The Effect of Na-K-Cl cotransporter inhibition on osteoclastogenesis induced by 1α,25-Dihydroxyvitamin D_3

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This certifies that the dissertation of

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Abstract

The Effect of Na-K-Cl cotransporter inhibition on osteoclastogenesis induced by 1α,25-Dihydroxyvitamin D_3

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(Directed by Prof. Jong-Gap Lee, D.D.S., Ph.D)

Na/K/Cl cotransporters (NKCC1) have been reported in numerous, diverse tissues, and several cellular functions. NKCC1 is involved in ion transport across the secretory and absorptive epithelia, regulation of cell volume, and possibly modulation of cell growth and development. Furthermore, recent studies showed that NKCC1 is present in osteoblasts. In this study physiological role of NKCC1 in the process of osteoclastogenesis was exploited in coculture system. 10 and 100 μM bumetanide, a specific inhibitor of NKCC1, decreased the number of tartrate-resistant acid phosphatase (TRAP) positive multinucleated cells. One micromolar bumetanide did not show any effect on the $1\alpha,25(OH)_2D_3$-induced osteoclast differentiation. To clarify the role of bumetanide on the inhibition of osteoclastogenesis, mRNA expression of RANKL and OPG was analyzed by RT-PCR.

Exposure of osteoblastic cell to the bumetanide treated medium resulted in the reduction of RANKL mRNA expression induced by $1\alpha,25(OH)_2D_3$, being dependent on the bumetanide concentration. On the other hand, the expression of OPG mRNA, a novel TNF receptor family member was increased. These results imply that 10 μM bumetanide inhibits osteoclast differentiation via inhibition of the RANKL mRNA expression and enhancement of the OPG mRNA expression in osteoblastic cell. However the expression of M-CSF mRNA which has been known to be a survival factor of osteoclasts was not changed at tested concentrations (1, 10, 100 μM). Also, we examined the expression and phosphorylation of c-Jun NH_2-terminal kinase (JNK). The phosphorylation of JNK was reduced. Furthermore, bumetanide caused the decrease in the relative cell volume up to 36% of the original volume. Taken all together, these results suggest that NKCC1 in osteoblastic cell has an important role in the osteoclast differentiation and the inhibition of NKCC1 activity reduced the osteoblastic cell volume change, which in turn results in the inhibition of osteoclastogenesis, decreasing the phosphorylation of JNK.

Key words : NKCC1, bumetanide, osteoclast, RANKL, OPG, JNK

I. Introduction

Bone remodeling is regulated by the activity of bone-forming osteoblasts and bone-resorbing osteoclasts. Both osteoblasts and osteoclasts are regulated by a variety of hormones and local factors. Osteoblasts stems from mesenchymal stem cells, whereas osteoclasts arise by the differentiation of osteoclasts precursors of monocyte/ macrophage lineage and their function is required not only for the development of the skeleton, but also for mineral homeostasis and normal remodeling of bone (Raise, L., 1998). An imbalance between bone formation and bone resorption causes such metabolic bone diseases like osteopetrosis and osteoporosis (Aubin, J.E., 1998). Therefore, osteoblasts and osteoclasts are known to have close relationships in the process of remodeling and the relationship is also continuous (Fig. 1).

A coculture system of spleen cells with osteoblastic cell or bone marrow stromal cells has been established to produce osteoclasts (Takahashi, N. 1999). In the coculture system, osteoclast-like cells are formed from spleen cell in the presence of such stimulators of bone resorption as interleukin 6 (IL-6), IL-11, parathyroid hormone (PTH), prostaglandin E_2 (PGE_2), and 1α,25-dihydroxyvitamin D_3 (Fuller, K. *et al*., 1998). Therefore, mature osteoclasts are produced by the action of osteoblastic cells as a results of many stimulators. The cell-to-cell interaction between osteoblasts/ stromal cells and osteoclast progenitors in the

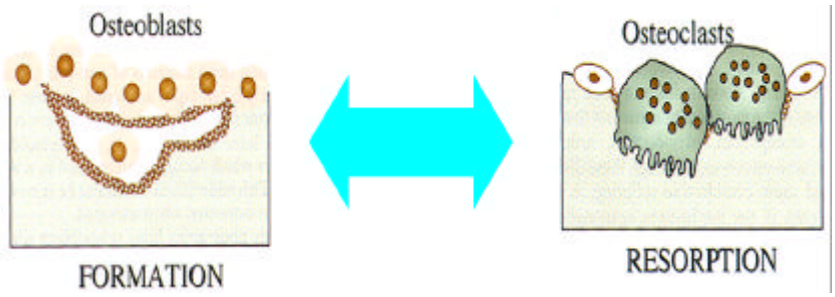

Fig. 1. General outline of bone remodeling. Bone remodeling is regulated by the activity of bone forming osteoblasts and bone resorbing osteoclasts.

cocultures has been found to be essential for the osteoclast formation (Fig. 2).

In the process of developing mature osteoclasts, certain kinds of signal molecules which are expressed from the osteoblasts have major role in osteoclastogenesis. Recently, many researchers have tried to elucidate the role of these signal molecules in the process of osteoclastogenesis. As a result of stimulation of osteotropic factors such as parathyroid hormone, signal molecules seem to act on osteoblast/ stromal cells to induce osteoclastogenesis hypothesized that a membrane-bound, designated as "receptor activator of NF-κB ligand (RANKL)," is expressed on osteoblasts/ stromal cells in response to osteotropic factors, and that it transduces, to osteoclast progenitors, a signal essential for osteoclastogenesis, through cell-to-cell interaction (Jimmi, E. *et al*., 1999). It is now known that in order to differentiate mature osteoclasts from osteoclast progenitors, RANKL which is produced from osteoblast and macrophage-colony stimulating factor (M-CSF) are required also. M-CSF is known to be the survival factor of the osteoclastogenesis and is essential for the macrophage to be transformed into osteoclasts. However, osteoprotegerin (OPG), a decoy receptor of RANKL is released from osteoblasts to inhibit the osteoclastogenesis. OPG is a secreted member of the tumor necrosis factor receptor (TNFR) family, and it inhibits osteoclastogenesis stimulated by $1,25(OH)_2D_3$, PTH, or IL-11

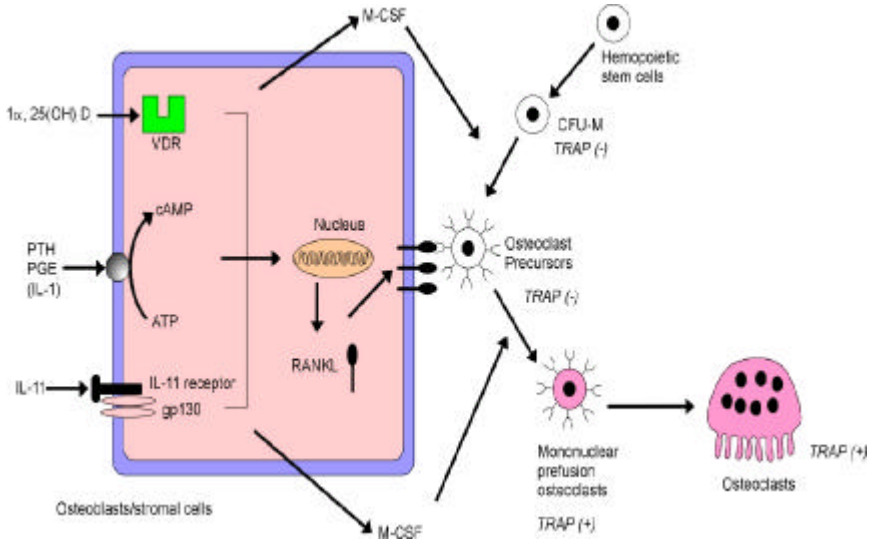

Fig. 2. Schematic diagram of osteoclast differentiation. Mature osteoclasts are formed from macrophage in the presence of stimulators as $1\alpha,25(OH)_2D_3$, PTH, PGE_2 or IL-11.

