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Clodronate stimulates osteoblast differentiation in ST2 and MC3T3-E1 cells and rat organ cultures

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Abstract

We investigated the direct effects of various bisphosphonates on osteoblasts. At 10^{-5} M, clodronate increased alkaline phosphatase activity in cultured MC3T3-E1 (osteoblast-like line) and ST2 (pluripotent mesenchymal line) cells. Etidronate significantly increased alkaline phosphatase activity at 10^{-5} M only in MC3T3-E1 cells. These effects were due to an increase in alkaline phosphatase-positive cell numbers, and the differentiation-enhanced cells were capable of mineralization (von Kossa stain). Other bisphosphonates (pamidronate, alendronate, and incadronate) did not increase alkaline phosphatase activity in either cell line. In cultured rat calvariae, clodronate stimulated the expression of genes for alkaline phosphatase and osteocalcin (osteoblast-differentiation markers), but decreased the expression of the gene for tartrate-resistant acid phosphatase (osteoclast marker). Clodronate, etidronate, and incadronate inhibited protein Tyr phosphatase and Ser/Thr phosphatase activities in MC3T3-E1 cells. These data suggest that clodronate acts directly on mesenchymal cells to enhance osteoblast differentiation, and this effect may be partly expressed through inhibition of protein Tyr phosphatase and/or Ser/Thr phosphatase activity.

Keywords: Bisphosphonate; Osteoblast; Differentiation; Alkaline phosphatase; Protein phosphatase; Protein tyrosine phosphatase

1. Introduction

Bisphosphonates, well-known bone antiresorptive agents, are used widely in the treatment of metabolic bone diseases such as Paget's disease, hypercalcemia, and osteoporosis (Fleish, 1991; Rodan and Balena, 1993). Bisphosphonates can be divided into two groups: those that do not contain an amino group in the molecule (nonamino-bisphosphonates, such as etidronate, clodronate, and tildronate) and those that do (amino-bisphosphonates, such as pamidronate, alendronate, incadronate, zoledronate and ibandronate). The two types of bisphosphonates differ in the effects they exert on osteoclasts (Russell and Rogers, 1999): Clodronate is metabolized to nonhydrolyzable analogs of ATP that inhibit ATP-dependent intracellular enzymes, while alendronate inhibits the mevalonate pathway.

The actions of bisphosphonates on osteoclasts have been the focus of attention because these drugs strongly inhibit osteoclastic bone resorption. However, there have been some studies of the ways in which bisphosphonates act on osteoblasts to modify bone metabolism. First, bisphosphonates stimulate bone formation. In in vitro experiments, clodronate was found to stimulate collagen synthesis in rabbit articular chondrocytes (Guenther et al., 1981) and rat bone cells (Gallagher et al., 1982; Guenther et al., 1981). Moreover, bisphosphonates have been shown to increase alkaline phosphatase activity in a chick periosteal osteogenesis model (Goziotis et al., 1995; Tenenbaum et al., 1992), a murine osteoblastic cell line (Igarashi et al., 1997), rat bone marrow cells (Klein et al., 1998), and human osteoblasts (Reinholz et al., 2000; Tsuchimoto et al., 1994). Furthermore, amino-bisphosphonates enhance osteoblast minerali-

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zation via an inhibition of the mevalonate pathway (Fujita et al., 2001; Reinholz et al., 2002). In in vivo experiments, 1 month's treatment with etidronate or alendronate has been found to increase the number of colony-forming units for fibroblasts (CFU-F), the early cell source of osteoblast progenitors, in mouse bone marrow (Giuliani et al., 1998). Moreover, etidronate treatment promoted closure of wounds created in the calvarial bone by surgery in rats (D'Aoust et al., 2000).

Second, bisphosphonates have inhibitory effects on bone resorption via actions on osteoblasts. Bisphosphonates act directly on osteoblasts, causing them to secrete a substance (subsequently named osteoprotegerin; Viereck et al., 2002) that inhibits both osteoclastic bone resorption and osteoclast formation (Nishikawa et al., 1996; Vitté et al., 1996).

Third, bisphosphonates inhibit apoptosis among osteoblasts and osteocytes, and these effects can be blocked by specific inhibitors of extracellular signal-regulated kinase (ERK) activation (Plotkin et al., 1999).

