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Inhibitory action of bisphosphonates on bone resorption does not involve the regulation of RANKL and OPG expression

Yong Hee Kim, Gwan-Shik Kim, and Jeong-Hwa Baek[1]

Department of Pharmacology and Dental Therapeutics, College of Dentistry, Seoul National University, Seoul, Korea
[1]Corresponding author: Tel, +82-2-740-8689; Fax, +82-2-741-3193; E-mail, baekjh@snu.ac.kr

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Abbreviations: RANKL, Receptor Activator of Nuclear Factor kB Ligand; OPG, Osteoprotegerin; TRAP, tartrate-resistant acid phosphatase; VNR, vitronectin receptor; CTR, calcitonin receptor; RT-PCR, reverse transcription-polymerase chain reaction; OCLs, osteoclast-like cells; 1,25-$(OH)_2VitD_3$, 1,25-dihydroxyvitaminD$_3$

Abstract

The mechanism of inhibitory action of bisphosphonates on bone resorption is not fully elucidated. Osteoclast formation and activity are regulated by osteoblast-derived factors such as the osteoclast differentiating factor, receptor activator of NF-kappaB ligand (RANKL) and the inhibitor, osteoprotegerin (OPG). To investigate *in vitro* effects of bisphosphonates on mouse osteoblastic cells, we examined the expression levels of RANKL and OPG in the cells treated with alendronate or pamidronate (10^{-8}~10^{-5} M) alone, or combined with 10 nM of 1,25-$(OH)_2VitD_3$ for 24 or 48 h. Various concentrations of alendronate and pamidronate did not change the mRNA expression of RANKL and OPG consistently irrespective of 1,25-$(OH)_2VitD_3$ presence. When added into cocultures of mouse osteoblastic cells and bone marrow cells, both alendronate and pamidronate inhibited osteoclast formation and bone resorption but failed to alter the RANKL and OPG mRNA expression. These results indicate that the inhibition of bone resorption by bisphosphonates is not mediated by the regulation of RANKL and OPG expression.

Key words: RANKL, OPG, alendronate, pamidronate, osteoclastogenesis

Introduction

Bisphosphonates, stable analogs of pyrophosphate, are potent inhibitors of bone resorption and have been used as effective therapeutic agents for the management of osteoporosis and other bone diseases such as Paget's disease (Papapoulos, 1996; Rodan and Fleisch, 1996; Fleisch, 1997). Osteoclasts, derived from hematopoietic precursors of monocyte/macrophage lineage, are primary bone resorbing cells and play a pivotal role in normal and pathologic bone remodeling in concert with osteoblasts (Roodman, 1996; Hayashi *et al*., 1998; Suda *et al*., 1999). Inhibition of bone resorption by bisphosphonates has been principally attributed to their inhibitory effect on osteoclasts. Bisphosphonates decrease the commitment of osteoclast progenitors into osteoclasts and osteoclast recruitment and promote apoptosis of mature osteoclasts (Hughes *et al*., 1989; Hughes *et al*., 1995; Parfitt *et al*., 1996; Jilka *et al*., 1998). In addition to direct effects on osteoclasts, it has been reported that bisphosphonates inhibit bone resorption indirectly through osteoblasts (Sahni *et al*., 1993; Nishikawa *et al*., 1996; Vitte *et al*., 1996; Plotkin *et al*., 1999). They have shown that bisphosphonates promote the release of factors from osteoblasts that inhibit the osteoclast formation and activity. The inhibitory factors released from bisphosphonate-treated osteoblasts, however, have not yet been identified.

RANKL, a member of the tumor necrosis factor (TNF) superfamily, is a membrane-bound protein produced by osteoblasts/stromal cells. It is known to be both sufficient and necessary for osteoclast formation *in vitro* in the presence of macrophage-colony stimulating factor (M-CSF) (Lacey *et al*., 1998; Quinn *et al*., 1998; Yasuda *et al*., 1998). Osteoprotegerin (OPG), a member of the TNF receptor superfamily, has been identified as a novel cytokine receptor secreted from osteoblasts (Simonet *et al*., 1997). OPG inhibits both differentiation and activation of osteoclasts by acting as a decoy receptor of RANKL (Lacey *et al*., 1998; Yasuda *et al*., 1998). It has been suggested that RANKL and OPG act as key mediators through which many osteotropic agents manifest their inhibitory or stimulatory effects on bone resorption (Suda *et al*., 1999; Takahashi *et al*., 1999; Aubin and Bonnelye, 2000; Hofbauer *et al*., 2000).

