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Alendronate inhibits cell invasion and MMP-2 secretion in human chondrosarcoma cell line[1]

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Key words

alendronate; bisphosphonate; chondrosarcoma; matrix metalloproteinase-2; invasion; migration

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Abstract

Aim: Chondrosarcoma is a malignant primary bone tumor that responds poorly to both chemotherapy and radiation therapy. The aim of the present study was to investigate the effect of alendronate, a bisphosphonate, on the invasion and migration of human chondrosarcoma cells (JJ012). **Methods:** JJ012 cells were treated with alendronate of various concentrations up to 100 μmol/L for a specified period, and then gelatin zymography and matrigel invasion assay was performed to study the effects of alendronate on matrix metalloproteinase (MMP)-2 activity and the invasion ability of JJ012 cells, respectively. **Results:** Our data showed that alendronate exerted a dose- and time-dependent inhibitory effect on the invasion and migration of JJ012 cells. Furthermore, gelatin zymography and RT-PCR showed that alendronate treatment decreased the activity and mRNA levels of MMP-2 in a concentration-dependent manner. **Conclusion:** Our findings suggest that alendronate may reduce MMP-2 secretion at the transcriptional and translational levels, and inhibit the invasion of chondrosarcoma cell. Therefore, alendronate may be a potential candidate for the systemic therapy of chondrosarcomas, as well as other malignant diseases.

Introduction

Chondrosarcoma is a malignant primary bone tumor with a poor response to currently-used chemotherapy or radiation treatment, making the management of chondrosarcomas a complicated challenge[1]. Clinically, surgical resection remains the primary mode of therapy for chondrosarcoma. Due to the absence of an effective adjuvant therapy, this mesenchymal malignancy has a poor prognosis[2] and therefore, it is important to explore a novel and adequate remedy.

Since chondrosarcoma is a type of highly malignant tumor with a potent capacity to invade locally and distant metastasis[3,4]. An approach decreasing the ability of invasion and metastasis may facilitate the development of effective adjuvant therapy. In the processing of chondrosarcoma invasion and metastasis, the degradation of extracellular matrix (ECM) and the components of the basement membrane caused by the concerted action of proteinases, such as matrix metalloproteinases (MMP), cathepsins, and plasminogen activator, is a critical step in tumor invasion and metastasis[3,5–8]. Among these enzymes, MMP-2 and MMP-9 degrade most components of the ECM directly and deeply involved in cancer invasion and metastasis[7,9]. Therefore, the inhibition of migration or invasion mediated by MMP-2 could be a preventive method of cancer metastasis[9].

Recently, there has been increasing evidence indicating that bisphosphonates (BP) have direct antitumor effects *in vivo* and *in vitro*, in addition to their therapeutic antiresorptive properties[10–19]. For example, Senaratne *et al* reported that BP could induce apoptosis in human breast cancer cell lines[17]. Forsea *et al* also demonstrated that BP had an inhibitory effect in cell cycle progression in human melanoma cells[13]. Furthermore, several studies have demonstrated that alendronate, a BP, could inhibit cell invasion in human epidermoid carcinoma cells[11] and osteosarcoma cells[12].

However, the inhibitory effect related to cell invasiveness of alendronate has not been studied yet. Therefore, in this study, the impact of alendronate on cell invasion and migration were examined *in vitro* in human chondrosarcoma cells (JJ012).

Materials and methods

Cell culture and alendronate treatment The human chondrosarcoma cell line (JJ012) was kindly provided from the laboratory of Dr Sean P SCULLY (University of Miami School of Medicine, Miami, FL, USA)[20]. The JJ012 cells were cultured in Dulbecco's modified Eagle's medium/modified Eagle's medium-α/Ham's F12 supplemented with 10% Fetal Bovine Serum and maintained at 37 ºC in a humidified atmosphere of 5% CO_2. Alendronate (4-amino-1-hydrocybutylidene) was a gift from Merck Sharp and Dohme (Tipperary, Ireland). For the alendronate treatment, appropriate amounts of stock solution (1 mmol/L in DMSO) were added into the culture medium to achieve the indicated concentrations. The cells were treated in the culture medium for 12, 24, and 48 h. The DMSO solution without alendronate was used as a blank reagent.

