EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 35, No. 3, 167-174, June 2003

High extracellular Ca^{2+} alone stimulates osteoclast formation but inhibits in the presence of other osteoclastogenic factors

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Accepted 23 April 2003

Abbreviations: CA2, carbonic anhydrase 2; Ca^{2+}_e, extracellular Ca^{2+}; CTR, calcitonin receptor; CTSK, cathepsin K; M-CSF, macrophage colony-stimulating factor; MMP-9, matrix metalloproteinase-9; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; RT-PCR, reverse transcription-PCR; TRAP-positive MNCs, tartrate-resistant acid phosphatase-positive multinucleated cells; VNR, vitronectin receptor; 1,25-$(OH)_2$vitD_3, 1,25-dihydroxyvitaminD_3

Abstract

High ambient Ca^{2+} at bone resorption sites have been implicated to play an important role in the regulation of bone remodeling. The present study was performed to clarify the mode of high extracellular Ca^{2+} (Ca^{2+}_e)-induced modulation of osteoclastogenesis and the expression of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG), thereby to define its role in osteoclast formation. Mouse bone marrow cells were cocultured with osteoblastic cells in the absence or presence of osteoclastogenic factors such as 1,25-dihydroxyvitaminD_3 (1,25-$(OH)_2$vitD_3) and macrophage colony-stimulating factor/soluble RANKL. Ca^{2+} concentration in media (1.8 mM) was adjusted to 3, 5, 7 or 10 mM. Osteoclast formation was confirmed by the appearance of tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells and the expression of osteoclast phenotypic markers (calcitonin receptor, vitronectin receptor, cathepsin K, matrix metalloproteinase-9, carbonic anhydrase 2). High Ca^{2+}_e alone significantly stimulated osteoclast formation in a dose-dependent manner. However, in the presence of highly osteoclastogenic factors, high Ca^{2+}_e significantly inhibited osteoclastogenesis. High Ca^{2+}_e alone continuously up-regulated RANKL expression while only transiently increased OPG expression. However, in the presence of 1,25-$(OH)_2$vitD_3, high Ca^{2+}_e did not change the 1,25-$(OH)_2$vitD_3-induced RANKL expression while increased OPG expression. Taken together, these findings suggest that high Ca^{2+}_e alone increase osteoclastogenesis but inhibit in the presence of other osteoclastogenic factors. In addition, high Ca^{2+}_e-induced osteoclastogenesis may be mediated by osteoblasts via up-regulation of RANKL expression. Meanwhile up-regulated OPG might participate in the inhibitory effect of high Ca^{2+}_e on 1,25-$(OH)_2$vitD_3-induced osteoclastogenesis.

Keywords: high extracellular calcium; osteoblast; osteoclast; osteoprotegerin; RANKL

Introduction

Bone remodeling involves both bone resorption and bone formation and should be accurately controlled to maintain bone mass (Canalis *et al.*, 1988). Osteoblasts are bone-forming cells, derived from mesenchymal stem cells. Osteoclasts are multinucleated cells primarily responsible for bone resorption and derived from hemopoietic cells. Various cytokines and hormones can induce the differentiation of hemopoietic cells into osteoclasts and the cell-to-cell interaction between osteoblasts/stromal cells and hemopoietic cells is required for osteoclast formation *in vitro* (Suda *et al.*, 1992). It is becoming apparent that receptor activator of nuclear factor-κB ligand (RANKL) on the surface of osteoblasts/stromal cells interacts with RANK, a receptor for RANKL, on the hemopoietic cell surface, resulting in the osteoclast differentiation (Lacey *et al.*, 1998; Yasuda *et al.*, 1998a; Hsu *et al.*, 1999). Osteoprotegerin (OPG) functions as a decoy receptor for RANKL and inhibits osteoclast differentiation by interrupting the RANKL-RANK interaction (Simonet *et al.*, 1997; Yasuda *et al.*, 1998b). OPG is also secreted by osteoblasts/stromal cells. Thus, the regulation of the relative expression of RANKL and OPG in osteoblasts/stromal cells is critical in the modulation

of osteoclastogenesis.

