Bisphosphonates Directly Regulate Cell Proliferation, Differentiation, and Gene Expression in Human Osteoblasts

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ABSTRACT

Bisphosphonates are widely used clinically to treat bone diseases in which bone resorption is in excess. However, the mechanism of bisphosphonate action on bone is not fully understood. Studies of direct action of bisphosphonates on bone have been limited mainly to their effects on bone-resorbing osteoclast cells, with implications that some activity may be mediated indirectly through paracrine factors produced by the bone-forming osteoblast cells. Little is known about the direct effects of bisphosphonates on osteoblasts. In this report, the direct actions of several bisphosphonates on cell proliferation, gene expression, and bone formation by cultured human fetal osteoblasts were examined. Osteoblast cell proliferation was decreased, and cytodifferentiation was increased in a dose-dependent manner in cultures treated with the bisphosphonate pamidronate. In addition, pamidronate treatment increased total cellular protein, alkaline phosphatase activity, and type I collagen secretion in osteoblasts. Consistent with the above-mentioned findings, the rate of bone formation was also increased in osteoblasts cultured with pamidronate. The actions of two other bisphosphonates, the weak-acting etidronate and the potent new analogue zolendronate, were also compared with the action of pamidronate on proliferation of immortalized human fetal osteoblast (hFOB) cells and rate of bone formation. Pamidronate and zolendronate decreased hFOB cell proliferation with equal potency, whereas etidronate decreased proliferation only at much higher concentrations. Studies comparing EDTA and etidronate indicate that etidronate may act indirectly on the hFOB cells by reducing free divalent ion concentrations, whereas pamidronate and zolendronate appear to act on the hFOB cells by a direct action. Both pamidronate and zolendronate increase hFOB cell bone formation, whereas no increase is observed with etidronate and EDTA. Taken together, these observations strongly suggest that treatment with pamidronate or zolendronate enhances the differentiation and bone-forming activities of osteoblasts.

INTRODUCTION

The bisphosphonates are a family of pyrophosphate analogues in which the oxygen linking the phosphates has been replaced by carbon (1). These compounds have high affinity for hydroxyapatite crystals (2) and are potent inhibitors of bone resorption (3). The bisphosphonates are widely used to treat bone diseases in which there is an excess of bone resorption (4). Several structurally related bisphosphonates have been synthesized by changing the two lateral chains on the carbon or by esterifying the phosphate groups (1). The resulting analogues vary extensively in antiresorptive potency, with analogues such as etidronate being the weakest, pamidronate being more potent, and the new analogue, zolendronate, being the most potent (1). The bisphosphonate pamidronate [(3-amino-1-hydroxypropylidene) bisphosphonate] is currently used for the treatment of hypercalcemia of malignancy, Paget’s disease, osteolytic bone metastasis of breast cancer, and osteolytic lesions of multiple myeloma. Pamidronate has intermediate antiresorptive potency between the weak-acting etidronate and the most potent analogue, zolendronate (1, 5).

Although the bisphosphonates are commonly used clinically to treat bone diseases, the mechanism of action of these compounds on bone is not completely understood. At the tissue level, treatment with bisphosphonates leads to an increase in bone mineral density that has been attributed to decreased bone turnover (3, 5–12). This observed decrease in bone turnover appears to be due to decreased frequency and resorption depth of the bone remodeling units (7, 10, 12). High doses of bisphosphonates can lead to impaired mineralization (6, 13). However, at lower levels of bisphosphonates, mineralization is normal, and net osteoblast function is unimpaired, leading to a positive bone balance (7, 10, 12, 14).

At the cellular level, bisphosphonates have been shown to have direct effects on osteoclasts. Bisphosphonates can reduce osteoclast numbers by inhibiting the proliferation and recruitment of osteoclast precursors (15–18) and inducing apoptosis in macrophages and mature osteoclast cells (19–22). In addition, bisphosphonates can directly inhibit the bone-resorbing activity of osteoclasts (23, 24). The mechanism by which bisphosphonates act directly on osteoclasts and osteoclast precursors has been reported to be due, at least in part, to inhibition of the mevalonate pathway (22, 25–30).

