International Journal of Oral Biology, Vol. 30, No. 1, March 31 2005, p. 23~30

International Journal of Oral Biology

Hypertonicity Down-regulates the 1α,25(OH)$_2$ Vitamin D$_3$-induced Osteoclastogenesis Via the Modulation of RANKL Expression in Osteoblast

Hyunjoo Jeong[1], Tian Yushun[1], Bohye Kim[1], Mi Young Nam[1], Hyun-A Lee[1], Yun-Jung Yoo[1,3,4], Jeong-Taeg Seo[1,2,3,4], Dong Min Shin[1,2,3,4], Seung-Ho Ohk[5], and Syng-Ill Lee[1,2,3,4]*

Department of Oral Biology[1], and Brain Korea 21 Project of Medical Sciences[2], Oral Science Research Center[3], Yonsei University College of Dentistry[4], Seoul 120-752, Korea and Department of Oral Microbiology, Dental Science Research Institute, Chonnam National University, Gwangju 500-757, Korea[5]

(Received February 25, 2005 ; Accepted March 11, 2005)

Bone remodeling is a process controlled by the action of two major bone cells; the bone forming osteoblast and the bone resorbing osteoclast. In the process of osteoclastogenesis, stromal cells and osteoblast produce RANKL, OPG, and M-CSF, which in turn regulate the osteoclastogenesis. During the bone resorption by activated osteoclasts, extracellular Ca^{2+}/PO_4^{2-} concentration and degraded organic materials goes up, providing the hypertonic microenvironment. In this study, we tested the effects of hypertonicity due to the degraded organic materials on osteoclastogenesis in co-culture system. It was examined the cellular response of osteoblastic cell in terms of osteoclastogenesis by applying the sucrose, and mannitol, as a substitute of degraded organic materials to co-culture system. Apart from the sucrose, mannitol, and NaCl was tested to be compared to the effect of organic osmotic particles. The addition of sucrose and mannitol (25, 50, 100, 150, or 200 mM) to co-culture medium inhibited the number of tartrate-resistant acid phosphatase (TRAP) positive multinucleated cells induced by 10 nM 1α,25(OH)$_2$vitaminD$_3$ (1α,25(OH)$_2$D$_3$). However, NaCl did exert harmful effect upon the cells in this co-culture system, which is attributed to DNA damage in high concentration of NaCl. To further investigate the mechanism by which hypertonicity inhibits 1α,25(OH)$_2$D$_3$-induced osteoclastogenesis, the mRNA expressions of receptor activator of nuclear factor (NF)-kB ligand (RANKL) and osteoprotegerin (OPG) were monitored by RT-PCR. In the presence of sucrose (50 mM), RANKL mRNA expression was decreased in a dose-dependent manner, while the change in OPG and M-CSF mRNA were not occurred in significantly. The RANKL mRNA expression was inhibited for 48 hours in the presence of sucrose (50 mM), but such a decrement recovered after 72 hours. However, there were no considerable changes in the expression of OPG and M-CSF mRNA. Conclusively, these findings strongly suggest that hypertonic stress down-regulates 1α,25(OH)$_2$D$_3$-induced osteoclastogenesis via RANKL signal pathway in osteoblastic cell, and may play a pivotal role as a regulator that modulates osteoclastogenesis.

Keywords: Hypertonicity, 1α,25(OH)$_2$D$_3$ induced osteoclastogenesis, RANKL, OPG, Cell shrinkage

*Corresponding author: Syng-Ill Lee, DDS, Ph. D. Department of Oral Biology, Yonsei University, College of Dentistry, 134 Shinchon-Dong, Seodaemoon-Gu, Seoul 120-752, Korea, Tel.: +82-2-361-8083, Fax: +82-2-364-1085, E-mail: lsi@yumc.yonsei.ac.kr

Introduction

Bone remodeling is a process controlled by the action of two major bone cells, bone forming osteoblast and bone resorbing osteoclast. The osteoblasts stem from mesenchymal stem cells, whereas osteoclasts are matured by the differentiation of osteoclast precursors of hematopoietic stem cells. Since these cells are influenced by mechanical and metabolic stimuli (Hofbauer *et al.*, 2000; Suda *et al.*, 1992), osteoblasts and osteoclasts are required not only for skeletal development, but also for mineral homeostasis and the normal remodeling of bone in adult (Raisz, 1999).

In the process of bone remodeling, osteoblasts and osteoclasts are known to be closely coupled to maintain the

constant bone mass (Suda *et al.*, 1992; Takahashi *et al.*, 1999; Tsukii *et al.*, 1998). However, an imbalance between bone formation and bone resorption causes metabolic bone diseases like osteopetrosis and osteoporosis (Aubin, 1998). Skeletal integrity is regulated by a variety of hormones such as parathyroid hormone (PTH), interleukin-1 (IL-1), and prostagrandin E_2 (PGE_2), and local factors like fluid movement, and located Ca^{2+} level (Burgess *et al.*, 1999; Sheikh-Hamad *et al.*, 1998; Kong *et al.*, 1999; Lacey *et al.*, 1998). In particular, a certain kinds of signal molecules, such as, receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG) and macrophage colony stimulating factor (M-CSF), expressed by osteoblasts, are involved in osteoclastogenesis and in osteoclast development (Tsukii *et al.*, 1998). For instance, when osteoblasts/stromal cells are stimulated by osteotropic factors such as parathyroid hormone, RANKL is expressed and induces the differentiation of osteoclast progenitors by binding to the receptor activator of NF-κB (RANK; also known as ODF receptor) (Jimi *et al.*, 1999). In addition, M-CSF is known to be essential for macrophages to be transformed into osteoclasts, while OPG, a decoy receptor of RANKL, is participated in the regulation of osteoclastogenesis (Lacey *et al.*, 1998).

