Positive Effects of Bisphosphonates on Osteogenic Differentiation in Patient-Derived Mesenchymal Stem Cells for the Treatment of Osteoporosis

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Abstract

BACKGROUND: Recent evidence from *in vitro* and *in vivo* studies indicates that bisphosphonates may promote osteoblastic bone formation and potentially inhibit osteoclast activity. However, little is known about the potential effect of bisphosphonates on the recruitment of osteoblastic precursors from patient-derived bone marrow stromal cells due to difficulties in accessing human bone marrow from healthy and disease subjects.

METHODS: In this study, we evaluated the potential of using FDA-approved and clinically utilized bisphosphonates such as alendronate, ibandronate, and zoledronate to enhance the development of bone forming osteoblasts from osteoporosis patient- and healthy-person derived hBMSCs (op-MSCs and hp-MSCs, respectively). hBMSCs were obtained from postmenopausal women without endocrine diseases or receiving hormone replacement therapy. Cells were treated with or without a bisphosphonate (alendronate, ibandronate, and zoledronate) and analyzed over 21 days of culture.

RESULTS: hBMSC from osteoporosis-patient with bisphosphonates treatment demonstrated a significant increase in Alizarin red staining after 7 days compared to that from healthy-person. Calcium contents and alkaline phosphatase (ALP) enzyme activity also demonstrated an increased propensity in hMSCs from osteoporosis-patient compared to those from healthy-person, although there were inter-individual variations. Gene expression levels varied among different donors. There were no significant differences in the effect on the osteoblastic differentiation of hBMSCs among alendronate, ibandronate, and zoledronate. Statistical significance in the osteoblastic differentiation of hBMSCs between the positive control group cultured in osteogenic medium alone and groups cultured in osteogenic medium supplemented with bisphosphonate was not shown either. These results might be due to various cell types of hBMSCs from individual clinical patients and concentrations of bisphosphonate used.

CONCLUSION: Our study using a clinically relevant *in vitro* model suggests that bisphosphonate treatment is more effective for patients with osteoporosis than its preventive effect for healthy person. In addition, patient-specific responses to bisphosphonates should be considered rather than bisphosphonate type prior to prescription. Further investigations are needed to determine how bisphosphonates influence hBMSCs function to mediate bone quality and turnover in osteoporotic patients. Such studies can generate novel approaches to treat age-related osteoporotic bone loss.

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1 Introduction

The aging segment of the population is rapidly expanding. Along with aging, osteoporosis has become a significant health concern. Age-related bone loss results from an imbalance between bone resorption and bone formation due to spatially or temporally uncoupled activity of bone resorbing osteoclasts and bone forming osteoblasts [1]. Mesenchymal stem cells (MSCs) are multipotent cells present in bone marrow that have the potential to differentiate into osteoblasts and form bone [2]. Aging is associated with a reduction in marrow MSC numbers and a deficiency in supportive mechanisms required for MSCs to augment bone formation [3, 4]. The decrease in the resident MSC population with advanced age might be the most important factor responsible for reduced bone formation and subsequent increase in bone fragility [5]. Therapeutic modalities that target bone formation by either increasing the number or the activity of osteoblasts are attractive approaches to enhance bone formation and promote bone regeneration. Bone regeneration through induction of MSCs can promote osteogenesis and provide a rational therapeutic strategy to prevent age-related osteoporosis.

Most of current treatments available for osteoporosis are directed toward preventing bone resorption, with bisphosphonate therapy being the most widely used antiresorptive approach to enhance bone strength of osteoporotic patients [6]. Bisphosphonates are a well-characterized class of synthetic compounds structurally related to pyrophosphate. It has been reported that bisphosphonates can mediate their antiresorptive actions primarily through inhibiting osteoclast activity [7]. Although the primary action of bisphosphonates is by inhibiting osteoclastic bone resorption [8], there is growing evidence that bisphosphonates also interact with osteoblasts. It has been reported that bisphosphonate does not affect radiographic or clinical outcomes in fracture healing or fusion surgery [9–13]. Prior evidence suggests that bisphosphonates can increase osteoblast proliferation and up-regulate the expression of genes involved in new bone formation [14–17]. However, the effect of bisphosphonates on early stages of osteogenic differentiation has not been fully understood yet. Moreover, little is known about the potential effect of bisphosphonates on patient-derived bone marrow stromal cells (BMSCs), although they are the primary target for bisphosphonate treatment. The need for such investigations is further highlighted by the fact that human BMSCs (hBMSCs) from osteoporotic patients are molecularly distinct from their non-osteoporotic counterparts. Thus,