Determination of cell viability The effects of alendronate on the viability of the JJ012 cells were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[21]. Briefly, the JJ012 cells were seeded in 24-well plates at a density of 3.5×10^4 cells/well and treated with an indicated concentration of alendronate at 37 ºC for 24 h. At the end of the indicated exposure period, the cells were washed with phosphate buffered solution and then incubated with MTT (0.5 mg/mL; Sigma Chemical Co, St. Louis, MO, USA) for 4 h. The viable cell number was directly proportional to the production of formazan following solubilization with isopropanol, which can be measured spectrophotometrically at 563 nm.

Cell invasion assay The cell invasion assay was performed to study the effects of alendronate on the invasion ability of the chondrosarcoma cell line, according to the methods described by Yuan *et al*[2]. Briefly, cell invasion was assayed in a modified Boyden chamber (Neuro Probe, Cabin John, MD, USA) with 8 μm pore size polycarbonate membrane filters. The filters were precoated with 10 μL matrigel (25 mg/50 mL; BD Biosciences, Bedford, MA, USA). After being treated with alendronate for 24 h, the surviving cells were harvested and seeded in a Boyden chamber at a density of 1×10^4 cells/well with serum-free medium, and then incubated for 24 h at 37 ºC. After the assay, the filters were air-dried for 5 h in a laminar flow hood. The invaded cells were fixed with methanol and stained with Giemsa. The cell numbers were counted under a light microscope.

Cell migration assay Cell migration experiments were performed using Bio-Coat cell migration chambers (Becton Dickinson, Meylan, France), which consisted of a 24-well companion plate with cell culture inserts containing 8 μm pore size filters. Briefly, untreated and alendronate-treated cells (1×10^4/500 μL) were added to each insert (upper chamber), and the chemoattractant (10% Fetal Calf Serum) was placed in each well of a 24-well companion plate (lower chamber). After incubation for 24 h at 37 ºC in a 5% CO_2 incubator, the upper surface of the filter was wiped with a cotton-tipped applicator to remove non-migratory cells. Cells that had migrated through the filter pores and attached on the under surface of the filter were fixed and stained. The cells from the microscopic fields were counted.

Determination of MMP-2 by gelatin zymography The activities of MMP-2 in the medium were measured by gelatin zymography protease assays as previously described[22]. Briefly, collected media of an appropriate volume were prepared with SDS sample buffer without boiling or reduction and subjected to 0.1% gelatin-8% SDS-PAGE electrophoresis. After electrophoresis, the gels were washed with 2.5% Triton X-100 and then incubated in a reaction buffer [40 mmol/L Tris-HCl (pH 8.0), 10 mmol/L $CaCl_2$, and 0.01% NaN_3] at 37 ºC for 12 h. Then the gels were stained with Coomassie brilliant blue R-250.

Preparation of RNA and RT-PCR Total RNA were extracted from the JJ012 cells by the Qiagen RNeasy Mini Kit (Valencia, CA, USA). For reverse transcription, 4 μg of total cellular RNA were used as the template in a 20 μL reaction containing 4 μL dNTP (2.5 mmol/L), 2.5 μL oligo dT (10 pmol/mL), and RTase (200 U/mL); the reaction was performed at 42 ºC for 1 h. Afterwards, 5 μL cDNA product was used as the template in PCR amplifications together with appropriate primers (sense of MMP-2, 5'-GGCCCTGTCACTCCTGAGAT-3'; antisense of MMP-2, 5'-GGCATCCAGGTTATCGGGGA-3'; sense of GAPDH, 5'-CGGAGTCAACGGATTTGGTCGT-AT-3'; antisense of GAPDH, 5'-AGCCTTCTCCATGGTTGG-TGAAGAC-3'). The final products were subjected to electrophoresis with 2% agarose gel and detected by ethidium bromide staining.