(Takahashi, N. *et al*., 1999). It is believed that RANKL, M-CSF and OPG which express from osteoblasts play an essential roles in regulation of osteoclastogenesis and osteoblast is the major factor in the process of bone remodeling (Fig. 3).

Recently, it is reported that Na/ K/ Cl cotransporter (NKCC1) is present in osteoblasts. Simply, it may be possible that NKCC1 is involved in the bone remodeling process. So, our question was what the role of NKCC1 is in osteoblast. Of course, NKCC1 is now known to be present in numerous, diverse tissues from a wide variety of animal species, and their functions are ion transport across secretory and absorptive epithelia, maintenance and regulation of cell volume and ion gradients, and possibly modulation of cell growth and development (Haas, M., Forbush, B., 1998). It can be hypothesized that NKCCl may cencern osteoclastogenesis in respect to the RANKL, OPG, M-CSF mRNA expression. Because there are several facts to be assumed that NKCC1 is involved in osteoclastogenesis, those are 1) NKCCl cotransporter exists in osteoblasts, 2) NKCCl transports the ion inward and outward across the epithelia and play an important role in regulating the cell size and volume, and 3) NKCCl affects the development and growth of the cells (Russell, J.M. *et al*., 2000). Nevertheless there is no plausible explanation for the role of NKCC1 in osteoblast in concern with osteoclastogenesis (Fig. 4). Therefore, we aimed at revealing the role of NKCC1 in osteoblast, inhibiting the NKCC1 activity using bumetanide which

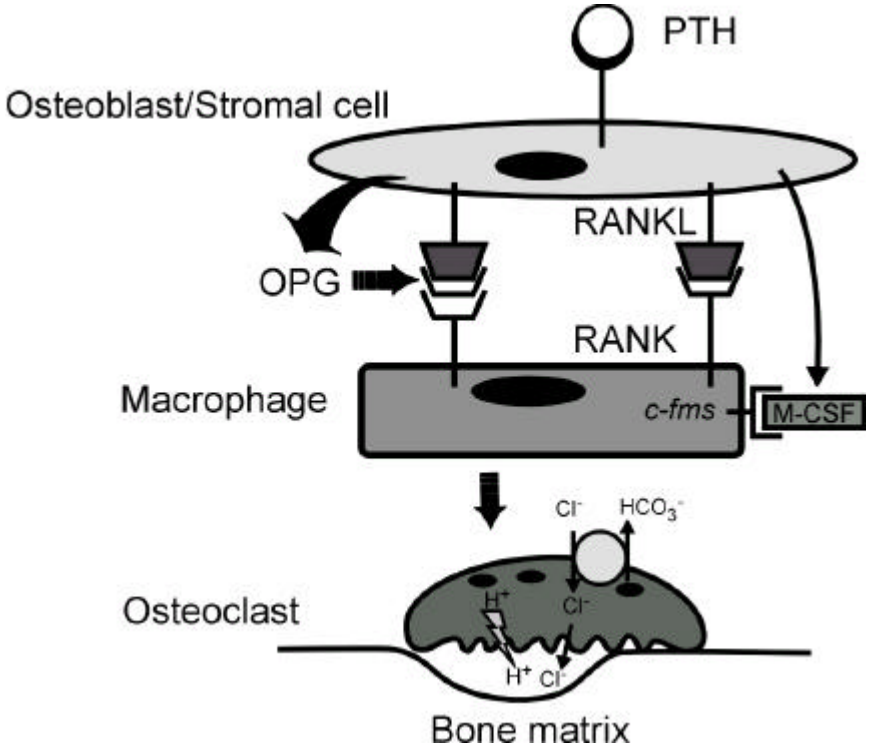

Fig. 3. Mechanisms of osteoclastogenesis and osteoclastic bone resorption. Stromal cells/ osteoblasts express RANKL and M-CSF, which are up-regulated by osteoclastogenic molecules such as PTH. PTH also blunts expression of OPG. RANKL and M-CSF, interacting with their receptors on monocyte-macrophage cells, induce osteoclast differentiation, a process inhibited by OPG. The differentiated osteoclast polarizes on the bone surface. After formation of the ruffled membrane, the osteoclast acidifies an extracellular microenvironment by means of proton pump. Intracellular pH is maintained by HCO_3^-/ Cl^- exchange.

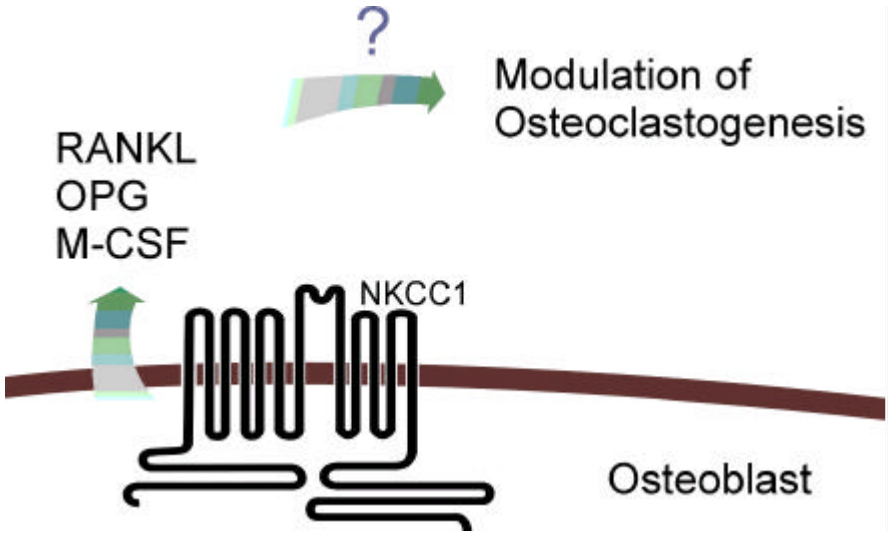

Fig. 4. Hypothetical model for modulation of osteoclastogenesis. The above model for NKCC1 and the osteoclastogenesis was inferred from the evidences that NKCC1 is exist in osteoblast, and play an important role in regulating the cellular size and volume. Therefore, blocking the NKCC1 might be a potent regulator of both bone formation and bone resorption.

is a specific inhibitor of NKCC1. Unexpectedly, we found that bumetanide inhibited the osteoclastogenesis in coculture system, osteoblast/ stromal cell. This fact give us the new clues that NKCC1 is probably responsible for the down-regulation of osteoclastogenesis. Therefore, in this study we applied osteoblast/ stromal cell coculture system to evaluate 1) Effect of bumetanide on $1\alpha,25(OH)_2D_3$-induced osteoclast formation, 2) Decrease in cell volume by bumetanide, 3) Changes in mRNA expression profile in RANKL, OPG, and M-CSF, 4) Effect of bumetanide on t-JNK and p-JNK.