There have been several reports that bisphosphonates inhibit protein Tyr phosphatase and thereby modulate bone metabolism (Endo et al., 1996; Murakami et al., 1997; Opas et al., 1997; Schmidt et al., 1996). In chick calvarial osteoblasts and osteoblast precursor cells, the protein Tyr phosphatase inhibitor orthovanadate increased both alkaline phosphatase activity and collagen synthesis (Lau et al., 1988). Furthermore, a protein phosphatase inhibitor, okadaic acid, has been shown to enhance fetal calf serum- and prostaglandin E_1-induced alkaline phosphatase activity in MC3T3-E1 cells, which are murine osteoblastic cells (Ito et al., 1996; Watanabe et al., 1994).

In this study, we set out to determine whether bisphosphonates act directly on osteoblast-like cells and pluripotent mesenchymal cells to accelerate osteoblast differentiation. In addition, we examined the effects of bisphosphonates on protein Tyr phosphatase and Ser/Thr phosphatase activities to try to elucidate the mechanisms underlying the effects of these drugs on osteoblasts.

2. Materials and methods

2.1. Bisphosphonates

Clodronate disodium tetrahydrate was obtained from Leiras Oy (Turku, Finland). Etidronate disodium tetrahydrate was purchased from Lion (Tokyo, Japan) and extracted by Kissei Pharmaceutical (Nagano, Japan). Alendronate monosodium trihydrate, pamidronate disodium hexahydrate, and incadronate disodium monohydrate were synthesized by Kissei Pharmaceutical.

2.2. Cell culture

MC3T3-E1 cells and ST2 cells (both murine) were purchased from the Riken cell bank (Ibaragi, Japan). Using 24-well plates, MC3T3-E1 cells were seeded (density, 1×10^4 cells/well) into α-minimum essential medium (MEM) containing 10% fetal bovine serum (Gibco, NY, USA). Drugs and/or ascorbic acid (50 μg/ml) were added 2 days after seeding. Fifteen days after drug additions, cells were washed with phosphate-buffered saline (PBS) and scraped off with 0.1% Triton X100. The cell lysate was sonicated and centrifuged, and the supernatant was stored at −30 °C. ST2 cells were seeded (24-well plates; density 2×10^4 cells/well) into RPMI 1640 medium containing 10% fetal calf serum, and drugs were added 2 days after seeding. After drug administration, ST2 cells were treated in the same way as MC3T3-E1 cells.

2.3. Alkaline phosphatase activity

Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate (SIGMA FAST® *p*-nitrophenyl phosphate tablet set; Sigma-Aldrich Japan K.K., Tokyo, Japan) as a substrate. Samples were incubated at 37 °C for 5 min, and absorption was measured at 410 nm, with *p*-nitrophenol being used as a standard. The protein concentration was measured by means of a bicinchoninic acid (BCA) protein-assay kit (Pierce Biotechnology, IL, USA).

2.4. Alkaline phosphatase stain

ST2 cells were seeded into eight-chamber slides (Nunc, NY, USA) at a density of 2.6×10^4 cells/cm^2 and cultured with RPMI 1640 medium containing 10% fetal calf serum. The next day, drugs and 50 μg/ml L-ascorbic acid were added, and culturing was allowed to proceed for 6 days. Cells were washed with PBS, then fixed with 10% formalin/PBS. The procedure used for alkaline phosphatase staining was based on a method previously described (Higuchi et al., 1979). Briefly, naphthol AS-MX phosphate (0.1 mg/ml: Sigma-Aldrich) and fast red–violet LB salt (0.2 mg/ml: Sigma-Aldrich) were dissolved in 0.1 M Tris buffer (pH 8.1) and then filtered, and this solution was used as the staining solution. Cells were incubated with the staining solution at room temperature, then washed well with distilled water. Images of the sections were captured into a computer via a CCD camera attached to a microscope. The stained area and the number of colonies were determined by use of a semiautomatic image-analyzing program (Mac Scope; Mitani, Fukui, Japan).

2.5. von Kossa stain

MC3T3-E1 cells were seeded into six-well plates at a density of 2.5×10^4 cells/well, and cultured with α-MEM medium containing 10% fetal calf serum. The next day, bisphosphonates, 50 μg/ml L-ascorbic acid, and 7 mM β-glycerophosphate (Sigma-Aldrich) were added, and culturing was allowed to proceed for 23 days. Bisphosphonates

were removed from the culture medium after 10 days. Cells were washed with HEPES-buffered saline (HBS) and fixed with 2.5% glutaraldehyde/HBS. Then, they were incubated with 5% silver nitrate at room temperature under UV light for 5 min. After being washed, cells were fixed using 5% sodium thiosulfate.