Osteoclasts dissolve the mineralized matrix and release Ca^{2+} to the extracellular fluid, getting Ca^{2+} concentration at the resorption sites as high as 40 mM (Silver *et al.*, 1988). Extracellular Ca^{2+} (Ca^{2+}_e) is involved in the regulation of many cellular processes including growth, differentiation, and hormone secretion (Brown and MacLeod, 2001). Since both osteoblasts and osteoclasts have Ca^{2+}_e-sensing mechanism (Kameda *et al.*, 1998; Yamaguchi *et al.*, 1998), it is expected that released high Ca^{2+}_e might affect bone remodeling. Previous reports showed that high Ca^{2+}_e increased DNA synthesis and chemotactic response in osteoblastic MC3T3-E1 cells but directly inhibited osteoclastic activity in isolated osteoclasts (Zaidi *et al.*, 1989; Yamaguchi *et al.*, 1998). However, conflicting results have been demonstrated with respect to the effects of high Ca^{2+}_e on the osteoclast formation. Sugimoto *et al.* (1993) reported that the exposure of osteoclast precursor cells to high Ca^{2+}_e inhibited 1,25-$(OH)_2$vitD_3-induced osteoclast formation. The same group of investigators subsequently showed that high Ca^{2+}_e stimulated osteoclast formation in mouse bone cell cultures containing osteoblasts (Kaji *et al.*, 1996). There were also other reports showing that high Ca^{2+}_e inhibited osteoclast formation in 1, 25-$(OH)_2$vitD_3- or parathyroid hormone (PTH)-treated mouse spleen cell culture, or in 1,25-$(OH)_2$vitD_3 plus dexamethasone- treated coculture of mouse marrow cells and MC3T3- E1 cells (Kanatani *et al.*,1999; Shirai *et al.*, 1999). These findings indicate that the modulating effect of Ca^{2+}_e on osteoclast formation may depend on the microenvironment of osteoclast precursor cells such as the presence of bone resorbing hormones or osteoblasts.

The present study was performed to clarify the mode of high Ca^{2+}_e-induced modulation on the osteoclast formation and the expression of RANKL and OPG, thereby to define its role in osteoclast formation as well as overall regulation of bone remodeling.

Material and Methods

Materials

ICR mice were purchased from Daehan Experimental Animal Center (Umsung-Kun, Korea). α-Minimum essential medium (α-MEM), fetal bovine serum (FBS), trypsin-EDTA, other cultural reagents and SUPERSCRIPT First-Strand Synthesis System were obtained from Gibco BRL (Gaithersburg, MD). Plastic culture wares were obtained from Corning (Corning, NY). 1,25-$(OH)_2D_3$ was obtained from Calbiochem-Novobiochem Corp. (San Diego, CA) and macrophagecolony-stimulating factor (M-CSF) and soluble RANKL (sRANKL) from Peprotech (London, UK). Primers for reverse transcription-PCR (RT-PCR) were synthesized at TaKaRa and TaKaRa *Taq* was purchased from TaKaRa (Otsu, Japan). easy-BLUE reagent was purchased from iNtRON (Seoul, Korea). Reagents for tartrate-resistant acid phosphatase (TRAP) staining and all other reagents were obtained from Sigma (St. Louis, MO).

Isolation of mouse calvarial osteoblastic cells and bone marrow cells

Neonatal mouse calvarial osteoblastic cells were obtained as previously described (Cohn and Wong, 1979). Briefly, frontal and parietal bones were dissected from two- to three-day-old ICR mice and digested consecutively six times with enzyme mixture containing 0.1% collagenase, 0.05% trypsin, and 0.5 mM EDTA for 10, 10, 10, 20, 20, and 20 min, respectively. Calvarial cells, released in later stages (IV to VI), which exhibit osteoblastic characteristics, were pooled and cultured in α-MEM supplemented with 10% FBS, and the cells from second passage were used for the following experiments.

Mouse bone marrow cells were obtained from four- to five-week-old ICR mice. Tibiae and femurs were aseptically isolated and dissected free of adhering tissues. Both ends of bone were cut off and the marrow cavity was flushed with 5 ml of α-MEM. Red blood cells were lysed and the remaining cells were used for coculture.

Coculture of osteoblastic cells and bone marrow cells

Coculture of mouse osteoblastic cells and bone marrow cells for *in vitro* osteoclast differentiation was previously described (Kim *et al.*, 2002). Briefly, mouse calvarial osteoblastic cells and bone marrow cells were plated together at a density of 1.5×10^4 cells and 3×10^5 cells per well, respectively, in 24-well culture plates and cultured for 7 days in α-MEM supplemented with 10% FBS. During the culture periods, cells were exposed to different concentrations of Ca^{2+}_e in the presence or absence of 1,25-$(OH)_2$vitD_3. As there was 1.8 mM Ca^{2+} in α-MEM, Ca^{2+} concentrations in culture media were adjusted to 3, 5, 7 or 10 mM by addition of $CaCl_2$.

Enzyme histochemistry for TRAP

Staining for TRAP was performed according to the modified method of Burstone (1958). At the end of culture, the cells were fixed with ethanol-acetone (50:50, v/v) and were incubated for 20 min in acetate buffer (0.1 M sodium acetate, pH 5.0) containing naphthol AS-MX phosphate, fast red violet LB salt, and 20 mM sodium tartrate. The numbers of TRAP-

positive multinucleated osteoclast-like cells (MNCs) that contain 3 or more nuclei were counted under light microscope.

