Bisphosphonates Inhibit Prostate and Breast Carcinoma Cell Adhesion to Unmineralized and Mineralized Bone Extracellular Matrices

Sandrine Boissier, Sandrine Magnetto, Lucien Frappart, Béatrice Cuzin, Frank H. Ebetino, Pierre D. Delmas, and Philippe Clézardin

Abstract

The molecular mechanisms by which tumor cells induce osteolytic metastases are likely to involve tumor cell adhesion to bone as well as the release of soluble mediators from tumor cells that stimulate osteoclast-mediated bone resorption. Bisphosphonates (BPs) are powerful inhibitors of the osteoclast activity and are, therefore, used in the treatment of cancer-associated osteolytic metastases. Here, we investigated the effect of BPs on breast and prostate carcinoma cell adhesion to unmineralized and mineralized bone extracellular matrices. BP pretreatment of tumor cells inhibited tumor cell adhesion to unmineralized and mineralized osteoblastic extracellular matrices in a dose-dependent manner. In contrast, BP did not affect adhesion of normal cells (fibroblasts) to extracellular matrices. The order of potency for four BPs in inhibiting tumor cell adhesion to extracellular matrices was found to be: ibandronate > NE-10244 (antiresorptive active pyridinium analogue of risedronate) > pamidronate > clodronate. BP did not affect [3H]thymidine incorporation by tumor cells, as assessed by a mitogen assay, indicating that BP did not exert any cytotoxic effect at concentrations used to inhibit tumor cell adhesion. NE-58051, the inactive pyridylpropyldenediene analogue of risedronate, had no inhibitory effect on tumor cell adhesion compared to that observed with its active counterpart NE-10244, suggesting that the mechanism of action of BP on tumor cells involved a stereospecific recognition step. Although integrins mediate cell-matrix interactions, BP recognition by tumor cells did not modulate cell surface integrin expression. In conclusion, our results provide evidence for a direct cellular effect of BP in preventing tumor cell adhesion to bone, suggesting that BPs may be useful agents for the prophylactic treatment of patients with cancer that is known to preferentially metastasize to bone.

Introduction

A very common metastatic site for prostate and breast carcinomas is bone (1, 2). In bone metastases, tumor cells reside in the bone marrow, and the bone marrow tumor nest is frequently surrounded by numerous osteoclasts that are resorbing bone (2). Tumor cells are also found adjacent to the resorbed endosteal bone surface where there is no evidence of resorbing osteoclasts, suggesting that tumor cells could directly cause bone destruction (1, 2). Although the molecular mechanisms by which tumor cells induce osteolytic metastases are not fully understood, some of these mechanisms are likely to involve tumor cell adhesion to bone extracellular matrix molecules as well as the release of soluble mediators from tumor cells that stimulate osteoclast-mediated bone resorption (2). Thus, any agent that could interfere with these mechanisms would be a major therapeutic advance in the treatment of bone metastases.

BPs are analogues of the naturally occurring compound pyrophosphate (3). They bind avidly to the bone mineral and are powerful inhibitors of bone resorption (3). They act directly on osteoclasts by preventing their recruitment from bone marrow, inhibiting their capacity to resorb bone, and shortening their life span (3). This provided the rationale for the use of BPs in the treatment of patients with osteolytic metastases. BPs (pamidronate and clodronate) reduce the occurrence of fractures and decrease the formation of newly osteolytic lesions in breast cancer patients (4, 5). Animal studies also demonstrate that pretreatment of nude mice with a potent antiresorptive BP, risedronate [2-(3-pyridyl)-1-hydroxyethylidene bisphosphonic acid], before breast carcinoma cell inoculation shows a marked reduction of the osteolytic lesions and a marked decrease in tumor burden in bone (6). BP pretreatment of cortical bone slices also partially inhibited breast carcinoma cell adhesion in vitro (7). Moreover, it has been recently shown that adjuvant treatment of breast cancer patients with the BP clodronate reduces the incidence and number of bone and nonbone metastases (8). These very important observations (6–8) suggest, therefore, that BPs not only act on osteoclasts but may also affect the invasive behavior of metastatic cells in bone. Here, we investigated the effect of BPs on tumor cell adhesion to bone. We have found that BPs inhibit tumor cell adhesion to unmineralized and mineralized bone extracellular matrices through a specific action on tumor cells. These results suggest that BPs may be useful agents for the prophylactic treatment of patients with cancer that is known to preferentially metastasize to bone.

