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 a 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 10% 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 10–10–10–8 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 AAC 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-
Dronate at high concentrations on the accumulation of Ca in the absence of 1,25(OH)2D3 was not investigated since the basal level of Ca in the cells was too low under such a condition. Similarly, etidronate potentiated the 1,25(OH)2D3-stimulated Ca accumulation of human osteoblastic cells at a concentration of 10⁻⁸ M and suppressed it at 10⁻⁶ M and above. The minimal effective concentration of etidronate in potentiation of Ca accumulation was about 10,000 times higher than that of alendronate, although suppression of Ca accumulation at higher concentrations was observed at almost the same concentrations of both bisphosphonates. The amount of Pi was similarly affected by the bisphosphonates (data not shown).

The suppressive effects on mineralization of human osteoblastic cells were compared in more detail among three bisphosphonates, alendronate, etidronate and pamidronate, at high concentrations (Fig. 2, a and b). Concentrations of alendronate, etidronate and pamidronate that showed 50% suppression of Ca accumulation stimulated with 50 ng/ml of 1,25(OH)2D3 in 14-day cultures were estimated to be 3 x 10⁻¹³, 4 x 10⁻¹¹ and 6 x 10⁻⁷ M, respectively (Fig. 2a). ALP activity was reduced only slightly (6-24%), not in a concentration-dependent manner but significantly, by these three bisphosphonates (Fig. 2c). DNA content was not significantly affected (Fig. 2d).

In another experiment, the minimal effective concentration 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 not observed at 10⁻¹⁴ M (Fig. 3, a and b). ALP activity was not affected by alendronate in the same concentration range (Fig. 3c).

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 in 10-day cultures, although it tended to be increased concentration-dependently in the absence of 1,25(OH)2D3 (Fig. 4c). Alendronate did not significantly affect the DNA content of the cells (Fig. 4d).
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)_{2}D_{3} (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)_{2}D_{3} : *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)_{2}D_{3} (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)_{2}D_{3}, 3, 7, 14 days, respectively; 7, 8, 9: 1,25(OH)_{2}D_{3} plus alendronate 10^{-13} M, 3, 7, 14 days, respectively; 10, 11, 12: 1,25(OH)_{2}D_{3} plus alendronate 10^{-11} M, 3, 7, 14 days, respectively.
no change of ALP activity was observed when we measured it at the end of culture.

In addition, in spite of the stimulation of pro $\alpha$1(I) collagen gene expression, $1,25$(OH)$_2$D$_3$ suppressed collagen synthesis in terms of protein level determined by measurement of hydroxyproline content. The reason for these paradoxical findings is not known.

Alendronate increased the amount of osteocalcin protein, but did not affect the gene expression of the matrix protein. Although there seems to be a possibility that a post-transcriptional process is modulated, it can not be excluded that the RT-PCR method is not sensitive enough to detect changes under the experimental conditions used.

Other investigators also described enhancement of osteoblast function with bisphosphonates (15–19). Among them, only Tenenbaum et al. (16) and Hankel et al. (19) evaluated the effect on mineralization. Tenenbaum et al. (16) used a chick periosteal osteogenesis model (tissue explant) in the absence of $1,25$(OH)$_2$D$_3$. In that model, pamidronate, but not etidronate, over a range of $3 \times 10^{-8}$ to $1 \times 10^{-6}$M, enhanced mineralization accompanied by an increase in ALP activity. In comparison with these figures, our data on the effective concentration for potentiation of mineralization were considerably low. In our studies, ALP activity was not evidently affected. Hankel et al. (19) indicated using a chick osteoblast cell culture system that alendronate ($10^{-9}$, $10^{-7}$M) and etidronate ($10^{-7}$M) increased Ca and Pi content per DNA content without alteration of cell layer collagen (hydroxyproline/DNA).

In contrast to our findings, the DNA content was reduced probably because bisphosphonate had been 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.

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. Kokhker 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 $[^{3}H]$-thymidine uptake, probably because bisphosphonates were added during the proliferation phase. Tenenbaum et al. (16) used a chick periosteal osteogenesis model (tissue explant) and observed that etidronate ($5 \times 10^{-6}$M and above) inhibited mineralization while it stimulated ALP activity. At the same time, pamidronate at low doses ($3 \times 10^{-8}$–$10^{-6}$M) enhanced both mineralization and ALP activity. Although no reason was given for the difference between the two bisphosphonates, it is not known whether or not there was a qualitative difference. In any case, in contrast to our results showing an inhibition of mineralization both by etidronate and by pamidronate, at least at $3 \times 10^{-7}$M, inhibition by pamidronate (in comparison with the control) was not observed even at $10^{-5}$M in their studies. It may be partly due to a difference in susceptibilities to bisphosphonates between our cell culture system and their explant culture system.

Thus, our data show for the first time that in a culture system of normal diploid human osteoblastic cells, mineralization is inhibited by bisphosphonates at relatively high concentrations.
References