they may not respond to bisphosphonate treatment in the same way [18–21].

Here, we evaluated the potential effect of FDA-approved and clinically utilized bisphosphonates (alendronate, ibandronate, and zoledronate) on osteogenic differentiation of hBMSCs isolated from aged osteoporosis patients- and healthy-person (op-BMSCs and hp-BMSCs, respectively). *In vitro* cultures of hBMSCs were incubated with bisphosphonates and their ability to differentiate toward osteoblasts was assessed using molecular and biochemical techniques. The differential capacity of op-BMSCs and hp-BMSCs to support mineralization was also investigated.

2 Materials and methods

2.1 Drugs and chemicals

Bisphosphonates used for this study were alendronate (A4978, Sigma, USA), ibandronate (15784, Sigma, USA), zoledronate (Novartis Pharmaceuticals Ltd., Australia). Osteogenic medium consisted of Dulbecco's modified Eagle's medium (DMEM) (Welgene, KO) supplemented with 10% fetal bovine serum (Gibco, USA), 1% antibiotics and antimycotics (Welgene, KO), beta-glycerophosphate (Sigma, USA), dexamethasone (Sigma, USA), and ascorbic acid (sigma, USA). Alkaline phosphatase activity was determined using Alkaline Phosphatase Kit (Sigma, USA) and Fast RR-salt (pH 5.4) (Sigma, USA). Quantichrom Ca^{++} assay (Bioassay, USA) was used for calcium assay. Calcium stain was conducted using Alizarin Red-S (Sigma, USA).

2.2 Subject

Healthy donor group and osteoporotic donor group consisted of postmenopausal women without endocrine diseases or receiving hormone replacement therapy. This study was conducted in accordance with Korea Good Clinical Practice (KGCP) and International Conference on Harmonisation (ICH) guidelines. It complied with the rights and safety of subjects under the Declaration of Helsinki. This study was approved by Institutional Review Board (IRB) of our institution (IRB Number: 06-2010-142). Bone marrow was collected from four osteoporosis patients (< -3.0 T-score of lumber, who did not take bisphosphonate or parathyroid hormone before) and four in the normal control group (> 1.0 T-score of lumber) by

Table 1 Baseline characteristics of patients

No.	sample	Age	Sex	BMD lumber	T-score lumber	BMD femur	T-score femur
1	Osteoporosis	80	F	0.632	− 4.3	0.641	− 2.8
2	Osteoporosis	71	F	0.728	− 3.3	0.692	− 2.3
3	Osteoporosis	82	F	0.626	− 4.4	0.553	− 3.5
4	Osteoporosis	56	F	0.658	− 3.7	0.640	− 2.5
5	Healthy	69	F	1.313	1.8	0.989	0.3
6	Healthy	68	F	1.100	0.0	0.970	0.1
7	Healthy	72	F	1.054	− 0.5	0.882	− 0.6
8	Healthy	71	F	1.152	0.3	1.105	1.2

injecting Manan needle to posterior superior iliac crest (Table 1).

2.3 Human mesenchymal stem cell culture

For separation of hBMSCs, collected bone marrow was diluted with PBS. After adding 4 ml of Lymphoprep (Axis-shield Co., Inc.) into the diluted bone marrow, the mixture was centrifuged. Separated hBMSCs were then washed with 10 ml of PBS and centrifuged at 600 g for 10 min. hBMSCs were then cultured with DMEM (low glucose) medium (Gibco Co., Inc.) containing 10% FBS and 1% antibiotic–antimycotic and incubated at 37 °C in a 5% CO₂ incubator. Culture medium was refreshed every 3–4 days.

