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Alendronate induces anti-migratory effects and inhibition of neutral phosphatases in UMR106 osteosarcoma cells

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Abstract

Bisphosphonates are nonhydrolysable pyrophosphate analogues that prevent bone loss in several types of cancer. However, the mechanisms of anticancer action of bisphosphonates are not completely known. We have previously shown that nitrogen-containing bisphosphonates directly inhibit alkaline phosphatase of UMR106 rat osteosarcoma cells. In this study, we evaluated the effects of alendronate on the migration of UMR106 osteosarcoma using a model of multicellular cell spheroids, as well as the alendronate effect on neutral phosphatases. Alendronate significantly inhibited the migration of osteoblasts in a dose-dependent manner (10^{-6}–10^{-4} M). This effect was also dependent on calcium availability. The spheroid morphology and distribution of actin fibers were also affected by alendronate treatment. Alendronate dose-dependently inhibited neutral phosphatase activity in cell-free osteoblastic extracts as well as in osteoblasts in culture. Our results show that alendronate inhibits cell migration through mechanisms dependent on calcium, and that seem to involve inhibition of phosphotyrosine-neutral-phosphatases and disassembly of actin stress fibers.

Keywords: Spheroid; Bisphosphonates; Phosphatase; Actin fibers; Osteosarcoma

1. Introduction

Breast and prostate cancer are diseases frequently characterized by widespread metastasis in the skeleton, inducing enhanced bone resorption by osteoclast activation (Roodman, 2004; Virtanen et al., 2002; Yin et al., 2005). Bisphosphonates are nonhydrolysable pyrophosphate analogues that strongly bind to hydroxyapatite (Green, 2005). This property explains their specific pharmacological action on mineralized tissues (Rodan and Fleisch, 1996). In particular, in bone tissue, bisphosphonates prevent bone loss by inhibiting osteoclast function as well as by stimulating osteoblast activation in vitro (Green, 2005; Rodan and Fleisch, 1996). Bisphosphonates are used in the treatment of osteoporosis, bone metastasis and multiple myeloma (Lipton, 2004). More recently, it has been shown that bisphosphonates can exert direct cytostatic and antiproliferative effects against a variety of tumor cells including osteosarcoma (Green, 2003; Kubista et al., 2006; Lipton, 2003; Virtanen et al., 2002). However, the mechanisms of anticancer action of bisphosphonates are not completely known and are a matter of intense investigation. Nitrogen-containing bisphosphonates have been demonstrated to inhibit mevalonate pathway, in particular through the farnesylation of small GTP-binding proteins (Fisher et al., 2000; Green, 2005; Luckman et al., 1998), thus affecting several basal cell functions and cell survival. We have previously shown that N-containing bisphosphonates (alendronate, pamidronate and zoledronate) directly inhibit the specific activity of bone–alkaline phosphatase (ALP) obtained from an extract of UMR106 rat osteosarcoma cells (Vaisman et al., 2005). This effect was observed in a concentration range to which this exoenzyme is probably exposed in vivo. Thus, bisphosphonates may affect the activity of various enzymes, in particular phosphatases involved in different transduction pathways.

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It has been previously demonstrated that bisphosphonates have direct effects on the invasion, adhesion and migration of cancer cells in monolayers (Boissier et al., 2000; Cheng et al., 2004). Alendronate, a nitrogen-containing biphosphonate, is a potent inhibitor of bone resorption used for the treatment of bone disorders such as osteoporosis, Paget's disease and hypercalcemia of malignancy (Chapurlat and Delmas, 2006). However, questions remain regarding the mechanisms by which alendronate inhibits invasion and migration of cancer cells.

Multicellular cell spheroids (MCS) are spherical symmetric aggregates of cells analogous to tissue with no artificial substrate for cell attachment. The advantage of this system is that it resemble more closely an in vivo situation, since it is a model of study with an intermediate complexity between monolayer cultures in vitro and tumors in vivo (Hamilton et al., 2001; Mueller-Klieser, 1997). In recent years MCS have become an adequate model to study tumor biology and to evaluate new antineoplastic therapies.