Here we examined whether the inhibitory effects of bisphosphonates on osteoclast formation and bone resorption is mediated through the regulation of RANKL and OPG expression in osteoblastic cells. Our results indicate that RANKL and OPG might not function as mediators of bisphosphonates action on bone resorption.

Table I. Primer sequences used in semiquantitative RT-PCR.

Murine RANKL	Forward	5'-ATCAGAAGACAGCACTCACT-3'
	Reverse	5'-ATCTAGGACATCCATGCTAATGTTC-3'
Murine OPG	Forward	5'-TGAGTGTGAGGAAGGGCGTTAC-3'
	Reverse	5'-TTCCTCGTTCTCTCAATCTC-3'
Murine β-actin	Forward	5'-GGACTCCTATGGTGGGTGACGAGG-3'
	Reverse	5'-GGGAGAGCATAGCCCTCGTAGAT-3'
Murine tartrate-resistant acid phosphatase (TRAP)	Forward	5'-TGACAAGAGGTTCCAGGA-3'
	Reverse	5'-AGCCAGGACAGCTGAGTG-3'
Murine vitronectin receptor (VNR)	Forward	5'-GCTCAGATGAGACTTTG-3
	Reverse	5'-ATCAACAATGAGCTGGA-3'
Murine calcitonin receptor (CTR)	Forward	5'-GTGAAAAGGCGGAATCT-3'
	Reverse	5'-AGGAACATGTGCTTGTG-3'

Materials and Methods

Animals and reagents

Newborn mice and 4-5 week-old mice, both ICR strain, were obtained from Daehan Bio Link (Eumsung, Choongchung, Korea). Neonatal mice were used for preparing osteoblastic cells and the latter were used for bone marrow cell cultures. The bisphosphonates, alendronate and pamidronate, were kindly provided from Merck & Co (Rahway, NJ, USA) and Hallim Pharm (Seoul, Korea), respectively. DMEM, α-MEM, fetal bovine serum (FBS), collagenase, Superscript™ First-Strand Synthesis System, and other culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA). 1,25-$(OH)_2VitD_3$ was purchased from Calbiochem (La Jolla, CA, USA). easy-BLUE™ reagent and TaKaRa Taq™ were purchased from iNtRON (Seoul, Korea) and TaKaRa (Otsu, Shiga, Japan), respectively. OAAS™ was obtained from OCT Inc. (Chunan, Chungnam, Korea). Leukocyte acid phosphatase assay kit was purchased from Sigma (St Louis, MO, USA).

Osteoblastic cell culture

Mouse osteoblastic cells were isolated from 30-40 neonatal ICR mice calvariae by enzymatic digestion as previously described (Kim *et al*. 1998). In brief, aseptically isolated frontal and parietal bones were digested with enzyme mixture (0.1% collagenase, 0.05% trypsin and 0.5 mM EDTA) for 10, 10, 10, 20, 20, and 20 min each. Osteoblastic cells, which were released in the later periods, were seeded in 60 mm dish in α-MEM containing 10% of FBS and 100 U/ml of penicillin and 100 μg/ml of streptomycin. Half million cells at the second passage were seeded in 60mm dish and cultured for 2 days at 37°C in 95% humidified air plus 5% CO_2. Then, cells were treated with various concentrations of alendronate or pamidronate in the presence or absence of 1,25-$(OH)_2VitD_3$ for 24 or 48 h.