In addition to the direct effects of bisphosphonates on osteoclasts, there is evidence that these compounds also act on the osteoclasts indirectly through the osteoblasts (31–33). Osteoblasts are key regulatory cells in bone that regulate bone cell differentiation and functions. It is likely that this indirect effect is due to modulation of osteoblast secretion of soluble paracrine factors that influence osteoclast activity (34–38). New studies also suggest that bisphosphonates can influence osteoblast function as well (39–43). However, the observed effects differ, depending on the bisphosphonate and the model system used.

Because the complete mechanism of action of bisphosphonates is not understood, and their actions on the important bone-forming osteoblasts are confusing, the effects of several bisphosphonates (etidronate, pamidronate, and zolendronate) on the latter regulatory cells were examined. The proliferation, differentiation, and bone formation (as measured by mineralization of nodules) by conditionally immortalized hFOB3 cells were examined and compared (44). In addition, the effects of EDTA on hFOB cell proliferation and mineralization were examined because some bisphosphonates are known to chelate divalent ions.

MATERIALS AND METHODS

Materials. Pamidronate was produced by Novartis Pharma AG (Basal, Switzerland). Etidronate was produced by MGI Pharm Inc. (Minnetonka, MN). Zolendronate was provided by Novartis Pharma AG. DMEM-Ham’s F-12 medium (1:1) and Alizarin Red S stain were purchased from Sigma (St. Louis, MO). Fetal bovine serum was purchased from Summit Biotechnology (Fort Collins, CO).

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3 The abbreviation used is: hFOB, human fetal osteoblast.
Cell Culture. The hFOB cells were developed previously and characterized in this laboratory (44). Briefly, hFOB cells were derived from primary cultures of fetal tissue and conditionally immortalized with a gene coding for the temperature-sensitive mutant (ts A58) of the SV40 large T-antigen. This cell line was isolated from primary cultures based on its osteoblast phenotype. Incubation of hFOB cells at the permissive temperature (34°C) results in rapid cell division, whereas little or no cell division occurs at the restrictive temperature (39°C). hFOB cells were maintained at 34°C in DMEM:Ham’s F-12 medium (1:1) supplemented with 10% (v/v) fetal bovine serum and 300 μg/ml Geneticin. Culture medium was removed and replaced with fresh medium every 3 or 4 days during experimentation.

Cell Proliferation. hFOB cells were seeded at 20,000 cells/cm² in 96-well plates and incubated at 34°C for 24 h in normal culture medium. The medium was then replaced with fresh medium containing various concentrations of pamidronate. The relative number of viable cells in each well was then determined at various times after treatment using the Cell Titer 96 AQ assay. One Solution Cell Proliferation Assay (Promega, Madison WI). Briefly, 20 μl of Cell Titer 96 AQ assay, One Solution were added to each well, including three wells containing only medium for background subtraction. The cells were then incubated at 37°C for 30 min. The absorbance at 490 nm in each well was then determined using a SpectraMax 340 plate reader/spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). This technique was used to determine the relative number of viable cells as determined directly in the 96-well plates using the above-mentioned proliferation assay.

Alkaline Phosphatase Activity. hFOB cells were seeded at 20,000 cells/cm² in 12-well and 96-well plates and incubated at 34°C for 24 h in normal culture medium. The medium was then replaced with fresh medium containing various concentrations of pamidronate. Alkaline phosphatase activity was determined in the 12-well plates using the Alkaline Phosphatase Kit (Sigma). The alkaline phosphatase activity values were normalized to the relative number of viable cells as determined directly in the 96-well plates using the above-mentioned proliferation assay.