2.6. Organ culture

Fetal rat calvariae were cultured by a previously described method with several modifications (Tsutsumi et al., 1994). Pregnant Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan) at 19 days of gestation were killed by decapitation under ether anesthesia. The calvarial bones were aseptically removed from the fetuses, and each was divided into two pieces. The two pieces were incubated for 2 h at 37 °C with BGJb-HW2 medium (Nissui Pharmaceutical, Tokyo, Japan), and then one was transferred to a culture tube and cultured for 4 days (37 °C; rotation speed 35 rph) with 1 ml of BGJb-HW2 medium containing a bisphosphonate. The other piece was cultured in the same way, but with BGJb-HW2 medium alone, and this sample was used as a control. Total RNA was purified from the cultured bone using Isogen (Nippon Gene, Tokyo, Japan).

2.7. Northern blot

Total RNA (20 μg) isolated from cultured calvariae was separated on formaldehyde agarose gels, transferred to a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL, USA), and subjected to hybridization with the indicated probes. After hybridization, the membranes were washed in a solution of 2 × standard sodium citrate (SSC; 0.3 M sodium chloride and 0.03 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS), and finally in 0.2 × SSC/0.1% SDS solution at 55–65 °C. They were then exposed to Image Plate (Fuji Photo Film, Tokyo, Japan). Radioactivity was measured using a bio-imaging analyzer (Mac-BAS; Fuji Photo Film).

Partial cDNAs of alkaline phosphatase (J03572; 174–1384 bp) and tartrate-resistant acid phosphatase (AF010306; 268–975 bp) were used as probes. A β-actin probe was purchased from Clontec (CA, USA). The cDNAs were labeled with [α-^{32}P]dCTP (NEN), using a BcaBEST labeling kit (Takara, Shiga, Japan).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (0.5 μg) was amplified using RT-PCR by means of a RNA PCR kit (Promega, WI, USA) and thermalcycler (GeneAmp PCR System 9700; PE Applied Biosystems, CA, USA). For the amplification of osteocalcin, the primers used were 5′-gcagacaccatgaggaccct-3′ and 5′-gacctctcatcggtttcgac-3′. For the amplification of β-actin, primers were purchased from Clontec. PCR products were separated by electrophoresis using agarose gel and stained with etidium bromide. Images of the bands were analyzed by using a semiautomatic image-analysis program (NIH Image ver. 1.61).

2.9. Ser/Thr phosphatase and protein Tyr phosphatase activities

When MC3T3-E1 cells had become subconfluent, they were scraped off and homogenized by sonication with homogenate buffer. The homogenate buffer for protein Tyr phosphatase activity was 500 mM acetate buffer (pH 5.2) containing 1 mM ethylenediaminetetraacetate (EDTA), 2 mM β-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride, while that for Ser/Thr phosphatase activity was 50 mM imidazole–HCl buffer (pH 7.2) containing 0.2 mM EGTA, 0.02% β-mercaptoethanol, 0.1 mg/ml bovine serum albumin, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 600 × *g*, and the supernatant was further centrifuged at 10,500 × *g*. The supernatant was mixed with Triton X-100 (final concentration, 0.1%) and used as the cytosol fraction. The pellet was resuspended in buffer containing 0.1% Triton X-100 and centrifuged at 100,000 × *g*. This supernatant was used as the membrane fraction.

Protein Tyr phosphatase and Ser/Thr phosphatase activities were determined by means of Tyr phosphatase assay system (Promega) and Ser/Thr phosphatase assay system (Promega), respectively. The phosphopeptides END(pY)INASL (Tyr-Phosphopeptide-1) (Daum et al., 1993) and DADE(pY)LIPQQG (Tyr-Phosphopeptide-2) (Zhang et al., 1993) serve as substrates for many PTPs. The phosphopeptide RRA(pT)VA (Ser/Thr Phosphopeptide) is compatible with several Ser/Thr phosphatases, such as protein phosphatase 2A, 2B, 2C (Donella Deana et al., 1990). The absence of Mg^{2+} provides suitable conditions to measure protein phosphatase-2A, while its presence is required to measure protein phosphatase-2C. However, the supplied Ser/Thr phosphopeptide is a poor substrate for Ser/Thr phosphatase-1 because of its more stringent structural requirements. The reactions were performed for 40 min at 37 °C for protein Tyr phosphatase, and for 20 min at 37 °C for Ser/Thr phosphatase.

