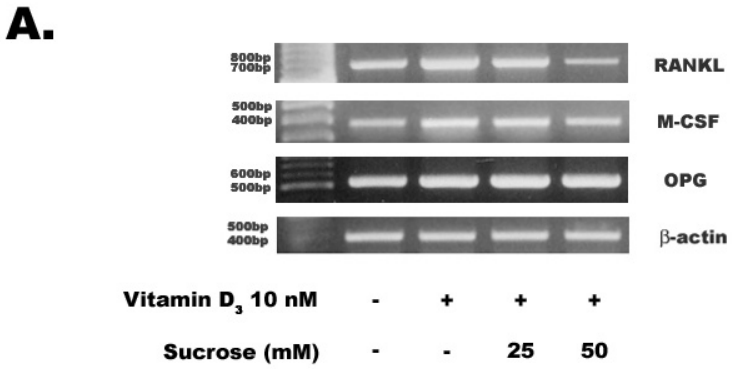


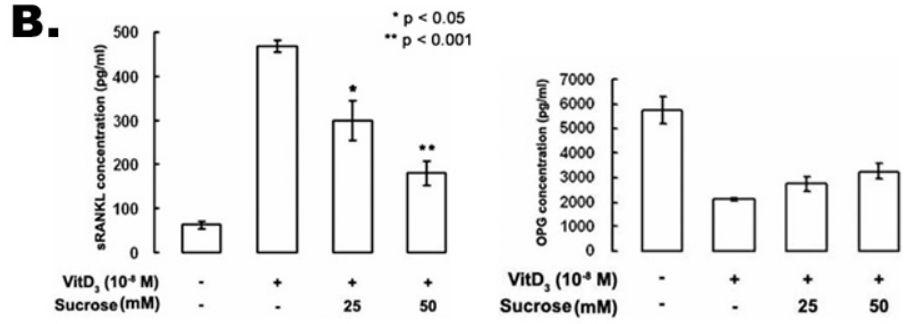
Figure 4. Osteoblast specific character was not changed by hypertonicity. Primary osteoblastic cells were seeded in 35-mm culture dishes (1×10^4 cells/dish) and grown to 4 days in α-MEM. After 4 days the cells were treated with each reagent and curtured for 4 days, and then the cells were collected and total RNA was extracted from the primary osteoblastic cells and was analyzed by RT-PCR. β-actin used for standardization for total RNA amounts remain unchanged.

5. Effects of hypertonicity on osteoclast differentiation related genes expression in mouse calvarial osteoblasts

Figure 5A depicts RANKL, OPG, and M-CSF mRNA expressions in osteoblastic cells. The β-actin mRNA levels used for standardization for total RNA amounts remained unchanged. The expression of mouse RANKL mRNA was markedly enhanced in calvarial osteoblastic cells treated with 1α, $25(OH)_2D_3$ alone but was decreased dose-dependently with sucrose. However, OPG and M-CSF gene are not changed upon 1α, $25(OH)_2D_3$ and sucrose treated. These findings indicate that addition of sucrose (25, 50 mM) inhibits osteoclast formation by down-regulating the expression of RANKL.

RANKL and OPG were also analyzed with ELISA using anti-RANKL and anti-OPG antibodies. RANKL was decreased with the increase of sucrose concentration (Figure 5B). On the other hand, the addition of sucrose did not change the OPG quantitatively which is consistent with OPG mRNA data (Figure 5B). RANKL expression were also detected with Western blot method (Figure 5C), obtained same results as well as ELISA assay. Consequently, the addition of sucrose inhibited RANKL mRNA and soluble RANKL, and led to altered osteoclastogenesis. In addition, such changes of RANKL mRNA and protein were dependent on the sucrose concentration.


A.
800bp
700bp
RANKL
500bp
400bp
M-CSF
600bp
500bp
OPG
500bp
400bp
β-actin
Vitamin D3 10 nM - + + +
Sucrose (mM) - - 25 50


B.
* p < 0.05
** p < 0.001
sRANKL concentration (pg/ml)
500
400
300
200
100
0
OPG concentration (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
VitD3 (10-8 M) - + + +
Sucrose (mM) - - 25 50
VitD3 (10-8 M) - + + +
Sucrose (mM) - - 25 50

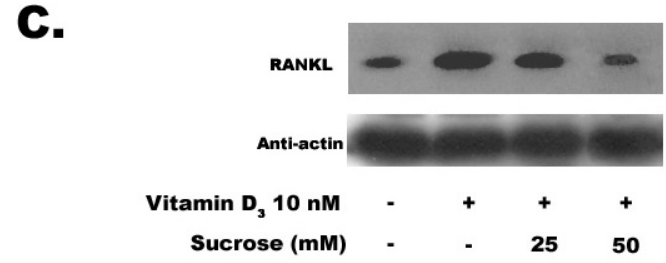

C.
RANKL
Anti-actin
Vitamin D3 10 nM - + + +
Sucrose (mM) - - 25 50

Figure 5. Hypertonicity caused down-regulation in RANKL. (A) Osteoblastic cells from nowborn mice calvariae were cultured 4 days. Then various concentrations of sucrose were added to the cells cultured with 10 nM 1α, $25(OH)_2D_3$. After indubation for 4 days, total RNAs were extracteds, and the expressions of RANKL, OPG, M-CSF mRNAs were analyzed by RT-PCR. (B) Osteoblastic cells were cultured at 37℃ with 5% CO_2. After 4 days, the cells were treated with each reagent and curtured for 4 days and then take the medium to analyze. The protein analysis using ELISA showed that addition of sucrose inhibited the expression of soluble RANKL level in osteoblasts, it was statistically significant. On the other hand OPG levels were increased slightly stimulated by sucrose, but it was not statistically significant. (C) Western blot analysis of the expression of the RANKL in primary osteoblasts isolated from calvariae after 4 days of culturing in absence or presence 1α,$25(OH)_2D_3$ and/or sucrose. The anti-actin protein levels used for standardization for total protein amounts remain unchanged. *, significantly different ($p < 0.05$) in compared to 10 nM 1α, $25(OH)_2D_3$ group; **, significantly different ($p < 0.001$) in compared to 10 nM 1α, $25(OH)_2D_3$ group.

6. Expression of Runx2 in calvarial osteoblastic cells under the hypertonic condition

From Fugure 5 results, we established that addition of sucrose inhibits osteoclast formation by down-regulating the expression of RANKL. RANKL gene basic promoter sequence has binding sites for Runx2 and Runx2 playing major role in RANKL expression. Therefore, we will examine the effect of hypertonicity on 1α, 25$(OH)_2D_3$-induced Runx2 expression. The osteoblastic cells from 1-day-old ICR mice calvarea were cultured at 37℃ with 5% CO_2 for 4 days and then treated with 10 nM 1α, 25$(OH)_2D_3$ and various concentration of sucrose. After 4 days, the cells were treated with lysis buffer and were investiged the Runx2 expression with Western blot analysis. The Runx2 level was increased significantly by 10 nM 1α, 25$(OH)_2D_3$ and were decreased dose-dependently with sucrose treatment (Figure 6).

