Alendronate Modulates Osteogenesis of Human Osteoblastic Cells In Vitro

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ABSTRACT—The bisphosphonates, which are carbon-substituted pyrophosphates, have been studied extensively both in vivo and in vitro to elucidate their effects on bone tissues and cells. However, because these agents were shown to have a potent inhibitory effect on bone resorption, the majority of studies have focused on only this aspect of bone metabolism. There appears to be less information regarding the direct effect of bisphosphonates on bone formation, so thus we undertook experiments to investigate the effects of bisphosphonates, especially alendronate, on the mineralization and matrix protein synthesis of human osteoblastic cells in vitro. The data show that the bisphosphonates, alendronate, etidronate and pamidronate, suppressed 1,25-dihydroxycholecalciferol (1,25(OH)2D3)-stimulated mineralization of human osteoblastic cells at high concentrations, while relatively lower concentrations of alendronate and etidronate potentiated mineralization of the cells in the presence of 1,25(OH)2D3. The potentiation of mineralization with alendronate was accompanied by increased synthesis of bone matrix proteins, osteocalcin and collagen, and the mRNA of pro α(I) collagen. These findings show that in addition to their well-known effects on bone resorption, bisphosphonates have significant and direct effects on osteogenesis in osteoblasts in vitro. The actual mechanism remains to be further investigated.

Keywords: Osteoblast (human), Bisphosphonate, Mineralization, Collagen, Osteocalcin

Bisphosphonates are carbon-substituted pyrophosphate analogues that include potent inhibitors of bone resorption (1), and they have been effectively used to control osteolysis or reduce bone loss in Paget’s disease (2), metastatic bone disease and hypercalcemia of malignancy (3), and osteoporosis (4–9).

The majority of the studies on these bisphosphonates that have potent inhibitory effect on bone resorption have focused on only this aspect of bone metabolism (10–14). However, there appears to be less information regarding the direct effect of bisphosphonates on bone formation. Indeed there seem to be some controversial findings in this regard showing that the bisphosphonates may stimulate alkaline phosphatase (ALP) activity (15, 16), prostaglandin E2 (PGE2) synthesis (17), collagen synthesis (18) and mineralization (16, 19) or inhibit ALP activity (16, 20), PGE2 synthesis (17), collagen synthesis (21) and mineralization (16, 21) of osteoblasts. This controversy may be a reflection of the problems associated with the use of varied and different model systems, including rat bone fragment (ex vivo) (21), rat calvaria cells (15, 17, 18), human osteoblast cells (20), chick periosteal osteogenesis model (in vitro) (16) and chick osteoblast cells (19).

In view of the above controversy, we undertook experiments to investigate the effects of bisphosphonates on the mineralization and matrix protein synthesis of human osteoblastic cells in vitro, using mainly alendronate, 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid, because this bisphosphonate is one of the most potent inhibitors of bone resorption in vitro (22) in experimental animals (23–28) and in patients with Paget’s disease (29–32) and osteoporosis (33).
MATERIALS AND METHODS

Materials

The following materials were obtained from the indicated commercial sources: Eagle’s α-minimum essential medium (MEM) (Life Technologies, Inc., Gaithersburg, MD, USA); fetal bovine serum (FBS) (Irvine Scientific, Santa Ana, CA, USA); α-glycerophosphate-2Na (α-GP) (Tokyo Kasei Co., Tokyo); trypsin, p-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO, USA); Gla-Osteocalcin assay kit (Takara Biomedicals, Kyoto). 1,25-Dihydroxycholecalciferol (1,25(OH)2D3), alendronate, etidronate and pamidronate were chemically synthesized at the Teijin Institute for Bio-Medical Research.

Cell culture

Human osteoblast-like periosteal cells, derived from the femur of 10-year-old boy, were generously supplied by Dr. Yasuko Koshihara of the Tokyo Metropolitan Institute of Gerontology. The cells were established in culture as previously reported (34–36). They demonstrated functional and morphological characteristics typical of osteoblasts, including high ALP activity, osteocalcin production and mineralization ability induced by α-GP plus 1,25(OH)2D3. Cells at 19 population doublings (PDL) in 6- or 12-well culture plates were cultured in α-MEM supplemented with 100% FBS at 37°C in 5% CO2 / 95% air. Two or three days after they reached confluence, the cells were cultured with or without 1,25(OH)2D3 in the presence of 2 mM α-GP for appropriate periods (3–14 days). Usually, the Ca content of the cells reaches a detectable level in 7-days cultures without 1,25(OH)2D3. However, the values of Ca content and the time at which the Ca content becomes detectable may vary between experiments. 1,25(OH)2D3 was dissolved in ethanol and added into culture medium at final concentrations of 10–100 ng/ml (the final ethanol concentration was 0.1% (v/v)). Each bisphosphonate was dissolved in phosphate-buffered saline (PBS) and added into culture medium at final concentrations of 10–14–10–5 M (the final PBS concentration was below 0.1% (v/v)). Culture media were replaced every 2 or 3 days.