2.10. Statistical analysis

All data are presented as the means ± S.E. (except where otherwise noted). Statistical analysis was carried out using SAS/STAT software ver. 6 (SAS Institute Japan, Tokyo, Japan). Time-course data were analyzed using Dunnett's multiple-comparison test (vs. Day 0). The differences between groups cultured with or without ascorbic acid (ascorbic acid+ and ascorbic acid −, respectively) were analyzed using a *t*-test procedure for individual time-points. The dose–response data were analyzed

using a parametric or non-parametric (normalized using control values) Dunnett's multiple-comparison test. Other numerical data were analyzed for the factor 'treatment' using the *t*-test procedure. IC_{50} values were calculated using GraphPad PRISM® ver. 3.00 (GraphPad Software, San Diego, CA, USA). The significance level was set at $P<0.05$.

3. Results

3.1. Effects of bisphosphonates on alkaline phosphatase activity and mineralization in stem cell- and osteoblast-like cell lines

MC3T3-E1 cells differentiated into osteoblast-like cells (to judge from their alkaline phosphatase activity) in the presence of 50 μg/ml ascorbic acid (Fig. 1), the level of alkaline phosphatase activity being significantly increased from Day 12 onwards and reaching a plateau on Day 15 (127 times higher than on Day 0). Alkaline phosphatase activity was only slightly increased in the absence of ascorbic acid (maximal, 19 times higher than on Day 0).

To examine the effects of bisphosphonates on osteoblast differentiation, MC3T3-E1 cells were cultured with bisphosphonates for 15 days, and alkaline phosphatase activity was measured as a marker of osteoblast differentiation. Clodronate and etidronate significantly increased alkaline phosphatase activity at 10^{-5} M (clodronate, 153% of control; etidronate, 111% of control), but other bisphosphonates did not (Fig. 2A). In ST2 cells, a pluripotent mesenchymal cell line, only clodronate (10^{-5} M) increased alkaline phosphatase activity (373% of control) (Fig. 2B).

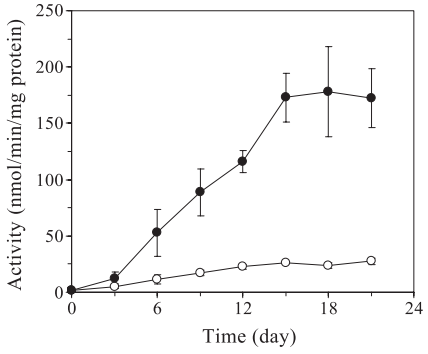

Fig. 1. Time course of changes in alkaline phosphatase activity in MC3T3-E1 cells in the presence or absence of ascorbic acid. ○: In the absence of ascorbic acid, ●: in the presence of ascorbic acid (50 μg/ml). Data represent means ± S.E. of three individual experiments. Each experiment was done in triplicate. Significant difference vs. Day 0: ascorbic acid −, from Days 9 to 21; ascorbic acid+, from Days 12 to 21. Significant difference between ascorbic acid − and ascorbic acid+: from Days 12 to 21 (except for Day 18).

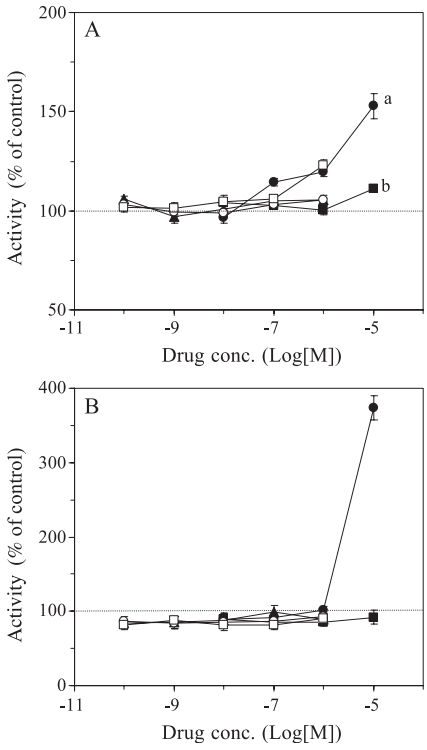

Fig. 2. Effects of bisphosphonates on alkaline phosphatase activity in MC3T3-E1 (A) or ST2 (B) cells. ●, clodronate; ■, etidronate; ▲, alendronate, ○, pamidronate; □, incadronate. Data represent means ± S.E. of three or four individual experiments. Each experiment was done in triplicate. The absolute values (nmol/min/mg protein) in control group were 178 ± 7, 148 ± 2, 122 ± 6 in MC3T3-E1 cells, and 1.01 ± 0.02, 1.64 ± 0.13, 2.46 ± 0.44, 3.52 ± 0.94 in ST2 cells. Significant difference vs. control: a, $P<0.05$; b, $P<0.01$.