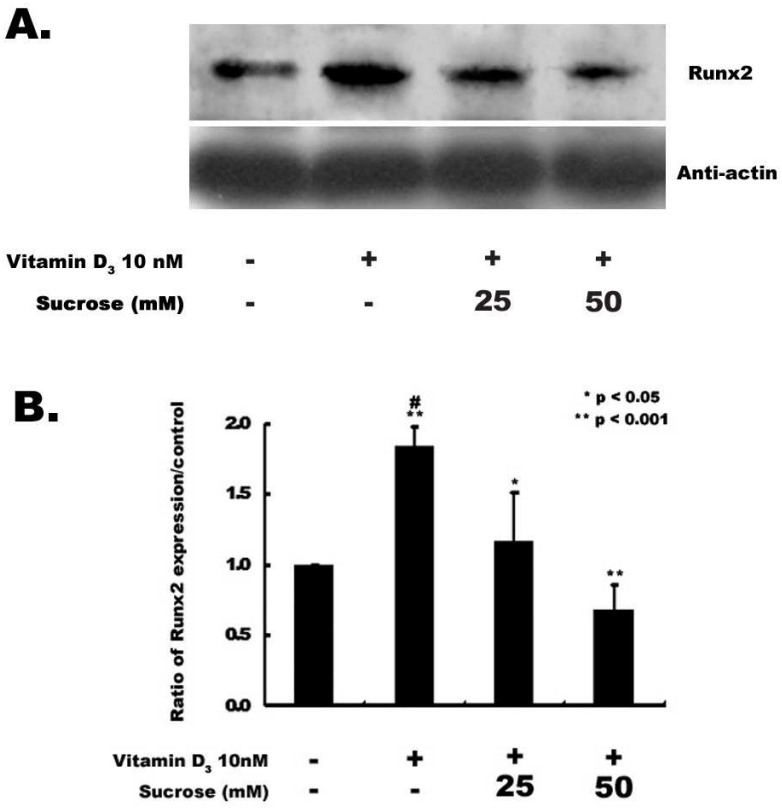


Figure 6. Hypertonicity suppressed 1α, 25(OH)$_2$D$_3$–induced Runx2 expression in osteoblastic cells. (A) Osteoblastic cells from nowborn mice calvariae were cultured 4 days and then treated with 10 nM 1α, 25(OH)$_2$D$_3$ and/or sucrose. After 4 days the expression of Runx2 was analyzed with Western blotting; (B) The density of each band were determined by Mata Morph Program and the values were normalized against control expression and are presented as fold induction. $^{\#}_{**}$, significantly different ($p < 0.001$) in compared to control group; *, significantly different ($p < 0.05$) in compared to 10 nM 1α, 25(OH)$_2$D$_3$ group; **, significantly different ($p < 0.001$) in compared to 10 nM 1α, 25(OH)$_2$D$_3$ group. Results represent means ± SEM of six independent experiments.

7. Effects of Runx2 for expression of RANKL mRNA and RANKL in calvarial osteoblastic cells

Above results indicated that Runx2 direct regulate RANKL expression probably. To confirm this Runx2 and RANKL relationship, we used siRNA technique. Namely, RANKL expressions were examined after disrupted Runx2 prodution by siRNA method. To silence the expression of Runx2 by RNA interference, we designed small interfering RNA (siRNA) duplex. The osteoblastic cells were transfected with RNA interference of Runx2, and were selected with G418-neomycin. Then these osteoblastic cells were changed with 10 nM 1α, $25(OH)_2D_3$ for 4 days. The results of expression of RANKL mRNA and protein by RT-PCR and Western blotting in osteoblastic cells were shown in Figure 7. These findings suggest that Runx2 gene may be involved in the regulation of RANKL expression at the upstream level.

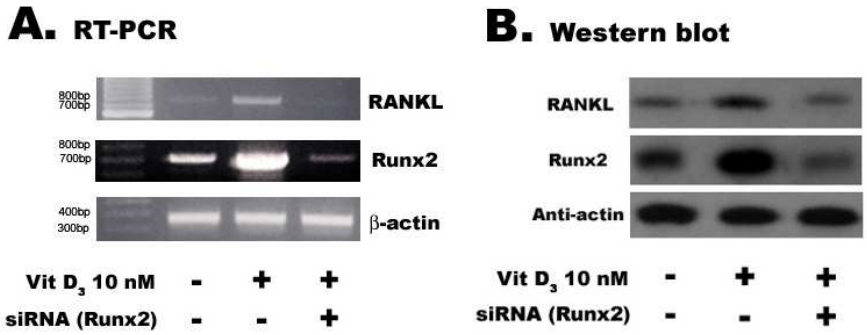


Figure 7. The 1*α*, 25(OH)$_2$D$_3$-induced RANKL mRNA and RANKL expressions were decreased by siRNA of Runx2 transfection in osteoblastic cells. Osteoblastic cells were transfected with RNA interference of Runx2 (siRNA of Runx2) gene and selected with G418-neomycin. The cells were untreated or treated with 10 nM 1α, 25(OH)$_2$D$_3$ for 4 days before mRNA isolation or western samples preparation. (A) The expression of RANKL mRNAs was analyzed by RT-PCR. β-actin used for standardization for total RNA amounts remain unchanged. (B) The expression of RANKL was analyzed with Western blot method. Anti-actin used for standardization for total protein amounts. Each experiment repeated three times.

8. Effect of Runx2 on the osteoclastogenesis in co-culture system.

The fact that Runx2 affect RANKL expression was demonstrated with siRNA method. In addition, RANKL plays an important role in osteoclastogenesis. Therefore, we will investigate that effect of Runx2 on the osteoclastogenesis. The primary osteoblastic cells on the 48-well plate were transfected with RNA interference of Runx2. Then select transfected osteoblastic cells and co-cultured with non-adherent bone marrow originated preosteoclastic cells and treated with 10 nM 1α, 25$(OH)_2D_3$. After 4 days, the cells were performed TRAP staining and cell viability test (Figure 8). These results suggest that osteoclastogenesis was down-regulated by disrupt Runx2 function with siRNA technique in osteoblastic cells. And did not shown toxic effect in this condition.

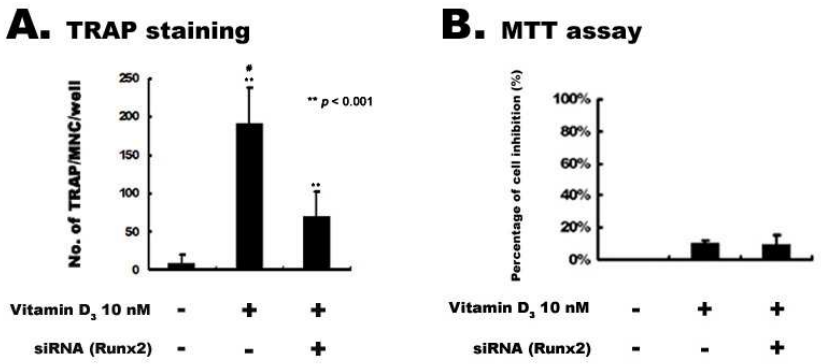


Figure 8. 1α, 25(OH)$_2$D$_3$–induced osteoclastogenesis was inhibited by siRNA of Runx2 transfection in co-culture system. Primer osteoblastic cells seeded 48-well plate and were transfected with RNA interference of Runx2 (siRNA of Runx2) gene and were selected with G418-neomycin treatment. Then added non-adherent preosteoclast cells to co-culture and treated with 10 nM 1α, 25(OH)$_2$D$_3$ for 4 days before TRAP staining and MTT assay. (A) The numbers of TRAP-positive cells/well were scored. (B) MTT test results. Data represent mean ± SEM of three wells. Analysis was performed with two tailed Student *t* test. $^{\#}_{*}$*, significantly different (p < 0.001) in compared to control group; **, significantly different (p < 0.001) in compared to 10 nM 1α, 25(OH)$_2$D$_3$ group. Similar results were obtained in four independent experiments, and representative data are shown.