In this study we evaluated the effects of alendronate on the migration of UMR106 MCS. We also investigated the relation between neutral phosphatases and cell migration. Our results show that alendronate inhibits cell migration through a mechanism that seems to involve inhibition of phosphotyrosine-neutral-phosphatases and disassembly of actin stress fibers.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA and fetal bovine serum (FBS) were from Gibco, (Life Technology, Buenos Aires, Argentina) and tissue culture disposable material was from Nunc (Buenos Aires, Argentina). Fluorogenic small substrate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) was obtained from Molecular Probes (Buenos Aires, Argentina). All other chemicals and reagents were obtained from commercial sources and were of analytical grade.

2.2. Cell culture and spheroid preparation

UMR106 rat osteosarcoma cells were grown in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2 atmosphere (Cortizo and Etcheverry, 1995). Cells were seeded on 75 cm^2 flasks and subcultured using trypsin-EDTA. For experiments, osteoblast-like cells were placed on multi-well plates and incubated in 10% FBS media. MCS were initiated by plating 10^4 cell/well in 10% FBS-DMEM on 24-well culture plates coated with 1% agar in DMEM/10% FBS (Ballangrud et al., 1999). One half of cell growth media was replaced every other day. After 5 days of culture, MCS with a size between 100–150 μm were selected to perform migration and immunofluorescence assays.

2.3. Assay of cell migration from the spheroid

MCS were plated on a multiwell cell culture plate and incubated during 24 h in DMEM plus 10% FBS, with or without different doses of alendronate. After this incubation period spheroids were fixed with absolute methanol and stained with Giemsa. Migration distance from the spheroid was measured using an ocular micrometer. To diminish errors in estimation of the migration distance two measures were assessed in each spheroid (Fig. 1) and at least 14 spheroids were evaluated per condition.

2.4. Inmunofluorescence assay of actin fibers

MCS were cultured in DMEM-10% FBS on coverslips in the presence or absence of different doses of alendronate, and allowed to migrate for 24 h at 37 °C in 5% CO_2 atmosphere. After this incubation period, MCS were fixed with 4% *p*-formaldehyde in PBS for 15 min, permeabilized with cold methanol for 4 min, and stained with fluorescein-labelled phalloidin (1:100) for 1 h at room temperature. Coverslips were mounted in a Vectastain mounting liquid and images were recorded and analyzed using a Nikon-5000 fluorescence microscope and a digital camera, in order to determine the actin structure of spheroid (Tzanakakis et al., 2001).

2.5. Fluorogenic phosphatase assay

In order to evaluate the activity of neutral phosphatases, a fluorogenic assay was carried out. A kinetic assay was performed using a 0.1% Triton-X100 osteoblast extract and the fluorogenic substrate DiFMUP as previously described (Cortizo et al., 2006; Welte et al., 2005). Different concentrations of Alendronate were pre-incubated with the osteoblastic extract (corresponding to 50 μg of protein) for 10 min. Then, the cell extract was incubated with 10 μM DiFMUP in phosphate buffer, pH 7.4, for 10 min. The resulting product was measured at 360/450 nm (excitation/emission wavelengths) using an Aminco-Bowman SPF100 spectrofluorometer. We also tested the effect of increasing doses of alendronate on UMR106 cell monolayer. This assay was carried out incubating confluent UMR106 monolayers with alendronate for 24 h. After that, an aliquot of cell lysates (50 μg of protein) were incubated with DiFUMP for 10 min and the fluorescence was determined as above.

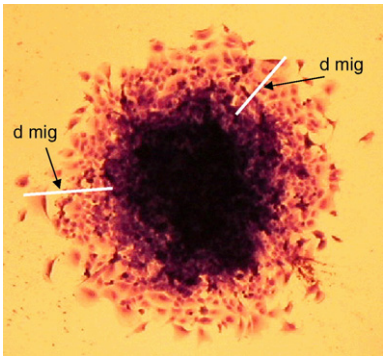

Fig. 1. UMR106 multicellular spheroids. Distance of migration was assessed using an ocular micrometer (d mig).