II. Materials and Methods

1. TRAP staining of osteoclasts

osteoblastic cells were isolated from 1 2 day-old newborn mice. 30 50 calvariae were subjected to digestions using 10 ml enzyme solution containing 0.2% collagenase (Wako, Japan) and 0.1% dispase (GiBCO BRL, U.S.A) for 20 minutes at 37。C in a shaking water bath. The supernatant was discarded and 10 ml enzyme solution was added. After shaking at 37。C for 20 minutes, the supernatant was then collected carefully and transferred into a new tube. This digestion of calvariae with collagenase-dispase was repeated three times. Collected supernatant (30 ml) was applied to centrifuge at 1,500×g for 10 minutes to collect the osteoblastic cells. Collected cells were resuspended in α-MEM containing 10% fetal calf serum (FCS) and cultured in 10 cm culture dishes at a concentration of 10^5 cells/ dish to confulence. Cells were then detached from culture dishes by trypsin-EDTA, suspended in α-MEM with 10% FCS and used for coculture as osteoblastic cells (Lacey, D. *et al*., 1998). Femorial and tibial bone marrow cells were collected from 4-week-old mice. Tibiae and femora were removed and dissected free of adhering tissues. The bone ends were removed and the marrow cavity was flushed by slowly injecting media in at one end using a 25-gauge needle. The collected calvariae and bone marrow cells were washed and used for the coculture (Fig. 5).

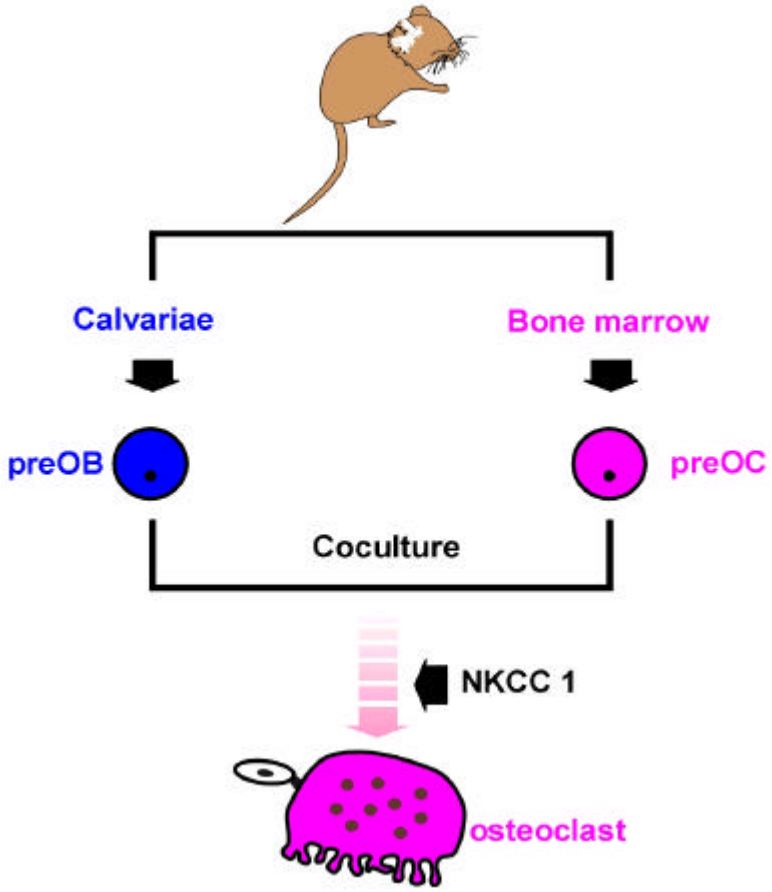

Fig. 5. Coculture system of osteoblastic cell and bone marrow. Mouse calvarial cells (1×10^4 cells/ well) were cocultured with bone marrow cells (1×10^5 cells/ well) in α-MEM containing 10% fetal calf serum in 48-well plates. All cultures were maintained at 37。C in a humidified atmosphere of 5% CO_2 in air. After treatment, the cells were subjected to tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) staining.

2. Induction of osteoclasts in coculture

Mouse osteoblastic cells (1×10^4 cells/well) were cocultured with bone marrow cells (1×10^5 cells/well) in α-MEM containing 10% fetal calf serum in 48-well plates (Corning Inc., Corning, NY). Culture volume was made up to 400 μl per well with α-MEM supplemented with 10% fetal calf serum (FCS), in the presence of $1\alpha,25(OH)_2D_3$ (10 nM), with or without bumetanide. All cultures were maintained at 37。C in a humidified atmosphere of 5% CO_2 in air. After treatment, the cells were subjected to tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) staining.

3. Viability test (MTT assays)

MTT (3-〔4,5-dimethylthiazol-2-yl-〕-2,5-diphenyltetrazolium bromide) test uses the principle which tetrazolium salts be reduced by reducing enzyme (succinate dehydrogenase) of mitochondria so that the toxicity of viable cells and the cellular differentiations can be measured. MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml and filtered to remove any insoluble residues (Sladowski, D. *et al*., 1993). The MTT solution was added directly to the assay plates at a rate of 10 μl to 100 μl cell culture medium and then the cells were incubated for a further 4 hours at 37。C. The purple formazan

crystals formed were dissolved by the addition of acidic isopropanol (100 μl 0.04 N HCl inisopropanol) followed by thorough mixing. The plates were subsequently read on a spectrophotometer at 570nm and 630 nm.

4. Measurement of mean cell volume

Poly-L-lysine coated cover glass on which cells were cultured was mounted on a thermal stage (37。C), which was set on the mechanical stage of an inverted microscope connected to a video-imaging system. The cells were perfused with the 0.01% w/v-L-lysine solution (McCarty, N.A., O'Neil, R., 1991). The images of the optical microscope were continuously recorded with a video-imaging system. To estimate cell volume, the area of osteoclasts in the video image (A) was measured. The averaged value of area measured in the first 2 min was used as the control (A_0). The relative cell volume, $V/V_0 = (A/A_0)^{1.5}$ was estimated, where V is the volume, A is the area, and subscript $_0$ is the value of the control.

5. **Reverse transcriptase-polymerase chain reaction (RT-PCR)**

cDNA can be polimerized from mRNA through reverse transcription procedure and then amplified by polymerase chain reaction (PCR). RT-PCR is used not only in the qualitative but

also in the quantitative analysis of mRNA expressed in cells.

RT-PCR experiment consists of RNA separation, cDNA polymerization and PCR amplification.