RT-PCR of osteoclast markers

To confirm the expression of osteoclast markers in this coculture system, total RNAs were isolated from cocultured cells using easy-BLUE reagent. One microgram of total cellular RNA was used for cDNA strand synthesis for RT-PCR (Superscrip First-Strand Synthesis System). PCR reactions (using 10% of first cDNA synthesis product) were carried out in 50 μl with TaKaRa *Taq* polymerase at the optimal temperature cycling protocol (Table 1). Amplified osteoclast markers were calcitonin receptor (CTR), vitronectin receptor (VNR), matrix metalloproteinase-9 (MMP-9), cathepsin K (CTSK), and carbonic anhydrase 2 (CA2). The sequences of PCR primers are presented in Table 2. Ten microliters of PCR products were analyzed by electrophoresis on a 1.2% agarose gel and visualized under the UV light after ethidium bromide staining.

Analysis of RANKL and OPG expression

To observe the effect of high Ca^{2+}_e on the expression of RANKL and OPG, mouse calvarial osteoblastic cells were exposed to various concentration of Ca^{2+}_e for 3, 24, 48 or 96 h in the presence or absence of 10 nM 1,25-$(OH)_2$vitD_3. Then total RNAs were isolated and RT-PCR was performed to detect the expression pattern of RANKL and OPG. PCR condition and PCR primer sequences are exhibited in Table 1 and 2. In addition, the expression of RANKL and OPG was also observed in the cells cocultured for 6 days.

Statistical analysis

All results were expressed as mean±SE The statistical significance was analyzed by Student's *t*-test. *P* values less than 0.01 were considered to indicate statistically significant difference.

Results

Effect of high Ca^{2+}_e on osteoclast formation in coculture of bone marrow cells and osteoblastic cells

First, we examined the effect of high Ca^{2+}_e on the osteoclast formation in the absence of added osteoclastogenic factors. As shown in Figure 1A, high Ca^{2+}_e significantly stimulated TRAP(+) MNCs formation in a dose-dependent manner. Treatment of cocultured cells with 10 nM 1,25-$(OH)_2$vitD_3 alone greatly stimulated TRAP(+) MNCs formation but increasing the media calcium concentration significantly suppressed 1,25-$(OH)_2$vitD_3-induced TRAP(+) MNCs formation (Figure 1B). Then we investigated the effect of 10 mM Ca^{2+}_e on the osteoclast formation in the presence of various concentrations of 1,25-$(OH)_2$vitD_3. As shown in Figure 2, 1,25-$(OH)_2$vitD_3 alone increased osteoclastogenesis dose-dependently in the range of 1 pM to 10 nM concentration. When the cells were exposed

Table 1. PCR condition.

CTR	32 cycles (30 s at 95°C, 30 s at 54.4°C, 1 min at 72°C)
VNR	28 cycles (30 s at 95°C, 30 s at 52.8°C, 1 min at 72°C)
MMP-9	28 cycles (30 s at 95°C, 30 s at 52.4°C, 1 min at 72°C)
CTSK	28 cycles (30 s at 95°C, 30 s at 55.6°C, 1 min at 72°C)
CA2	28 cycles (30 s at 95°C, 30 s at 53.1°C, 1 min at 72°C)
OPG	32 cycles (30 s at 95°C, 30 s at 45.5°C, 1 min at 72°C)
RANKL	28 cycles (30 s at 95°C, 30 s at 45.3°C 1 min at 72°C)
β-actin	23 cycles (30 s at 95°C, 30 s at 58°C, 1 min at 72°C)

Table 2. Sequences of PCR primers and the expected sizes of PCR products.

CTR	Forward:	5'-TGAAAAGGCGGAATCT-3'
(595 bp)	Reverse:	5'-AGGAACATGTGCTTGTG-3'
VNR	Forward:	5'-GCTCAGATGAGACTTTG-3'
(961 bp)	Reverse:	5'-ATCAACAATGAGCTGGA-3'
MMP-9	Forward:	5'-CGAGACATGATCGATGA-3'
(841 bp)	Reverse:	5'-ACGCTGGAATGATCTAA-3'
CTSK	Forward:	5'-TCAAGGTTCTGCTGCTA-3'
(923 bp)	Reverse:	5'-GAGCCAAGAGAGCATAT-3'
CA2	Forward:	5'-CACCCTTCCAAGATCTTATA-3'
(196 bp)	Reverse:	5'-ATCCATTGTGTTGTGGTATG-3'
OPG	Forward:	5'-TGAGTGTGAGGAAGGGCGTTAC-3'
(636 bp)	Reverse:	5'-TTCCTCGTTCTCTCAATCTC-3'
RANKL	Forward:	5'-ATCAGAAGACAGCACTCACT-3'
(750 bp)	Reverse:	5'-ATCTAGGACATCCATGCTAATGTTC-3'
β-actin	Forward:	5'-GGACTCCTATGGTGGGTGACGAGG-3'
(366 bp)	Reverse:	5'-GGGAGAGCATAGCCCTCGTAGAT-3'