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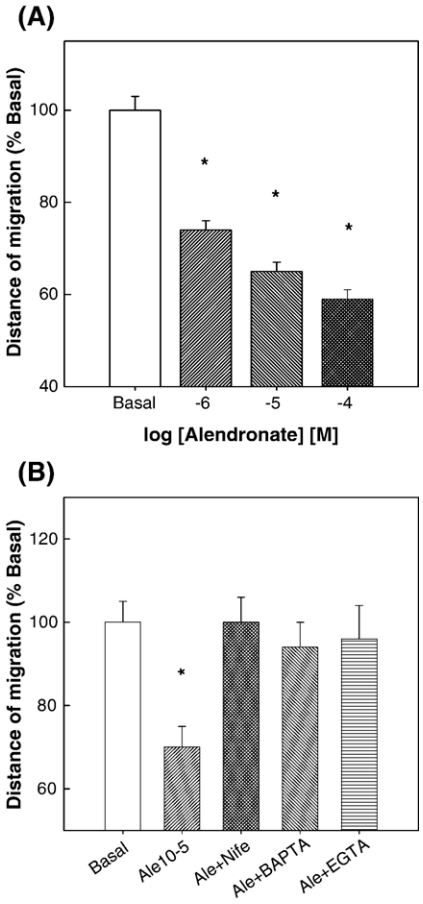

Fig. 2. Effect of alendronate on cell migration (A) and effect of different calcium inhibitors (B). MCS were incubated with different doses of alendronate with or without different agents for 24 h. The distance of migration was estimated with a graduated ocular. Results are expressed as % Basal and represents the media±S.E.M ($n=14$). Differences versus basal are: *$p<0.001$.

2.6. Statistical analysis

Three independent experiments were run for each experimental condition. Results are expressed as the mean±S.E.M. Statistical analysis of the data was performed by Student's t test. Values of P less than 0.05 were considered to be significant.

3. Results

3.1. Cell migration from spheroids

The effect of 24 h-alendronate treatment on the migration of UMR106 osteoblasts was assessed using a three-dimensional model of spheroids. Alendronate significantly inhibited ($p<0.001$) the migration of osteoblasts in a dose-dependent manner (Fig. 2A). In order to evaluate the role of calcium on the inhibitory effect of alendronate on cell migration, spheroids were treated with 1 μM nifedipine (a L-type calcium channel blocker), 0.1 nM 1, 2-bis (2-aminophenoxy) ethane-*N*, *N*, *N*′, *N*′-tetraacetic acid-acetoxymethyl ester (BAPTA) (an intracellular calcium chelator) or 1 μM EGTA (an extracellular calcium chelator). Neither nifedipin, BAPTA nor EGTA modified cell migration (data not shown). However, the inhibitory effect of 10^{-5} M alendronate on migration was completely prevented by the addition of each agent (Fig. 2B). We also investigated the effect of vanadate, a well known tyrosine phosphatase inhibitor, on the UMR106 MCS migration. We found that vanadate inhibited cell migration in a dose dependent fashion (Table 1). This effect was neither potentiated nor enhanced by the co-incubation with 10^{-4} M alendronate (data not shown). All together these results suggest that alendronate decreases cell migration by controlling the availability of calcium, with a degree of migration inhibition similar to that induced by vanadate.

Table 1
Effect of vanadate doses on cell migration

	Vanadate [μM]			
	0	2.5	5	10
Migration distance (% Basal)	100±4	72±4 a	65±3 a	48±2 a

Data represents the media±S.E.M., $n=14$. a $P<0.001$.