Trizole reagent, chloroform, isopropyl and ethanol were used to separate RNA from cells. Quantitative analysis of extracted tRNA for the purity analysis was done. Next step is to prime the mRNA for cDNA synthesis. In the first, a 3' (antisense) gene-specific primer is annealed to the mRNA and extended with reverse transcriptase. This generates a cDNA template for the 5' (sense) primer. When priming cDNA with a gene-specific primer, a number of experimental parameters may need to be optimized, including primer concentration and annealing temperature. Five to ten microliter dilute cDNA is used for each 50 μl PCR reaction. In order to compare the results easily, the data were run through denstomery. In that way, the density of results of RT-PCR can be easily distinguished.

6. Western blot

The protein extracts from osteoblastic cells were prepared. The bone marrow cell were resuspended in the prewarmed cracking buffer (80 mM Urea, 0.05% SDS, 0.4% 10 mM Tris-HCl, 1 μM EDTA, 40 μg/ml Bromophenol blue, 10 μl/ml β-Mercaptoethanol, 70μl/ml protease inhibitor solution, 50 μι 100× PMSF, 60。C), and transferred to a tube containing glass

beads (425-600 μm ; Sigmas #G-8772) to disrupt the bone marrow cells. The cell resuspensions with glass beads were lysed by heating at 70。C for 10 min followed by vigorous vortexing for 1 min. The lysates as protein extracts were separated from unbroken cells by centrifugation at 14,000 rpm for 5 min. For a western blot analysis, protein extracts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad). The blot was incubated in a blocking solution containing 4% bovine serum albumin (BSA) and 10% normal goat serum (NGS) in phosphate buffered saline plus 0.15% Tween-20 (PBS-T) at RT for 2 hrs. The blot was then probed with the primary antibody (monoclonal antibody of p-JNK or t-JNK, Clontech) for overnight at 4。C, followed by detection with a horseradish peroxidase-linked goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc) and enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

7. Data analysis

Comparison between groups was performed by the Student's *t*-test. Significant differences from each other were determined at $P < 0.05$.

III. Results

1. Effect of bumetanide on 1α,25(OH)$_2$D$_3$-induced osteoclast formation

To investigate the effect on the differentiation of osteoclast, 1, 10, and 100 μM bumetanide were added to the osteoblastic cell/ bone marrow coculture with 1α,25(OH)$_2$D$_3$. 1α,25(OH)$_2$D$_3$ was used to induce osteoclast differentiation. Ten nanomolar 1α,25(OH)$_2$D$_3$ was added to the coculture, and 42 TRAP positive multinucleated cells were formed whereas no TRAP positive cell was detected when coculture was incubated in media only. The addition of 10 or 100 μM bumetanide decreased the number of TRAP positive multinucleated cells up to 15 or 8 cells per well, respectively. However, 1 μM bumetanide did not change 1α,25(OH)$_2$D$_3$-induced osteoclast differentiation (Figs. 6, 7). The effect of bumetanide on differentiation of osteoclasts showed in dose dependent manner.

2. Evaluation of changes in cell viability by bumetanide

To demonstrate the effect of bumetanide on the cell proliferation, MTT assay was performed according to the method of Ferrari *et al.* 1989. One, ten, and a hundred micromolar bumetanide was added to the coculture and incubated for 3 days

10 nM 1α,25(OH)$_2$D$_3$ 10 nM 1α,25(OH)$_2$D$_3$ media only
+ 10 μM bumetanide

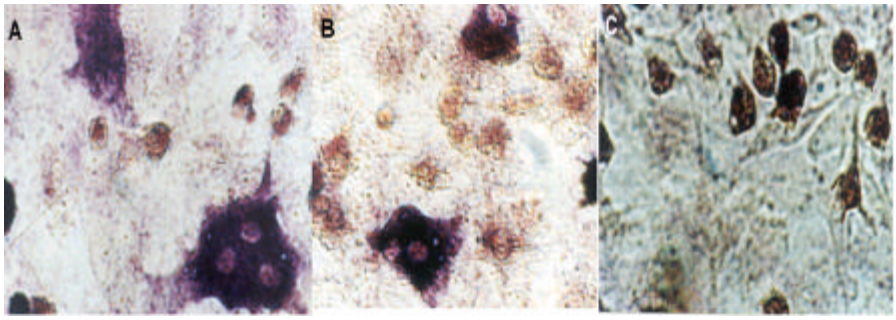

Fig. 6. Inhibition of osteoclast differentiation by 10 μM bumetanide. Ten micromolar bumetanide was added to osteoblastic cell/ bone marrow coculture to block NKCC1. Cells were treated with bumetanide and/ or 1α,25(OH)$_2$D$_3$. After incubation for 4 days, cells were stained with TRAP staining method. Panels A, 10 nM 1α,25(OH)$_2$D$_3$; B, 10 nM 1α,25(OH)$_2$D$_3$ and 10 μM bumetanide; C, media only.

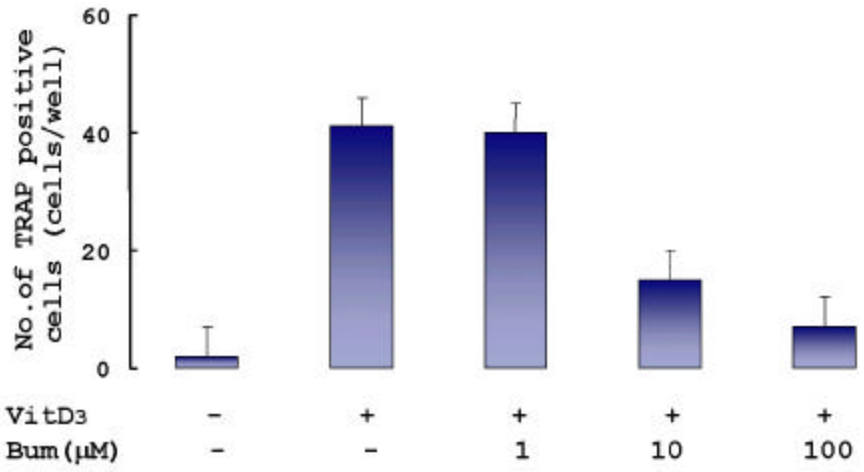

Fig. 7. Changes in $VitD_3$-induced TRAP positive cells in response to bumetanide (Bum). Bumetanide (1, 10, 100 μM) was added to the osteoblastic cell/ bone marrow coculture in the presence of 10 nM $1\alpha,25(OH)_2D_3$. After incubation for 4 days, cells were stained by TRAP staining method. TRAP positive multinucleated cells that have more than 3 nucleus were counted.

(Fig. 8). The inhibitions of cellular proliferation by bumetanide were less than 10% of control which was incubated with medium only. This implies that bumetanide did not show toxic effect when added up to 100 μM to the coculture. Therefore, the results mean that the effect of bumetanide on differentiation was caused by controlling NKCC1, not by its toxic effects on the cell.

3. Decrease of cell volume by bumetanide

Ten micromolar bumetanide reduced the relative cell volume to the half level (Fig. 9). During the first 24 minutes, normal media was perfused through the cover glass for stabilization of osteoclasts, and then during the next 20 minutes, 10 μM bumetanide was perfused. The relative cell volume was reduced from one to 0.6. The original volume of osteoclasts was represented as V_0 and testing volume, as V. V/V_0 was less than 1, it was regarded as the reduction of cell volume. As a result, the relative cell volume was reduced from 1 to about 0.6 level, suggesting that the bumetanide reduced the relative volume of the osteoclasts. During the last 20 minutes normal media was perfused again for the recovery of cell volume. Under the circumstance of cell perfused with normal media, cell volume was not completely recovered to the original volume. However it is obscure what happens in the cell volume regulation before and after bumetanide treatment.