Materials and Methods

BPs. Five BPs were used. Clodronate [dichloromethylene bisphosphonic acid] was obtained from Rhône-Poulenc RORER Laboratories (France). Pamidronate [3-amino-1-hydroxypropyldenediene bisphosphonic acid] was obtained from Ciba-Geigy (Germany). Ibandronate [1-hydroxy-3(methylpentylamino)-propyldenediene bisphosphonic acid] was a gift from Dr. F. Bauss (Boehringer-Mannheim, Germany). Disodium 2-(N-methyl-3-pyridinyl)-1-hydroxyethylidene bisphosphonic acid (NE-10244), a potent antiresorptive analogue of risedronate, and 3-(3-pyridyl)-1-hydroxyethylidene bisphosphonic acid (NE-58051), an inactive analogue of risedronate, were obtained from Procter & Gamble Pharmaceuticals.

Cell Lines. Human breast carcinoma (MCF-7 and MDA-MB-231), prostate carcinoma (PC3), and osteosarcoma (MG-63) cell lines were obtained from the American Type Culture Collection. Cell line PmPC3 is a highly metastatic variant of human prostate carcinoma cell line PC3. Human foreskin fibroblasts were kindly provided by Dr. D. Schmitt (Hôpital Edouard Herriot). Human osteoblasts were isolated from trabecular bone and cultured using methods as described previously (9).

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2 To whom requests for reprints should be addressed, at INSERM Research Unit 403, Pavillon F, Hôpital Edouard Herriot, Place d'Arsonval, 69437 Lyon Cédex 03, France.

3 The abbreviation used is: BP, bisphosphonate.
Antibodies. Mouse monoclonal antibodies directed against $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, and $\beta_3$ integrins were purchased from Immunotech (Luminy, France). Goat antimouse IgG conjugated with FITC was from Coulter Immunology.

Flow Cytometry. Cell surface expression of integrins by prostate and breast carcinoma cells was analyzed on a FACScan flow cytometer (Becton Dickinson) as described previously (10).

BP Treatment of Tumor Cell Lines. For cell adhesion assays, tumor cell monolayers reaching 90% confluency were washed with serum-free RPMI 1640 and treated for 24 h with BPs diluted in RPMI containing 1% (w/v) BSA. At the end of the incubation, cell monolayers were washed with RPMI to remove BPs, and cells were harvested with trypsin-EDTA then resuspended in RPMI containing 0.2% (w/v) BSA. After 90-min incubation at 37°C to allow cells to recover from trypsin-EDTA treatment, untreated and BP-treated cells were seeded for cell adhesion assays. The cell viability before the experiments was measured by trypan blue exclusion. BPs never had any effect on cell viability.

Tumor Cell Adhesion Assay to Extracellular Matrices Produced in Vitro by Cultured Human Osteoblastic Cells. Human osteoblasts and MG-63 cells were grown in six-well culture plates. After the cell cultures had reached confluency and the formation of an extracellular matrix had started, cells were washed in PBS and lysed by sequential exposure to distilled water at 4°C following a procedure described by Kirk and Kahn (11). Exposed extracellular matrix was then rinsed, saturated with 0.1% (w/v) heat-inactivated BSA, and washed with RPMI. Untreated and BP-treated tumor cells (10$^5$ cells/ml) were plated in six-well culture plates (2 ml/well) containing extracellular matrices and incubated for 3 h at 37°C in a 5% CO$_2$ incubator. At the end of the incubation, nonattached cells were removed by three PBS washes, and attached cells were fixed in PBS containing 0.25% glutaraldehyde. Cells were then observed by microscopy and counted microscopically using an eyepiece reticle (Zeiss, plate no. VI).

Tumor Cell Adhesion Assay to Bovine Cortical Bone Slices. Bovine cortical bone slices were cut with an Isomet low-speed saw (Buehler, Lake Bluff, IL), cleaned by sonication, and washed with serum-free RPMI. Untreated and BP-treated tumor cells (10$^5$ cells/ml) were seeded on the top of bone slices and incubated for 1 h at 37°C in a 5% CO$_2$ incubator. The remainder of the experiment was performed as described by van der Pluijm et al. (7). Cells that adhered within five nonoverlapping microscopic fields (0.3 mm$^2$ each) were counted for each bone slice, and results were expressed as the number of cells/mm$^2$.