Osteoclast differentiation and bone resorption assay

Bone marrow cells were prepared from tibiae and femurs from 4-5 week-old ICR mice by flushing bone marrow cavity with α-MEM and lysing erythrocytes with 0.83% NH_4Cl in 10 mM Tris-HCl pH 7.4. To induce osteoclast formation *in vitro*, coculture of osteoblastic cells (7.5×10^3 cells/well) and bone marrow cells (1.5×10^5 cells/well) was performed in a 48-well plate coated with bone mineral-like apatite crystal (OAAS™). Cells were cultured in 0.3 ml of α-MEM containing 10% FBS and 10 nM of 1,25-$(OH)_2VitD_3$ for 6 days. The medium was changed every other day and bisphosphonates were added at the beginning of culture and at the time of medium change. At the end of the cultures, the cells were subjected to a tartrate-resistant acid phosphatase (TRAP) staining using leukocyte acid phosphatase assay kit following manufacturer's protocol. TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclast-like cells (OCLs). After counting the number of OCLs, the culture plate was washed twice with PBS and treated with 12% sodium hypochlorite to remove cells and resorption area was measured by using Global Lab image system (Data Translation Inc. Marlboro, MA, USA).

For reverse transcription-polymerase chain reaction (RT-PCR), coculture was performed by using bone marrow cells (3×10^6 cells) and mouse osteoblastic cells (1.5×10^4 cells). In the same culture condition as above, cells were cultured for indicated periods and lysed with easy-BLUE™ reagent for the isolation of total cellular RNA.

Semiquantitative RT-PCR

cDNA was synthesized from 1 μg of total RNA by extension of random primers with 1 U of Superscript RT. PCR was performed in a final volume of 50 μl containing 2 U of Takara Taq, 1 × PCR buffer, 0.8 mM dNTP mixture, and 100 pM of specific primers. The reactions were: denaturation at 95°C for 30 s, annealing at 53°C

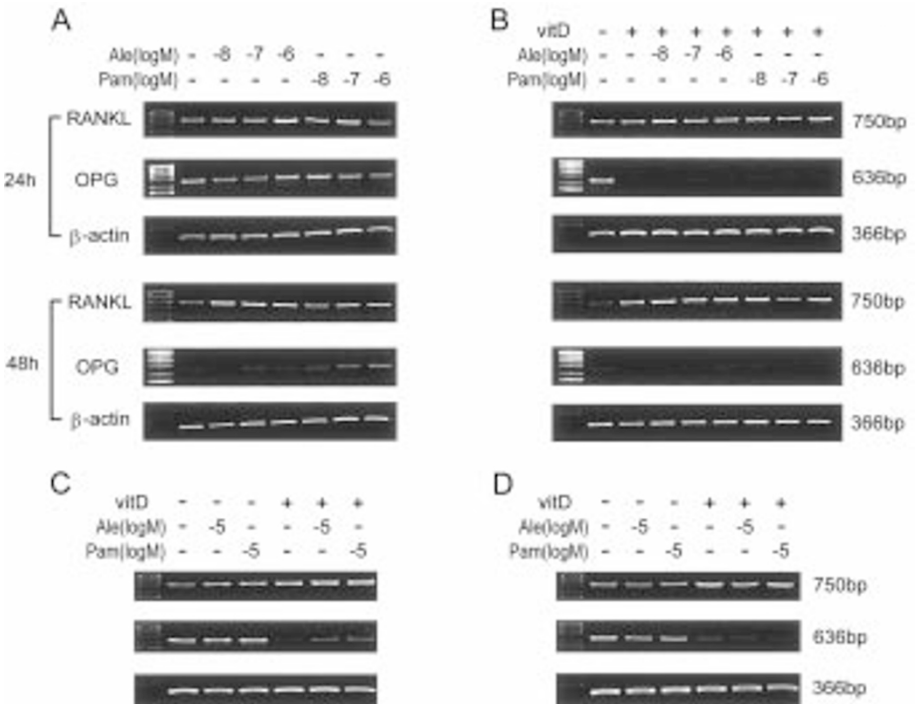

Figure 1. Effect of bisphosphonates on the RANKL and OPG mRNA expression. Mouse osteoblastic cells were exposed to 10^{-8}~10^{-6} M alendronate or pamidronate for 24 or 48 h in the presence (B) or absence (A) of 1,25-$(OH)_2VitD_3$. C, D) Mouse osteoblastic cells were cultured with 10^{-5} M of alendronate or pamidronate in the presence of 1,25-$(OH)_2VitD_3$ for 24 h (C) or 48 h (D). Ale:alendronate, Pam: pamidronate.