Consequently, it is believed that RANKL, M-CSF and OPG, which are expressed by osteoblasts, are associated with osteoclastogenesis, and that osteoblasts may play a major role in the osteoclast differentiation. This RANKL signals via a membrane-bound tumor necrosis factor (TNF) receptor super family member named TRANCE/RANK in osteoclast, differentiating the osteoclast precursor cells into the matured osteoclast (Takahashi *et al.*, 1999). In another words, the RANKL/RANK axis mediates the osteoclast activity associated with RANKL, and eventually leads to the demineralization of bone matrix. 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 degradation of inorganic matrix precedes the degradation of organic matrix, which mostly takes place in extracellular resorption lacunae (Jones *et al.*, 1987). 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, 1988; Blair *et al.*, 1989; Väänänen *et al.*, 1990). Mainly, proteolytic enzymes of osteoclasts, interstitial collagenase, lysosomal enzymes and cysteine proteases function together to digest organic matrix (Salo *et al.*, 1996).

Therefore when bone is actively resorbed, it might be possible that high osmolality is provided due to the high Ca^{2+}/PO_4^{2-} (as high as 40 mM; Silver *et al.* 1998) and the degraded organic materials (mainly collagen) in the extracellular fluid around bone cells. Of these high osmotic particles, Ca^{2+}/PO_4^{2-} induces apoptosis of osteoclast and may change the function of osteoblast through the cell shrinkage (Kameda *et al.* 1995; Lorget *et al.* 2000; Rani *et al.*, 2000). On the other hand, is not examined how the degraded organic materials from the resorbed bone affect the bone cells (osteoblast and osteoclast) in terms of high osmolality. Here, we hypothesized that the hypertonicity by the degraded organic materials in bone could be one of the regulating factors for bone remodeling. In the present study, we examined the cellular response of osteoblastic cell in terms of osteoclastogenesis by applying the sucrose, and mannitol, as a substitute of degraded organic materials to co-culture system. Apart from the sucrose, mannitol, and NaCl was tested to be compared to the effect of organic osmotic particles. Experimentally, the number of osteoclast formation was determined by counting tartrate-resistant acid phosphatase (TRAP)-positive cells. To confirm the effect of organic osmotic particles on the osteoclastogenesis, we assayed RANKL, OPG and M-CSF mRNA expression profiles in osteoblasts.

Materials and Methods

Materials

Routine cell culture media were obtained from GIBCO/BRL (Grand Island, NY). The Tartrate-Resistant Acid Phosphatase Staining Kit and sucrose were purchased from the Sigma Chemical Co., Ltd. (St. Louis, MO). Trizol was from Invitrogen Corp. (Carlsbad, CA), and the ICR mice were from Samtacho Co., Ltd. (Seoul, Korea). All other chemicals were of the highest grade commercially available.

In vitro formation assay of osteoclast

The osteoclast formation assay was carried out as previously reported by Choi *et al.* (2001). Briefly, the osteoblasts were obtained by growing calvarial cells from 1- 2-day-old newborn mice for 4 days in (-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS). Bone marrow cells were obtained by femur and tibiae from 4-week-old ICR mice. The tibiae and femur were removed and dissected free of adhering tissues. Mouse calvarial cells (1×10^4 cells/well) were co-cultured for 4 days with bone marrow cells (1×10^5 cells/well) in α-MEM containing 10% FBS. The co-culture cells in 48-well plates (Corning Inc., Corning, NY) were stimulated with $1\alpha,25(OH)_2D_3$ (10 nM) in the presence and absence of sucrose (25, 50, 100, 150, or 200 mM). All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cells were subjected to tartrate-resistant acid phosphatase (TRAP, an osteoclast marker enzyme) staining. We counted TRAP positive multinucleated (more than three) cells as osteoclasts. *In vitro* formation assay of osteoclast was repeated four times.

Viability test

The MTT (3-[4, 5-dimethylthiazol-2-yl-]-2, 5-diphenyl-

tetrazolium bromide) test is based on the principle of that tetrazolium salts were reduced by mitochondrial enzymes (succinate, and dehydrogenase), which allows the toxicity of viable cells and the level of cellular differentiation to be measured. MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml and filtered to remove any insoluble residue. MTT solution was added directly to the assay plates. The cells were subsequently incubated for an additional 4 hours at 37°C. The purple formazan crystals that formed were dissolved in DMSO (dimethyl solfoxide), and the plates were read on a spectrophotometer at 570 nm. This assay was repeated four times.