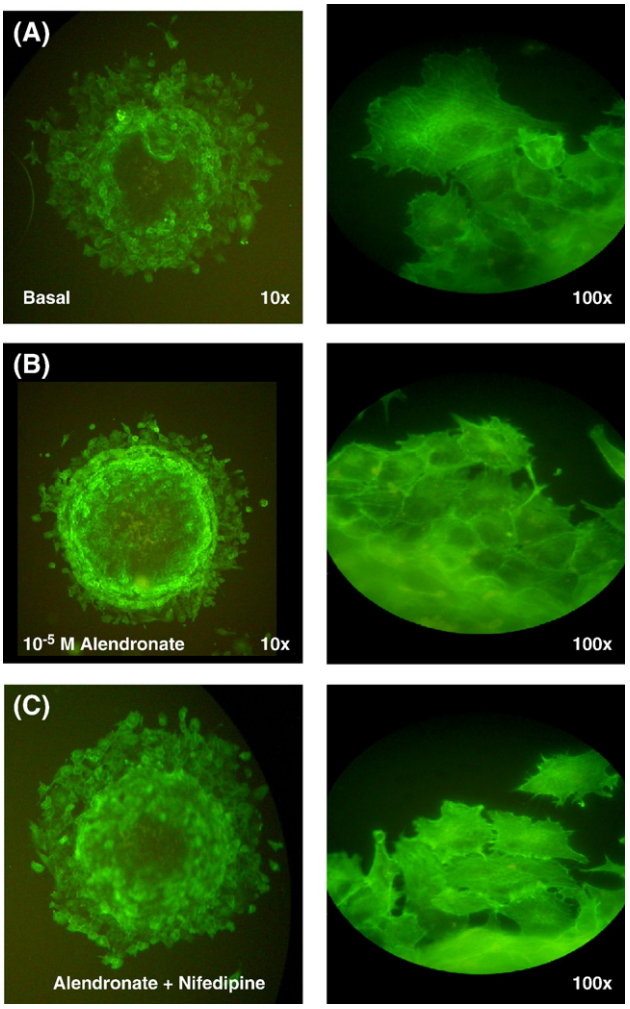

Fig. 3. Effect of alendronate on actin stress fibers. MCS were incubated with (B) or without (A) alendronate plus 1 μM nifedipine (C) for 24 h. Actin stress fibers were evaluated by staining with phalloidin—FITC. Magnification: 10× (left panels) and 100× (right panels).

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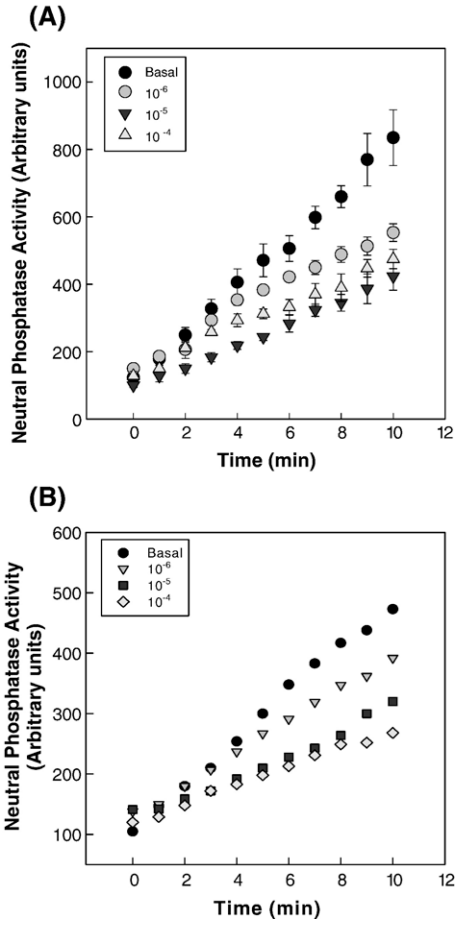

Fig. 4. Effect of alendronate on inhibition of protein tyrosine phosphatases activity from osteosarcoma extract (A) and UMR106 cell culture (B). An aliquot corresponding to 50 μg protein was incubated with the fluorescent substrate DiFMUP in PBS plus different doses of alendronate for 10 min. The formation of the fluorescent product was evaluated at 360/450 nm and expressed as Arbitrary units.