(for mVNR and mCTR), at 55°C (for mRANKL and mTRAP) or at 60°C (for mOPG and mβ-actin) for 30 s, and extension at 72°C for 1 min. All PCRs were within the exponential amplification range. The primer sequences are shown in Table I. PCR products were electrophoresed on a 1.2 % agarose gel and visualized under the UV light after ethidium bromide staining. For semiquantitative estimation, the gel was analyzed with Quantity One (BIO-RAD. Hercules, CA, USA).

Results and Discussion

Alendronate and pamidronate, the nitrogen containing bisphosphonates, have been known to be effective against Pagets disease, osteoporosis, tumor-associated bone disease, and glucocorticoid-induced osteoporosis (Frijlink *et al*., 1979; Reid *et al*., 1994; Berenson *et al*., 1996; Black *et al*., 1996; Saag *et al*., 1999; Orwoll *et al*., 2000).

To investigate whether alendronate and pamidronate affect the mRNA expression of RANKL and OPG in osteoblasts, we performed semiquantitative RT-PCR by using total RNA from mouse osteoblastic cells treated with various concentrations of alendronate or pamidronate. Since the basal level of RANKL mRNA expression in primary osteoblastic cells was too low to be detected by northern blot analysis, we employed RT-PCR analysis instead. As shown in Figure 1A, neither alendronate nor pamidronate induced any consistent change in RANKL and OPG mRNA level after 24 h. Unexpectedly, after 48 h treatment, both alendronate and pamidronate rather increased RANKL mRNA expression. OPG mRNA expression level was slightly increased only by 10^{-7} and 10^{-6} M pamidronate. According to the previous reports, osteotropic agents modulate osteoclast formation through the regulation of the ratio of RANKL/OPG expression rather than increasing or decreasing RANKL and/or OPG alone (Hofbauer *et al*., 1999; Atkins *et al*., 2000; Hofbauer *et al*., 2000). The increase in the RANKL/OPG ratio means condition favoring increased osteoclast formation and vice versa. In the present study, the RANKL/OPG ratio, calculated from data obtained by densitometric analysis, was rather increased in all experimental groups except in the group of pamidronate treatment for 48 h (data not shown).

Next, we performed the same experiments in the presence of 1,25-$(OH)_2VitD_3$ to examine the effects of bisphosphonates under the osteoclastogenic environment. 1,25-$(OH)_2VitD_3$ is well known to effectively induce osteoclastogenesis in cocultures of mouse spleen cells and osteoblast-rich populations (Udagawa *et al*., 1989) and to increase RANKL expression and to decrease OPG expression (Horwood *et al*., 1998; Murakami *et al*., 1998; Yasuda *et al*., 1998). Consistent with the previous reports, 1,25-$(OH)_2VitD_3$ upregulated RANKL expression and downregulated OPG expression