Statistical analysis In a set of experiments, each assay was performed in duplicate. Each experiment was repeated independently at least 3 times. All data are presented as mean±SD. Statistical significance was determined by the Student's *t*-test. A P-value <0.05 was considered to be statistically significant.

Results

Effect of alendronate on the viability of JJ012 cells The cytotoxic effect of various concentrations of alendronate (0–100 μmol/L) on JJ012 cells by MTT assay was assessed as shown in Figure 1. It was clear that 24 h treatment of alendronate, even at a concentration as high as 100 μmol/L, had no cytotoxicity to the JJ012 cells. This concentration range was then applied in all subsequent experiments.

Inhibition on invasion and migration of JJ012 cells by alendronate Using a cell invasion assay with a Boyden chamber, the number of invading cells was significantly reduced in JJ012 cells following alendronate treatment. Furthermore, alendronate inhibited the invasive and migration ability in a dose- and time-dependent manner (Figure 2A–2D). At a dose of 100 μmol/L, alendronate reduced the invasion ability of the JJ012 cells to 21.3% of that of the controls (=untreated cells, set to 100%).

Inhibitory effects of alendronate on MMP-2 By the use of gelatin zymography assays, it was shown that alendronate prevented a time-dependent increase in MMP-2 enzyme activity (Figure 3A,3B). There was only 23.4% of MMP-2 activity left after 100 μmol/L alendronate treatment, as compared with that of non-alendronate-treated cells.

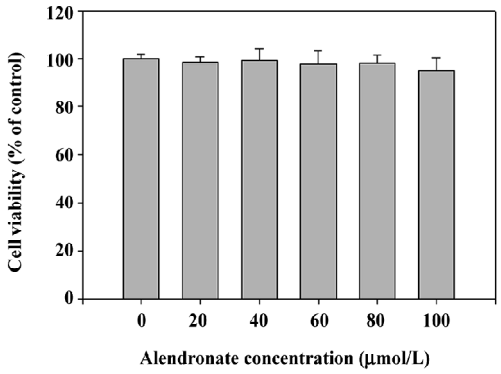

Figure 1. Effect of alendronate on the viability of JJ012 cells. JJ012 cells were treated with an indicated concentration of alendronate for 24 h before being subjected to a MTT assay for cell viability. Values represented as the mean±SD of at least 3 independent experiments.

Inhibitory effects of alendronate on the mRNA levels of MMP-2 To further evaluate if the significant regulatory ef-