Type I Collagen Secretion. hFOB cells were seeded at 20,000 cells/cm² in 12-well and 96-well plates and incubated at 34°C for 24 h in normal culture medium. The medium was then replaced with fresh medium containing various concentrations of pamidronate. The amount of collagen type I COOH-terminal propeptide was determined in the conditioned media using the Prolagen-C assay (Metra Biosystems, Inc., Mountain View, CA). The type I collagen values were normalized to the relative number of viable cells as determined directly in the 96-well plates using the above-mentioned proliferation assay.

Mineralization. hFOB cells were seeded at 20,000 cells/cm² in 12-well and 96-well plates and incubated at 34°C for 24 h in normal culture medium. The medium was then replaced with fresh medium containing various concentrations of pamidronate. The degree of mineralization was determined in the 12-well plates using Alizarin Red staining. Briefly, medium was aspirated from the wells, and the cells were rinsed twice with PBS. The cells were fixed with ice-cold 70% (v/v) ethanol for 1 h. The ethanol was removed, and the cells were rinsed twice with deionized water. The cells were then stained with 40 mM Alizarin Red S in deionized water (adjusted to pH 4.2) for 10 min at room temperature. The Alizarin Red S solution was removed by aspiration, and the cells were rinsed five times with deionized water. The water was removed by aspiration, and the cells were incubated in PBS for 15 min at room temperature on an orbital rotator. The PBS was removed, and the cells were rinsed once with fresh PBS. The cells were then destained for 15 min with 10% (v/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 405 nm was measured using a SpectraMax 340 plate reader/spectrophotometer (Molecular Devices Corp.). The concentration of Alizarin Red S staining in the samples was determined by comparing the absorbance values with those obtained from Alizarin Red S standards. The mineralization values were normalized to the relative number of viable cells as determined directly in the 96-well plates using the above-mentioned proliferation assay.

Statistical Analysis. Significance was determined using the two-tailed Student’s t test.

RESULTS

Effect of Pamidronate on hFOB Cell Proliferation. Dose-response and time-course experiments were performed to determine the effects of pamidronate on hFOB cell proliferation. As shown in Fig. 1A, treatment of hFOB cells with pamidronate for 6 days decreased the number of viable hFOB cells in the cultures in a dose-dependent manner compared with vehicle-treated cells. A maximum 95% reduction in viable hFOB cells was observed at the 25 μg/ml dose level. The differences in viable cell numbers were significant at the 0.5 μg/ml dose level, with P < 0.05 and P < 1 × 10⁻⁹ at the highest dose (100 μg/ml). The effect of pamidronate (0, 2.5, 10, and 25 μg/ml) on the proliferation of hFOB cells in culture over time is illustrated in Fig. 1B. Whereas the control osteoblasts continue to proliferate rapidly through 10 days of culture, the proliferation of the osteoblasts cultured with 2.5 and 10 μg/ml pamidronate is slowed. At concentrations of pamidronate greater than 10 μg/ml, the cells become rounded and detached, and significant osteoblast cell death is observed.

![Graph A](image1.png)

**Fig. 1.** Effect of pamidronate on hFOB cell proliferation. hFOB cells were seeded in 96-well plates and cultured at 34°C in normal growth medium with or without pamidronate. hFOB cell proliferation was then assessed as described in “Materials and Methods.” A, dose response to pamidronate analyzed at day 6 (++, P < 0.05; ***, P < 1 × 10⁻⁹; ppp, P < 1 × 10⁻¹⁰) compared with vehicle treatment). B, time course of pamidronate treatment analyzed at days 0, 3, 6, and 10. ○, vehicle; ■, 2.5 μg/ml; △, 10 μg/ml; and ●, 25 μg/ml pamidronate (+, P < 0.05; **, P < 0.0001; ppp, P < 1 × 10⁻⁹ compared to vehicle treatment). The data represent the mean values (n = 4). Error bars, SDs from the mean values.
Effect of Pamidronate on Total Protein Levels in hFOB Cells. Fig. 2A illustrates the effect of increasing concentrations of pamidronate on total protein in hFOB cells. As shown, the total cellular protein levels in the cultured hFOB cells are increased with increasing concentrations of pamidronate after treatment for 4 days. This change in total cellular protein levels becomes significant at 1.0 μg/ml pamidronate ($P < 0.05$), and a maximum increase of 82% is observed at 10 μg/ml pamidronate. As demonstrated in Fig. 2B, the increase in total cellular protein induced by pamidronate (10 μg/ml) is significant by day 3 ($P < 0.001$), reaches a maximum on day 5, and begins to decrease on day 7.