IV. Discussion

During osteoclastic bone resorption, crystal hydroxyapatite is dissolved into free Ca^{2+}/PO_4^{2-} ions in the resorptive hemivacuole where it can reach concentrations as high as 40 mM [Silver *et al.*, 1988; Xu *et al.*, 2005]. High extracellular Ca^{2+} has been shown to trigger an increase in cytosolic Ca^{2+}, through Ca^{2+} release from internal stores and/or Ca^{2+} influx, and this results in the inhibition of bone resorption [Zaidi *et al.*, 1999a; Zaidi *et al.*, 1999b] and induction of osteoclast apoptosis [Lorget *et al.*, 2000]. On the other hand, bone resorption leads to the localized degradation of fully mineralized bone matrix, including removal of both inorganic and organic matrix components [Jones SJ, Boyde A., 1987] [Salo *et al.*, 1996]. Therefore when bone is actively resorbed, it might be possible that high osmolality is provided due to the high concentration of Ca^{2+}/PO_4^{2-} and the degraded organic materials (mainly collagen) in the extracellular fluid around bone cells. Hypertonicity is a genotoxic agent. Acute exposure to a hypertonic environment causes immediate water efflux and results cell shrinkage. Here, we hypothesized that the hypertonicity in bone could be one of the regulating factors for bone remodeling. In this report, we demonstrated the effects of hypertonicity on osteoclastogenesis in osteoblast-preosteoclast co-culture system for the purpose of understanding at the molecular level of hypertonicity on bone metabolism. Hypertonic condition was made by addition of sucrose. We have shown for the first time that hypertonicity

down-regulated 1α, $25(OH)_2D_3$-induced osteoclastogenesis in the co-culture system. Interestingly, we found that according to increase of the hypertonicity, the number of 1α, $25(OH)_2D_3$-induced TRAP positive multinucleated cells were reduced up to about 70% in 50 mM sucrose, compared to 10 nM 1α, $25(OH)_2D_3$ group in co-culture system (Figure 2). Nevertheless, it could be argued that the inhibition of osteoclastogenesis by sucrose might be caused not due to the physiological intervention of sucrose in the normal process of osteoclastogenesis but due to the cell damage. To rule out the possibility that sucrose causes non-physiological cell damage, an MTT viability test was performed. The test showed that sucrose under 50 mM did not exert any harmful effect upon the cells in this co-culture system (Figure 2C). It was the effects of hypertonicity without a toxic effect upon the cells to reduction of the formation of TRAP positive cells. Furthermore, hypertonicity inhibited 1α, $25(OH)_2D_3$-induced pit formation using the OAAS plates (Figure 3). The area of resorption in OAAS plates was remarkably decreased by hypertonicity. We suggested that hypertonicity plays a role as a down-regulated modulator in 1α, $25(OH)_2D_3$-induced osteoclastogenesis. In the co-culture system, osteoblasts were prepared from new born ICR mice calvariea and preosteoclasts were obtained from the femurs and tibiae of 4-weeks-old ICR male mice. To analyze the effect of hypertonicity on osteoclastogenesis by co-culture system, we first confirmed the osteoblastic cells. The expression of osteoblastic markers, including collagen 1, alkaline phosphatase, osteopontin, and osteocalcin, are upregulated according to the osteoblast maturation [Liu et al., 2001]. In the

present study, we used ALP staining and RT-PCR methods to confirm the cells from new borm ICR mice calvariae are osteoblastic cells (Figure 1). From ALP staining result, we found a large number of spindle-shaped alkaline phosphatase positive osteoblastic cells [Quinn et al., 2002]. And, from RT-PCR results, we found this cell express osteoblastic marker genes as well as collagen 1, osteopontin, and osteocalcin (late stage marker gene). Therefore, from above results we firmly believe that from calvariae cells are osteoblastic cells and more differentiated cells (from osteocalcin expression).

Since osteoclast differentiation was mediated by several factors, such as RANKL, OPG, and M-CSF [Udagawa *et al.*, 1990; Yoshida *et al.*, 1990; Tanaka *et al.*, 1993; Kameda *et al.*, 1995; Lacey *et al.*, 1998; Fuller *et al.*, 1998; Kanatani *et al.*, 1999; Burgess *et al.*, 1999], we used an osteoblast cell to evaluate whether the mRNA expression profiles of osteoclast differentiation related genes as well as RANKL, OPG, and M-CSF induced by 1α, 25$(OH)_2D_3$, were changed or not by hypertonicity. Simultanously, we also analyzed the osteoblast marker genes expression, because hypertonicity inhibite osteoclastogenesis maybe through arrest osteoblast differentiation. In the hypertonic condition, osteoblast marker gene (collagen 1, osteopontin, osteocalcin) expressions were not changed (Figure 4). This result indicated that osteoblasts specific character was not changed by hypertonicity. The expression of RANKL mRNA was down-regulated upon increasing the hypertonicity, and the expressions of OPG and M-CSF were not changed significantly (Figure 5). In addition, the expression of sRANKL was decreased with hypertonicity in the

process of 1α, $25(OH)_2D_3$-induced osteoclastogenesis, being in consistency with the decrease in RANKL mRNA expression (Figure 5). On the other hand, OPG was slightly increased with hypertonicity, but it was not statistically significant (Figure 5). On the basis of such findings, inhibition mechanism of osteoclastogenesis by hypertonicity might be associated with modulating RANKL expression in differentiated osteoblasts.

The co-culture system containing two kind cells, one is osteoblasts, the other one is preosteoclast. How could hypertonicity affect on preosteoclast? The answer was known through study before, thogh the data not shown in this report. We tested that hypertonicity inhibited RANKL-induced osteoclastogenesis in RAW264.7 cell (preosteoclast cell line). Hypertonicity reduced the number of TRAP positive multinucleated cells without toxic effect, but the reducing effect is not so significant and hypertonicity not affect RANKL-RANK mediated signaling of osteoclast differentiation such as MAP Kinases (ERK, JNK, and p38) and NF- κB. Putting these results together, we suggested that hypertonicity affected RANKL synthesis pathway in osteoblastic cell is more important for inhibition of osteoclastogenesis by hypertonic condition.