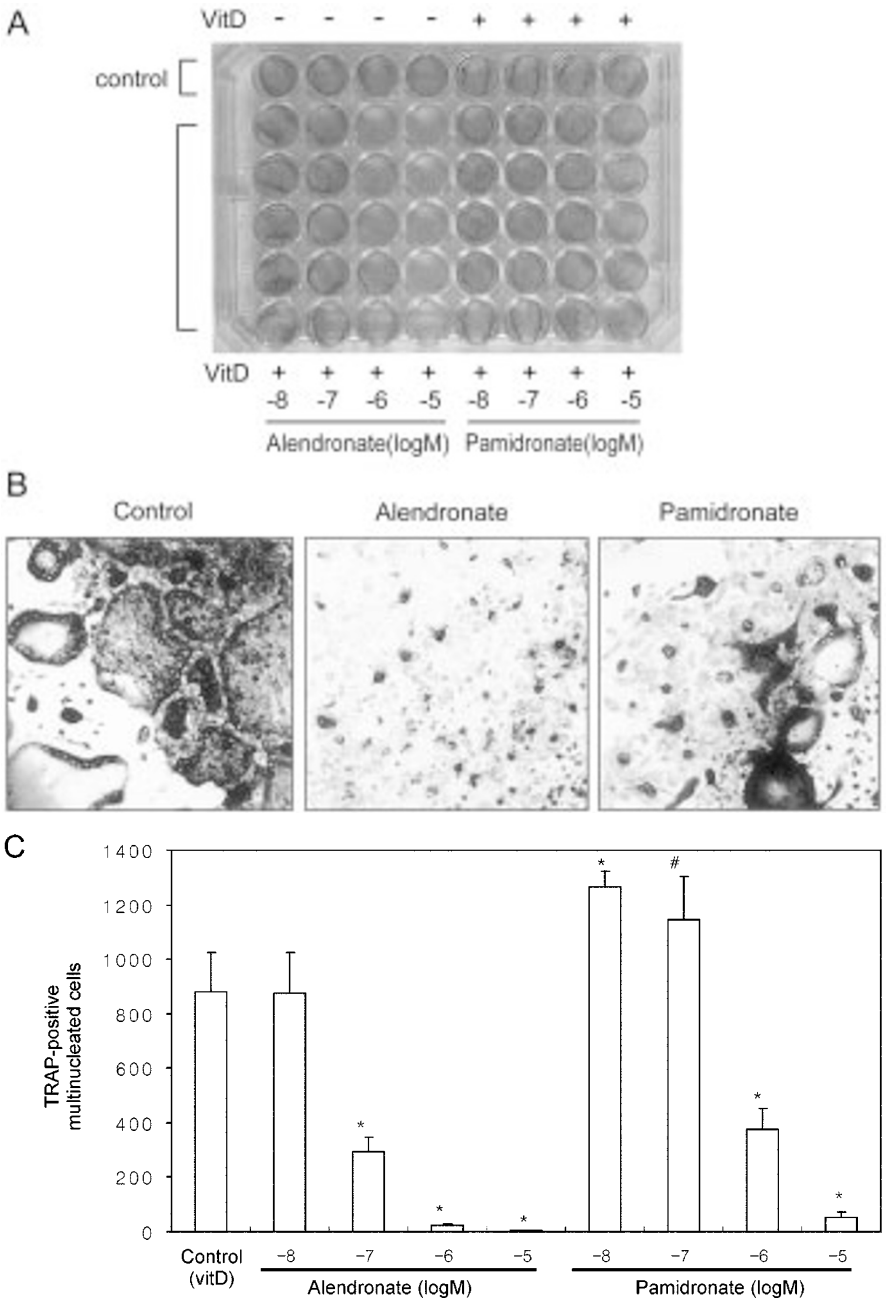

Figure 2. Effect of bisphosphonates on the osteoclast formation. Mouse osteoblastic cells (7.5×10^3 cells/well) and bone marrow cells (1.5×10^5 cells/well) were cocultured in the presence of 10 nM of 1,25-$(OH)_2VitD_3$ for 6 days. Various concentrations of alendronate or pamidronate were added over the culture period. A) TRAP-stained 48-well culture plate. B) Osteoclast-like cells (OCLs) formed in the culture were identified by TRAP staining. Representative areas are shown here. Original magnification × 100. C) The numbers of OCLs containing 3 or more nuclei were counted. Data represent mean ±S.D. (n=5) # $p < 0.05$, compared to control, * $p < 0.01$, compared to control.

in mouse osteoblastic cells (Figure 1B) and increased RANKL/OPG ratio upto~10 folds. The addition of alendronate or pamidronate for 24 h did not affect the altered expression level of RANKL and OPG by 1,25-$(OH)_2VitD_3$. After 48 h treatment, bis-phosphonates still did not alter RANKL expression level. OPG expression, reduced by 1,25-$(OH)_2VitD_3$, was increased by bisphosphonates but was still lower than that of control. To rule out the possibility that the failure of bisphosphonates to alter the RANKL and OPG mRNA expression might be due to the low con-centration of the agents, we repeated experiment with 10^{-5} M alendronate or pamidronate. As shown in Figure 1C and D, higher concentration of alendronate and pamidronate