Cell Proliferation Assay. Quiescent tumor cells cultured in RPMI containing 1% (w/v) BSA were treated in the presence or absence of increasing concentrations of BPs. After a 24-h incubation at 37°C, cells were stimulated with fetal calf serum and pulsed with [3H]thymidine as previously described (12).

Results

BP Inhibit Tumor Cell Adhesion to Extracellular Matrices Produced by Cultured Osteoblastic Cells. Human prostate and breast carcinoma cells adhered to a similar extent to extracellular matrices produced by human osteoblasts and MG-63 osteosarcoma cells (results not shown). The effect of BPs on tumor cell adhesion was, therefore, conducted with extracellular matrices produced by MG-63 cells. As shown in Fig. 1, pretreatment of PC3 cells with increasing concentrations of BPs inhibited tumor cell adhesion to osteoblastic extracellular matrices in a dose-dependent manner. The order of potency for four BPs in inhibiting tumor cell adhesion to extracellular matrices was found to be: ibandronate > NE-10244 (active pyridinium analogue of risedronate) > pamidronate > clodronate. Ibandronate, NE-10244, pamidronate, and clodronate inhibited PC3 cell adhesion with half-maximal inhibition (IC$_{50}$) of 5 $\times$ 10$^{-12}$, 10$^{-10}$, 10$^{-9}$, and 10$^{-5}$ M, respectively. In contrast, pretreatment of PC3 cells with NE-58051 (the inactive pyridyl propyliendane analogue of risedronate) did not inhibit tumor cell adhesion to osteoblastic extracellular matrices (Fig. 1). BP pretreatment of PmPC3, MCF-7, and MDA-MB-231 cells at IC$_{50}$ concentrations also inhibited tumor cell adhesion to osteoblastic extracellular matrices, whereas BP pretreatment of extracellular matrices rather than tumor cells was inef-
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Fig. 2. BPs inhibit MCF-7 and MDA-MB-231 breast carcinoma cell adhesion but not fibroblast adhesion to unmineralized bone extracellular matrices. MCF-7, MDA-MB-231 cells, and fibroblasts were treated with BPs using concentrations that induced half-maximal inhibition (IC₅₀) of prostate carcinoma cell attachment to osteoblastic extracellular matrices. IC₅₀ concentrations for ibandronate, NE-10244, pamidronate, and clodronate were 5 × 10⁻¹², 10⁻¹⁰, 10⁻⁸, and 10⁻⁶ M, respectively. Columns, means of three to five experiments with BPs; bars, SD.

Fig. 3. BPs inhibit Prostate carcinoma cell adhesion to cortical bone slices. Cortical bone slices or tumor cells were treated with clodronate, ibandronate, NE-10244 (active risedronate analogue), and NE-58051 (inactive risedronate analogue) for 24 h. Cells that adhered to cortical slices were counted microscopically. The attachment of untreated cells to untreated cortical slices was used as a positive control. Columns, mean percentage of positive control (set to 100%; three to four separate experiments); bars, SE. Significances were calculated using one-way ANOVA followed by a Fisher’s PLSD test. Attachment of clodronate-, NE-10244-, and ibandronate-treated tumor cells to cortical slices was significantly lower than that observed with untreated tumor cells to clodronate-, NE-10244-, and ibandronate-treated cortical slices, respectively. Attachment of NE-58051-treated tumor cells to cortical slices did not differ significantly from the control. Attachment of tumor cells to NE-58051-treated cortical slices was significantly lower than that observed with NE-58051-treated tumor cells.

BPs Do Not Inhibit Adhesion of Human Fibroblasts to Unmineralized Osteoblastic Extracellular Matrices. To further investigate the effect of BPs on cell adhesion, experiments were conducted with normal cells. BP pretreatment of fibroblasts at concentrations that induced half-maximal inhibition of tumor cell adhesion did not inhibit fibroblast adhesion to osteoblastic extracellular matrices (Fig. 2).