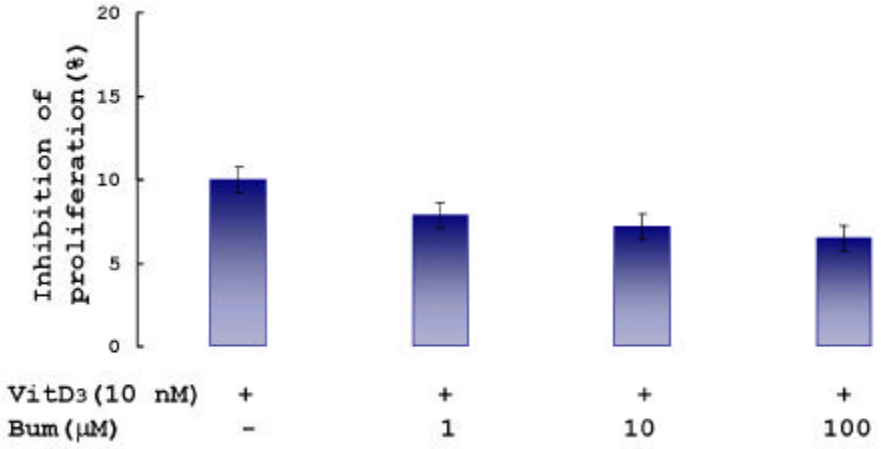

Fig. 8. Effect of bumetanide on cell proliferation in coculture. Bumetanide (1, 10, 100 μM) was added to the osteoblastic cell/ bone marrow coculture in the presence of 10 nM 1α,25(OH)$_2$D$_3$. After incubation for additional 4 days, MTT assays were carried out according to the method described in materials and methods.

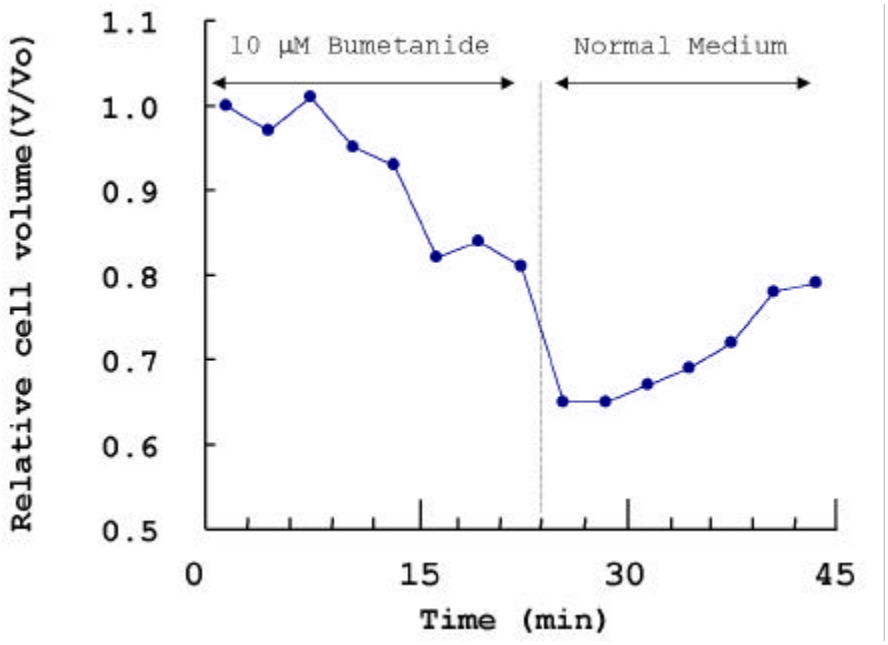

Fig. 9. Effect of bumetanide on osteoblastic cell volume. Ten micromolar bumetanide was treated to osteoblastic cell. In the presence of bumetanide, cell volume was decreased suggesting that the blockade of NKCC1 activity caused the decrease in osteoblastic cell volume. After exposing the osteoblastic cell to the normal media for 30 min, 10 μM bumetanide was treated to osteoblastic cell.

4. Changes in mRNA expression profile in RANKL, OPG, and M-CSF

Osteoclastogenesis is known to be mediated and modulated by the secretion of RANKL, OPG, and M-CSF by osteoblastic cells (Figs. 10, 11, 12). Therefore, bumetanide was added to the osteoblastic cell culture and incubated for 3 days. Total RNA was isolated and the expressions of RANKL, OPG, and M-CSF were monitored by RT-PCR. As the bumetanide concentration added to the osteoblastic cell culture increased, the expression of RANKL mRNA induced by $1\alpha,25(OH)_2D_3$ was down-regulated. The decrease of RANKL expression was dependent on the increase in bumetanide concentration. Moreover, the expression of OPG mRNA, a novel TNF receptor family member was increased. These findings indicate that bumetanide inhibits osteoclast differentiation via inhibiting the expression of RANKL mRNA and enhancing the expression of OPG mRNA. However the expression of M-CSF, a survival factor of osteoclasts, was not changed regardless of bumetanide concentration. β-actin was used as the control of the mRNA expression in the osteoblastic cells.

5. Ratio of RANKL/OPG mRNA in response to bumetanide

Ratio of RANKL/ OPG mRNA was illustrated in Figure 13. As the bumetanide concentration was increased, the ratio of

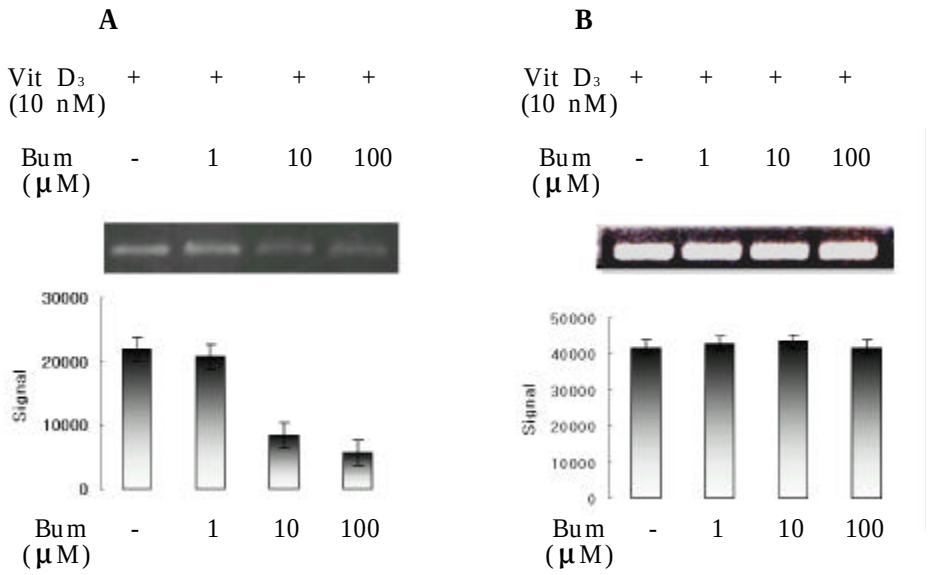

Fig. 10. Effect of bumetanide on the expression of RANKL mRNA in osteoblastic cell. Panels A, RANKL; B, β-actin. Bumetanide (1, 10, 100 μM) were added to the osteoblastic cell in the presence of 10 nM 1α,25(OH)$_2$D$_3$. After incubation for 2 days, total RNA was isolated and monitored RANKL mRNA using RT-PCR.