RT-PCR

Total RNA was prepared from human osteoblastic cells by the AGPC method (37). The cDNA was synthesized from 1 μg of total RNA at 37°C for 60 min. By use of HCOLA-1A (5'-CCA CCG ACC AAG AAA CCA-3') and HCOLA-1B (5'-GCT CAC CAG GAC GAC CAG-3') for the pro α1(I) collagen gene, HBGP-1A (5'-CCT CAC ACT CCT CGC CCT ATT-3') and HBGP-1B (5'-ATA GGC CTC CTG AAA GCC GAT-3') for the osteocalcin gene as specific primers, PCR amplification was carried out in a thermocycler oven (MiniCycler PTC-150; MJ Research, Watertown, MA, USA). Denaturation for 30 cycles at 94°C for 1 min, annealing at 55°C (osteocalcin) or at 60°C (pro α1[I] collagen) for 2 min and extension at 72°C for 3 min were routinely performed after an initial 2-min denaturation. After electrophoresis on agarose gels, the PCR products were stained with ethidium bromide and photographed (Polaroid ACMEL M-085 Auto).

Measurement of calcium, phosphorus, ALP activity, osteocalcin and collagen production and DNA content

ALP activity was assayed by the method of Maio and de Carli (38) with p-nitrophenyl phosphate as the substrate. At the end of culture, the cells were washed three times with saline and agitated for 15–20 min in a solution of 10 mM p-nitrophenyl phosphate in 1 mM MgCl2 / 0.1 M carbonate buffer (pH 10.0). The reacted solution was then withdrawn and its optical density measured at 415 nm.

Cell layers were washed again with saline and incubated twice with 5% perchloric acid (PCA) in an ice bath for 15 min to extract calcium (Ca) and phosphorus (Pi). The amounts of Ca and Pi were measured by the o-cresolphthalein complexone (OCPC) method (39) and according to Chen et al. (40), respectively.

Osteocalcin was extracted by sonication of the cells in 20% formic acid; the supernatant containing osteocalcin was then lyophilized and stored at −80°C until assayed. Osteocalcin was determined by means of an enzyme-linked immunoassay using a Gla-Osteocalcin assay kit.

The amount of collagen accumulated in the cell layer was estimated from the hydroxyproline content determined by the method of Kivirikko et al. (41) after hydrolysis by constant boiling HCl (6 N) at 130°C for 30 min in an autoclave.

DNA content was measured by the method of Burton (42) after extraction with PCA.

Statistical analyses

Statistical analyses were performed by the two-tailed Dunnett’s test (multiple comparisons).

RESULTS

Effects of bisphosphonates in a wide range of concentration on mineralization of human osteoblastic cells

Alendronate at concentrations of 10–12–10–8 M potentiated the accumulation of Ca by human osteoblastic cells in 7-day cultures in the presence of 100 ng/ml of 1,25-(OH)2D3 and, conversely, suppressed it at 10–6 M and above (Fig. 1). The potentiative effect was maximal at around 10–9 M and not observed in the absence of 1,25-(OH)2D3 (data not shown). The suppressive effect of alen-
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Fig. 1. Effects of alendronate and etidronate on Ca accumulation of human osteoblastic cells. Confluent human osteoblastic cells at 20 PDL were not treated or treated with bisphosphonate at the indicated concentrations in the presence of 1,25(OH)₂D₃ (100 ng/ml) and 2 mM α-glycerophosphate for 7 days. Each column shows a mean±S.E.M. (n=3). ■, alendronate; □, etidronate. Calcium content was not measurable in the cells without 1,25(OH)₂D₃. Statistical significance compared with the control (1,25(OH)₂D₃ alone): *P<0.05 and **P<0.01.