Effect of Pamidronate on Alkaline Phosphatase Activity in hFOB Cells. Dose-response and time-course experiments were performed to examine the effects of pamidronate on alkaline phosphatase activity in hFOB cells. As shown in Fig. 3A, pamidronate treatment for 4 days increased the alkaline phosphatase activity per viable hFOB cell in a dose-dependent manner. The increase in alkaline phosphatase activity was significant at 2.5 μg/ml pamidronate ($P < 0.002$) and reached a maximum of 38% over vehicle-treated cells at 10.0 μg/ml pamidronate ($P < 1 \times 10^{-5}$). Fig. 3B shows the effect of pamidronate (10 μg/ml) treatment of hFOB cells on alkaline phosphatase activity over time. The increased alkaline phosphatase activity was observed by day 2 and reached a maximum of 82% on day 5. After day 5, the pamidronate-induced alkaline phosphatase activity begins to decrease.

Effect of Pamidronate on Type I Collagen Secretion from hFOB Cells. Fig. 4A demonstrates the effect of pamidronate treatment for 4 days on type I collagen secretion from hFOB cells. Pamidronate treatment caused a dose-dependent increase in type I collagen secretion per viable hFOB cell. The minimally effective concentration was 2.5 μg/ml pamidronate ($P < 0.01$), and a maximum increase of 65% was observed at the highest concentration used, 10.0 μg/ml pamidronate ($P < 0.01$). Fig. 4B demonstrates the effect of pamidronate (10 μg/ml) treatment on type I collagen secretion from hFOB cells over time. Although type I collagen secretion in pamidronate-treated cultures was initially less than control in the experiment shown, type I collagen secretion gradually increased over the 7-day culture period to levels above those of control cultures.

Effect of Pamidronate on hFOB Cell Mineralization. To examine the effects of pamidronate on bone formation, hFOB cells were stained for calcium incorporation using Alizarin Red. As shown in Fig. 5A, treatment with pamidronate for 7 days increased the amount of staining per viable cell in a dose-dependent manner. The maximum effect on mineralization was achieved at 10 μg/ml pamidronate. At this concentration of pamidronate, an increase of 81% in Alizarin Red staining per viable cell was observed ($P < 1 \times 10^{-5}$). As demonstrated in Fig. 5B, the increase in mineralization induced by pamid-
ronate (2.5 \mu g/ml) occurs between day 4 and day 10 of culture and begins to decrease by day 14.

Comparative Effects of Etidronate, Pamidronate Zoledronate, and EDTA on hFOB Cell Proliferation. Because the bisphosphonates can form insoluble complexes with divalent ions, the effects of the bisphosphonates etidronate, pamidronate, and zoledronate on hFOB cells were compared with those of EDTA on an equivalent molarity basis. As shown in Fig. 6A, these compounds inhibit hFOB cell proliferation with different potencies. Pamidronate and zoledronate were approximately equally potent with 50% effective doses (ED50) of $4.2 \times 10^{-5}$ and $4.0 \times 10^{-5}$ M, respectively. This finding does not correlate with the reported 100-fold higher in vivo potency reported for zoledronate compared with pamidronate (5). Etidronate was approximately 180-fold less potent than zoledronate and pamidronate, with an ED50 of $7.5 \times 10^{-3}$ M. The potency difference between etidronate and pamidronate is very similar to the reported in vivo antiresorptive potency difference for these compounds (1). The antiproliferative potency of EDTA was between that of zoledronate/pamidronate and etidronate, with an ED50 of $5.6 \times 10^{-4}$ M.