To examine whether the increase in alkaline phosphatase activity was due to an increase in alkaline phosphatase activity per cell or to an increase in the number of alkaline phosphatase-positive cells, ST2 cells were stained for alkaline phosphatase activity. The alkaline phosphatase-positive area was significantly increased by clodronate (267% of control) but decreased by alendronate (to 60% of control) when each was used at a concentration of 10^{-5} M (Table 1).

MC3T3-E1 cells were cultured in the presence or absence of bisphosphonates and then stained by von

Table 1
Alkaline phosphatase-positive area in ST2 cells cultured with clodronate or alendronate (10^{-5} M)

	Area (arbitrary unit)
Control	10837 ± 2719
Clodronate	28943 ± 6757[a]
Alendronate	6496 ± 1998[b]

Data represent means ± S.E. of three experiments. Each experiment was done in quadruplicate.
Significant difference vs. control: [a]$P<0.05$; [b]$P<0.01$.

Table 2
Von Kossa stain-positive area and colony number in MC3T3-E1 cells cultured with bisphosphonates

		Area (mm^2/well)	Colony number (/well)
Control		1.95 ± 1.96	3.33 ± 2.31
Clodronate	3×10^{-7} M	1.52 ± 1.88	2.67 ± 1.53
	3×10^{-6} M	3.37 ± 0.97	7.67 ± 1.53
	3×10^{-5} M	33.69 ± 18.42[a]	68.67 ± 15.04[b]
Etidronate	1×10^{-5} M	0.39 ± 0.22	1.67 ± 0.58
Alendronate	3×10^{-6} M	1.55 ± 1.16	4.33 ± 1.53
Pamidronate	3×10^{-6} M	1.12 ± 0.83	3.67 ± 2.08
Incadronate	3×10^{-6} M	1.16 ± 0.94	3.00 ± 1.73

Data represent means ± S.E. of individual three experiments. Each experiment was done in triplicate.
Significant difference vs. control: [a]$P<0.05$; [b]$P<0.01$.

Kossa stain to examine whether differentiation-stimulated cells had the potential for mineralization. Both the number of mineralized nodules and the area showing mineralization were significantly increased by clodronate at 3×10^{-5} M (each to about 20 times higher than control) (Table 2), but the other bisphosphonates were ineffective.

3.2. Effects of bisphosphonates on gene expression in cultured calvariae

We next examined whether bisphosphonates stimulated osteoblast differentiation in cultured rat calvariae (an experimental system more similar to natural conditions than cultured cell lines). Gene expression was used as an index of cell differentiation and of the suppressive effects on osteoclasts. The expression of alkaline phosphatase and that of the osteocalcin gene, markers of osteoblast differentiation, were stimulated by clodronate at a concentration of 10^{-5} M (alkaline phosphatase, 198% of control; osteocalcin, 281% of control) (Fig. 3A and B). However, the other bisphosphonates used in this experiment were ineffective. The expression of the tartrate-resistant acid phosphatase gene was significantly decreased by all bisphosphonates tested (to 20–50% of control at 10^{-5} M) (Fig. 3C).

3.3. Effects of bisphosphonates on phosphatase activity

Because bisphosphonates are analogues of pyrophosphate, their inhibitory effects on phosphatase activity were examined. For this, we used MC3T3-E1 cells and chemically synthesized peptides as sources of enzymes and substrate. When Tyr-Phosphopeptide-1 or -2 was used as substrate, clodronate had a stronger inhibitory effect than the other bisphosphonates (3.5–63 times more potent than etidronate and 8.4–24 times more potent than incadronate) (Table 3). The inhibitory effect of clodronate on protein Tyr phosphatase activity was 7.9–27 times more potent than that of orthovanadate (which is known to be a protein Tyr phosphatase inhibitor) when Tyr-Phosphopeptide-1 was used as substrate (data not shown). The corresponding factor was 1.2–3.2 times for Tyr-Phosphopeptide-2. Alendronate and pamidronate did not inhibit protein Tyr phosphatase activity.