3.2. Actin stress fibers

Actin cytoskeleton reorganization is a key event in cell migration process (Burridge et al., 1988). Thus, we analyzed the morphology of actin stress fibers by inmunofluorescence. Spheroids were labeled with FITC-phalloidin to detect F-actin. Untreated MCS cultures expressed very few and weakly stained stress fibers, largely confined to the cell membrane and showing a circumferential pattern of actin (Fig. 3A). Higher magnification showed well developed actin fibers throughout the cytoplasm, with cytoplasmic protrusions to the edge of migration. After 10^{-5} M alendronate treatment for 24 h, an intense actin staining in the MCS was observed (Fig. 3B). Cells protruding from the alendronate-treated MCS showed a diffuse pattern of FITC-phalloidin and a more intense fluorescence in the cell–cell contacts without a directional migration (Fig. 3B, 100×). Co-treatment of MCS with nifedipine and alendronate (Fig. 3C) expressed a pattern and staining of actin stress fibers similar to that observed in the basal conditions for both migrating cells and those in the body of the MCS. These observations suggest that nifedipine prevents the alendronate-induced disassembly of actin fibers.

3.3. Effect of alendronate on neutral phosphatases activity of UMR106 cells

First we investigated the protein tyrosine phosphatases using a fluorogenic kinetic assay in a Triton-X100 cell extract of UMR106 osteoblasts. Aliquots of cell extracts were pre-incubated with different doses of alendronate in PBS buffer for 10 min, followed by the addition of the fluorogenic DiFMUP substrate and the activity of total neutral protein tyrosine phosphatases evaluated during the next 10 min. Control untreated (basal) extracts showed a linear response during this incubation period (Fig. 4A). Treatment with alendronate significantly inhibited protein tyrosine phosphatases activity in a dose-dependent fashion, with a maximal inhibition obtained at 10^{-5} M (47% inhibition) (Fig. 4A). Similarly, 100 μM vanadate also significantly inhibited protein tyrosine phosphatases activity (45% inhibition) (data not shown). Alendronate inhibition of protein tyrosine phosphatases activity was not modified by the co-incubation with 100 μM vanadate. Thus, alendronate seems to inhibit tyrosine-phosphatases in the UMR106 cell extracts.

We also investigated the direct effect of alendronate on protein tyrosine phosphatases in the intact cell. UMR106 osteoblasts were cultured in the presence of different doses of alendronate for 24 h, after which the protein tyrosine phosphatases activity was assessed as before. In the basal culture, protein tyrosine phosphatases exhibited a linear response (Fig. 4B). In treated cells, alendronate inhibited protein tyrosine phosphatases in a dose-response manner with a maximal inhibitory effect observed at a concentration of 10^{-4} M (57% of basal, $p<0.01$). These results suggest that alendronate is affecting phosphorylation/dephosphorylation pathways in parallel with its inhibition of migration.

4. Discussion

Diverse pathologies such as osteolytic metastasis, Paget's disease and osteoporosis cause a loss in bone mass, and as a result there is skeletal pain and pathologic fracture increasing the morbidity for the patient (Hamdy et al., 1993; Manolagas and Jilka, 1995; Rodan and Martin, 2000). As evidenced for bone metastasis, a vicious cycle between osteoclasts, stromal cells/osteoblasts, and cancer cells has been hypothesized during the progression of primary bone tumors (Chirgwin and Guise, 2000). Bisphosphonates are currently used in the treatment as well as in co-adjuvant therapy for these pathologies (Horie et al., 2006; Jagdev et al., 2001; Matsumoto et al., 2005) because they have a high affinity for the hydroxyapatite mineral in bone and are taken up selectively and adsorbed to mineral surfaces at sites of increased bone turnover where they inhibit osteoclast activity (Kellinsalmi et al., 2005; Rodan and Fleisch, 1996). Moreover, bisphosphonates can inhibit the development and viability of mature osteoclasts and in addition, they may exert their actions indirectly by affecting neoplastic cells (Evdokiou et al., 2003; Heymann et al., 2005). Using a model of three-dimensional