Because EDTA was as effective at similar concentrations as the weak bisphosphonate etidronate, it is possible that some of the ob-

![Fig. 4. Effect of pamidronate on type I collagen secretion from hFOB cells. hFOB cells were seeded in 12-well and 96-well plates and cultured at 34°C in normal growth medium or without pamidronate. The amount of type I COOH-terminal propeptide was determined in the conditioned media from 12-well plates and normalized to the relative number of viable cells as determined directly in the 96-well plates as described in “Materials and Methods.” A, dose response to pamidronate analyzed on day 4 (*, $P < 0.01$ compared to vehicle). B, time course of pamidronate (10 \mu g/ml) treatment analyzed on days 1, 2, 3, 4, 5, 6, and 7 (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0002$ compared with vehicle treatment). The data shown represent the mean values ($n = 4$). Error bars, SDs from the mean values. ■, data normalized to relative cell number; □, data not normalized.

Fig. 5. Effect of pamidronate on hFOB cell mineralization. hFOB cells were seeded in 12-well and 96-well plates and cultured in normal growth medium with or without pamidronate. The degree of mineralization was determined in the 12-well plates using Alizarin Red staining and normalized to the relative number of viable cells as determined directly in the 96-well plates as described in “Materials and Methods.” A, dose response to pamidronate analyzed at day 7 (*, $P < 0.02$; **, $P < 0.005$; and ***, $P < 0.001$; and ***, $P < 1 \times 10^{-5}$ compared with vehicle treatment). B, time course of pamidronate (2.5 \mu g/ml) treatment analyzed on days 4, 6, 8, 10, and 14 (*, $P < 0.005$; **, $P < 0.0005$; and ***, $P < 1 \times 10^{-5}$ compared with vehicle treatment). The data shown represent the mean values ($n = 3$ or 4). Error bars, SDs from the mean values. ■, data normalized to relative cell number; □, data not normalized.

The inhibition of hFOB cell proliferation by chelating the essential divalent ions is the case, we hypothesized that the addition of divalent ions to the bisphosphonate-treated culture medium would reverse the effects of the bisphosphonates on the osteoblast cells. To test this hypothesis, hFOB cells were treated with vehicle, etidronate, pamidronate, zoledronate, or EDTA at the respective ED50 concentrations described above. The culture media were then treated with increasing concentrations of calcium and magnesium chlorides, and proliferation of the hFOB cells was measured after 7 days of culture. As shown in Fig. 6B, the addition of increasing concentrations of divalent ions to the vehicle- and EDTA-treated culture media caused a dose-dependent increase in hFOB cell proliferation, suggesting that etidronate was inhibiting the hFOB cell proliferation by chelating the essential divalent ions. Interestingly, similar to the vehicle-treated cultures, the addition of increasing concentrations of divalent ions to the etidronate- and EDTA-treated culture media caused a further decrease in hFOB cell proliferation. We conclude from this experiment that the antiproliferative effects of both EDTA and etidronate on hFOB cells are likely due to reductions in the free divalent ion concentrations available in the culture medium caused by these compounds. However, the antiproliferative effects of the more potent bisphosphonates,
pamidronate and zoledronate, appear to be due to a mechanism other than reduction of free divalent ions in the culture medium.

The effects of etidronate, pamidronate, zoledronate, and EDTA on the rate of bone formation as measured by hFOB cell mineralization of the osteoblast-produced matrix was also examined. As shown in Fig. 6C, the addition of pamidronate or zoledronate to the culture medium produced a marked increase the Alizarin Red stain per viable cell. Similar to the results in the proliferation experiments, both pamidronate and zoledronate displayed similar potencies in increasing mineralization in hFOB cells.