When Ser/Thr phosphopeptide was used as substrate, inhibitory effects were observed only in the absence of Mg^{2+} (Table 3), the rank order of potency being clodronate>etidronate (1/23 of clodronate)>incadronate (1/171 of clodronate)>pamidronate (1/236 of clodronate). The potency of clodronate was 864 times weaker than that of okadaic

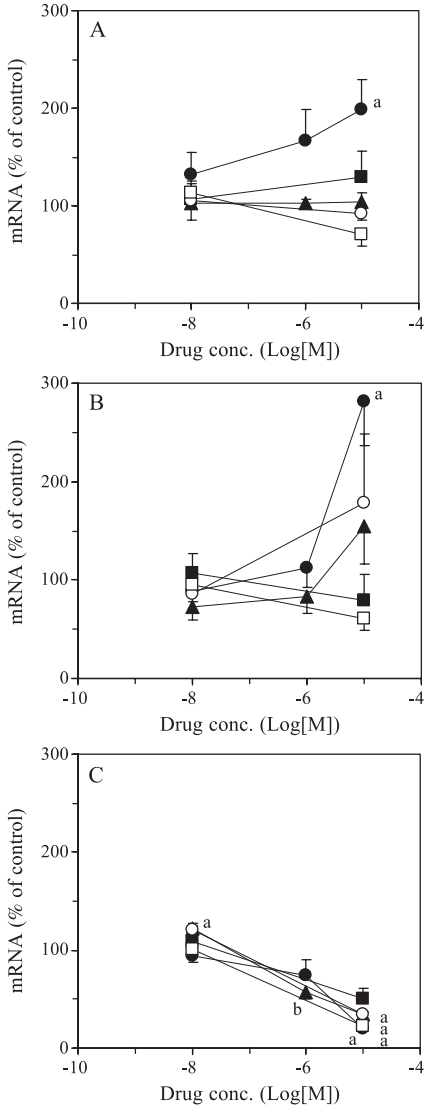

Fig. 3. Effects of bisphosphonates on gene expression in cultured rat calvariae. (A), alkaline phosphatase; (B), osteocalcin; (C), tartrate-resistant acid phosphatase. ●, clodronate; ■, etidronate; ▲, alendronate, ○, pamidronate; □, incadronate. Data represent means ± S.E. of four individual experiments. In each experiment, four pieces of calvariae were used in one group. Significant difference vs. control: a, $P<0.05$; b, $P<0.01$.

Table 3
IC_{50} values for effects of bisphosphonates on phosphatase activity (μM)

	Clodronate	Etidronate	Alendronate	Pamidronate	Incadronate
Tyr-Phosphopeptide-1 (END(pY)INASL)					
Membrane	0.381 (0.0963–0.986)	23.8 (4.09–139)	>100	>100	3.21 (0.582–17.7)
Cytosol	2.78 (0.69–11.2)	–	>100	>100	>100
Tyr-Phosphopeptide-2 (DADE(pY)LIPQQG)					
Membrane	1.00 (0.568–1.76)	3.45 (0.293–40.7)	>100	>100	23.8 (8.11–70.1)
Cytosol	2.70 (1.82–4.01)	>1000	>100	>100	32.4 (2.73–384)
Ser/Thr Phosphopeptide (RRA(pT)VA)					
$-Mg^{2+}$	0.198 (0.162–0.241)	4.49 (1.51–13.4)	>100	46.5 (31.2–69.4)	33.9 (22.7–50.6)
$+Mg^{2+}$	>10	>1000	>100	>100	>100

Data represent the IC_{50} values (95% confidence limits).

acid, which is known to be a Ser/Thr phosphatase inhibitor (data not shown).

4. Discussion

In this study, we have shown that clodronate stimulates osteoblast differentiation in osteoblast-like and pluripotent mesenchymal cells (Fig. 2 and Table 1). The differentiation-stimulated cells not only expressed markers of differentiation but also had the potential for mineralization, which is an important osteoblast function (Table 2). Moreover, this stimulatory effect of clodronate was observed under crude conditions (cultured rat calvariae) in which other kinds of cells, such as osteoclasts, osteocytes, and bone marrow cells, coexisted (Fig. 3A and B). These data suggest that clodronate has the ability to induce bone formation, and that this effect can be expressed when there is also an inhibition of osteoclastic bone resorption.