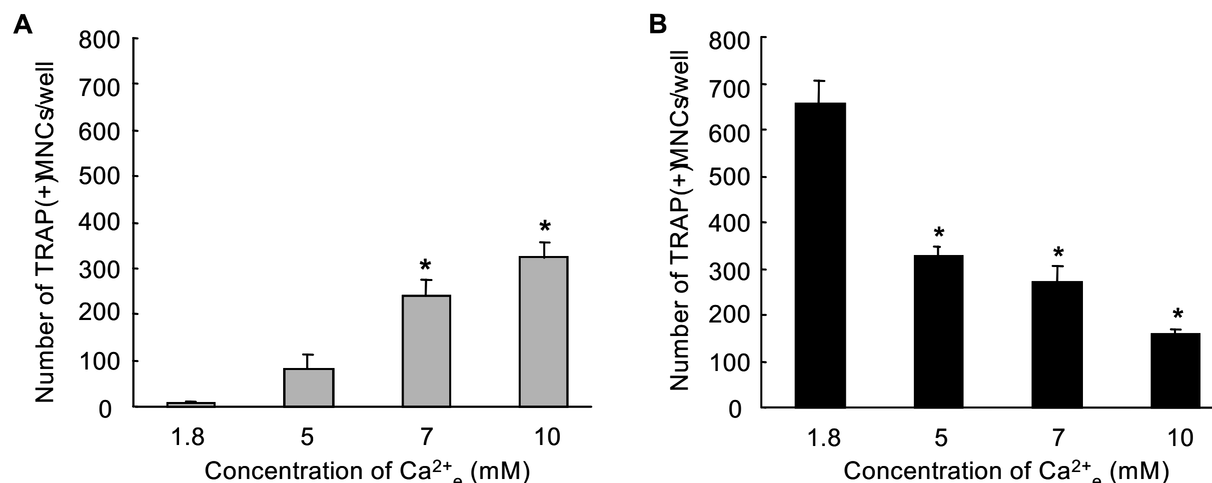

Figure 1. Effect of high Ca^{2+}_e on the osteoclast formation. Mouse calvarial osteoblastic cells and bone marrow cells were cocultured for 7 days in the absence (A) or presence (B) of $1,25\text{-}(OH)_2vitD_3$ and TRAP staining was performed. Data represent mean ± SE ($N = 6$). *$P < 0.01$, compared to the corresponding control (1.8 mM Ca^{2+}_e), TRAP(+) MNCs; tartrate-resistant acid phosphatase-positive multinucleated cells.

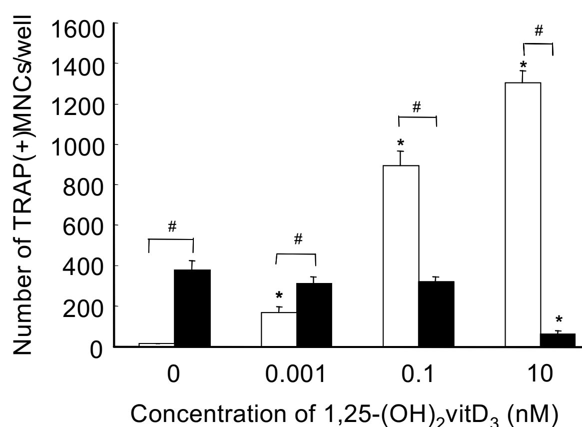

Figure 2. Effect of high Ca^{2+}_e on the osteoclast formation in the presence of various concentrations of $1,25\text{-}(OH)_2vitD_3$. Cocultured cells were exposed to 1.8 mM Ca^{2+}_e (open bar) or 10 mM Ca^{2+}_e (closed bar) for the entire culture period. Data represent mean ± SE ($N = 6$). *$P < 0.01$, compared to control [the absence of $1,25\text{-}(OH)_2vitD_3$], #$P < 0.01$, significantly different from corresponding cultures in 1.8 mM Ca^{2+}_e.

