사람 U2OS 골육종 세포에서 Matrix Metalloproteinase의 발현에 Ibandronate가 미치는 영향

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배경: 골육종은 소아 및 청소년기에 가장 흔하게 발생하는 악성 골종양 중 하나이다. 최근 수술적 치료와 항암 화학요법을 병행하여 생존율이 증가하였지만, 아직까지도 항암제는 항암제 내성 및 이차성 악성 종양의 발생 등 여러 문제점을 가지고 있다. 일부 종양은 matrix metalloproteinase(MMPs)의 발현이 증가되어 있고, MMP inhibitor에 대한 연구가 진행되고 있다. 반면 bisphosphonate(BPs) 제제는 골흡수를 억제하는 능력이 있으며, 파골세포와 관계된 골 병변에 광범위하게 사용되고 있다. 또 bisphosphonate 제제는 직접적인 항암 효과를 가지고 있는 것으로 알려져 있다.

대상 및 방법: 골육종 세포주(U2OS)를 ibandronate(0, 0.1, 1, 10M)를 이용하여 48시간 동안 처치하였다. 세포의 생존능은 MTT assay를 이용하여, MMP-2와 MT1-MMP의 mRNA level은 reverse-transcition polymerase chain reaction을 이용하여 측정하였으며, MMP-2와 MT1-MMP 단백의 양은 Westernblot을, MMP-2의 활성은 Gelatin zymography를 이용하여 측정하였다. 또, ibandronate 처치 전후의 골육종 세포주의 침습성은 Matrigel invasion assay를 이용하여 측정하였다.

결과: 48시간 ibandronate 처치 후 U2OS 세포주의 침습력은 ibandronate에 대해 용량의존적으로 감소하였다. 특히 10M 이내의 ibandronate는 세포독성이 나타나지 않았다. 젤라틴 용해능과 MMP-2 및 MT1-MMP의 단백 및 mRNA 정도 역시 ibandronate 농도가 증가함수록 감소하였다.

결론: Ibandronate는 골육종 세포주의 MMP-2 및 MT1-MMP의 발현을 억제하였으며, 종양세포의 침습력을 감소시켰다. Bisphosphonate의 종양세포 침습 억제 능력은 새로운 전이 억제제의 개발에 도움이 될 것으로 사료된다.

색인 단어: 골육종, Matrix metalloproteinase, Ibandronate
Background

Bisphosphonates (BPs) are stable analogues of pyrophosphonate, and widely used and potent drugs in the fight against osteoclast-mediated\(^3,5\). Furthermore, they are widely used to treat metabolic bone diseases, such as, Paget’s disease\(^28\) and hypercalcemia\(^10\) and to treat postmenopausal osteoporosis\(^27\). Preclinical evidence suggest that BPs have direct antitumor effects on a variety of human cancer cells\(^29\), and it is known that they decrease cell proliferation in human osteosarcoma cell line panels, disturb the cell cycle, and induce the apoptosis of SaOS-2 cells\(^9,17\). These findings suggest that BPs could play a beneficial adjuvant role in the treatment of osteosarcoma.

Osteosarcoma is one of the most common primary malignant tumors of bone and occurs mainly in adolescents and young adults\(^13,34\). Recently, the prognosis of these patients has improved substantially due to the development of various adjuvant chemotherapies. However, these chemotherapies are not fully effective, and as a result, 20% of all osteosarcoma patients still die as a result tumors metastasis\(^2,20,36\). Despite the advances made at improving survival over the last three decades, a limit appears to have been reached\(^25\). Therefore new approaches are required to improve the prognosis. In the present study, we examined the effects of the third-generation BPs, ibandronate, on osteosarcoma cell invasion.

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that remodel and degrade extracellular matrix (ECM). More than 25 MMPs have been identified to date, and are classified based on their substrate specificities and structural characteristics\(^4,8,37\). Tumor growth, invasion, and metastasis require tumor cell proliferation, proteolytic digestion of the extracellular matrix (ECM), cell migration through basement membranes into the circulatory system, and extravasation and growth at metastatic sites\(^23\). MMPs contribute to this metastatic process by degrading basement membrane. In addition, MMPs can, due to their proteolytic activities, promote tumor growth by increasing the bioavailabilities of growth factors in the ECM\(^16\). Furthermore, it is becoming increasingly clear that MMPs play a central role in ECM degradation\(^19\). Among MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), are present in large quantities in cancer tissues\(^12,22\), and accumulating evidence indicates that MMP-2 and MMP-9 play critical role during tumor invasion and metastasis\(^1,11,16,22,33,35\). Furthermore, MMP-2 is secreted from cells as a zymogen (pro-MMP-2) and is activated posttranslationally by a trans-membrane MMP designated as membrane type I MMP (MT1-MMP). The activation of pro-MMP-2 is regulated by a complex mechanism involving the formation of a trimolecular complex with MT-1-MMP and TIMP2\(^26\).