Stimulatory effects of bisphosphonates on osteoblast differentiation and mineralization have been reported previously for the bisphosphonates used in this study, and also for zoledronate. For example, bisphosphonates increased alkaline phosphatase activity in a chick periosteal osteogenesis model (Goziotis et al., 1995; Tenenbaum et al., 1992), a murine osteoblastic cell line (Igarashi et al., 1997), rat bone marrow cells (Klein et al., 1998), and human osteoblasts (Reinholz et al., 2000; Tsuchimoto et al., 1994). A few years ago, nonamino-bisphosphonates (such as clodronate and etidronate) were shown to be metabolized to nonhydrolyzable analogs of ATP, which inhibit ATP-dependent intracellular enzymes, while amino-bisphosphonates (such as alendronate, pamidronate, incadronate and zoledronate) were found to inhibit the mevalonate pathway (Russell and Rogers, 1999). Fujita et al. (2001) recently reported that bisphosphonates act on phosphate-sensitized MC4 cells to accelerate mineralization via the non-RAS-MEK-ERK1/2-Cbfa1 transactivation pathway, with incadronate having an additional inhibitory action on the mevalonate pathway. Then, Reinholz et al. (2002) showed that zoledronate induced human osteoblast differentiation via inhibition of the mevalonate pathway. In contrast, in the present study, only clodronate had a clear stimulatory effect on osteoblast differentiation. The reasons for this discrepancy are unclear, but the different cell lines and culture conditions used may be important factors.

Some years ago, a protein Tyr phosphatase inhibitor, orthovanadate, was found to increase alkaline phosphatase activity and collagen synthesis in chick calvarial osteoblasts and osteoblast precursor cells (Lau et al., 1988). Further, a Ser/Thr phosphatase inhibitor, okadaic acid, was shown to enhance fetal calf serum- and prostaglandin E1-induced alkaline phosphatase activity in MC3T3-E1 cells (Ito et al., 1996; Watanabe et al., 1994). Okadaic acid may act downstream of protein kinase A (Ito et al., 1996) and protein kinase C (Watanabe et al., 1994). Inhibitory effects of bisphosphonates on protein Tyr phosphatase activity have also been reported (Endo et al., 1996; Murakami et al., 1997; Opas et al., 1997; Schmidt et al., 1996). For example, in calvaria-derived osteoblasts, alendronate and etidronate inhibited protein Tyr phosphatase-σ and stimulated cellular proliferation, which is the opposite of differentiation in general (Endo et al., 1996). For that reason, we examined the inhibitory potency of bisphosphonates on protein Tyr phosphatase and Ser/Thr phosphatase activities in MC3T3-E1 cells (Table 3). In our study, clodronate had the most potent effects on both protein Tyr phosphatase and Ser/Thr phosphatase activities among the bisphosphonates used. Clodronate inhibited membrane-associated protein Tyr phosphatase more powerfully than cytosolic protein Tyr phosphatase and was approximately equal in potency to orthovanadate when Tyr-Phosphopeptide-2 was used as a substrate. The rank order of potency was clodronate>incadronate>etidronate≫alendronate and pamidronate, and this order seemed closely related to the potency for causing osteoblast differentiation in MC3T3-E1. Clodronate also strongly inhibited protein phosphatase 2C-like phosphatase, and the rank order of potency was clodronate>etidronate>incadronate>pamidronate≫alendronate. Considering the rank order of potency, inhibition of protein Tyr phosphatase activity might be related more to osteoblast differentiation than inhibition of Ser/Thr phosphatase activity in MC3T3-

E1 cells. Alendronate did not inhibit protein Tyr phosphatase activity. This result was consistent with the potency with which these bisphosphonates affected osteoblast differentiation in MC3T3-E1 cells. However, it has been reported by others that several protein Try phosphatases are inhibited by alendronate (Endo et al., 1996; Opas et al., 1997; Schmidt et al., 1996). The reason for this discrepancy is unclear at present; however, the different enzyme sources and substrates may be relevant factors.

In MC3T3-E1 cells, etidronate increased alkaline phosphatase activity significantly at 10^{-5} M, and incadronate increased alkaline phosphatase activity to the same level as clodronate when both were used at 10^{-6} M (Fig. 2A). However, neither etidronate nor incadronate increased alkaline phosphatase activity in ST2 cells or gene expression in cultured rat calvariae (Figs. 2 and 3). In ST2 cells, the IC_{50} values of clodronate, etidronate and incadronate for membrane-associated protein Tyr phosphatase activity were 1.54, 66.2, and 21.2 μM when Tyr-Phosphopeptide-1 was used as substrate (data not shown). These values in ST2 cells were higher than those in MC3T3-E1 cells (2.2–4.0 times). These data suggested that 2×10^{-5}–7×10^{-5} M was the critical IC_{50} value for bisphosphonates to affect osteoblast differentiation.