RT-PCR

The expressions of RANKL, OPG, M-CSF and β-actin were evaluated by RT-PCR using total RNA isolated from mice osteoblastic cells. Total RNA was isolated using Trizol reagent, chloroform, isoprophyl alcohol. First-strand cDNA was synthesized from total RNA with random hexamers using the Advantage™ RT-for-PCR Kit (Clontech Laboratories Inc. Palo Alto, USA.). And then, cDNA subjected to PCR amplification with Ampli*Tag* DNA polymerase (Applied Biosystems, CA, USA). The primers used were: for mouse RANKL (750 bp), 5'-ATCAGAAGACAGCACTCACT-3' (forward), 5'-ATCTAGGACATCCATGCTAATGTTC-3' (reverse); for mouse OPG (636bp), 5'-TGAGTGTG AGGAAGGGCGTTAC-3' (forward) 5'-TTCCTCGTT CTCTCAATCTC-3' (reverse); for β-actin (366 bp), 5'-GGACTCCTATGGTGGGTGACGAGG-3' (forward), and 5'-GGGAGAGCATAGCCCTCGTAGAT-3' (reverse); for mouse M-CSF (395bp), 5'-CATGACAAGGCCTGCGTCCGA-3' (forward), and 5'-AAGCTCTGGCAGGTGCTCCTG -3' (reverse1), 5'-GCCGCCTCCACCTGTAGAACA-3' (reverse2). The polymerase chain reactions were performed on a T gradient 96 PCR machine (Biometra Co., Gottingen, Germany). The PCR program initially started with a 95°C denaturation for 5 min, followed by 25 to 38 cycles of 95°C/1 min, Ta/1 min, 72°C/1 min (Ta, annealing temperature; 45.3°C for RANKL, 47.9°C for OPG, and 58°C for β-actin and M-CSF). Linear amplification range for each gene was tested on the adjusted cDNA. The less expressed transcripts of RANKL and OPG required 35 cycles of PCR for detection. For M-CSF and β-actin, 30 and 25 cycles of PCR were performed, respectively. The PCR samples were electrophoresed on 1.5% agarose gels. The gels were stained with ethidium bromide 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

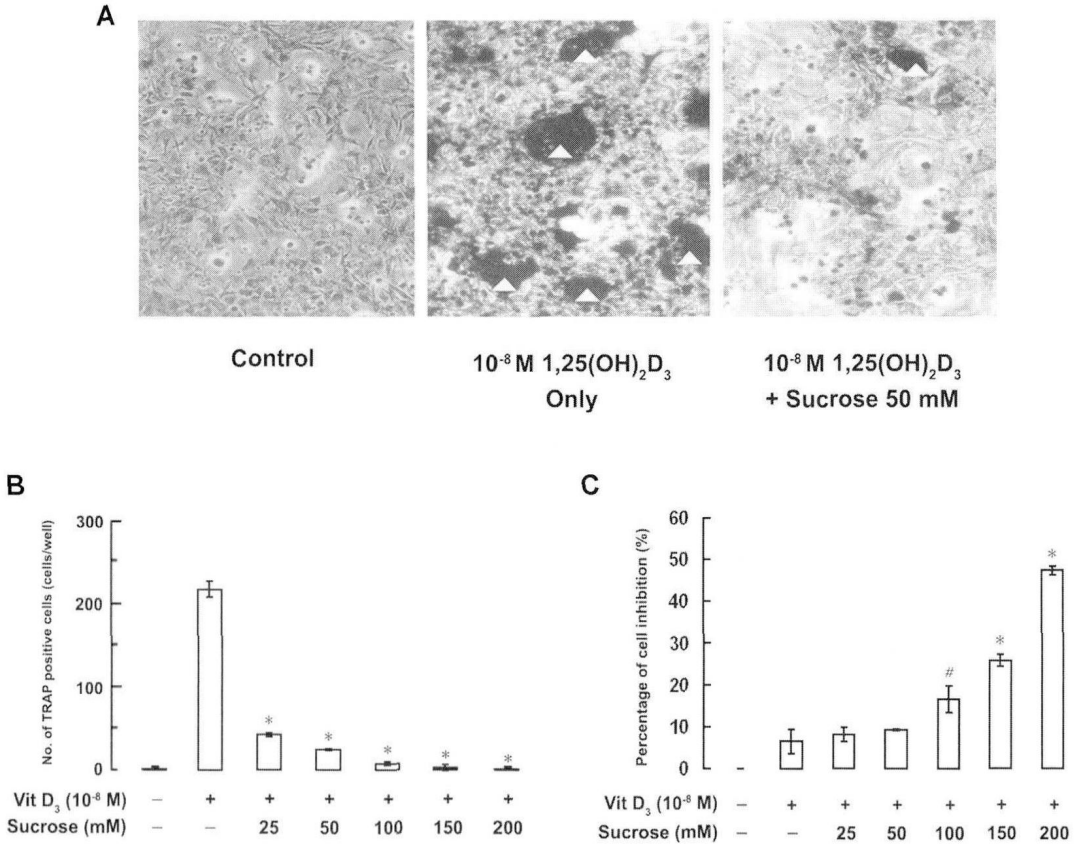

Fig. 1. The Osteoclast differentiation inhibits in the presence of sucrose at various concentrations. (A), In the presence of sucrose, 10 nM $1\alpha,25(OH)_2D_3$ induced osteoclast differenciation was reduced (×400). The yellow triangles indicated osteoclast cells. (B) TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts (C) MTT assay. Sucrose did not affect viability of the co-cultured cells up to 100 μM concentrations. The statistical significance of differences between the groups was determined using the one-way ANOVA test. Data are shown as mean ± S.E. *p value < 0.01, $^{\#}p$ value < 0.05, significant differences from the $1\alpha,25(OH)_2D_3$ only.

ladder standards.