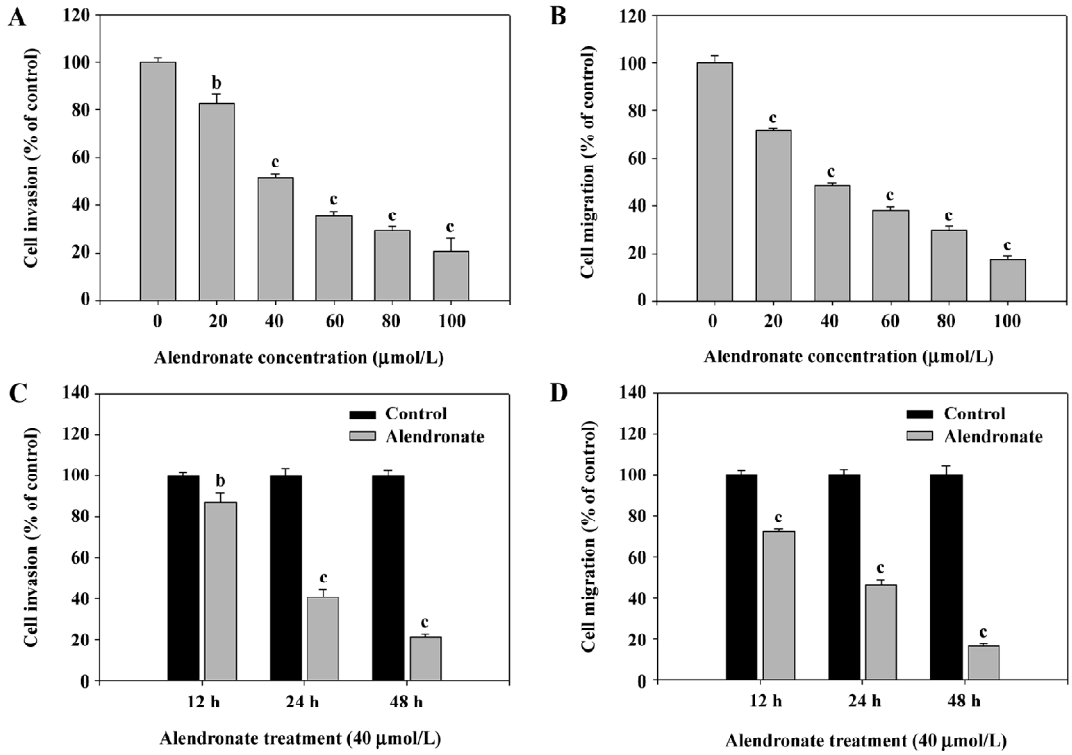

Figure 2. Concentration- and time-dependent effects of alendronate on invasion and migration of JJ012 cells. In concentration-dependent assays (A, B), JJ012 cells were treated with alendronate at an indicated concentration for 24 h, and in time-dependent assays (C, D), cells were treated with 40 μmol/L alendronate for 12, 24, and 48 h. Treated cells were then subjected to analyses for invasion and migration. Data represent the mean±SD of at least 3 independent experiments. ($^bP<0.05$; $^cP<0.01$).

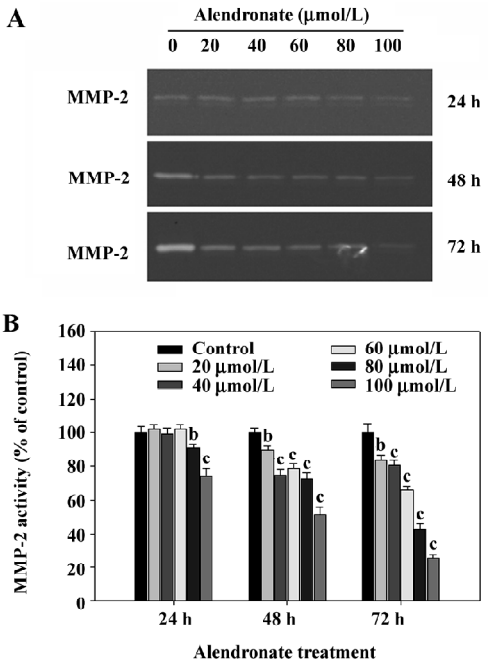

Figure 3. Effects of alendronate on the activity of MMP-2 in JJ012 cells. (A) JJ012 cells were treated with an indicated concentration of alendronate for 24, 48, and 72 h and then subjected to gelatin zymography to analyze the activity of MMP-2. (B) determined activity of MMP-2 was subsequently quantified by densitometric analysis with that of the control being 100% as shown just below the gel data ($^bP<0.05$; $^cP<0.01$).