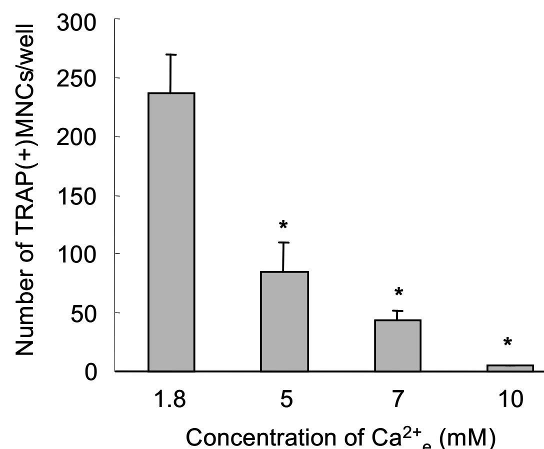

Figure 3. Effect of high Ca^{2+}_e on the osteoclast formation in the presence of M-CSF and sRANKL. Mouse calvarial osteoblasts and bone marrow cells were cocultured in the presence of 30 ng/ml M-CSF and 60 ng/ml sRANKL for 7 days. Data represent mean ± SE ($n = 6$). *$P < 0.01$, compared to control (1.8 mM Ca^{2+}_e).

to both high Ca^{2+}_e and $1,25\text{-}(OH)_2vitD_3$, the effect of high Ca^{2+}_e depended on the concentration of $1,25\text{-}(OH)_2vitD_3$. In the presence of 1 pM and 0.1 nM $1,25\text{-}(OH)_2vitD_3$, the number of TRAP(+) MNCs was similar to that of 10 mM Ca^{2+}_e alone. That is, in less osteoclastogenic condition (1 pM $1,25\text{-}(OH)_2vitD_3$) high Ca^{2+}_e increased osteoclast formation to the level of its own, while in more osteoclastogenic condition (0.1 nM $1,25\text{-}(OH)_2vitD_3$), it suppressed osteoclastogenesis to the level of its own. Moreover, in highly osteoclastogenic condition (10 nM $1,25\text{-}(OH)_2vitD_3$), high Ca^{2+}_e even more greatly suppressed osteoclast formation. The number of TRAP(+) MNCs was less than 20% of that in high Ca^{2+}_e alone.

From the above results, we speculated that the presence of highly osteoclastogenic factors was the prerequisite for the inhibitory effect of high Ca^{2+}_e. So we investigated whether the inhibitory effect of high Ca^{2+}_e would occur when osteoclastogenesis was induced by M-CSF and sRANKL in coculture system. As shown in Figure 3, high Ca^{2+}_e significantly suppressed osteoclast formation in a concentration-dependent manner. This finding indicated that the inhibitory effect of high Ca^{2+}_e was not confined to $1,25\text{-}(OH)_2vitD_3$-induced osteoclastogenesis but was more

universal.

Next, we observed the expression pattern of osteoclastic markers in cocultured cells to confirm whether formed TRAP(+) MNCs were authentic osteoclasts. Transcripts of CTR, VNR, CTSK, MMP-9 and CA2 were identified by RT-PCR. As shown in Figure 4, the expression pattern was similar whether osteoclastogenesis was induced by high Ca^{2+}_e or by 1,25-$(OH)_2vitD_3$, although the transcripts levels in high Ca^{2+}_e-treated culture were lower than those in 1,25-$(OH)_2vitD_3$-treated culture, coinciding with the TRAP staining results. When the cells were cultured with both 10 mM Ca^{2+}_e and 10 nM 1,25-$(OH)_2vitD_3$, the osteoclast markers expression decreased.

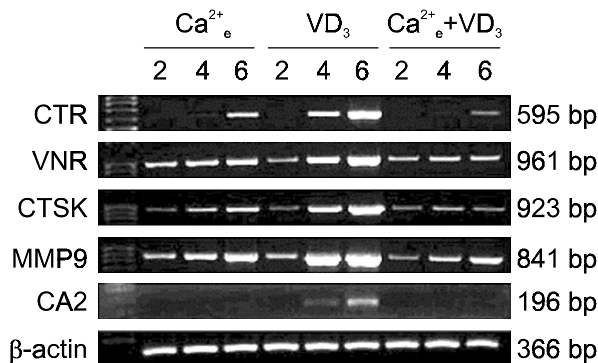

Figure 4. Expression of osteoclast markers. Mouse bone marrow and osteoblastic cells were cocultured in the presence of 10 mM Ca^{2+}_e, 10 nM 1,25-$(OH)_2vitD_3$, or both for 2, 4, and 6 days and RT-PCR was performed as described in Materials and Methods. VD_3; 1,25-$(OH)_2vitD_3$.