Data analysis and statistics

The results are expressed as the mean± S.E. The statistical significances of differences between the groups were determined using the Kruskal-Wallis and Bonferroni's test. In statistical tests, the p value < 0.05 was considered to be significant.

Results

Inhibition of 1α,25(OH)$_2$D$_3$-induced osteoclast formation by the addition of sucrose in co-culture system

To investigate whether the hypertonic stress affects the osteoclastogenesis, mouse calvarial osteoblast cells were co-cultured with bone marrow stromal cells. When 10 nM of 1α,25(OH)$_2$D$_3$ was added to the co-culture, TRAP positive multinucleated cells were formed, whereas no TRAP positive cells were detected in media only. When adding the sucrose to normal medium, 1α,25(OH)$_2$D$_3$-induced osteoclast formation was reduced as shown in Fig. 1A. In detail, the addition of 25, 50, 100, 150, and 200 mM of sucrose dramatically reduced the number of TRAP positive multinucleated cells up to about 65% in 50 mM sucrose (Fig. 1B). Nevertheless, it could be possible that sucrose caused cell damage directly without intervening on the normal differentiation of osteoclasts. To rule out the above possibility, we used a viability test. As shown in Fig. 1C, sucrose did not show a toxic effect to co-culture cells even if treated up to 100 mM. These results suggest that hypertonic stress due to the addition of sucrose by itself inhibits the 1α,25(OH)$_2$D$_3$-induced osteoclast formation, not by its direct toxic effect upon the cells.

Inhibitory effects of the addition of mannitol and NaCl on 1α,25(OH)$_2$D$_3$-induced osteoclast formation in co-culture system

To clarify the above sucrose effect as a hypertonic stress, mannitol or NaCl replaced to provide a hypertonic environment instead of sucrose. The addition of mannitol (the same of sucrose concentrations) also decreased the formation of TRAP positive multinucleated osteoclast cells although the inhibition was less effective than in the case of sucrose. As shown in Fig. 2A, hypertonic stress by the addition of mannitol resulted in the decreased TRAP positive multinucleated osteoclast formation in dose dependent manner. Mannitol did show the toxic effect to the cells on the basis of viability test (Fig. 2B). When several concentrations of the NaCl (12.5, 25, 50, 75, and 150 mM) were added to the co-culture,