Effects of alendronate at lower concentrations on osteocalcin and collagen synthesis

Alendronate was tested at the minimal (10⁻¹³ M) and sub-maximal (10⁻¹¹ M) effective concentrations for the potentiation of mineralization. Alendronate enhanced the 1,25(OH)₂D₃ (10 ng/ml)-stimulated Ca accumulation of human osteoblastic cells in 10-day cultures (Fig. 4a). The effect was not yet obvious in 5-day cultures because the Ca content was not detectable with any treatment (data not shown). 1,25(OH)₂D₃ stimulated the osteocalcin synthesis of the cells, while it inhibited collagen synthesis (Fig. 4, b and c). Alendronate evidently increased osteocalcin synthesis only in the presence of 1,25(OH)₂D₃ in 10-day cultures, but no concentration dependence was observed (Fig. 4b). Collagen synthesis was significantly increased by alendronate only at 10⁻¹¹ M in the presence of 1,25(OH)₂D₃ (100 ng/ml) in 14-day cultures, and no effect on mineralization was observed at 10⁻¹⁴ M (Fig. 3, a and b). ALP activity was not affected by alendronate in the same concentration range (Fig. 3c).

Fig. 1. Effects of alendronate and etidronate on Ca accumulation of human osteoblastic cells. Confluent human osteoblastic cells at 20 PDL were not treated or treated with bisphosphonate at the indicated concentrations in the presence of 1,25(OH)₂D₃ (100 ng/ml) and 2 mM α-glycerophosphate for 7 days. Each column shows a mean±S.E.M. (n=3). ■, alendronate; □, etidronate. Calcium content was not measurable in the cells without 1,25(OH)₂D₃. Statistical significance compared with the control (1,25(OH)₂D₃ alone): *P<0.05 and **P<0.01.
Fig. 2. Comparison of effects on accumulations of Ca (a) and Pi (b), ALP activity (c) and DNA content (d) of human osteoblastic cells among alendronate, etidronate and pamidronate. The cells were not treated or treated with bisphosphonate in the presence of 1,25(OH)2D3 (50 ng/ml) for 14 days. Each point (△, etidronate; ●, alendronate; □, pamidronate) or column (□, etidronate; ■, alendronate; □, pamidronate) shows a mean±S.E.M. (n=4). Statistical significance compared with the control (1,25(OH)2D3 alone): *P<0.05 and **P<0.01.
Fig. 3. Effects of alendronate on accumulations of Ca (a) and Pi (b) and ALP activity (c) of human osteoblastic cells. The cells were not treated or treated with alendronate in the presence of 1,25(OH)₂D₃ (100 ng/ml) for 14 days. Each column shows a mean±S.E.M (n=6). Statistical significance compared with the control (1,25(OH)₂D₃ alone): **P<0.01.

Effects of alendronate at lower concentrations on gene expression of osteocalcin and of pro α1(I) collagen

Effects of alendronate on gene expression of osteocalcin and of pro α1(I) collagen were studied in the presence of 1,25(OH)₂D₃ (10 ng/ml) in 3-, 7- and 14-day cultures containing 10⁻¹³ M and 10⁻¹¹ M of the bisphosphonate. 1,25(OH)₂D₃ dramatically stimulated osteocalcin gene expression in 3- and 7-day cultures, but alendronate did not potentiate the gene expression at any time (Fig. 5a). 1,25(OH)₂D₃ also stimulated the gene expression of pro α1(I) collagen despite its suppressive effect on collagen synthesis as indicated above (Fig. 5b). This stimulatory effect was already evident in 3-day cultures, and thereafter the gene expression was gradually reduced. Alendronate potentiated the gene expression stimulated with 1,25(OH)₂D₃ at each time in a concentration-dependent manner.

DISCUSSION

Relatively low concentrations of alendronate and etidronate potentiated mineralization of human osteoblastic cells only in the presence of 1,25(OH)₂D₃. This somewhat unexpected action seemed not to be due to its physicochemical action, since potentiation of mineralization with alendronate was accompanied by increases in bone matrix proteins, osteocalcin and collagen, and the mRNA of pro α1(I) collagen. At this time, the actual mechanism remains to be further investigated. Although the expression of pro α1(I) collagen mRNA was observed to be increased by the treatment with alendronate, a direct effect on gene expression has not been proven. Thus the increase may be mediated indirectly via cytokines such as IL-4 (43), IGF-I (44, 45) or TGF-β (46, 47), resulting in cell differentiation or a change in cell function.

It is not known at present why mineralization is potentiated only in the presence of 1,25(OH)₂D₃. This vitamin induces differentiation characterized by high levels of ALP activity and osteocalcin production, as well as deposition of Ca and Pi (34, 35, 48, 49), in osteoblast-like cells. 1,25(OH)₂D₃ also stimulates osteocalcin gene expression and synthesis directly (50, 51), while it shows opposite effects on collagen gene expression and synthesis, apparently depending on the culture system used (36, 52–55). In addition, 1,25(OH)₂D₃ was suggested to have a direct stimulatory effect on the mineralization process; this effect might be associated with its effects on phosphatidylinerine synthesis or metabolism of matrix protein including proteoglycan (49). Alendronate may modulate one of these action steps of 1,25(OH)₂D₃ mentioned above or the specific receptor of the hormone, so this situation remains to be clarified.