Protein phosphorylation is closely involved in osteoblast differentiation. MC3T3-E1 cells constitutively express bone morphogenetic protein-2 (BMP-2), BMP-4, and BMP-7 (Xiao et al., 2002), and BMPs transduce signals through a unique class of Ser/Thr kinase receptors, which phosphorylate Smad proteins. Smad1, Smad5, and Smad8, when activated via BMP receptors, form heteromeric complexes with Smad4 and translocate into the nucleus, where they activate the transcription of specific genes such as Runt-related gene 2/core-binding factor α1 (Runx2/Cbfa1). The activity of focal adhesion kinase (FAK), which is immediately downstream of β1-integrin, is essential if BMP-Smad signaling is to stimulate osteoblast differentiation (Tamura et al., 2001). Therefore, protein phosphorylation is very important for osteoblast differentiation. Hence, clodronate-stimulated osteoblast differentiation may occur partly or entirely via protein Tyr phosphatase and/or Ser/Thr phosphatase inhibition.

Generally, bone formation is tightly coupled to bone resorption, and bone formation is decreased when bone resorption is strongly inhibited by bisphosphonates. In our previous animal studies, a high dose of clodronate inhibited bone resorption and formation simultaneously (Itoh et al., 1999, 2002). However, the inhibitory effects of clodronate on bone mineralization and formation are reportedly weaker than those of etidronate (Itoh et al., 1999; Lepola et al., 1996). In our calvarial experiment, the expression of genes for alkaline phosphatase and osteocalcin was enhanced by clodronate simultaneously with suppression of tartrate-resistant acid phosphatase-gene expression (Fig. 3). Therefore, a stimulatory effect of clodronate on bone formation might be partly responsible for the apparent weakness of clodronate as an inhibitor of bone resorption-coupled bone formation.

In this study, a stimulatory effect of clodronate on osteoblast differentiation was observed at 10^{-5} M or more. In clinical use, blood concentrations of clodronate also reach 10^{-5} M or more. For example, after infusion of 300 mg/kg of clodronate in patients with Paget's disease or prostatic cancer, the maximum serum concentration reached was 5.7–10.1 mg/l ($1.97–3.50 \times 10^{-5}$ M) (Hanhijärvi et al., 1989), while after oral administration of 800 mg clodronate to healthy volunteers, the maximum serum concentration was 443–482 ng/ml ($1.53–1.67 \times 10^{-6}$ M) (Laitinen et al., 2000). Therefore, on the basis of our data, an effect of clodronate on bone formation can be expected at clinically used doses.

In this study, the amino-bisphosphonates, alendronate, pamidronate, and incadronate were only examined at 10^{-6} M or less in MC3T3-E1 and ST2 cells, because their inhibitory action on bone resorption is up to 1000 times more potent than that of clodronate. However, in experiments involving von Kossa staining of MC3T3-E1 cells, alkaline phosphatase staining of ST2 cells, and cultured rat calvariae, we used concentrations of amino-bisphosphonates greater than 10^{-6} M, yet no stimulatory effects were observed. These data suggest that bisphosphonates other than clodronate (at least among those we tested) have little or no effect on osteoblast differentiation.

In conclusion, clodronate stimulated osteoblast differentiation in osteoblast-like and pluripotent mesenchymal cells (Fig. 2 and Table 1). The differentiation-stimulated cells not only expressed markers of differentiation but also had a potential for mineralization (Table 2). Moreover, such stimulation by clodronate was also observed in cultured rat calvariae (Fig. 3A and B), in which tartrate-resistant acid phosphatase, a marker of osteoclasts, was suppressed by all the bisphosphonates used (Fig. 3C). Clodronate also inhibited protein Tyr phosphatase and Ser/Thr phosphatase activities in MC3T3-E1 cells. These data suggest that clodronate acts directly on osteoblastic and mesenchymal cells and enhances osteoblast differentiation, and that this effect may be expressed in part through inhibition of protein Tyr phosphatase and/or Ser/Thr phosphatase activities.

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