Interestingly, RANKL genes contain functional Runx2 binding sites and mutation of these sites abrogates the transcriptional activity of the RANKL promoter [O'Brien *et al.*, 1998] [Kitazawa *et al.*, 1999]. $Runx2^{-/-}$ mice die just after birth, due to a failure to breath [Komori *et al.*, 1997; Otto *et al.*, 1997]. These mice completely lack both endochondrial and intramembranous ossification due to the absence of osteoblasts, demonstrating that Runx2 is an

essential factor for osteoblast differentiation. Runx2 plays important roles in multiple processed of endochondral ossification, including chondrocyte maturation, vascular invasion into the cartilage, osteoclast differentiation, and osteoblast differentiation [Komori T., 2002]. In addition, a lack of osteoclasts in $Runx2^{-/-}$ mice suggests a potential role of Runx2 in osteoclastogenesis [Komori *et al.*, 1997]. The 5'flanking region of the mouse and human RANKL/TRANCE/OPGL/ODF gene basic promoter contains inverted TATA- and CAAT- boxes and have putavive Runx2 binding sites, and RANKL expression is severely decreased in $Runx2^{-/-}$ mice [Gao *et al.*, 1998; Kitazawa *et al.*, 1999; Kitazawa *et al.*, 2003; Kabe *et at.*, 2005]. In addition to explaining the osteoblast-specific expression of RANKL, the requirement of Runx2 for RANKL gene expression may constitute the molecular mechanism of the linkage between osteoblastogenesis and osteoclatogenesis [Porte *et al.*, 1999]. More extensive studies on the regulation of RANKL by Runx2 will provide insight into the molecular mechanism involved in the classical hypothesis proposed by Rodan and Martin [Rodan GA, Martin TJ, 1981] concerning the interaction between osteoblasts and osteoclasts during bone remodeling. Runx2 through its effects on osteoblast lineage commitment and function could also directly or indirectly regulate the bone resorption process [Kannan *et al.*, 2000]. As previously reported [Kitazawa R., Kitazawa S., 2002], one VDRE (-935) is located further upstream in the region of the mouse RANKL gene. Further, Runx2 interacts with other transcription factors, transcriptional cofactors, and transcriptional repressors, and the interactions greatly influence

Runx2 function. Therefore, it is possible that Runx2 regulates RANKL expression in cooperation with other factors, which also determine the level of RANKL expression. Indeed, the retinoid X receptor (RXR)-vitamin D receptor (VDR) [Farach-Carson MC, Ridall AL., 1998; Javed *et al.*, 1999] complex is one of the candidates, because 1α,25$(OH)_2D_3$ was required for the induction of RANKL by Runx2 [Kitazawa R., Kitazawa S., 2002; Kitazawa *et al.*, 2003; Enomoto *et al.*, 2003]. Authors [Enomoto *et al.*, 2003] isolated $Runx2^{-/-}$ calvarial cell lines CA120-4 cells, most of which expressed OPG strongly but RANKL barely. It is consistent with a previous report [Gao *et al.*, 1998] that OPG was detected, but RANKL was undetectable in $Runx2^{-/-}$ mice. Interestingly, the induction of RANKL in CA120-4 cells by Runx2 was dependent on the presence of 1α, 25$(OH)_2D_3$. Neither Runx2 nor 1α,25$(OH)_2D_3$ alone could induce RANKL expression in this cell line. Therefore, Runx2 is required for the induction of RANKL by 1α, 25$(OH)_2D_3$, indicating a cooperative action of Runx2 and 1α,25$(OH)_2D_3$. So the basic structure of RANKL promoter contains of inverted TATA- and CAAT-boxes and a consensus binding sites of Runx2 flanked by VDRE, and when was stimulated by 1α,25$(OH)_2D_3$ that formed VDR-RXR heterodimers and binding to VDRE is important to regulate synthesizing of RANKL.

On the other hand, Runx2 transgenic mice indicated that Runx2 promotes osteoblast differentiation at an early stage, but inhibits osteoblast differentiation at a late stage [Viereck *et al.*, 2002; Toshihisa K., 2003]. In addition, regulation of RANKL expression by Runx2 seems to be dependent on the maturational

stage of osteoblast lineage cells [Komori T., 2002]. Others [Geoffroy *et al.*, 2002] reported that overexpression of Runx2 in cells of the osteoblastic lineage does not necessarily induced a substantial increase in bone formation in the adult skeleton but has a positive effect on osteoclast differentiation *in vitro* and can also dramatically enhance bone resorption *in vivo*, possibly through increased RANKL expression.

Therefore, we will to analyze 1α, 25$(OH)_2D_3$-induced Runx2 expression in osteoblastic cells, because Runx2 is related with RANKL expression and it plays a potential role in osteoclastogenesis, probably. Through analysis, we found that the 1α, 25$(OH)_2D_3$-induced Runx2 expression was reduced with the hypertonicity in osteoblastic cells (Figure 6). But the mechanism of inhibited Runx2 expression in hypertonic condition was not clear. Mounting evidence suggests a broad role for SFKs (Src family kinases) that cytoplasmic kinases in the cell response to hypertonic and hypotonic stress, and in the ensuing regulatory volume increase or decrease. Many, and perhaps all, SFKs are influenced by anisotonicity. Hypertonicity activates as well as Yes [Reinehr *et al.*, 2004] and exerts a variable effect upon Src [David M. Cohen, 2005]. Hypermosmotic exposure (406 mosm) resulted in a rapid activation of the Src kinase family members Yes in hepatocytes [Reinehr *et al.*, 2004]. Src/Yes tyrosine kinase signaling contributes to the regulation of bone homeostasis. Yes-associated protein (YAP), a mediator of Src/Yes signaling, interacts with the native Runx2 protein, and suppresses Runx2 transcriptional activity [Zaidi *et al.*, 2001; Lian *et al.*, 2003; Zaidi *et al.*, 2004]. YAP, in turn, contains a ww domain

in the N-terminus [Sudol M., 1994] that recognizes a proline-rich motif (PPxY) present in a broad range of proteins including Runx factors [Sudol *et al.*, 1995; Sudol M., Hunter T., 2000]. And find that several osteoblast-related genes are regulated by the Runx2-YAP complex [Zaidi *et al.*, 2004]. However, the relationship of Runx2 and Yes in osteoblastic cells by hypertonicity was not detected, and the regulation of Runx2-YAP complex on RANKL expression was not clear. It is remain to be made further investigation.

Through above study, we found that 1α, $25(OH)_2D_3$–induced Runx2 expression was inhibited and 1α, $25(OH)_2D_3$–induced RANKL expression was blunted in osteoblastic cells by hypertonicity. Therefore, we will investigate the role of Runx2 on synthesize of RANKL by siRNA technique. Osteoblastic cells were transfected with RNA interference of Runx2 and stimulated with 10 nM 1α, $25(OH)_2D_3$. Silencing of Runx2 in osteoblastic cells inhibited the expression of RANKL mRNA and protein level (Figure 7) and osteoclastogenesis (Figure 8). On osteoteoclast formation experiment, osteoblstic cells were transfected with siRNA (Runx2) and co-cultured with non-adherent preosteoclasts. Because the residual adherent bone marrow cells from which the BMM precursors were contained a small but significant proportion (1.9±1.7%) of osteoblastic cells [Quinn *et al.*, 2002]. If use whole bone marrow cells, the small amount osteoblastic cells could affect on the result of siRNA (Runx2) treated osteoclastogenesis by 1α, $25(OH)_2D_3$ treatment. This siRNA experiment results indicated that Runx2 was related on the regulation of RANKL expression and osteoclast formation in co-culture system. But the changes of 1α, $25(OH)_2D_3$–

induced RANKL expression and osteoclast formation by hypertonicity remains to be further investigated.