Effect of high Ca^{2+}_e on the expression of RANKL and OPG

In order to investigate the mechanism of high Ca^{2+}_e-induced osteoclast formation, the effect on RANKL and OPG expression was observed in high Ca^{2+}_e-treated osteoblastic cells. As shown in Figure 5A, 10 mM Ca^{2+}_e significantly up-regulated RANKL expression and the increased transcripts level was maintained during culture period. However, high Ca^{2+}_e transiently increased OPG expression and after 24 h, OPG expression level was recovered to the control level (Figure 5A). Next we observed the effect of high Ca^{2+}_e on RANKL and OPG expression in the presence of 10 nM 1,25-$(OH)_2vitD_3$. 1,25-$(OH)_2vitD_3$ alone significantly increased RANKL expression, while decreased OPG expression (data not shown). High

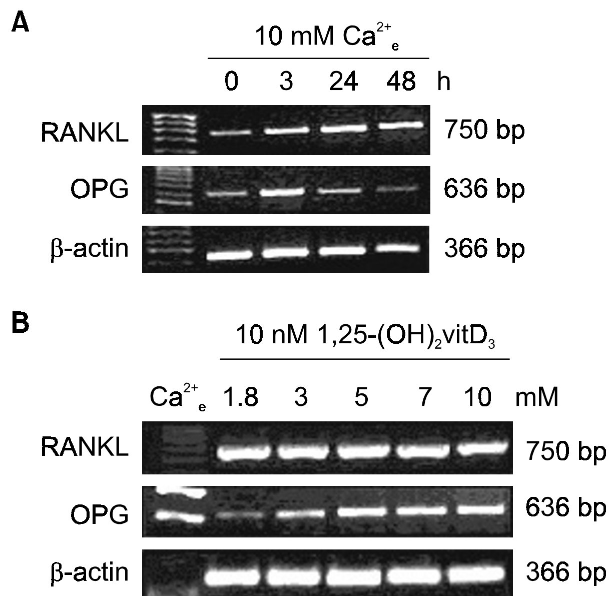

Figure 5. Effect of high Ca^{2+}_e on the expression of RANKL and OPG in mouse calvarial osteoblastic cells. (A) Cells were cultured in 10 mM Ca^{2+}_e for 0, 3, 24, and 48 h. (B) Cells were cultured in 10 nM 1,25-$(OH)_2vitD_3$ alone or with added calcium to 3, 5, 7, and 10 mM for 48 h.

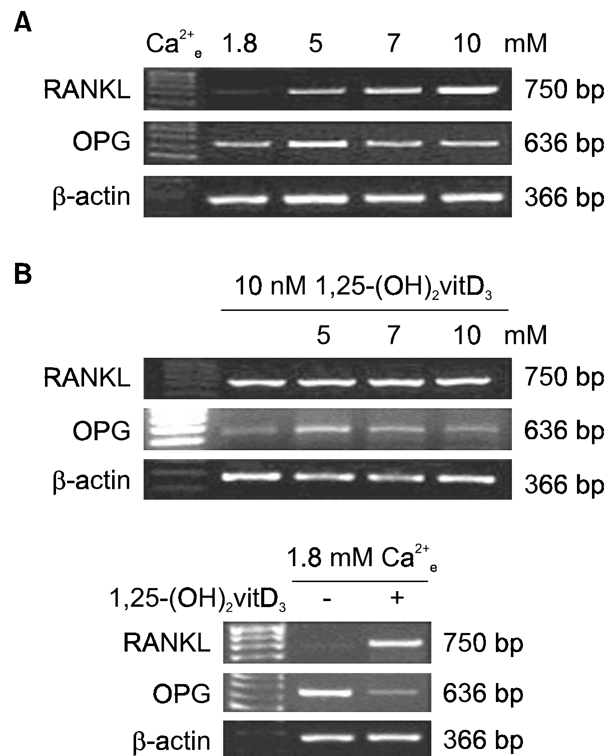

Figure 6. Effect of high Ca^{2+}_e on the expression of RANKL and OPG in coculture of mouse bone marrow and osteoblastic cells. (A) Cells were cultured with added calcium to 5, 7, and 10 mM for 6 days. (B) In upper panel, cells were cultured in 10 nM 1,25-$(OH)_2vitD_3$ alone or with added calcium to 5, 7, and 10 mM for 6 days. In lower panel, cells were cultured in the absence or presence of 10 nM 1,25-$(OH)_2vitD_3$ for 6 days.