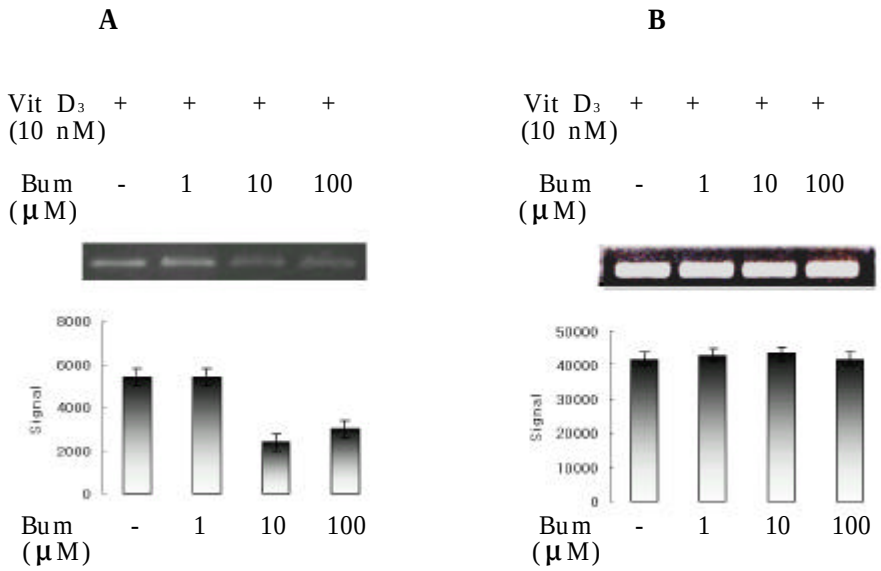

Fig. 11. Effect of bumetanide on the expression of M-CSF mRNA in osteoblastic cell. Panels A, M-CSF; B, β-actin. Bumetanide (1, 10, 100 μM) were added to the osteoblastic cell in the presence of 10 nM $1\alpha,25(OH)_2D_3$. After incubation for 2 days, total RNA was isolated and monitored M-CSF mRNA using RT-PCR.

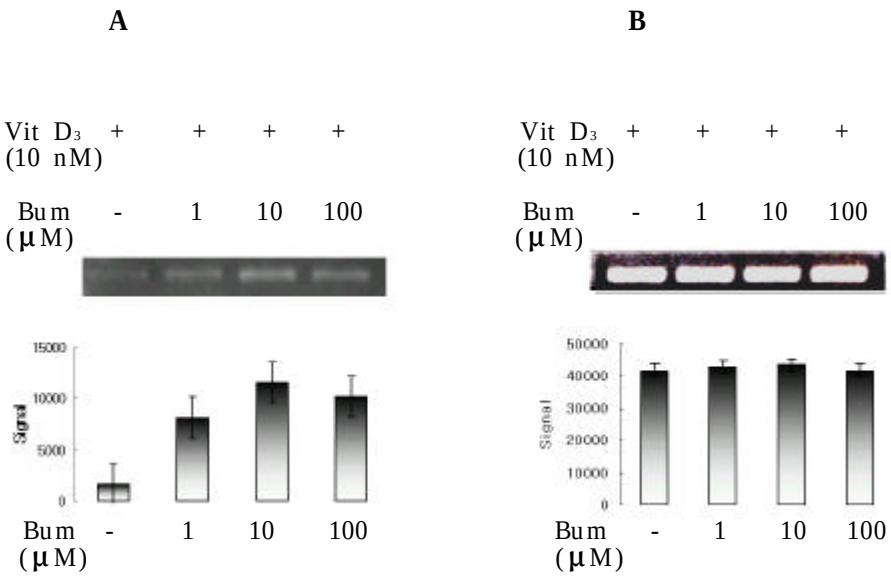

Fig. 12. Effect of bumetanide on the expression of OPG mRNA in osteoblastic cell. Panels A, OPG; B, β-actin. Bumetanide (1, 10, 100 μM) were added to the osteoblastic cell in the presence of 10 nM $1\alpha,25(OH)_2D_3$. After incubation for 2 days, total RNA was isolated and monitored OPG mRNA using RT-PCR.

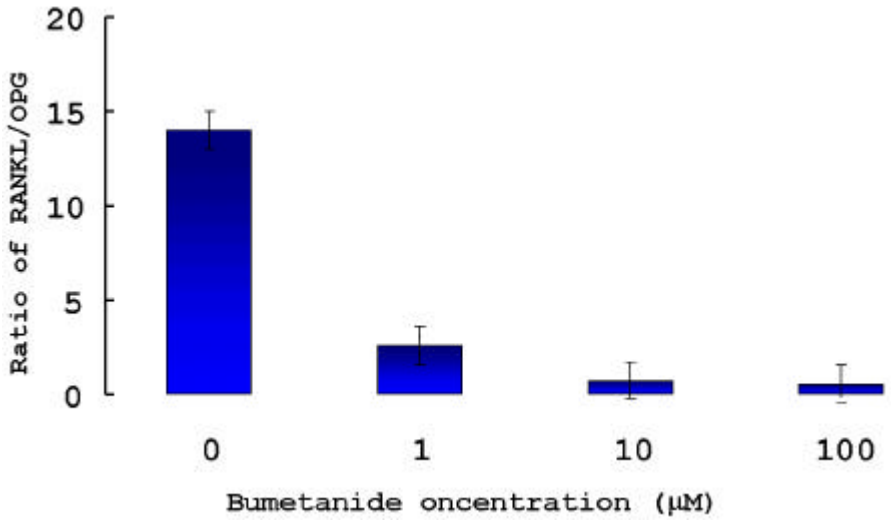

Fig. 13. Relative expression of RANKL and OPG mRNA in osteoblastic cells. Agarose gel electrophoresis was carried out and densities of the bands were analyzed with densitometer. Relative ratio of the RANKL and OPG mRNA was calculated based on the density of each band.

RANKL/ OPG mRNA was decreased. This means that bumetanide caused the changes in mRNA expression (RANKL and OPG) of signal molecules which are closely linked to the osteoclastogenesis. Consequently, bumetanide inhibited osteoclastogenesis, leading to the alteration of RANKL, OPG mRNA expression. Also such changes in expression levels of signal molecules were dependent on bumetanide concentration.

6. The effect of bumetanide on t-JNK and p-JNK

c-Jun NH_2-terminal kinase (JNK), one of the mitogen activated protein kinase (MAPK) family, has been known to exhibit activation by hyper osmolarity and cellular volume change (Janet *et. al.*, 1999). To investigate the effect of bumetanide on the expression and phosphorylation of JNK (p-JNK), bumetanide was added to the osteoblastic cells and incubated for 3 days.