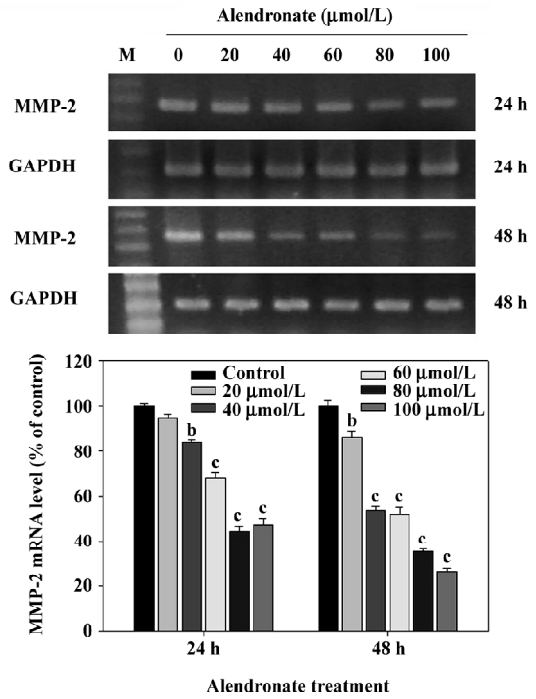

Figure 4. Effects of alendronate on the mRNA levels of MMP-2. JJ012 cells were treated with alendronate at an indicated concentration for 24 and 48 h, and then the RNA samples were extracted and subjected to a semiquantitative RT-PCR for MMP-2 with GADPH being an internal control. The PCR products were quantitated by densitometric analysis with that of the untreated group being 100%. Data represent the mean±SD of at least 3 independent experiments. ($^bP<0.05$; $^cP<0.01$).

fects of alendronate on MMP-2 in the JJ012 cells are on the mRNA level, a semiquantitative RT-PCR analysis was performed. With GADPH as an internal control, the mRNA levels of MMP-2 were significantly reduced by alendronate in a concentration- and time-dependent manner (Figure 4). It was therefore clear that the regulation of MMP-2 expressions by alendronate was, at least in part, on the transcriptional level.

Discussion

Our study demonstrates that alendronate could significantly reduce MMP-2 secretion in JJ012 cells in a time- and dose-dependent manner and also inhibit invasion ability in a time- and dose-dependent manner, without showing any cytotoxity or apoptosis induction. Although the beneficial effect of BP in the treatment of metastatic bone disease could be due to their anti-osteoclastic properties[23], preclinical and clinical trials and several *in vitro* and *in vivo* reports have suggested that BP might also have specific anticancer action on several types of tumors, such as osteosarcoma, breast, prostate, and melanoma cancer[12–16]. They act either by inhibiting cell viability and/or inducing apoptosis, inhibiting cell adhesion and/or invasiveness, or anti-angiogenic potential[24–26].

BPs are well tolerated by patients of all ages and categories. Alendronate is a relatively less potent class of BP with minimal side effects[27]. However, to our knowledge, it is the first time that the inhibitory effects of alendronate related to cell invasiveness have been investigated and analyzed in human chondrosarcoma cell (JJ012). In the present study, alendronate was shown to inhibit *in vitro* tumor cell invasion in the chondrosarcoma cell line. It is therefore highly likely that the inhibitory effect of alendronate on chondrosarcoma cell invasion may primarily be due to MMP-2 inhibition. One of the key steps in the metastatic cascade involves the disruption of ECM and basement membranes, permitting tumor cells to access a distant metastatic site. Furthermore, the different inhibitory effects on MMP-2 secretion and the mRNA level may indicate that the regulation of MMP-2 expression by alendronate in JJ012 cells was more involved at the transcriptional level than the translational or post-translational levels. However, it warrants

further study to elucidate the underlying mechanism.

Tissue invasion requires the expression of proteinases that are specific for interstitial ECM. In particular, the 72 kDa gelatinase A/Type IV collagenase (MMP-2) is a well-studied member of the MMP family, which plays a critical role in tumor invasion, metastasis, and angiogenesis[4,28]. Therefore, the inhibition of chondrosarcoma cell-derived MMP-2 production may significantly reduce local spread and invasion.

In the present study, our findings identified that alendronate may reduce the activity and mRNA levels of MMP-2 and inhibit cell invasion in the chondrosarcoma cell line. Therefore, alendronate may be a potential candidate for the systemic therapy of chondrosarcomas, as well as other malignant diseases.

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