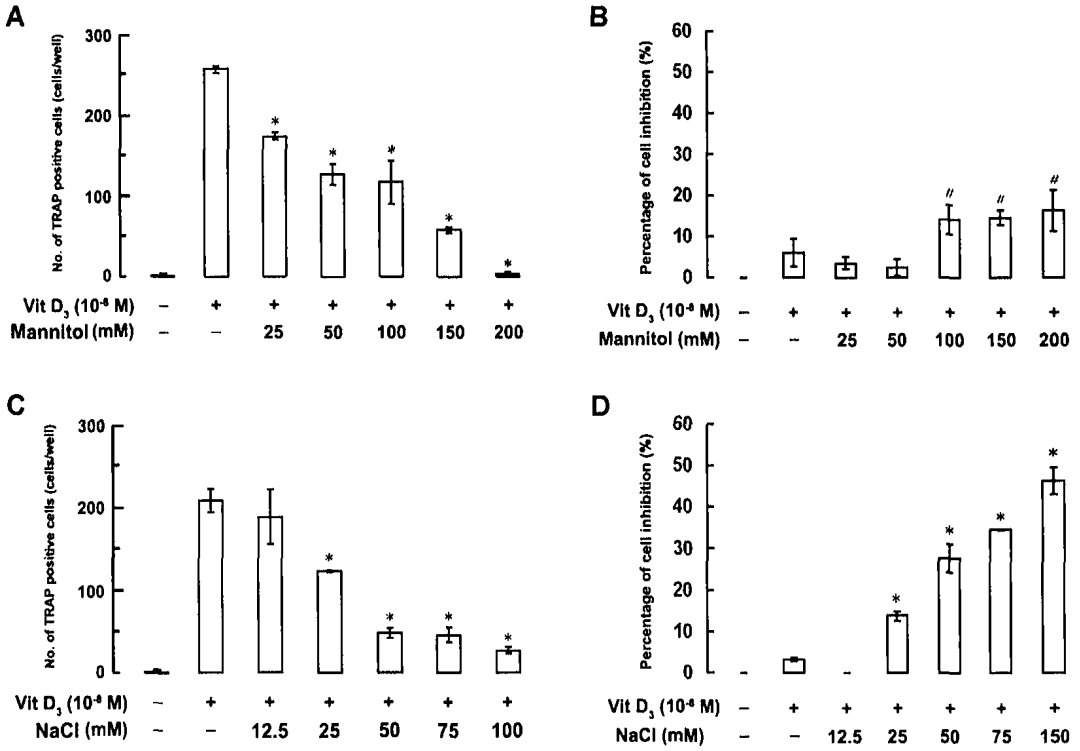

Fig. 2. The osteoclast differentiation inhibits in the hypertonic stress substituting mannitol or NaCl for sucrose. (A) Mannitol was added to the osteoblast/bone marrow coculture with 10 nM 1α, 25(OH)$_2$D$_3$. After incubation for an additional 4 days, the cells were stained using a TRAP staining method. Only the TRAP positive multinucleated cells containing more than 3 nuclei were counted. (B) The MTT assays were carried out according to the method described in materials and methods. (C) NaCl was added to the osteoblast/bone marrow co-culture with 10 nM 1α, 25(OH)$_2$D$_3$. (D) The MTT assays were carried out according to the method described in materials and methods. The statistical significance of differences between the groups was determined using the one-way ANOVA test. Data are shown as mean ± S.E. *p value < 0.01, #p value < 0.05, significant differences from the 1α,25(OH)$_2$D$_3$ only.

the number of TRAP positive multinucleated osteoclast cells also decreased in a dose dependent manner (Fig. 2C). As shown in Fig. 2D, however, NaCl did show the toxic effect to the cells on the basis of viability test.

Effects of hypertonic stress by the addition sucrose on RANKL, OPG, and M-CSF expression in mouse calvarial osteoblastic cells

Since osteoclastogenesis is modulated through the secretion of OPG, RANKL, and M-CSF by osteoblastic cells, the expressions of RANKL, OPG and M-CSF mRNA in osteoblasts were monitored by RT-PCR in the presence and absence of sucrose (Fig. 3). As the sucrose concentrations (25, 50, and 100 mM) in the culture medium were increased, the $1\alpha,25(OH)_2D_3$ -induced expression of RANKL mRNA was down-regulated. The decrement of RANKL expression was dependent on the sucrose concentration. However, OPG and M-CSF mRNA expression did not change. The effect of sucrose on RANKL mRNA expression in a period of the incubation time was also monitored by adding 50 mM sucrose with $1\alpha,25(OH)_2D_3$. The RANKL mRNA expression induced by $1\alpha,25(OH)_2D_3$ was reduced until 48 hours after the addition of 50 mM sucrose, but was recovered after 72 hours (Fig. 4). These findings indicate that hypertonic stress by the addition of sucrose (25, 50, and 100 mM) inhibits osteoclast formation by down-regulating the expression of RANKL mRNA.

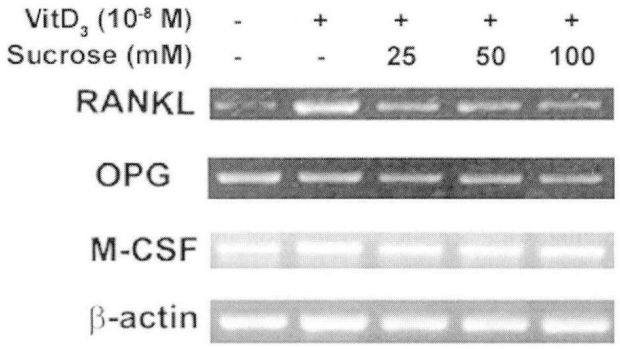

Fig. 3. Sucrose caused changes in RANKL, OPG and M-CSF mRNA expression. Indicated concentrations of sucrose were added to the mouse calvarial osteoblasts culture with 10 nM $1\alpha,25(OH)_2D_3$. After incubation for 2 days, total RNA was then extracted from osteoblasts, and the expression of RANKL, OPG and M-CSF mRNAs were analyzed by RT-PCR. RANKL, OPG, and M-CSF were 750 bp, 636 bp, and 395 bp, respectively.

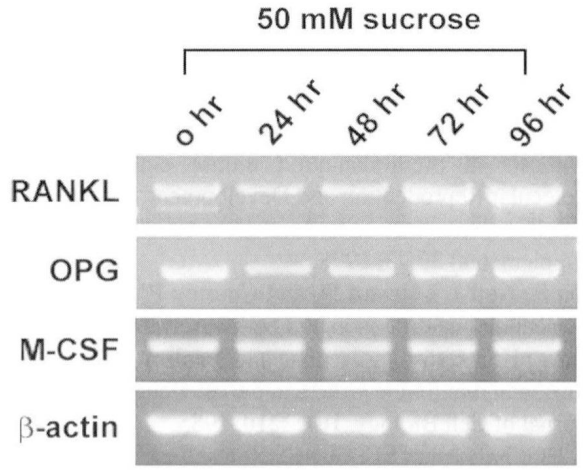

Fig. 4. In the presence of 50 mM sucrose, the RANKL mRNA expression was changed in a period of the time. The different RANKL expression levels according to the incubation time suggest that the inhibitory effect of hypertonicity on the osteoclastogenesis 50 mM sucrose was added to the osteoblast culture with 10 nM of $1\alpha,25(OH)_2D_3$. Total RNA was isolated after incubation of the indicated time intervals. mRNAs were amplified according to the method described in materials and methods.