Methods

1. Reagents

Ibandronate, 3-(N-methyl-N-pentyl) amino-1-hydroxypropane-1, 1-diposphonic acid was purchased from (GlaxoSmithKline, Korea). A stock solution of ibandronate was prepared in phosphate–buffer saline (PBS). All other chemicals and reagents used were of analytical grade.
2. Cell Culture

U2OS was purchased from the Korean Cell Line Bank (KCLB). Cells were cultivated in Dulbecco’s Minimum Essential Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY). Cultures were maintained at 37°C in a 5% CO2/95% air atmosphere. The medium was changed every 2~3 days, and cells were passaged twice a week.

3. Ibandronate treatment of U2OS cells

U2OS cells were seeded in 6-well plates at a density of 2×10^5 cells/well in DMEM/10% FBS overnight. The cells were then washed and treated with different concentrations of ibandronate (0, 0.1, 1, 10 μM) for 48 h at 37°C in 5% CO2. Conditioned media were then collected and cells were harvested.

4. MTT cell viability assay

U2OS cells were seeded onto a 96-well culture plate at a density of 1×10^4 cells/well in 100 μl of complete DMEM. On the second day of culture, media were replaced with 100 μl of serum-free DMEM and ibandronate at concentrations of 0-10 μM. On the third day, 100 μl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, USA) was added to each well and incubated for 4 h. Media were then discarded and 100 μl of dimethyl sulfoxide (DMSO; Sigma) was added. Absorbance was measured at 570 nm using an plate reader.

5. In vitro invasion

U2OS cells (4×10^4) in 300 μl of serum-free MEM were seeded into the upper chamber of a 10-well chemotaxis chamber (Neuro Probe, USA) and complete MEM was placed in the lower chamber, and a Matrigel-coated membrane was inserted between the two chambers. Following overnight incubation at 37°C, the medium in the upper chamber was replaced with serum-free MEM and cells were treated with ibandronate at 0, 0.1, 1 and 10 μM for 48 hours incubation at 37°C in a 5% CO2 atmosphere. Finally, membranes were fixed and stained using a Hemacolor rapid staining kit (Merck, Germany), and the cells from 5 random microscopic fields (200×magnification) were counted.

6. Gelatin zymography

Protein concentrations in conditioned media were determined using the bicinchoninic acid method (BCA kit) (Pierce, IL, USA). Conditioned media was mixed with a equal volume of 4x sample buffer (200 mM Tris-HCl, 8% SDS, 0.4% bromophenol blue, 40% glycerol), and electrophoresed on 8% SDS polyacrylamide gels containing 2 mg/ml of gelatin (type A, Sigma, St. Louis, MO, USA). Gels were then washed twice for 30 min in 2.5% Triton X-100 at room temperature, and incubated for 18 hours at 37°C in incubation buffer (50 mM Tris-HCl (pH 7.5), 5mM CaCl2, and 200 mM NaCl). Gels were then stained for 1 hour with 0.25% (w/v) Coomassie brilliant blue R-250 (Bio-Rad) and then destained in destaining buffer (10% acetic acid and 20% methanol).

7. Western blot analysis

Cells were treated with ibandronate (0, 0.1, 1, 10 μM) for 48 h, scraped into 1X cell lysis buffer (Cell Signaling, USA), and
incubated for 10 min on ice. The resulting cell lysates were cleared by centrifugation at 6,700×g at 4°C for 5 min. Supernatants, which contained cytosolic proteins, were collected and protein concentrations were measured using the bicinchoninic acid method (BCA kit) (Pierce, IL, USA). Cell lysates, containing same amounts of protein, were mixed with equal volumes of 4X sample loading buffer, boiled for 5 min, cooled on ice for 5 min, and then analyzed by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane (Amersham Life Science, UK), and then the membrane was blocked with 5% skimmed milk in 1X TBST [0.01 M Tris (pH 7.6), 0.1 M NaCl and 0.1% Tween-20] for 1 h at room temperature with shaking and incubated with indicated primary antibodies followed by HRP-conjugated secondary antibody. The immunoreactive protein bands were developed using the Enhanced Chemiluminescence (ECL Plus) system (Amersham Bioscience, UK).