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osteosarcoma UMR106 culture, we found that alendronate, a nitrogen-containing bisphosphonate, diminishes cell migration in a dose-dependent manner. Our results are in agreement with previous reports demonstrating that bisphosphonates inhibit cell migration in other cellular systems (Boissier et al., 2000; Cheng et al., 2004; Virtanen et al., 2002). In these reports, the authors carried out assays in monolayer cultures of different cancer cells. Although the molecular mechanism involved in the antimigratory activity of bisphosphonates are still not clear, several signalling pathways may be involved, such as extracellular signal-regulated kinases and calcium channels (Mathov et al., 2001). For instance, it has been shown that different bisphosphonates rapidly modulate cytosolic calcium levels (Colucci et al., 1998, Vazquez et al., 2003). In the present study, we attempted to determine the role of calcium and the mechanisms by which alendronate inhibits cell migration. Our results suggest that the inhibition of cell migration could be mediated by an increase in intracellular calcium levels, since nifedipine, a blocker of L-type calcium channels, EGTA, an extracellular calcium chelator, and BAPTA, an intracellular calcium chelator, all reversed the inhibition of cell migration caused by alendronate. These observations agree well with the previous report of Vazquez demonstrating that nifedipine and verapamil reduced cystosolic calcium levels in the ROS 17/2.8 osteosarcoma line induced by bisphosphonates (Vazquez et al., 2003). Thus, our results seem to indicate that the alendronate-inhibited cell migration is mediated by the availability of intracellular calcium. However, further experiments with electrophysiology and calcium-sensitive fluorochromes would be needed to prove our hypothesis.

Cell migration is a complex event that involves multiple simultaneous processes such as focal adhesion formation, actin cytoskeletal reorganization and activation of many intracellular signaling pathways (Howe, 2004; Li et al., 2005; Watanabe et al., 2005; Wehrle-Haller and Imhof, 2003). Calcium, as well as the Rho small GTP binding family of phosphatases, play an important role in cell motility (Chaudhuri et al., 2003; Wheeler and Ridley, 2004). Calcium flux plays a critical role in cell migration and it is a very well regulated process. A highly regulated $[Ca^{+2}]_i$ transient influx must occur for normal migration, while a sustained increase in $[Ca^{+2}]_i$ inhibits cell migration (Chaudhuri et al., 2003). We found that alendronate causes a reorganization of actin cytoskeleton and this effect was reversed by co-treatment with nifedipine. On the other hand, we also found that incubation of intact osteosarcoma cells or cell extracts with alendronate causes a decrease in neutral phosphatases activity as determined using the fluorogenic substrate DiFMUP. Our results further suggest that this effect was mainly mediated by tyrosine phosphatases since vanadate showed a similar and non-additive effect to alendronate. These results are in agreement with previous observations demonstrating that alendronate inhibits protein tyrosine phosphatases (Endo et al., 1996; Opas et al., 1997; Schmidt et al., 1996; Skorey et al., 1997). Previous data suggest that the Rho family of GTPases activate kinases that are important in cytoskeletal-mediated changes affecting motility (Mackesky and Hall, 1997; Schmitz et al., 2000). The Rho family of GTPases are post-transcriptionally modified by prenylation. The prenyl-group anchors GTPases onto membranes and constitutes an essential modification for cell growth, transformation and cytoskeletal reorganization (Wheeler and Ridley, 2004). It has previously been demonstrated that alendronate inhibits the mevalonate pathway and in consequence inhibits protein prenylation (Fisher et al., 1999). In particular, alendronate prevents geranylgeranylation of Rho, thus inhibiting cancer cell invasion (Sawada et al., 2002; Virtanen et al., 2002).

In conclusion, our results demonstrate that alendronate inhibits MCS cell migration by mechanisms that involve calcium mobilization, actin-cytoskeleton reorganization as well as inhibition of neutral phosphatases. These studies contribute to a better understanding of the mechanisms by which alendronate can reduce osteosarcoma invasion.

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