Bumetanide did not have any effect on the expression of JNK. Total amount of JNK (t-JNK) was remained constant in the range of bumetanide concentration to be used. However, bumetanide augmented the phosphorylation of JNK in the dose dependent manner, indicating that bumetanide activated the JNK in the process of osteoclastogenesis. (Fig. 14).

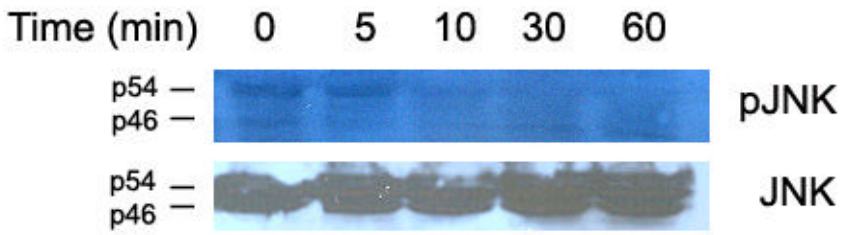

Fig. 14. Effect of bumetanide on the activation of JNK. Osteoblastic cells were exposed to 10 μM bumetanide for indicated time. Anti-phospho-JNK or anti-JNK antibody was used as a primary antibody for the Western blot.

IV. Discussion

In the process of developing mature osteoclasts, the functions of osteoblasts and osteoclasts are intimately related. During skeletal development and bone remodeling process, osteoblasts synthesize and secrete signal molecules that control osteoclast differentiation (Ducy *et al*., 2000) and cell-to-cell interactions are necessary during the whole procedure. This is a direct and crucial interaction that has been well established *in vivo* (Burgess *et al*., 1999). Thus osteoblasts play a crucial role in differentiating the macrophage/ monocyte to mature osteoclasts. However, it has been only 4-5 year since clear mechanism of maturation of macrophages into osteoclasts has been identified. There are two molecules that are essential and sufficient to promote osteoclastogenesis: M-CSF and RANKL (Teitelbaum, S., 2000). M-CSF binds to its receptor, c-fms, on macrophage to provide signals required for their survival and proliferation. OPG is known to be a soluble "decoy" receptor that competes with RANK for RANKL. It has been described that the balance between the expression of the stimulator of osteoclastogenesis, RANKL, and of the inhibitor, OPG, is the crucial factor that governs the amount of bone resorbed.

In the conjuction of machineries in osteoblast, it has been reported that Na/ K/ Cl cotransporter (NKCC1) is present in osteoblasts recently. We still do not know the role of NKCC1 in

osteoblast. Nevertheless, it may be possible to hypothesize that the NKCC1 is involved in the bone remodeling process, judging from the facts that 1) NKCC1 cotransporter exists in osteoblasts, 2) NKCC1 transports the ion inward and outward across the epithelia and play an important role in regulating the cell size and volume, and 3) NKCC1 affects the development and growth of the cells (Russell, J.M. *et al*., 2000). However, there is no plausible explanation for the role of NKCC1 in osteoblast in concern with osteoclastogenesis (Fig. 4). Therefore, we aimed at revealing the role of NKCC1 in osteoblast, inhibiting the NKCC1 activity using bumetanide.

Unexpectedly, we found that bumetanide inhibited the osteoclastogenesis in coculture system, osteoblast/ stromal cell. This fact give us the new clues that NKCC1 is probably responsible for the down-regulation of osteoclastogenesis. Therefore, in this study we applied osteoblast/ stromal cell coculture system to evaluate 1) effect of bumetanide on $1\alpha,25(OH)_2D_3$-induced osteoclast formation, 2) decrease in cell volume by bumetanide, 3) changes in mRNA expression profile in RANKL, OPG, and M-CSF, 4) Effect of bumetanide on t-JNK and p-JNK.

As shown in Fig 6 and 7, when 10 and 100 μM bumetanide was applied to the coculture system, the number of TRAP positive cells were markedly reduced in a dose dependent manner compare to the control group. However, 1 μM

bumetanide did not change 1α,25(OH)$_2$D$_3$-induced osteoclast differentiation. Moreover, cell viability test showed that bumetanide did not show toxic effect when added up to 100 μM to the coculture (Fig. 8). It is clear that bumetanide has some effects on formation of TRAP positive cell with no toxic effects. So, cell volume was measured to determine whether bumetanide has any effect on cell volume since NKCC1 plays a major role in controlling the volume of cells. As shown in Fig. 9, 10 μM of bumetanide reduced the relative cell volume to the half level.

Osteoclastic bone resorption consists of multiple steps such as the differentiation of osteoclast precursors into mononuclear prefusion osteoclasts (pOC), the fusion of pOCs to form multinucleated osteoclasts, and the activation of osteoclasts to resorb bone. Several factors related in the differentiation, maturation and activation of osteoclasts have been reported, which include RANKL, OPG and M-CSF. Since osteoclast differentiation has been reported to be mediated by these factors, mRNA expressions were investigated. The expression of RANKL mRNA induced by 1α,25(OH)$_2$D$_3$ was down-regulated with the increase of bumetanide concentration. Moreover, the expression of OPG mRNA, a novel TNF receptor family member was increased. These findings indicate that bumetanide inhibits osteoclast differentiation via inhibiting the expression of RANKL mRNA and enhancing the expression of OPG mRNA.

On the other hand, as an inhibitor of NKCC1, bumetanide

inhibits cellular uptake of Na^+, K^+, and Cl^- ions, which results in the cellular volume change. O'Donnell *et. al.* demonstrated that phosphatase inhibition results in the elevation of endothelial cell volume, thus suggesting that resting endothelial cell volume is also determined by the integrated activities of kinases and phosphatases. The stimulation of co-transporter by cell shrinkage was not enhanced by phosphatase inhibition, indicating that the effects of shrinkage and phosphatase inhibition were not additive, suggesting that shrinkage inhibits the phosphatase. In this study, bumetanide did not have any effect on the expression of JNK. Total amount of JNK (t-JNK) was remained constant in the range of bumetanide concentration to be used but the activated form of JNK, phosphorylated JNK (p-JNK), was decreased according to the increase of bumetanide concentration. JNK is one of the mitogen activated protein kinase (MARK) family. It has been known to exhibit activation by hyper-osmolarity and cellular volume change. The decrease of p-JNK indicates that the cell volume change of osteoclasts are due to activation of JNK in osteoclasts.

From these results, it might be inferred that cellular shrinkage of osteoblastic cell induced by the addition of bumetanide, which results the inhibition of NKCC1 activity, is an important modulator in regulating osteoclastogenesis.