DISCUSSION

This study describes the effects of several bisphosphonates on the proliferation and differentiation of cultured hFOB cells. The hFOB cells are unique among the osteoblast models currently used in that they are conditionally immortalized normal human osteoblasts (44). Karyotype analysis on these cells revealed that they have only minor chromosomal translocations and deletions, which is in sharp contrast to the major chromosomal abnormalities that we observed in MG-63 osteosarcoma cells by comparison. Thus, the hFOB cells make an excellent model system to study the function of osteoblasts and the effects of agents such as bisphosphonates on osteoblasts in vitro.

The actual concentration ranges of the bisphosphonates that osteoblasts and other cells in the body are exposed to under pharmacological conditions are unknown. Therefore, it is difficult to design in vitro experiments that can directly correlate to physiological conditions. The concentrations of bisphosphonates used in these experiments were chosen based on reported levels in patient sera after i.v. administration of pamidronate (45–49) that reached transient concentrations ranging up to 4.29 μg/ml or 10^{-5} M in sera. Whereas these peak serum levels are transient, bisphosphonates accumulate rapidly and at high concentrations in bone (1). One report has also estimated that pharmacological doses as of one bisphosphonate, alendronate, could give rise to local concentrations as high as 1 mM (10^{-3} M) alendronate in the resorption space (50). Using concentrations similar to the reported peak serum concentrations, we have observed direct effects of pamidronate on cultured hFOB cells.

The proliferation of hFOB cells was decreased in a dose-dependent manner in cultures treated with pamidronate. This observation is not surprising because similar effects of bisphosphonates have been reported with many other cell types including osteoclasts (20–22), intestinal epithelial cells (51), lymphocytes (52), macrophages (16, 19) myelomas (53, 54) breast cancer cells (55), and primary osteoblasts (39, 41). Recent experiments in this laboratory confirm this inhibition, i.e., pamidronate treatment decreased the proliferation of cultured MCF-7 and T47D breast cancer cells as well as LnCAP prostate cancer cells. In contrast, bisphosphonates have been reported to induce proliferation of marrow osteoprogenitors (40) and inhibit apoptosis of osteocytes and osteoblasts (43). The reason for these opposing effects is unknown but may be due to differences in the cell
types, duration of treatment, the bisphosphonate analogue used, and the concentrations of bisphosphonates used in these other experiments.

Although pamidronate treatment decreased the proliferation of hFOB cells in culture, it increased total cellular protein, alkaline phosphatase activity, and type I collagen secretion. These observations are an indication that pamidronate treatment enhances the differentiation of the osteoblasts from the proliferation stage to development into the nonproliferating matrix maturation stage (56). In addition, bone formation, as measured by mineralization, was also increased in hFOB cells treated with pamidronate. Thus, pamidronate further augments the development of the cultured osteoblasts from the matrix maturation stage to the mineralization stage (56). Taken together, these results strongly suggest that pamidronate treatment induces the differentiation of cultured hFOB cells into more mature bone-forming cells that may in part explain the positive effects of bisphosphonates on overall bone mineral balance (7, 10, 12, 14).

Because the bisphosphonates can form insoluble complexes with divalent ions, we also compared the effects of these compounds to EDTA and by the addition of divalent ions to the media. Pamidronate and zoledronate were approximately equal in antiproliferative potency, whereas etidronate and EDTA were less potent. The data presented in this study support that the effects of EDTA and etidronate on hFOB cell proliferation involve the reduction of the free divalent ion concentration in the media. In contrast, the actions of the more potent pamidronate and zoledronate analogues appear to be the result of a more direct action on osteoblasts. Pamidronate and zoledronate also increased the rate of hFOB cell bone formation, whereas the less potent etidronate did not alter this process. The observed differences in the effects of the more potent nitrogen-containing bisphosphonates (pamidronate and zoledronate) compared with the less potent etidronate on osteoblasts may help explain the differences in in vivo potencies between these compounds. However, the only slight differences in osteoblast effects were observed between pamidronate and zoledronate and did not correlate with the differences between these two analogues as measured in vitro (5). This infers that action on other cell types (e.g., osteoclasts) or noncellular (e.g., whole body) mechanisms, such as the half-life and metabolism, may be responsible for the potency differences between these two potent compounds.

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