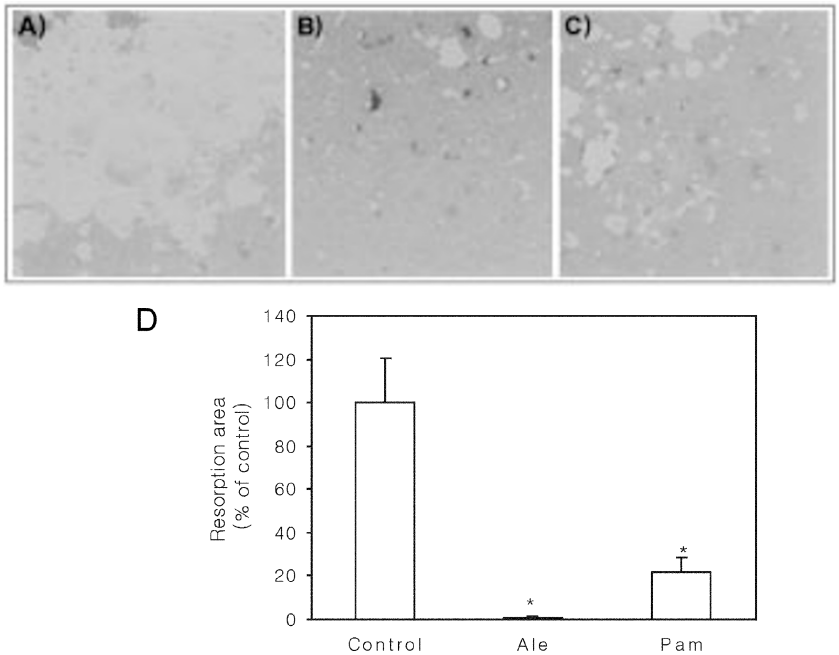

Figure 3. Effect of bisphosphonates on the bone resorption by osteoclasts. Mouse osteoblastic cells (7.5×10^3 cells/well) and bone marrow cells (1.5×10^5 cells/well) were cocultured in a 48-well plate coated with bone mineral-like apatite crystal (OAAS^{TM}) in the presence of 10 nM of 1, 25-$(OH)_2VitD_3$ for 6 days and 10^{-5} M alendronate or pamidronate was added over the culture period. At the end of the culture, cells were removed and resorption area was measured by using Global Lab image system. Representative areas are shown here. Original magnification ×40. A) Control, B) Alendronate (10^{-5} M), C) Pamidronate (10^{-5} M), D) The areas resorbed by OCLs are plotted as percent of control. Data represent means ±S.D. (n=4) * < 0.01, compared to control. Ale: alendronate, Pam: pamidronate

also failed to upregulate the OPG expression or downregualte RANKL expression irres-pective of the presence of 1,25-$(OH)_2VitD_3$. These results indicate that alendronate or pamidronate did not regulate RANKL and OPG expression in mouse os-teoblasts in favor of inhibition of osteoclastogenesis. To rule out the possibility that these results were due to the inactivity of these bisphosphonates in our experimental system, we confirmed the inhibitory effect of these compounds on *in vitro* osteoclastogenesis and os-teoclast function.

To examine the inhibitory effects of alendronate and pamidronate on the *in vitro* osteoclast formation, we cocultured mouse bone marrow cells with osteoblastic cells in the presence of 1,25-$(OH)_2VitD_3$ with various concentrations of alendronate or pamidronate. Both alendronate and pamidronate inhibited the osteoclast formation in a dose-dependent manner (Figure 2). The number of TRAP(+) OCLs were reduced by 10^{-7}~10^{-5} M alendronate. Pamidronate also decreased osteoclast formation at 10^{-6} and 10^{-5} M. Interestingly, 10^{-8} and 10^{-7} M pamidronate increased the number of TRAP(+) OCLs, which appeared to be contradictory to the inhibitory effect of bisphosphonates. The majority of 1,25-$(OH)_2VitD_3$-induced multinucleated TRAP(+) cells were OCLs containing more than 10 nuclei, which are thought to be more active than OCLs containing smaller numbers of nuclei (Figure 2B). In contrast, TRAP(+) OCLs formed in the presence of 10^{-8} and 10^{-7} M pamidronate, are largely made up with OCLs containing only 3 or 4 nuclei, indicating that pamidronate at these concentrations might inhibit fusion of osteoclast precursors into large multinucleated cells (Figure 2B).