Discussion

Several signal molecules, which express from osteoblasts such as RANKL, OPG, and M-CSF is involved in osteoclastogenesis. This osteoclastogenesis is controlled by metabolic (nutrition, drugs, and disease) and mechanical stimuli (e.g., normal daily activity, exercise, physical force, fluid movement). Of mechanical stimuli, osmolality as a novel regulator for osteoclastogenesis could be hypothesized since the activated osteoclast enhances extracellular Ca^{2+}/PO_4^{2-} concentration up to 40 mM as well as degraded organic materials (mainly collagen) during the bone resorption (Gravallese *et al.*, 2001). Therefore, in this study, we introduced the hypertonic challenge (sucrose and mannitol as a substitute of a degraded organic materials) to vitro osteoclast formation system to pursuit the cellular response. As a function of sucrose concentration, the number of TRAP positive multinucleated cells was reduced up to about 65%, compared with positive control (10 nM $1\alpha,25(OH)_2D_3$ only) in co-culture system (Fig. 1). Nevertheless, it could be argued that the inhibition of osteoclastogenesis by sucrose addition might be caused not due to the physiological intervention of sucrose in the process of osteoclastogenesis, but due to the cell damage. As previously described in results, sucrose concentration under 50 mM did not exert any harmful effect upon the cells in this co-culture system, confirming that down-regulation of $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis by sucrose addition (at least up to 50 mM) was stemmed from the effects of hypertonic stress rather than a direct toxic effect upon the cells. From these results, it might be speculated that high sucrose concentrations (not in the case of over 50 mM) induced reversible cellular shrinkage (data not shown) and led to interfere the process of osteoclastogenesis. Recent reports suggest that a cell volume change can denote various cellular functions such as transportation, metabolic control, hormone release, cell proliferation and death. It has also been reported that hypertonic shrinkage inhibits cell proliferation and osmotic swelling accelerates it (Yancey *et al.*, 1990; Anbari *et al.*, 1993). These facts mean that changes in cell volume may participate in cell growth and proliferation. Furthermore,

this inhibition of osteoclast formation by hypertonic stress was confirmed by substituting mannitol or NaCl for sucrose. The addition of mannitol or NaCl inhibited the differentiation of osteoclasts in a similar manner of sucrose addition, however, the inhibitory effect of NaCl was less remarkable than sucrose. Unlikely sucrose and mannitol, NaCl is ionized in water, then permeable to cell membrane, causing not only toxic effect on the basis of viability but also DNA damage (Kultz *et al.*, 2001). From these results, we suggested that hypertonicity plays a role as a new modulator in $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis. If so, how does the hypertonic stress lead to the modulation of $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis?

It has been reported that osteoclast differentiation is mediated by several factors, such as RANKL, OPG and M-CSF. Of cause, RANKL is involved in osteoclast differentiation, maturation, and activation induced by osteoblastic cells treated with parathyroid hormones (Takahashi *et al.*, 1999), and is essential for the osteoblast-mediated activation of mature osteoclasts. To clarify the role of hypertonicity in the process $1\alpha,25(OH)_2D_3$-induced osteoclastogenesis in co-culture system, the mRNA expression profiles of RANKL, OPG, and M-CSF were examined. The expression of RANKL mRNA was down-regulated as increasing the sucrose concentration, however, the expression of OPG and M-CSF mRNA were not changed significantly (Fig. 3). In particular, when 10 nM $1\alpha,25(OH)_2D_3$ was added to the medium, the expression of RANKL mRNA in osteoblastic cells was decreased within exposure for 48 hours by 50 mM sucrose, then increased after exposure for 72 hours. In fact, the hypertonic stress in the osteoblastic cells by sucrose addition did not have any long-term effect. Here, our question is what makes the difference between them in terms of time course. Possibly, such a different RANKL expression levels according to the incubation time suggest that the inhibitory effect of hypertonicity on the osteoclastogenesis by $1\alpha,25(OH)_2D_3$ might be involved in the early differentiation stage of the prefusion of osteoclast precursors rather than the later period of osteoclast differentiation or the maturation and activation of TRAP-positive multinucleated cells. In the results, it could be delineated that hypertonicity affects RANKL production in osteoblastic cell.