From our study, we confirmed that Runx2 might be related with RANKL expression in hypertonic condition. But it has been controversial whether Runx2 regulates RANKL expression. It has been shown that Runx2 does not stimulated the 0.7-kb 5'-flanding region of the RANKL gene [O'Brien *et al.*, 2002], which contains two putative Runx2 binding sites, and neither Runx2 nor the dominant negative form of Runx2 expression has an effect on RANKL expression in a stromal/osteoblastic cell line, UAMS-32. Therefore, the regulatory region for Runx2 in RANKL gene may be outside of the 0.7-kb 5'-flanking region. As UAMS-32 cells express Runx2 strongly in a steady state, it may be difficult to induce or inhibit RANKL expression by the introduction of Runx2 or its dominant negative form. In the recent report [Notoya *et al.*, 2004], Runx2 is considered as not essential for the vitamin D-regulated expression of RANKL and osteoprotegerin in osteoblastic cells. They used C2 cells and C6 cells, which are derived from calvariae of Runx2-deficien mice, and in the presence of 100 nM 1α, $25(OH)_2D_3$, C2 cells and C6 cells induced the formation of osteoclast-like cells in co-culture with mouse spleen cells. This result to opposite a previous report [Gao *et al.*, 1998] that calvarial cells from Runx2-dificient E18.5 mice have a significantly reduced ability to support the formation of osteoclasts with 10 nM 1α, $25(OH)_2D_3$. It is possible that Notoya *et al.* used spleen cells maybe contain osteoblastic cells and these cells stimulate osteoclastogenesis in very high concentration of 1α, $25(OH)_2D_3$. Also, Runx2

might be not completely disrupted in this C2 and C6 cells probably, so were affected by so high concentration of 100 nM 1α, $25(OH)_2D_3$ stimulation.

Taken all together, our findings reveal that hypertonicity may be novel candidate for the regulation of bone resoption, doing the down-regulation of 1α, $25(OH)_2D_3$-induced osteoclastogenesis via Runx2 in co-culture system. Our curren view on the hypothetical effect of hypertonicity on the 1α, $25(OH)_2D_3$–induced osteoclastogenesis pathway is schematically depicted in Figure 9.

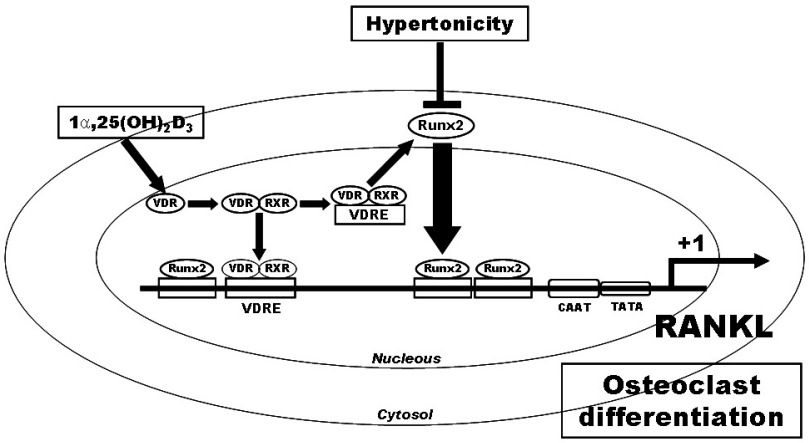


Figure 9. Hypothesisful regulation mechanism of hypertonicity on 1α, $25(OH)_2D_3$–induced osteoclastogenesis. Hypertonicity down-regulates 1α, $25(OH)_2D_3$-sitimulated Runx2 expression and then affected RANKL synthesis in osteoblasts and inhibited osteoclastogenesis.

Further studies are needed to clarify the exact and detailed 1α, $25(OH)_2D_3$-induced RANKL expression and the inhibition by hypertonicity through Runx2 signaling mechanisms.

V. Conclusion

In summary, we have provided the first evidence that hypertonicity inhibited not only osteoclast formation in co-culture system but also osteoclast function (bone resorption activity) in 1α, 25$(OH)_2D_3$–induced RANKL-RANK mediated osteoclastogenesis. And the expressions of RANKL mRNA and soluble RANKL were down-regulated upon increasing the hypertonicity, but the expressions of OPG and M-CSF were not changed significantly in mouse osteoblastic cells. Hypertonicity inhibited 1α, 25$(OH)_2D_3$–induced Runx2 expression in differentiated osteoblastic cells. When siRNA of Runx2 transfected primary osteoblastic cells were treated with 10 nM 1α, 25$(OH)_2D_3$, the mRANKL and RANKL expressions were blunted and osteoclastogenesis was inhibited in co-culture system.

Therefore, hypertonicity may be a novel candidate for the regulation of bone resoption, doing the down-regulation of 1α, 25$(OH)_2D_3$-induced osteoclastogenesis via Runx2 reduction.

References

Rodan GA, Martin TJ.: Role of osteobalsts in hormonal control of bone resorption-a hypothesis. *Calcif Tissue Int* 33: 349-351, 1981

Minkin C.: Bone acid phosphatase: tartrate-resistant acid phosphatase as a marker of osteoclast function. *Calcif Tissue Int* 72:285-290, 1982

Mosmann T.: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth* 65: 55-63, 1983

Baron Roland, Neff Lynn, Louvard Danier., and Courtoy Pierre J.: Cell-mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. *J. Cell Biol.* 101: 2210-2222, 1985

Jones SJ, Boyde A.: Scanning microscopic observations on dental caries. *Scanning Microsc.* 1: 1991-2002, 1987

Silver IA, Murrills RJ, Etherington DJ.: Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. *Exp Cell Res* 175: 266-276, 1988

Takahashi N., Akatsu T., Udagawa N., Sasaki T., Yamaguchi A., Moseley JM, Martin TJ and Suda T.: Osteoblastic cells are involved in osteoclast formation. *Endocrinology* 123: 2600-2602, 1988

Vaes G..: Cellular biology and biochemical mechanism of bone resorption. A review of recent developments on the formation, activation, and mode of action of osteoclasts. *Clin Orthop Relat Res.* 231: 239-271, 1988

Blair HC, Teitelbaum SL, Ghiselli R, Gluck S.: Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science* 245: 855-7, 1989

Udagawa N., Takahashi N., Akatru T., Tanaka H., Sasaki T., Nishihara T., Koga T., Martin T.-J., and Suda T.: Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proc.Natl. Acad. Sci. U.S.A* 87: 7260-7264, 1990

Väänänen H. K., Karhukorpi E.-K., Sundquist K., Wallmark B., Roininen I., Hentunen T., Tuukkanen J., and Lakkakorpi P.: Evidence for the presence of a proton pump of the vacuolar H^+-ATPase type in the ruffled borders of osteoclasts. *The Journal of Cell Biology* 111: 1305-1311, 1990

Yoshida H., Hayashi S-I, Kunisada T., Ogawa M., Nishikawa S., Okamura H., Suda T., Shultz LD., & Nishikawa S-I.: The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* 345: 442-444, 1990

Owen TA, Aronow MS, Barone LM, Bettencourt B, Stein GS, Lian JB**:** Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in normal rat osteoblast cultures. *Endocrinology* 128: 1496-1504, 1991

Shalhoub V, Conlon D, Tassinari M, Quinn C, Partridge N, Stein GS, Lian JB: Glucocorticoids promote development of the osteoblast phenotype by selectively modulating expression of cell growth and differentiation associated genes. *J Cell Biochem* 50: 425-440, 1992

Suda T., Takahashi N., and Martin T.J.: Modulation of osteoclast differentiation. *Endocrine Reviews* 13: 66-80, 1992

Tanaka, S., Takahashi, N., Udagawa, N., Tamura, T., Akatsu, T., Stanley, E. R., Kurokawa, T., and Suda, T.: Macrophage colony-stimulating factor is indispensable for both proliferation and differentiation of osteoclast progenitors. *J. Clin. Invest.* 91: 257-263, 1993

Sudol M.: Yes-associated protein (YAP^{65}) is a praline-rich phosphoprotein that binds to the SH3 domain of the Yes protooncogene product. *Oncogene* 9: 2145-2152, 1994