To further investigate the effect of bisphosphonates on

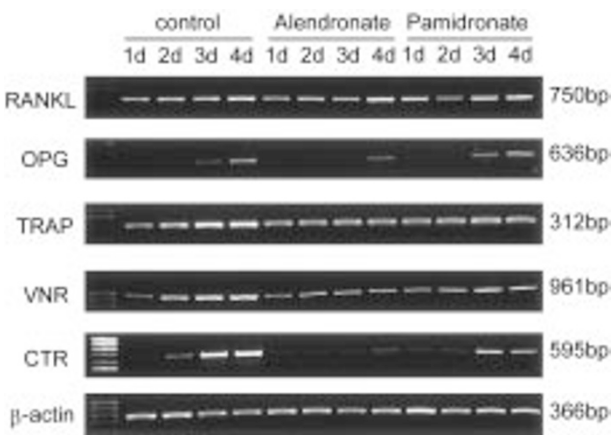

Figure 4. Effect of bisphosphonates on the mRNA expression of RANKL, OPG, and osteoclast markers in cocultures. Mouse bone marrow cells (3×10^6 cells) and osteoblastic cells (1.5×10^4 cells) were cocultured in 60 mm dish in the presence of 10 nM of 1,25-$(OH)_2VitD_3$. 10^{-5} M Alendronate or pamidronate was added over the culture period. After indicated culture periods, total RNA was isolated and used for semiquantitative RT-PCR. TRAP, tartrate-resistant acid phosphatase; VNR, vitronectin receptor; CTR, calcitonin receptor

the *in vitro* osteoclast activity, we measured the area of bone mineral-like apatite crystal resorbed by OCLs. As shown in Figure 3, both alendronate and pamidronate reduced the area resorbed by OCLs. In this study, the reduction of resorption area by bisphosphonates seems to be attributable to the decrease in the number of OCLs rather than the direct effect on the resorption machinery, since both alendronate and pamidronate inhibited osteoclast formation almost completely at 10^{-5} M.

The coculture system used in this study has different microenvironment from the culture of osteoblasts alone in that the interactions between two cell population could affect each other. Thus, it needs to explore whether bisphosphonates regulate RANKL or OPG ex-pression in this coculture system. We performed semiquantitative RT-PCR by using the total RNA from the cocultures treated with alendronate or pamidronate for the indicated times during coculture. In the presence of 1,25-$(OH)_2VitD_3$, the mRNA expression of os-teoclastic markers, such as TRAP, VNR, and CTR, were increased according to the differentiation (Figure 4). In cocultures treated with alendronate or pamidronate, however, the markers were hardly upregulated, consistent with the TRAP staining result. In control cul-ture, 1,25-$(OH)_2VitD_3$-induced RANKL mRNA ex-pression was maintained during the culture period, while suppression of OPG mRNA expression by 1,25-$(OH)_2VitD_3$ was relieved according to the progression of culture periods. Both alendronate and pamidronate failed to alter the mRNA expression level of RANKL and OPG over the culture period. These results show that the inhibition of osteoclast formation by alendronate and pamidronate is not associated with the regulation of RANKL or OPG mRNA expression.

Bisphosphonates manifest their anti-resorptive action via several mechanisms including direct effects on osteoclasts and indirect actions through osteoblasts. They have been suggested to inhibit differentiation, survival, and activity of osteoclast through osteoblast-secreted osteoclast-inhibiting factor, which has not yet been identified (Vitte *et al*., 1996; Yu *et al*., 1996). Recently, Mackie *et al*. reported that pamidronate reduced RANKL mRNA expression in UMR-106 osteosarcoma cell line after a 6-day culture, but did not alter OPG expression, which is different from our results (Mackie *et al*., 2001). The discrepancy might be attributed to the differences in the source of cells such as species and primary vs. cancer cells. Although our results demonstrated that bisphosphonates did not significantly affected RANKL and OPG mRNA levels, it could not be ruled out that they would modulate RANKL and OPG expression at the post-transcriptional level, necessitating further study at the protein level.

In conclusion, the present study showed that nitrogen-containing bisphosphonates, alendronate and pamid-ronate, did not alter the RANKL and OPG mRNA expression in favor of the inhibition of the osteoclast formation. Although alendronate and pamidronate in-hibited osteoclast formation and bone resorption in cocultures of mouse osteoblastic cells and bone marrow cells, these inhibitory effects did not appear to be linked to the regulation of RANKL and OPG mRNA expression. Taken together, it could be suggested that RANKL and OPG might not be the main target of bisphosphonates to inhibit bone resorption.

Acknowledgements

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