Next question is what signal molecules are linked to the hypertonicity and lead to the decrease in RANKL expression. About this fundamental question, we could not speculate the clear understanding. One of the plausible explanations may draw from the renal tubular cells, which is always exposed to the high osmotic environment. In the kidney, tubular cells adapt to the hypertonicity by accumulating compatible osmolytes such as betaine, inositol, taurine, sorbitol, and glycerophosphorylcholine (Handler *et al.*, 2001). To produce these osmolytes, tubular cells activated genes encoding the regulation of compatible osmolyte transporter; sodium/myo-inositol co-transporter (SMIT), the sodium/chloride/betaine co-transporter (BGT-1), and aldose reductase (AR), which transcriptional activator is TonEBP (Tonicity-responsive enhancer binding protein) (Kultz *et al.*, 1999; Woo *et al.*, 2001). TonEBP plays a central role in the cellular accumulation of compatible osmolytes (Woo *et al*, 2001). Apart from some knowledge of the general functions of hypertonicity in a kidney as well as other the tissue (Woo *et al.*, 2001; Handler *et al.*, 2001), there are no experimental evidences whether the hypertonic stress is related to osteoclastogenesis at the cellular level. With respect to osteoclastogenesis, we expect that there is the effect of TonEBP on the bone cells by hypertonic stress. This question remains to be solved.

In conclusion, unexpectively, hypertonicity, such as the addition of sucrose and mannitol, inhibited $1\alpha,25(OH)_2D_3$ induced osteoclast formation in co-culture system. And the expression of RANKL mRNA was down-regulated upon increasing the hypertonic stress, but the expression of OPG and M-CSF mRNA was not changed significantly, indicating that the hypertonic stress might be closely related to the regulation of RANKL expression in osteoblastic cells. Taken all together, it could be suggested that hypertonicity plays a pivotal role in the process $1\alpha,25(OH)_2D_3$ induced osteoclastogenesis that associated with RANKL signal. Further studies will be necessary to elucidate how hypertonicity modulates the RANKL expression in osteoblastic cells.

Acknowledgement

This work was supported by a grant Yonsei University College of Dentistry Research Fund in 2004.

References

Al-Habori M.: Cell volume and ion transport regulation. International journal of Biochemistry and cell biology. **26**:319, 1994.

Anbari K. and Schultz RM.: Effect of sodium and betaine in culture media on development and relative rates of protein synthesis in preimplantation mouse embryos in vitro. Molecular Reproduction and Development. **35**(1):24, 1993.

Aubin JE.: Bone stem cells. Journal of cellular biochemistry. (supplements) **30**:73, 1998.

Baron R, Neff I, Louvard D. and Courtoy PJ.: 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. Journal of Cell Biology. **101**:2210, 1985.

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

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

Choi BK, Ohk SH, Lee HJ, Kang JH, Jeong GJ and Yoo YJ.: Effects of whole cell sonicateds of Treponema lecithinolyticum on osteoclast differentiation. Journal of Periodontology. **72**:1172, 2001.

Cossins AR.: Cell physiology. A sense of cell size. Nature. **352**:667, 1991.

Dmitrieva NI, Cai Q and Burg MB. : Cells adapted to high NaCl have many DNA breaks and impaired DNA repair both in cell culture and in vivo. Proc. Natl. Acad. Sci. USA. **101**(8):2317, 2004.

Ducy P, Schinke T and Karsenty G.: The osteoblast : A sophisticated fibroblast under central surveillance. Science. **289**:1501, 2000.

Eveloff JL and Warnock DG. : Activation of ion transport systems during cell volume regulation. American journal of Physiology. **252**:10, 1987.

Everts V, Delaisse JM, Korper W, Niehof A, Vaes G and Beertsen W.: Degradation of collagen in the bone-resorbing compartment underlying the osteoclast involves both cysteine-proteinases and matrix metalloproteinases. Journal of Cellular Physiology. **150**(2):221, 1992.

Fuller K, Wong B, Fox S, Choi Y and Chambers TJ.: TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. Journal of Experimental Medicine. **188**:997, 1998.

Gravallese EM, Galson DL, Goldring SR and Auron PE.: The role of TNF-receptor family members and other TRAF-dependent receptors in bone resorption. Arthritis Research. **3**(1):6, 2001.

Handler J.S. and Kwon H.M.: Transcriptional regulation by changes in tonicity. Kidney International. **60**(2):408, 2001.

Hernandez CJ, Beaupre GS and Carter DR.: A model of nuclear factor-kB ligand and osteoprotegerin in the pathogenesis and treatment of metabolic bone diseases. Journal of Rehablilitation Researh and Development. **37**(2):234, 2000.

Hofbauer LC and Heufelder AE. : Clinical review 114: hot topic. The role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in the pathogenesis and treatment of metabolic bone diseases. The Journal of Clinical Endocrinology & Metabolism. **85**(7):2355, 2000.

Hoffmann EK and Dunham PB. : Membrane mechanisms and intracellular signalling in cell volume regulation. International Review of Cytology. **161**:173, 1995.

Jimi E, Nakamura I, Duong LT, Ikebe T, Takahashi N, Rodan GA and Suda T.: Interleukin-1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. Experimental Cell Research. **247**(1): 84, 1999.

Jimi E, Nakamura I, Ikebe T, Akiyama S, Takahashi N and Suda T.: Activation of NF-kappaB is involved in the survival of osteoclasts promoted by interleukin-1. The Journal of Biological Chemistry. **273**(15):8799, 1998.

Jimi E, Shuto T and Koga T : Macrophage colony-stimulating factor and interleukin-1 alpha maintain the survival of osteoclast-like cells. Endocrinology. **136**(2):808, 1995.