2.4 Exposure to bisphosphonate

hBMSCs obtained from osteoporosis patients or healthy patients were cultured in 24-well cell culture plate at a density of 3×10^4 cells/well for 24 h. Subsequently, the culture medium was removed and replaced by osteogenic medium (OM). Alendronate, ibandronate, or zoledronate was then added into the osteogenic medium at a concentration of 50 nM for experimental group. Positive control (PC) cells were cultured in the osteogenic medium without bisphosphonate supplement while negative control (NC) cells were cultured in the basal medium (BM).

2.5 Alkaline phosphatase (ALP) activity assay

After 7 days of culture, ALP activity as an early marker of osteogenic differentiation was assayed utilizing the conversion of a colorless *p*-nitrophenyl phosphate to colored *p*-nitrophenol. Cells were washed twice with DPBS and lysed with 0.2% Triton X-100. Cell lysates were assayed for ALP activity using *p*-nitrophenylphosphate as a substrate. The activity was defined as the amount of *p*-nitrophenol released after incubation for 30 min at room temperature. Color change was measured spectrometrically at wavelength of 405 nm.

2.6 ALP staining

For ALP staining, cells were washed with DPBS. Fast blue RR salt was then added to each well. After incubated for 10 min at room temperature, results were observed by optical microscopy.

2.7 Alizarin red-S staining

The degree of mineralization was determined using Alizarin Red staining. After 7 and 14 days of culture, cells were washed twice with DPBS. Cells were THEN stained with 40 mM Alizarin Red S in deionized water (adjusted to pH 4.2) for 10 min at room temperature.

2.8 Calcium assay

Cells of each group were solubilized in 500 µL of 0.1% HCl for 5 min at room temperature. Cell lysates (10 µL) were mixed with phenolsulfonphthalein dye and assayed in triplicates using 96-well plates. The color intensity was measured at 612 nm.

2.9 Quantitative reverse transcription polymerase chain reaction (PCR)

hBMSCs were seeded into 24-well plate at density of 2×10^4 cells/well. At the end of week 1 and week 3, total RNA was obtained using easy-blue RNA isolation kit (Intron Co., Inc.). Total RNA was quantified using nano-drop and cDNA was synthesized from 2 µg of RNA using superscript III (Invitrogen Co., Inc.). After cDNA synthesis, gene specific PCR was carried out for ALP, BSP, and GAPDH. All PCR products were visualized on 1.5% agarose gel. Qualitative analysis for relative gene expression was performed using a gel doc system.

2.10 Statistical analysis

After determining normal distribution of each group, differences in expression of ALP and calcium content

between groups were determined by analysis of variance with Bonferroni collection for post hoc analysis. Statistical significance was considered at $p < 0.05$. Differences between osteoporosis group and healthy group were analyzed with Student's t test.

3 Results and discussion

3.1 Characterization of hBMSCs isolated from osteoporosis patients and healthy persons

To closely mimic *in vivo* setting, hBMSCs were isolated from bone marrow collected from patients. According to their bone mineral density, four patients

(72.3 ± 11.8 years) were allocated into the osteoporosis group while four patients (70.0 ± 1.83 years) were assigned into the healthy group. There was no significant difference in average age between the two groups (Table 1). However, bone mineral densities of lumbar spine and femur in the osteoporosis group were significantly lower than those in the healthy control group (both $p < 0.05$). Medication history of both groups was evaluated within 1 week after bone marrow harvesting. Two patients in the osteoporosis group were treated with cox-2 inhibitor and one patient was treated with aspirin. In the healthy control group, two patients were treated with aspirin. Both groups did not take bisphosphonate, parathyroid hormone, or steroids within 1 month after bone marrow harvesting.