$Ca^{2+}{}_e$ did not further enhance RANKL expression which was already up-regulated by 1,25-$(OH)_2$vitD_3 (Figure 5B). However, as the medium calcium concentration increased in the presence of 1,25-$(OH)_2$vitD_3, the level of OPG transcript which was down-regulated by 1,25-$(OH)_2$vitD_3, increased significantly (Figure 5B). This stimulatory effect on OPG expression was observed even after 96 h incubation though the degree was lessened (data not shown). Then we observed RANKL and OPG expression in cocultured cells for 6 days. As shown in Figure 6A, in the absence of 1,25-$(OH)_2$vitD_3, high $Ca^{2+}{}_e$-induced increase in RANKL expression was still evident after 6 days, while OPG expression was not different from that of control. In the presence of 1,25-$(OH)_2$vitD_3, increasing medium calcium concentration did not further change the expression level of RANKL or OPG after 6 days (Figure 6B).

Discussion

Our previous study revealed that high $Ca^{2+}{}_e$ manifested the biphasic effect on osteoclast formation depending on the presence or absence of 1,25-$(OH)_2$vitD_3 in cultured mouse bone marrow cells (Kim *et al.*, 1998). However, other reports have showed quite conflicting results regarding the effect of high $Ca^{2+}{}_e$ on the osteoclast formation depending on culture condition and/or added bone resorbing agents. To clarify the mode of modulating effect of high $Ca^{2+}{}_e$ on the osteoclast formation, we used coculture system of mouse bone marrow cells with osteoblastic cells in this study. It would be more reasonable to evaluate the effect of high $Ca^{2+}{}_e$ on the osteoclast formation in the presence of osteoblastic cells since osteoblasts co-exist in bone microenvironment under physiological condition. In addition, osteoblasts mediate various bone-resorbing factor-stimulated osteoclast formation through the regulation of RANKL and/or OPG expression (Suda *et al.*, 1999; Takahashi *et al.*, 1999). Osteoclast formation could be confirmed by morphological characteristics such as TRAP staining positivity and multinuclearity, and the expression of osteoclast phenotypic markers including CTR, VNR, MMP-9, CTSK, and CA2. Morphological characteristics and phenotypic marker expression pattern were similar whether osteoclast formation was induced by 1,25-$(OH)_2$vitD_3 or high $Ca^{2+}{}_e$.

Present study demonstrated that high $Ca^{2+}{}_e$ significantly stimulated osteoclast formation in the absence of any added osteoclastogenic agent. However, in the presence of highly osteoclastogenic agents such as 1,25-$(OH)_2$vitD_3 and M-CSF/sRANKL, high $Ca^{2+}{}_e$ significantly inhibited osteoclast formation. These findings are consistent with the previous reports that the stimulatory effect of high $Ca^{2+}{}_e$ on the osteoclast formation was observed in osteoblasts-containing bone marrow cultures without other osteoclastogenic factors (Kaji *et al.*, 1996; Kim *et al.*, 1998; Takami *et al.*, 2000), while high $Ca^{2+}{}_e$ or Ca^{2+}-sensing receptor agonists inhibited osteoclast formation in coculture of mouse bone marrow cells and osteoblasts/stromal cells in the presence of osteoclastogenic factors such as 1,25-$(OH)_2$vitD_3 and dexamethasone (Takeyama *et al.*, 2000). On the other hand, Sugimoto and coworkers (Sugimoto *et al.*, 1993; Kanatani *et al.*, 1999; Dempster *et al.*, 2000) reported that high $Ca^{2+}{}_e$ inhibited osteoclast differentiation which was induced by 1,25-$(OH)_2$vitD_3, PTH, PGE_2, or M-CSF/sRANKL and they suggested that this inhibitory effect was the result from the direct action on osteoclast precursors because their culture condition did not contain osteoblasts/stromal cells. Collectively, our study and other previous reports demonstrated that high $Ca^{2+}{}_e$ inhibited the osteoclast formation stimulated by highly osteoclastogenic factors with no exception regardless of the concomitant presence of osteoblasts/stromal cells. Meanwhile high $Ca^{2+}{}_e$-induced stimulation in osteoclast formation had been observed only when the precursor cells were cocultured with osteoblasts/stromal cells in the absence of osteoclastogenic factors. These findings led us to speculate that the modulation mode of high $Ca^{2+}{}_e$ on the osteoclast formation *in vitro* may be biphasic according to the culture condition, *i.e.,* the presence or absence of osteoclastogenic factor(s) and osteoblasts/stromal cells.

Since RANKL and OPG are the key regulators of osteoclast differentiation (Suda *et al.*, 1999; Takahashi *et al.*, 1999), we examined the effect of high $Ca^{2+}{}_e$ on RANKL and OPG expression. High $Ca^{2+}{}_e$ alone significantly increased RANKL expression and those stimulatory effects were maintained after 6 days. Meanwhile OPG expression was increased only transiently. These findings were similar to previous report showing that calcium ionophores and high $Ca^{2+}{}_e$ up-regulated RANKL and OPG mRNA expression but the effect on RANKL was more profound (Takami *et al.*, 2000). These results indicate that high $Ca^{2+}{}_e$-induced osteoclast formation may be mediated by osteoblasts via up-regulation of RANKL expression, coinciding with the observations that high $Ca^{2+}{}_e$ stimulated osteoclastogenesis only in the presence of osteoblasts/stromal cells.