Ducy, P. and Karsenty, G.: Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol Cell Biol* 15: 1858-1869, 1995

Kameda T, Ishikawa H, Tsutsui T.: Detection and characterization of apoptosis in osteoclasts in vitro. *Biochem Biophy. Res Commun* 207(2): 753-760, 1995

Sudol M., Bork P., Einbond A., Kastury K., Druck T., Negrini M., Huebner K., Lehman D.: Characterization of the mammalian YAP (Yes-association protein) gene and its role in defining a novel protein module, the WW domain. *J Biol Chem* 270: 14733-14741, 1995

Gerstenfeld LC, Zurakowski D, Schaffer JL, Nichols DP, Toma CD, Broess M, Bruder SP, Caplan AI Variable hormone responsiveness of osteoblast populations isolated at different stages of embryogenesis and its relationship to the osteogenic lineage. *Endocrinology* 137: 3957-3968, 1996

Salo J., Metsikkö K., Palokangas H., Lehenkari P., and Väänänen H. K.: Bone-resorbing osteoclasts reveal a dynamic division of basal plasma membrane into two different domains. *Journal of Cell Science* 109: 301-307, 1996

Tezuka K-I, Denhardt D. T., Rodan G A., and Harada S-I.: Stimulation of mouse osteopontin promoter by v-Src is mediated by a CCAAT box-binding factor. *J. Biol. Chem.* 271: 22713-22717, 1996

Ducy, P., Zhang R., Geoffroy V., Ridall A. L., and Karsenty G.: Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* 89: 747-754, 1997

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T.: Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 89: 755-764, 1997

Lian, J.B., Shalhoub, V., Aslam, F., Frenkel, B., Green, J., Hamrah, M., Stein, G. S., and Stein, J.L.: Speciesspecific glucocorticoid and 1,25-dihydroxyvitamin D responsiveness in mouse MC3T3-E1 osteoblasts: Dexamethasone inhibits osteoblast differentiation and vitamin D downregulates osteocalcin gene expression. *Endocrinology* 138: 2117-2127, 1997

Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W. H., Beddington, R. S. P., Mundlos, S., Olsen, B. R., Selby, P. B., and Owen, M. J.: *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* 89: 765-771, 1997

Simonet W. S., Lacey D. L., Dunstan C. Kelley R., M., Chang M.-S., Lüthy R., Nguyen H.Q., Wooden S., Bennett L., Boone T.: Osteoprotegerin: A Novel Secreted Protein Involved in the Regulation of Bone Density. *cell* 9: 309-319, 1997

Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Bartlett 3rd FS, Frankel WN, Lee SY, Choi Y.: TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J Biol Chem* 272: 25190-25194, 1997

David S-H, John DM, Wadi N.S., Robert S., Bruns A., Watts III, and Daine R.: P38 kinase activity is essential for osmotic induction of mRNAs for HSP70 and transpoter for organic solute betaine in Madin-Darby Canine kidney cells. *J. Biological Chemistry* 273: 1832-1837, 1998

Farach-Carson MC, Ridall AL.: Dual 1, 25-dihydroxyvitamin D_3 signal response pathways in osteoblasts: cross-talk between genomic and membrane initiated pathways. *Am J Kidney Dis* 31: 729-742, 1998

Fuller K., Wong B., Fox S., Choi YW, and Chambers Tim J.: TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. *J Exp Med.* 188(5): 997-1001, 1998

Gao, YH., Shinki T., Yuasa T., Kataoka-Enomoto H., Komori T., Suda T., and Yamaguchi A.: Potential role of Cbfa1, an essential transcriptional factor for osteoblast differentiation, in osteocalstogenesis: regulation of mRNA expression of osteoclast differentiation factor(ODF). *Biochem Biophy Res Commun* 252: 697-702, 1998

Lacey, D., Timms, E., Tan, H., Kelley, M., Dunstan, C., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivban, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Daufman, S., Sarosi, I., Shalhoub, V., Senal, G., Guo, J., Delaney, J.: Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* 93: 164-176, 1998

O'Brien CA, Farrar NC, Manolagas SC.: Identification of a binding site in the murine RANKL/OPGL gene promoter: a potential link between osteoblastogenesis and osteoclastogenesis. *Bone* 23 (Suppl): S149 (abstra.), 1998

Sato, M., Mori E., Komori T., Kawahatao H., Sugimoto M., Terai K., Shimizu H., Yasui T., Ogihara H., Yasui N., Ochi T., Kitamura Y., Ito Y., and Nomura S.: Transcriptional regulation of osteopontin gene in vivo by PEBP2alphaA/CBFA1 and ETS1 in skeletal tissues. *Oncogene* 17: 1517–1525, 1998

Yasuda H., Shima N., Nakagawa N., Yamaguchi K., Kinosaki M., Mochizuki S-I, Tomoyasu A., Yano K., Goto M., Murakami A., Tsuda E., Morinaga T., Higashio K., Udagawa N., Takahashi N., and Suda T.: Osteoclast differentiation factor is a ligand for

osteoprotegeriny osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. USA*. 95: 3597-3602, 1998

Benson, M. D., Aubin J. E., Xiao G., Thomas P. E., and Franceschi R. T.: Cloning of a 2.5 kb murine bone sialoprotein promoter fragment and functional analysis of putative Osf2 binding sites. *J. Bone Miner. Res.* 14: 396–405, 1999

Burgess TL., Qian Y-X, Kaufman S., Ring BD., Van G., Capparelli C., Kelley M., Hsu H., Boyle WJ., Dunstan CR., Hu Sylvia, and Lacey DL.. The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J Cell Biol.* 145(3): 527-538, 1999

Javed A., Gutierrez S., Montecino M., van Wijnen AJ, Stein JL, Stein GS, Lian JB.: Multiple Cbfa/AML sites in the rat osteocalcin promoter are required for basal and vitamin D responsive transcription and contribute to chromatin organization. *Mol Cell Biol* 19: 7491-7500, 1999

Jimenez, M. J. G., Balbin, M., Lopez, J. M., Alvarez, J., Komori, T., and Lopez-Otin, C.: Collagenase 3 is a target of Cbfa1, a transcription factor of the *runt g*ene family involved in bone formation. *Mol. Cell Biol.* 19: 4431-4442, 1999

Jimi E., Akiyama S., Tsurukai T., Okahashi N., Kobayashi K., Udagawa N., Nishihara T., Takahashi N., and Suda T.: Osteoclast differentiation factor acts as a multifunctional regulator in murine osteoclast differentiation and function. *J. Immunology* 163: 434-442, 1999

Kanatani, M., Sugimoto T., and Kanzawa M.: High extracellular calcium inhibits osteoclast-like cell formation by directly acting on the calcium sensing receptor existing in osteoclast precursor cells. *Biochemical and Biophysical Reseach Communications* 261: 144-148, 1999

Kitazawa R., Kitazawa S., Maeda S.: Promotor structure of mouse RANKL/TRANCE/ OPGL/ODF gene. *Biochim Biophys Acta* 1445: 134-141, 1999

Porte, D., Tuckermann, J., Becker, M., Baumann, B., Teurich, S., Higgins, T., Owen, M. J., Schorpp-Kistner, M., and Angel, P.: Both AP-1 and Cbfa1-like factors are required for the induction of interstitial collagenase by parathyroid hormone. *Oncogene* 18: 667-678, 1999