Jones SJ and Boyde A.: Scanning microscopic observations on dental caries. Scanning Microsc. **1**(4):1991, 1987.

Kameda T., Ishikawa H. and Tsutsui T.: Detection and characterization of apoptosis in osteoclasts in vitro. Biochemical and Biophysical Research Communications. **207**(2):753, 1995.

Kanatani M, Sugimoto T, Kanzawa M, Yano S and Chihara K.: High extracellular calcium inhibits osteoclast-like cell formation by directly acting on the calcium-sensing receptor existing in osteoclast precursor cells. Biochemical and Biophysical Research Communications. **261**(1):144, 1999.

Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ and Penninger JM.: OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature. **397**:315, 1999.

Kultz D and Chakravarty D.: Hyperosmolality in the form of elevated NaCl but not urea causes DNA damage in murine kidney cells. Proc. Natl. Acad. Sci. USA. **98**(4):1999, 2001

Kultz D and Csonka L.: What sets the TonE during osmotic stress? Proc. Natl. Acad. Sci. USA. **96**(5):1814, 1999.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J and Boyle WJ.: Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell, **93**(2):165, 1998.

Lorget F, Kamel S, Mentaverri R, Wattel A, Naassila M, Maamer M and Brazier M.: High extracellular calcium concentrations directly stimulate osteoclast apoptosis. Biochemical and Biophysical Research Communications. **268**(3):899, 2000.

Malgaroli A, Meldolesi J, Zallone AZ and Teti A.: Control of cytosolic free calcium in rat and chicken osteoclasts. The role of extracellular calcium and calcitonin. Journal of Biological Chemistry. **264**(24):14342, 1989.

Raisz LG: Physiology and pathophysiology of bone remodeling. Clinical Chemistry. **45**:1353, 1999.

Rani CS and MacDougall M.: Dental cells express factors that regulate bone resorption. Molecular Cell Biology Research Communication. **3**(3):145, 2000.

Roodman GD.: Biology of osteoclast activation in cancer. Journal of clinical Oncology. **19**(15):3562, 2001.

Sarkadi, B. and Parker, J.C.: Activation of ion transport pathways by changes in cell volume. Biochimica et Biophysica Acta. **1071**(4):407.

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

Sheikh-Hamad D, Di Mari J, Suki WN, Safirstein R, Watts BA 3rd and Rouse D.: p38 kinase activity is essential for osmotic induction of mRNAs for HPS 70 and transpoter for organic solute betaine in Madin-Darby Canine Kidney cells. The Journal of Biological Chemistry. **273**(3):1832, 1998.

Shirai Y, Yoshimura Y, Yawaka Y, Hasegawa T, Kikuiri T, Takeyama S, Matsumoto A and Oguchi H.: Effect of extracellular calcium concentrations on osteoclast differentiation in vitro. Biochemical and Biophysical Research Communi-

cations. **265**(2):484, 1999.

Silver IA, Murrills RJ and Etherington DJ.: Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. Experimental Cell Reserch. **175**(2):266, 1988.

Strange K, Emma F and Jackson PS.: Cellular and molecular physiology of volume-sensitive anion channels. American Journal of Physiology. **270**:C711, 1996.

Suda T, Takahashi N and Martin TJ.: Modulation of osteoclast differentiation. Endocrine Review. **13**(1):66, 1992.

Suda T, Udagawa N, Nakamura I, Miyaura C and Takahashi N.: Modulation of osteoclast differentiation by local factors. Bone, **17**:87, 1995.

Takahashi N, Udagawa, N and Suda T.: A new member of tumor necrosis factor ligand family, ODF/OPGL/TRANCE/RANKL, regulates osteoclast differentiation and function. Biochemical and Biophysical Research Communications. **256**:449, 1999.

Takeyama, S., Yoshimura, Y., Shirai, Y., Deyama, Y., Hasegawa, T., Yawaka, Y., Kikuiri, T., Matsumoto, A. and Fukuda, H.: Low calcium environment effects osteoprotegerin ligand/osteoclast differentiation factor. Biochemical and Biophysical Research Communications. **276**:524, 2000.

Tsukii K, Shima N, Mochizuki S, Yamaguchi K, Kinosaki M, Yano K, Shibata O, Udagawa N, Yasuda H, Suda T and Higashio K.: Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1 alpha,25-dihydroxyvitamin D3, prostaglandin E2, or parathyroid hormone in the microenvironment of bone. Biochemical and Biophysical Research Communications **146**:337, 1998.

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. Clinical Orthopaedics and Related Research. **231**:239, 1988.

Väänänen HK, Karhukorpi EK, 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. Journal of Cell Biology. **111**:1305, 1990.

Woo SK, Lee SD and Kwon HM.: TonEBP transcriptional activator in the cellular response to increased osmolality. Pflügers Archiv European Journal of Physiology. **444**(5): 579, 2002.

Yancey PH, Burg MB and Bagnasco SM.: Effects of NaCl, glucose, and aldose reductase inhibitors on cloning efficiency of renal medullary cells. (1990) American Journal of Physiology. **258**:198, 1990.