8. Reverse transcription-polymerase chain reaction

Cells treated with ibandronate(0, 0.1, 1, 10 μM) for 48 h and washed with ice-cold 1X phosphate buffered saline (PBS) twice. Total RNA was extracted using TRIzol Reagent (Invitrogen, USA), according to the manufacturer’s instructions. RNA(1 μg) was reverse-transcribed using the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen, San Diego) at 37°C. The following primers were used to determine target gene levels. β-actin (sense 5’-CTCAGATCCGTGGAGATCT-3’ and anti-sense 5’-CTTGGTTCTCCAGCT-TCAGG-3’) and MT1-MMP (sense 5’-GGGCT-GGCTGCTGCCATCAACA-3’ and anti-sense 5’-GCCGCCCTCCCTCGTCCACCTCAAT-3’). All primers were checked against the GeneBank Database to ensure no cross-reactivity with other known human DNA sequences. PCR cycles were performed using the following sequence: 94°C for 5 min, then 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C (for MMP-2) or 58°C (for MMP-9) for 1 minute, and polymerization at 72°C for 1 minute), and followed by 72°C for 7 minutes. RT-PCR products were visualized on 1.2% agarose gels electrophoresed in 0.5 TAE buffer containing 0.5 μg/ml ethidium bromide.

9. Statistical analysis

Band Intensities were quantified using Multi Gauge V3.0 and Scion Image software. Results are expressed as means ± standard deviations. Statistical significance was accepted for p values of <0.05 by the student t-test, and all statistical analyses were reviewed independently by a statistician.

Results

1. The antiproliferative effects of ibandronate on U2OS cells

MTT assays were used to determine the effects of ibandronate on osteosarcoma cell growth. Ibandronate treatment at 0 to 10 μM for 48-hours did not significantly inhibit the growth of U2OS cell line (Fig. 1), demonstrating that it has no significant effect on U2OS survival at a concentration
of 10 μM. Thus, we performed all subsequent experiments using ibandronate concentrations between 0 and 10 μM.

2. Ibandronate suppressed the invasive capacities of U2OS cells

We carried out Matrigel invasion assays after treating U2OS cells with ibandronate. Ibandronate was found to inhibit the invasive activities of the cell line dose-dependently (Fig. 2).

3. Ibandronate reduced MMP-2 activities in U2OS cells

Since MMP-2 play a critical role in tumor cell invasiveness, we examined the effect of ibandronate on the enzyme activities of MMP-2. Accordingly, gelatin zymography was conducted using conditioned media harvested from ibandronate treated U2OS cells. The gelatinolytic activities of MMP-2 were found to be reduced in the cell line after treatment with increasing concentrations of ibandronate, which suggested that the reductions in cell invasion by ibandronate is a consequence of reductions in the activities of MMP-2. (p<0.05) (Fig. 3).

4. Ibandronate reduced MMP-2 and MT1-MMP protein levels in U2OS cell line

To investigate whether ibandronate inhibits the expressions of MMP-2 and MT1-MMP, U2OS cells were treated with ibandronate and MMP-2 and MT1-MMP protein levels were determined by Western blotting. As shown in Fig. 4, Western blotting revealed that ibandronate inhibit MMP-2 and MT1-MMP protein levels (p<0.05).

5. Risedronate suppressed MMP-2 and MT1-MMP mRNA levels in U2OS cell line

RT-PCR was used to determine whether ibandronate suppresses MMP-2 and MT1-MMP at the transcription levels. Ibandronate was found to attenuate MMP-2 and MT1-MMP mRNA levels dose-dependently (p<0.05) (Fig. 5).

Discussion

A number of studies have demonstrated the beneficial effects of bisphosphonates on bone metastases from different solid tumors, such as, those of the breast, prostate and renal cell carcinoma. In the majority of previous studies, first or second-generation bisphosphonates have been examined at the relatively high concentrations required to inhibit the cell proliferation of osteosarcoma
cells. In addition, third-generation bisphosphonates have been reported to induce osteosarcoma cell apoptosis. Tadahiko Kubo and Shoji Shimose reported that minodronate and incadronate perturb the cell cycle and induce the apoptosis of SaOS-2 cells. Evdokiou and colleagues studied the third-generation bisphosphonate, zoledronic acid (ZOL), and found that it dose- and time-dependently decreased cell proliferation in a panel of human osteosarcoma cell lines. However, the molecular mechanism underlying inhibition by BPs has not been determined. Cheng YY et al. reported that alendronate reduces MMP-2 secretion and induces tumor cell apoptosis in osteosarcoma, but the molecular targets and modes of action of MMP-2 and MT1-MMP inhibitors, like ibandronate, are substantially unknown. In the present study, we found that ibandronate suppresses cell invasion and the gelatinolytic activities and protein and mRNA expressions of MMP-2 and MT1-MMP in the U2OS osteosarcoma cell line. Pia Heikkilä, in a study on the inhibition and downregulation of MT1-MMP by clodronate (a non-nitrogen-containing bisphosphonate) suggested that these activities are related to reductions in MG-63 osteosarcoma cell invasion and spread. Pro-MMP-2 can be activated by several mechanisms depending on stimulators and cell types. Initially, pro-MMP-2 can be activated by the action of highly expressed MT1-MMP and the adequate expression of TIMP-2. In this situation, the balance between MT1-MMP and