Suda T., Takahashi N., Udagawa N., Jimi E., Gillespie MT., and Martin JT.: Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocrine Reviews* 20(3): 345-357, 1999

Zaidi M, Adebanjo OA, Moonga BS, Sun L, Huang CL.: Emerging insights into the role of calcium ions in osteoclast regulation. *J Bone Miner Res* 14: 669-674, 1999a

Zaidi M, Moonga BS, Adebanjo OA.: Novel mechanisms of calcium handling by the osteoclast: A review- hypothesis. *Proc Assoc Am Physicians* 111: 319-327, 1999b

Ducy P.: Cbfa1: a molecular switch in osteoblast biology. *Developmental Dynamics* 219: 461-471, 2000

Kannan T., David LH., Rebecca R.H., Yang XH., Rachelle J.S.G., Chandrasekhar S., T. John Martin, and Jude E. Onyia.: The osteoblast-specific transcription factor Cbfa1 contributes to the expression of osteoprotegerin, a potent inhibitor of osteoclast differentiation and function. *The Journal of Biological Chemistry* 275: 25163-25172, 2000

Lorget F, Kamel S, Mentaverri R, Wattel A, Naassila M, Maamer M, Brazier M.: High extracellular calcium concentrations directly stimulate osteoclast apoptosis. *Biochem Biophys Res Commun* 268: 899-903, 2000

Sudol M., Hunter T.: New wrinkles for an old domain. *Cell* 103: 1001-1004, 2000

Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, Koshihara Y, Oda H, Nakamura K, Tanaka S.: Involvement of receptor activator of nuclear factor kB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum* 43: 259-269, 2000

Teitelbaum, S. L.: Bone resorption by osteoclasts. *Science* 289: 1504-1508, 2000

Thirunavukkarasu, K., Halladay, D. L., Miles, R. R., Yang, X., Galvin, R. J., Chandrasekhar, S., Martin, T. J., and Onyia, J. E.: The osteoblast-specific transcription factor Cbfa1 contributes to the expression of osteoprotegerin, a potent inhibitor of osteoclast differentiation and function. *J Biol Chem* 275: 25163-25172, 2000

Tsurukai, T., Udagawa N., Matsuzaki, K., Takahashi, N., and Suda, T.: Roles of macrophage-colony stimulating factor and osteoclast differentiation factor in osteoclastogenesis. *J Bone Miner Metab* 18: 177-184, 2000

Choi, B. K., Ohk S. H., Lee H. J., Kang J. H., Jeon G. J., and Yoo Y. J.: Effects of whole cell sonicates of *Treponema lecithinolyticum* on osteoclast differentiation. *Journal of Periodontology* 72: 1172-1177, 2001

Choi YN, Woo KM, Ko S-H, Lee YJ, Park S-J, Kim H-M, and Kwon B S.: Osteoclastogenesis is enhanced by activated B cells but suppressed by activated CD8+ T cells. *Eur. J. Immunol*. 31: 2179-2188, 2001

Karsenty, G.: Transcriptional control of osteoblast differentiation. *Endocrinology* 142: 2731-2733, 2001

Liu WG. Toyosawa S., Furuichi T., Kanatani N., Yoshida C., Liu Y., Himeno M., Narai S., Yamaguchi A., Komori T.: Overexpression of Cbfa1 in osteoblasts inhibits osteoblast maturation and causes osteopenia with multiple fractures. *J Cell Biol* 155: 157-166, 2001

Zaidi, S. K., Sullivan, A. J., Ito, Y., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B.: The Yes-associated protein (YAP) is a suppressor of Runx2/Cbfa1 dependent activation of the bone specific osteocalcin gene. *J. Bone Miner. Res.* 16: S205 (abstract), 2001

Drissi H., Pouliot A., Koolloos C., Stein JL., Lian J.B., Stein GS., and Wijnen André J. van: 1,25-$(OH)_2$-vitamin D3 suppresses the bone-related Runx2/Cbfa1 gene promoter. *Experimental Cell Reseach* 274: 323-333, 2002

Geoffroy V., Kneissel M., Fournier B., Boyde. A., and Matthias P.: High bone resorption in adult aging transgenic mice overexpression Cbfa1/Runx2 in cells of the osteoblastic lineage. *Mol. Cell. Biol.* 22: 6222-6233, 2002

Gerard K. and Erwin F.-W.: Reaching a genetic and molecular understanding of skeletal development. *Developmental Cell* 2: 389–406, 2002

Kitazawa R., Kitazawa S.: Vitamin D3 augments osteoclastogenesis via vitamin D-responsive element of mouse RANKL gene promoter. *Biochem Biophys Res Commun* 290: 650-655, 2002

Kitazawa S. and Kitazawa R.: RANK ligand is a prerequisite for cancer-associated osteolytic lesions. *J Pathol* 198: 228-236, 2002

Komori T.: Runx2, a multifunctional transcription factor in skeletal development. *J. Cell. Biochem* 87: 1-8, 2002

O'Brien, C. A., Kern, B., Gubrij, I., Karsenty, G., and Manolagas, S. C.: Cbfa1 does not regulate RANKL gene activity in stromal/osteoblastic cells. *Bone* 30(3): 453-462, 2002

Quinn J. M. W., Whitty G. A., Byrne R. J., Gillespie M. T., and Hamilton J. A.: The generation of highly enriched osteoclast-lineage cell populations. *Bone* 30: 164-170, 2002

Viereck V., Siggelkow H., Tauber S., Raddatz D., Schutze N., and Hüfner M.: Differential regulation of Cbfa1/Run2 and osteocalcin gene expression by vitamin-D3, Dexamethasone, and local growth factors in primary human osteoblasts. *Journal of Cellular Biochemistry* 86: 348-356, 2002

Enomoto H., Shiojiri S., Hoshi K., Furuichi T., Fukuyama R., Yoshida C. A., Kanatani N., Nakamura R., Mizuno A., Zanma A., Yano K., Yasuda H., Higashio K., Takada K., and Komori T.: Induction of osteoclast differentiation by Runx2 through receptor activator of nuclear factor-kB ligand (RANKL) and osteoprotegerin regulation and partial rescue of osteoclastogenesis in Runx2$^{-/-}$ mice by RANKL transgene. *The Journal of Biological Chemistry* 278: 23971-23977, 2003

Kim H-J, Kim J-H, Bae S-C, Choi J-Y, Kim H-J, and Ryoo H-M.: The protein kinase C pathway plays a central role in the fibroblast growth factor-stimulated expression and transactivation activity of Runx2. *The journal of Biological Chemistry* 278(1): 319-326, 2003

Kitazawa S., Kajimoto K., Kondo T., and Kitazawa R.: Vitamin D_3 supports osteoclastogenesis via functional vitamin D response element of human RANKL gene promoter. *Journal of Cellular Biochemistry* 89: 771-777, 2003

Lian JB., Stein JL., Stein GS., van Wijnen AJ., Montecino M, Javed A., Gutierrez S., Shen JL, Zaidi S. K., and Drissi H.: Runx2/Cbfa1 functions: Diverse regulation of gene transcription by chromatin remodeling and co-regulatory protein interactions. *Connective Tissue Research* 44 (Suppl. 1): 141-148, 2003

Toshihisa K.: Requisite roles of Runx2 and Cbfb in skeletal development. *J. Bone Miner. Metab* 21: 193-197, 2003

Notoya M., Otsuka E., Yamaguchi A., and Hagiwara H.: Runx-2 is not essential for the vitamin D-regulated expression of RANKL and osteoprotegerin in osteoblastic cells.