BPs Inhibit Tumor Cell Adhesion to Cortical Bone Slices. The number of highly metastatic cells (PmPC3 and MDA-MB-231 cells) that adhered to bone slices was always significantly higher than that observed with poorly metastatic cells (PC3 and MCF-7 cells). Pretreatment of bone slices for 24 h with clodronate, NE-10244 (active
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Untreated cells

Ibandronate-treated cells

Fig. 4. Ibandronate does not modulate tumor cell surface integrin expression. Untreated and ibandronate (10^{-6} M, 24 h)-treated MDA-MB-231 cells were harvested with PBS-EDTA, incubated for 2 h at 4°C with an anti-integrin mouse monoclonal antibody followed by incubation (30 min; 4°C) with fluorescein-conjugated goat antimouse IgG. Staining was analyzed on a FACSscan flow cytometer. x axis fluorescent intensity; y axis, full-scale counts.

Discussion

The present study was designed to investigate the effect of five BPs (ibandronate, pamidronate, NE-10244, NE-58051, and clodronate) on prostate and breast carcinoma cell adhesion to bone. To fulfill this purpose, two distinct cell adhesion models were used: unmineralized extracellular matrices produced by cultured osteoblastic cells and mineralized bone cortical slices. We found that pretreatment of tumor cells with BPs inhibited their adhesion to osteoblastic extracellular matrices in a dose-dependent manner. By contrast, pretreatment of these unmineralized extracellular matrices with BPs did not inhibit tumor cell adhesion, suggesting that the inhibitory activity of BPs was mediated through a direct action on tumor cells. Such an inhibition of tumor cell adhesion to osteoblastic extracellular matrices did not result from a cytotoxic effect of BPs because they did not affect [3H]thymidine incorporation (nor trypan blue exclusion) by tumor cells. Moreover, tumor cells appeared to be more susceptible to BP treatment than normal cells (as exemplified by fibroblasts). As shown in Fig. 1 (inset), minor changes in risendronate chain length dramatically affected its inhibitory activity on tumor cell adhesion, relative to the NE-10244 analogue. Indeed, the inactive analogue of risendronate (NE-58051) is 10,000-fold less potent than NE-10244 as an antiresorptive agent in vivo (14). In this respect, it has been suggested that the mechanism of action of BPs on osteoclasts involves a stereospecific recognition step (14). The large potency variations between NE-10244 and NE-58051 observed in our study were also suggestive of the involvement of a stereospecific recognition of bisphosphonates by tumor cells. To further address this question, we studied the effect of BPs on tumor cell adhesion to mineralized bone matrices. As exemplified with the effects of clodronate, NE 10244, and ibandronate on adhesion of PmPC3 cells to cortical bone slices, we...
observed that exposure of tumor cells with these BPs ($10^{-6}$–$10^{-4}$ M) drastically inhibited tumor cell adhesion. By contrast, BP-coated bone slices only partially inhibited tumor cell adhesion. Interestingly, it has been previously shown that BPs, including ibandronate ($10^{-9}$ M) and pamidronate ($10^{-4}$ M), coated to cortical and trabecular bone matrices partially inhibit the adhesion of MDA-MB-231 cells (7). Our results are in accordance with those obtained by van der Pluijm et al. (7). However, as aforementioned for the inhibitory effect of bisphosphonates on tumor cell adhesion to unmineralized extracellular matrices, pretreatment of tumor cells with NE-58051 (the inactive analogue of risedronate) did not inhibit tumor cell adhesion to mineralized matrices, whereas NE-10244, the active risedronate analogue, did. In contrast, both NE-58051- and NE-10244-coated bone slices inhibited tumor cell adhesion to a similar extent. It is possible that BPs coated to cortical slices modulate tumor cell adhesion by steric exclusion, whereas the recognition of BPs by tumor cells induces the inhibition of specific intracellular signal transduction mechanisms. This would explain why the extent of inhibition of tumor cell adhesion to BP-coated mineralized matrices was constantly lower than that observed with the adhesion of BP-treated tumor cells to untreated bone slices. Although BPs did not affect tumor cell surface integrin expression (this study), our results provide evidence for a direct cellular effect of BPs, independent of the need for a contact of tumor cells with BP-coated mineral. These findings are consistent with the observation that clodronate reduces the incidence of nonbone metastases in patients with breast cancer (8). Our results, therefore, raise the interesting possibility that BPs may be useful agents for the prophylactic treatment of patients with cancer that is known to preferentially metastasize to bone.

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References

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