In the present study, 1,25(OH)₂D₃ stimulated both gene expressions of osteocalcin and pro α1(I) collagen in 3- and 7-day cultures, but the gene expressions were reduced in 14-day cultures. These might reflect the process of mineralization of osteoblasts which seems to progress sequentially in the order of increases of ALP activity, collagen synthesis, osteocalcin synthesis, and accumulations of Ca and Pi and gradual decreases of ALP activity and matrix protein synthesis after the mineralization of the cells develops to a certain degree. Therefore, it is also likely that
Fig. 4. Effects of alendronate on accumulations of Ca (a), bone matrix proteins, osteocalcin (b) and collagen (c) and DNA content (d) of human osteoblastic cells. The cells were not treated or treated with alendronate in the presence or absence of 1,25(OH)₂D₃ (10 ng/ml) for 5 or 10 days. Data for Ca are only those in the 10-day cultures since Ca was not detectable with any treatment in the 5-day cultures. Each column shows a mean±S.E.M. (n=3 or 4). "n.d." means "not detectable". Statistical significance compared with each reference culture without alendronate in the presence or absence of 1,25(OH)₂D₃: *P<0.05 and **P<0.01.

Fig. 5. Effects of alendronate on gene expression of osteocalcin (a) and of pro α 1(I) collagen (b) of human osteoblastic cells. The cells were treated with alendronate in the presence of 1,25(OH)₂D₃ (10 ng/ml) for the indicated periods. The lanes are: 1, 2, 3: no additives, 3, 7, 14 days, respectively; 4, 5, 6: 1,25(OH)₂D₃, 3, 7, 14 days, respectively; 7, 8, 9: 1,25(OH)₂D₃ plus alendronate 10⁻¹³ M, 3, 7, 14 days, respectively; 10, 11, 12: 1,25(OH)₂D₃ plus alendronate 10⁻¹¹ M, 3, 7, 14 days, respectively.
Bisphosphonates inhibit bone mineralization in vivo (10, 11, 56–62), probably by a combination of the following three mechanisms: 1) a physicochemical mechanism leading to inhibition of crystal growth; 2) a direct inhibitory effect on bone formation in osteoblasts; and 3) as a result of the homeostatic coupling in bone, an inhibition of resorption is followed by an inhibition of formation; i.e., bone turnover is slowed down. The present data did not result from mechanism 3 at least since our human osteoblastic cell culture system contains no osteoclasts. Comparison among the three bisphosphonates with respect to the concentration showing 50% suppression of accumulation of Ca indicated the following sequence of potency: alendronate > etidronate > pamidronate. However, the largest difference was only twice as much between alendronate and pamidronate. Since alendronate is about 10 and 1,000 times more effective in inhibiting bone resorption than pamidronate and etidronate, respectively (23, 63, 64), there is no correlation between the inhibitory effect on bone resorption and that on mineralization by osteoblastic cells observed in the present study.

The inhibitory effect of bisphosphonates on osteoblast function in vitro has been suggested previously (16, 17, 20, 21, 65). However, it is not clear if such inhibition is due to direct effects on osteoblasts or not, because the culture systems included not only osteoblasts but also other cell types. Khokher and Dandona (20) used human osteoblasts, but did not evaluate mineralization. In contrast to our observations, the authors indicated the inhibition of ALP secretion with relatively low concentrations of bisphosphonates (approximately 10^{-8} M etidronate or pamidronate) accompanied by inhibition of [3H]-thymidine uptake, probably because bisphosphonates were added during the proliferation phase.

Thus, our results indicate for the first time that bisphosphonate directly promotes mineralization accompanied by an increase of bone matrix synthesis in a human osteoblastic cell culture system. However, whether this effect of alendronate contributes to its pharmacological effect in vivo is not known at present. There is no evidence that indicates bisphosphonates directly promote bone formation in vivo.

On the other hand, all three bisphosphonates tested, alendronate, etidronate and pamidronate, suppressed 1,25(OH)_{2}D_{3}-stimulated mineralization of human osteoblastic cells at relatively higher concentrations. Hankel et al. (19) described that 10^{-5} M alendronate, but not the same concentration of etidronate, was cytotoxic in a chick osteoblast cell culture system. In our studies, however, the suppression of mineralization was observed with at least a 3\times 10^{-7} M concentration of any of the three bisphosphonates, showing only a slight effect on ALP activity and without changing the DNA content. These data demonstrate that the suppression of mineralization with bisphosphonates is not due to cytotoxicity.
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