Biochemical and Biophysical Reseach Communications 324: 655-660, 2004

Reinehr R., Becker S., Höngen A., and Häussinger D.: The Src family kinase Yes triggers hyperosmotic activation of the epidermal growth factor and CD95. *The Journal of Biological Chemistry* 279: 23977-23987, 2004

Yun J-H, Pang E-K, Kim C-S, Yoo Y-J, Cho K-S, Chai J-K, Kim C-K, Choi S-H.: Inhibitory effects of green tea polyphenol(-)-epigallocatechin gallate on the expression of matrix metalloproteinase-9 and on the formation of osteoclasts. *J Periodont Res* 39: 300-307, 2004

Zaidi S K, Sullivan A J, Medina R., Ito Y., van Wijnen AJ, Stein JL, Lian JB, and Stein GS.: Tyrosine phosphorylation controls Runx2-mediated subnuclear targeting of YAP to repress transcription. *The EMBO Journal* 23: 790-799, 2004

David M. Cohen.: SRC family kinases in cell volume regulation. *Am J Physiol Cell Physiol* 288: 483-493, 2005

Kabe Y., Yamade J., Uga H., Yamaguchi Y., Wada T., and Handa H.: NF-Y is essential for the recruitment of RNA polymerase II and inducible transcription of several CCAAT box-containing genes. *Molecular and Cellular Biology* 25: 512-522, 2005

Xu J., Wang C., Han R., Pavlos N., Phan T., Steer JH., Bakker AJ., Joyce DA., and Zheng MH.: Evidence of reciprocal regulation between the high extracellular calcium and RANKL signal transduction pathways in RAW cell derived osteoclasts. *J. Cell. Physiol.* 202: 554-562, 2005

Abstract (in Korean)

뼈모세포와 뼈파괴전구세포 혼합배양에서 hyperto-nicity 에 의한 1α, 25-dihydroxyvitamin D_3-induced osteoclastogenesis 의 억제와 Runx2 의 역할

<지도교수 이승일>

연세대학교 대학원 치의학과

전옥순

뼈개조는 뼈를 형성하는 뼈모세포 (osteoblast)와 뼈흡수를 유발하는 뼈파괴세포 (osteoclast)에 의해 조절된다. 뼈파괴세포는 뼈파괴전구세포 (preosteoclast)로부터 분화되는데, 이 과정에 뼈모세포는 뼈파괴세포분화 촉진 인자인 RANKL 와 M-CSF, 그리고 RANKL 의 저해제인 OPG 를 분비하여 뼈파괴세포 분화를 이끌어간다. 그러나 뼈파괴세포에 의해 뼈의 흡수가 일어나면, 뼈무기질의 분해산물로서 1) Ca^{2+}이나 인산염, 2) 뼈유기질 성분인 아교질이나 osteonectin 등의 분해산물이 증가하게 되어 이차적인 뼈대사 조절이 유도된다. 특히 뼈흡수 과정에서 만들어진 여러 가지 분해산물에 의해 뼈모세포, 뼈파괴세포의 주위에 삼투압이 높은 이른바 hypertonic 한 미세환경이 형성된다. 따라서 이 실험에서는 높은

삼투압이 뼈파괴세포 분화과정에 미치는 영향과 뼈대사에 미치는 영향을 분석하였다.

1α, 25-dihydroxyvitamin D_3 [1α, 25(OH)$_2D_3$] (10 nM)로 뼈모세포와 뼈파괴전구세포를 혼합배양하여 뼈파괴세포 분화를 유도한 후에 삼투압을 증가시키기 위하여 sucrose (25, 50, 100, 150, 200 mM)로 처리하였다. TRAP 염색법으로 핵이 3 개 혹은 그 이상 존재하는 뼈파괴세포 수를 측정한 결과, sucrose 의 농도가 증가함에 따라 그 수가 점차 감소하였으며, 뼈의 주요성분인 calcium phosphate nano crystal 로 초박막 코팅된 OAAS 판을 이용하여 뼈흡수 능력을 비교한 결과에서도 뼈흡수 능력이 sucrose 농도 증가에 비례하여 점차 감소하는 것을 확인하였다. 한편 세포의 독성능 검사 결과 sucrose 50 mM 까지 세포에 독성이 없었으며, 이는 sucrose 50 mM 이하의 농도에서는 1α, 25(OH)$_2D_3$ 에 의해 감소된 뼈파괴세포 형성이 삼투압 증가에 따른 결과라는 것을 의미한다. 높은 삼투압 환경이 뼈모세포의 특성에 미치는 영향을 확인하기 위하여 역전사중합효소연쇄반응법 (RT-PCR)으로 뼈모세포의 분화에 관여하는 유전자 (osteocalcin, osteopontin, collagen 1)를 분석하였다. 높은 삼투압은 뼈모세포의 특성에는 영향을 미치지 않았다. 그러나 뼈모세포에서 만들어지면서 뼈파괴세포 형성에 결정적 인자인 RANKL 과 M-CSF, OPG 를 측정한 결과, sucrose 의 농도가 증가함에 따라 RANKL mRNA 가 점차적으로 감소하였고 M-CSF, OPG 의 발현에는 변화가 없었다. 이를 효소면역측정법 (ELISA assay)과 면역검색법 (western blot)으로 RANKL 의 변화를 정량적으로 검색한 결과 RT-PCR 결과와 마찬가지로 RANKL 이 sucrose 농도가 증가함에 따라 감소하였다. 이는

뼈모세포에서 높은 삼투압이 RANKL 발현을 억제하여 뼈파괴세포 분화를 억제한다는 것을 의미한다.

이와 같이 높은 삼투압이 RANKL 발현을 억제하여 뼈파괴세포 분화를 억제하였지만, RANKL 유전자의 촉진염기서열 (promoter)에는 Runx2 결합위치가 있고 이 결합부위를 돌연변이 (mutation)시키면 RANKL 의 전사가 이루어지지 않는다. 이는 RANKL 발현에 Runx2 가 밀접하게 관련되어 있음을 나타내는 결과로 받아들이고, 면역검색법을 통하여 뼈모세포의 1α, $25(OH)_2D_3$ 에 의해 증가되었던 Runx2 발현이 높은 삼투압에 의해 감소되는 것을 확인하였다. 또한 Runx2 간섭유전자 (RNA interference)를 뼈모세포에 이입 (transfection) 시켰을 때 1α, $25(OH)_2D_3$ 에 의해 발현되는 RANKL mRNA 나 단백질의 양이 현저히 감소되었으며, 간섭유전자처리를 거친 뼈모세포를 뼈파괴전구세포와 혼합배양 시, 뼈파괴세포 형성이 현저히 억제되었다.

이상의 사실로 미루어 보아 뼈흡수가 활발히 일어나 형성된 세포주위의 삼투압 증가는 뼈파괴세포 형성을 억제하는데, 이는 뼈모세포의 Runx2 발현을 억제하여 이차적으로 RANKL 발현을 떨어뜨리기 때문에 발생되는 결과로 해석된다. 따라서 hypertonicity 는 뼈파괴를 조절할 수 있는 새로운 뼈대사 조절인자로 생각된다.

핵심되는 말: hypertonicity, 뼈파괴세포분화, 뼈모세포, RANKL, Runx2, 1α, $25(OH)_2D_3$