Next, we observed the effect of high $Ca^{2+}{}_e$ on RANKL and OPG expression in the presence of 10 nM 1,25-$(OH)_2$vitD_3. As previously reported (Yasuda *et al.*, 1998a,b), 1,25-$(OH)_2$vitD_3 significantly increased RANKL expression, while decreased OPG expression. As the medium calcium concentration increased, the level of OPG transcript which was down-regulated by 1,25-$(OH)_2$vitD_3, increased significantly and this effect

was maintained even after 96 h incubation though the degree was lessened. But this stimulatory effect was observed no more in cocultured cells after 6 days. Meanwhile high Ca^{2+}_e did not further enhanced RANKL expression which was already up-regulated by 1,25-$(OH)_2vitD_3$. During the preparation of this manuscript, Takahashi *et al.* (2002) reported closely similar findings that high Ca^{2+}_e treatment for the initial 24 h in the presence of 1,25-$(OH)_2vitD_3$ reduced the TRAP(+) MNCs formation but high Ca^{2+}_e treatment at the later stage of osteoclastogenesis (the last 2 days of culture) stimulated the formation of TRAP(+) MNCs. They suggested that the inhibitory effect of high Ca^{2+}_e was mediated by up-regulation of OPG expression while the stimulatory effect by up-regulation of RANKL and down-regulation of OPG. Takeyama *et al.* (2000) also demonstrated that Ca^{2+}-sensing receptor agonists diminished RANKL expression in the presence of 1,25-$(OH)_2vitD_3$ and dexamethasone. The reason for the discrepancy in the effect on RANKL expression is not clear now. But, at least, these results indicate that up-regulated OPG might participate in the inhibitory effect of high Ca^{2+}_e on 1,25-$(OH)_2vitD_3$-induced osteoclastogenesis. High Ca^{2+}_e was also known to up-regulate OPG expression in osteoclasts as well as osteoblasts. Although we did not confirm in this study, Moonga *et al.* (1999) previously demonstrated that osteoclasts themselves expressed OPG mRNA and high Ca^{2+}_e enhanced OPG expression. On the basis of this report, the previous findings that high Ca^{2+}_e inhibited osteoclast formation by directly acting on osteoclast precursor cells (Sugimoto *et al.*, 1993; Kanatani *et al.*, 1999; Dempster *et al.*, 2000) may be explained. That is, enhanced OPG production by high Ca^{2+}_e in osteoclastic cells may counteract the effects of bone resorbing factors such as 1,25-$(OH)_2vitD_3$, PTH, PGE_2, or M-CSF/sRANKL, though the mechanism is not clear now.

The local concentration of Ca^{2+} at resorption sites was reported to rise as high as to 40 mM (Silver *et al.*, 1988). Since high Ca^{2+}_e inhibited bone resorption by mature osteoclasts, it has been considered as a negative feedback mechanism to prevent excessive resorption (Malgaroli *et al.*, 1989; Zaidi *et al.*, 1989). However, since most osteoclasts at bone resorption site are persistently exposed to relatively high Ca^{2+}_e, the sustained inhibition by high Ca^{2+}_e should result in excessive inhibition of bone resorption and inactive phase of bone remodeling. Therefore, there should be some modulating mechanism to prevent not only excessive bone resorption but also excessive inhibition of bone resorption. It seems that high Ca^{2+}_e might be a candidate for such modulating mechanism because high Ca^{2+}_e exhibited biphasic effects on osteoclast formation depending on culture condition such as co-existence of bone resorbing agents. And those biphasic effects were partially mediated through the regulation of RANKL and OPG expression level. Up to now, why and how such biphasic effects occur are not clear, but several reports suggested that there were some modulating interactions between Ca^{2+}-sensing signaling and hormone or cytokine signaling. That is, high Ca^{2+}_e inhibited or enhanced PTH- and PGE_2-induced cAMP generation, while hormone-induced increase in cAMP or IL-6 receptor-mediated signals attenuated Ca^{2+}-sensing and reversed Ca^{2+}-mediated effects in bone cells (Peck *et al.*, 1981; Hartle *et al.*, 1996; Zaidi *et al.*, 1996; Adebanjo *et al.*, 1998). Further study will be needed to clarify the mechanism of biphasic regulation of osteoclast formation by high Ca^{2+}_e.

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