**Fig. 2.** Ibandronate impedes the invasiveness of U2OS cells. Stained areas represented numbers of migrating cells. The number in the panels show the concentration of ibandronate added. Images are representative of three independent experiments.
TIMP-2 is important. At low concentration, TIMP-2 binds to the catalytic site of some activated MT1-MMP molecules, generating receptors for pro-MMP-2, thereby promoting MMP-2 activation. In our study, ibandronate downregulated both MMP-2 and MT1-MMP expression in U2OS cell line, therefore, further work about TIMP-2 is required to clarify this issue. Accordingly, our findings demonstrate that ibandronate

**Fig. 3.** Ibandronate inhibited the gelatinolytic activities of MMP-2. (A) Conditioned media harvested from U2OS cells treated for 48 h with the indicated concentrations of ibandronate were analyzed by gelatin zymography. The white bands represent MMP-mediated gelatin digestion. The image is representative of three independent experiments. MMPs activities (expressed as percentages of controls) are shown in B (n=3). Numbers in boxes represent the concentration of ibandronate (in μM) added to cells. Bars represent the MMPs activities (expressed as percentages of controls) of each band ± standard deviation.

**Fig. 4.** Ibandronate reduced the expressions of MMP-2 and MT1-MMP proteins. (A) Beta-actin was used as a loading control. Images are representative of three independent experiments. (B) shows MMPs protein levels (expressed as percentages of controls) (n=3). Numbers in the box represent the concentration of ibandronate in μM added to the cells. Bars represent MMPs protein levels (expressed as percentages of controls) of each band ± standard deviation.
has anti-invasive and antimetastatic activity via the inhibition of MMP-2 and MT1-MMP activities in human osteosarcoma cells. On the other hand, Ichinose et al found that bisphosphonates alone do not influence the amount of MMP-2 produced by human osteoblasts, which suggests that bisphosphonates suppress expression of MMPs in osteosarcoma cells but not in normal human osteoblasts.

According our MTT assay results, ibandronate at up to 10 μM had no significant cytotoxic effect on U2OS cells. Therefore, given the known importance of MMP-2 and MT1-MMP in tumor invasion, our findings suggest that the inhibitory effect of ibandronate on osteosarcoma cell invasion is probably due to MMP inhibition rather than tumor cell death.

Conclusions

This study suggests that ibandronate reduce the MMP-2 and MT1-MMP secretion in osteosarcoma line, which may contribute to the reduction of invasive potential of the osteosarcoma cells. However, a pilot clinical trial will be necessary before ibandronate is to be considered as possible adjuvant in the multi-drug treatment of osteosarcoma.

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Effects of Ibandronate on the Expression of Matrix Metalloproteinases in Human U2OS Osteosarcoma Cells

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Background: Osteosarcoma is one of the most common primary malignant tumors of bone occurring mainly in children and adolescents. Although surgery combined with chemotherapy has markedly improved patient survival during the last years, the use of anticancer drugs is still associated with serious problem, such as the frequent acquisition of drug-resistant phenotypes and occurrence of “secondary malignancies”. Several solid tumors display enhanced expression of matrix metalloproteinases (MMPs), and recently clinical trials have been initiated on MMP-inhibitors. On the other hand, bisphosphonates (BPs) are inhibitors of bone resorption, and widely used to treat osteoclast-mediated bone diseases. Also they appear to possess direct antitumor activity.

Methods: One osteosarcoma cell line (U2OS) was treated with ibandronate (0, 0.1, 1, 10 $\mu$M) for 48 hours. Cell viabilities were determined using MTT assay, the mRNA levels of MMP-2 and MT1-MMP were detected by reverse-transcription polymerase chain reaction, the amount of MMP-2 and MT1-MMP protein were measured by Westernblot, the activities of MMP-2 were observed by Gelatin zymography, and Matrigel invasion assays were used to investigate the invasive potential of osteosarcoma cell lines before and after ibandronate treatment.

Results: The invasiveness of U2OS cell line was reduced dose-dependently following 48 hour treatment of up to 10 $\mu$M of the ibandronate at which concentration no cytotoxicity occurred. Furthermore, the gelatinolytic activities and protein and mRNA levels of MMP-2 and MT1-MMP were also suppressed by increasing ibandronate concentrations.

Conclusion: Given that MMP-2 is instrumental in tumor cell invasion, it is very likely that the reduction in osteosarcoma cell invasion by ibandronate is a consequence, at least in part, of suppressed expression of both MMP-2 and MT1-MMP. Isolation of a molecule (s) responsible for the bisphosphonate inhibition of tumor cell invasion would pave the way for the development of a new generation of metastasis inhibitors.

Key Words: Osteosarcoma, Matrix metalloproteinase, Ibandronate

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