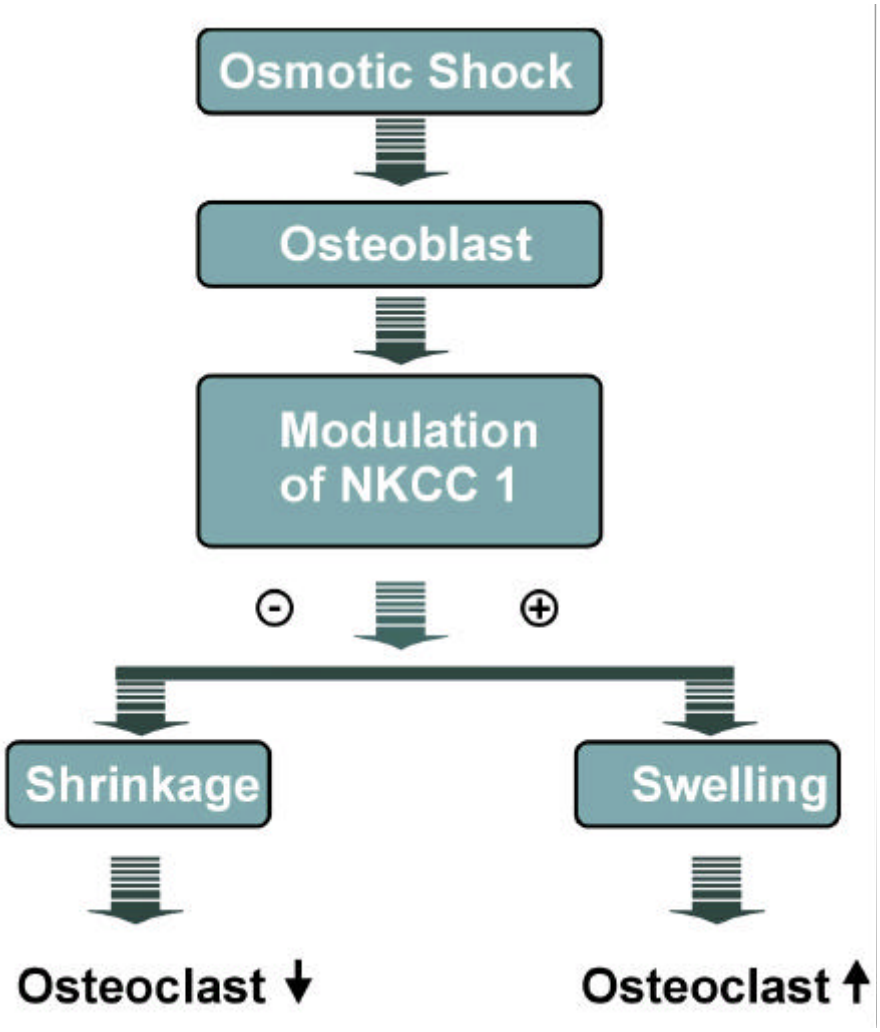

Fig. 15. Proposed model for the alteration of osteoclastogenesis via NKCC1 modulation. The osmotic shock on osteoblastic cell results in modulation of NKCC1. It is proved that the shirinkage in osteoblasts result in decrease of osteoclast activity.

V. Conclusion

In the process of osteoclastogenesis, there are several factors that inhibit or stimulate the whole process. The mature osteoclasts are produced in the presence of osteoblasts and osteoclast precursor cell together with cell-to-cell interaction. Several signal molecules, known as RANKL, OPG, and M-CSF which expressed from osteoblasts have important functions in the process of osteoclastogenesis.

There are several ways to control osteoblasts in the process of osteoclastogenesis but the role of Na-K-Cl cotransporter of osteoblasts related to osteoclast differenciation has not been clarified. Since it is reported that the presence of NKCC1 in the membrane of osteoblasts and the fact that it controls the ion transports and cell volume, possibilities of NKCC1 engaged in the process of osteoclastogenesis might be presumed.

Therefore, bumetanide, a specific inhibitor of NKCC1, was used in the coculture system of osteoblasts and osteoclasts to evaluate the effect of NKCC1 on osteoclastogenesis,. When 10 and 100 μM of bumetanide was used, inhibition of osteoclasts was noted. The results were run through MTT test to evaluate the toxic effect of bumetanide on osteoclasts and proved that bumetanide concentration lower than 100 μM does not effect the cell proliferation.

The changes in the cellular volume of osteoclasts which

resulted from the effect of bumetanide were measured. The results showed that the relative cell volume was reduced to the half level. To confirm the cell volume changes of osteoclasts, the modulator for cell volume change, the JNK of osteoclasts was analyzed. The result showed that the activated form of JNK was increased according to increase of bumetanide concentration. Moreover, the mRNA expression of the signal molecules, known as RANKL, OPG, and M-CSF were measured. The results showed that the mRNA expression of RANKL and M-CSF were decreased and that of OPG was increased.

These results suggest that when bumetanide, a specific inhibitor of NKCC1, is used, the process of osteoclastogenesis is inhibited by controlling the mRNA expressions of signal molecules such as RANKL and OPG in osteoblasts and also by reducing the relative cellular volume of osteoclasts.

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국문 요약

Na-K-Cl cotransporter의 비활성화가 비타민 D_3에 의해 유도되는 파골세포의 분화에 미치는 영향

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김은영

Na-K-Cl cotransporter (NKCC1)은 다양한 세포와 조직에 분포하며 NKCC1의 기능은 세포 내외로 이온을 전달하고 세포의 부피와 이온의 균형을 이루고 또한 세포의 성장과 발육에 영향을 준다. 이 연구에서는 비타민 D_3에 의해 유도되는 파골세포에 NKCC1이 미치는 영향을 검토하였다. NKCC1의 선택적 억제제인 bumetanide를 10, 100 μM 첨가하였을 경우 TRAP 양성 세포의 수는 각 well 당 평균 42에서 15로 감소하였다. Bumetanide 1 μM를 첨가하였을 경우 비타민 D_3에 의해 유도되는 파골세포의 분화에는 영향을 주지 못하였다. 결과적으로 NKCC1의 억제로 인하여 파골세포의 분화가 억제된 것을 알 수 있다. Bumetanide의 억제 효과를 확인하기 위하여 RT-PCR을 통한 RANKL, OPG 그리고 M-CSF의 mRNA의 발현을 측정한 결과 조골세포에 대한 bumetanide의 농도가 높아질수록 RANKL과 M-CSF의 발현 정도는 감소하였고 RANKL의 decoy receptor인 OPG의 발현 정도는 증가하였다. 이러한 결과로 부터 조골세포에 bumetanide를 첨가하는 경우 조골세포내의 RANKL, OPG, M-CSF의 발현을 조절하여 파골세포로의 분화가 억제됨을 확인하였다. 또한 bumetanide를 첨가하여 NKCC1을 억제할 경우 원

래 파골세포의 부피에 비하여 상대적인 파골세포의 부피가 36% 정도까지 감소하였으며, 이러한 변화의 기전을 밝히기 위하여 세포의 용적 변화의 매개 물질인 JNK를 검사한 결과 약물의 농도가 증가할수록 활성화된 JNK가 증가하는 것을 확인할 수 있었다.

결과적으로 NKCC1은 파골세포의 분화에 중요한 역할을 가지고 있으며, bumetanide에 의하여 억제된 NKCC1의 활성은 조골세포의 부피의 변화를 초래하고, 파골세포 분화 조절 인자인 RANKL, M-CSF의 감소 및 OPG의 증가를 유발한다.

핵심되는 말 : NKCC1, bumetanide, 파골 세포, RANKL, OPG, JNK