Fig. 1 Optical microscopy of osteogenic differentiation with culture time. **A** At 7 days after culture. **B** At 21 days after culture. After 7 days after culture, op-MSCs appeared to have induced deposition of mineral like particles. A dramatic course of mineralization was

observed over 21 days of culture. Mineralization of hp-MSCs was detected over 21 days of culture. PC positive control, NC negative control, ALN alendronate, IBN ibandronate, ZON zoledronate

3.2 Effect of bisphosphonate on mineral formation

hBMSCs obtained from osteoporosis patients and healthy patients were evaluated for their ability to undergo osteogenic differentiation and subsequent mineralization following their incubation in OM supplemented with or without 50 nM of alendronate (ALN), ibandronate (IBN), and zoledronate (ZON) for up to 21 days. As shown in Fig. 1, deposition of mineral-like particles was observed at 7 days after culture of op-MSCs (Fig. 1A) and at 21 days after culture of hp-MSCs (Fig. 1B). In contrast, no mineral-like particles were observed in the negative control group. These results indicated that bisphosphonate treatment had a positive influence on osteogenic differentiation of op-MSCs and hp-MSCs. Particularly, all bisphosphonates more effectively induced the osteogenic differentiation of op-MSCs than that of hp-MSCs.

The mineralization capacity was evaluated by Alizarin Red staining. Mineralization was observed in op-MSCs after 7 days of culture (Fig. 2A). Increased staining was observed over 21 days of culture (Fig. 2B). However, mineralization in hp-MSCs was observed after more than 21 days of culture. Calcium contents in op-MSCs were considerably higher than those in hp-MSCs at an early stage (7 days of culture). They were considerably increased in hp-MSCs after 21 days of culture (Fig. 2C, D). Calcium content pattern for each bisphosphonate treatment was similar to that observed for the positive control. However, op-MSCs from #60 patient showed noticeably lower

calcium contents than op-MSCs from other osteoporosis patients.

3.3 Comparison between osteogenic differentiation of hBMSCs isolated from osteoporosis patients and healthy patients

To ascertain whether bisphosphonate treatment had stimulatory effects on hBMSC osteogenic activity, we analyzed both ALP protein activity and gene expression level in cultures of hBMSCs undergoing osteogenic differentiation. Enhanced alkaline phosphatase (ALP) staining was observed in op-MSCs compared to that in hp-MSCs despite donor-specific variations (Fig. 3A). The level of ALP enzyme activity evaluated by an end-point assay showed significant difference depending on individual samples of op-MSCs and hp-MSCs when assays were performed under identical treatment conditions (Fig. 3B). ALP enzyme activity patterns of op-MSCs and hp-MSCs in response to each bisphosphonate treatment were almost similar to those of the positive control. Similar trend was also observed for mineralization capacity (Fig. 2). Interestingly, ALP enzyme activity of op-MSCs from #60 patient was significantly increased in response to zoledronate treatment.

Conventional RT-PCR analysis was performed to assess progression of hBMSCs toward osteoblast lineage at mRNA expression level (Fig. 4). At 7 days after culture of op-MSCs from #20 patient, IBN and ZON mediated ALP gene expression levels were more than 10-fold higher than

Fig. 2 Mineralization capacity. Mineralization was effectively induced in op-MSCs treated with bisphosphonates. **A** Alizarin Red staining after 7 days culture. **B** Alizarin Red staining after 21 days of

culture. **C** Calcium concentration measured after 7 days culture and **D** 21 days culture. *PC* positive control, *NC* negative control, *ALN* alendronate, *IBN* ibandronate, *ZON* zoledronate

Fig. 3 **A** ALP staining at 7 days after culture. **B** ALP activity at 7 days after culture. Enhanced ALP activity in op-MSCs compared to that in hp-MSCs was observed. There were also donor-specific

variations. *PC* positive control, *NC* negative control, *ALN* alendronate, *IBN* ibandronate, *ZON* zoledronate

PC. This resulted in strong up-regulation of IBN mediated BSP gene expression at 21 days after culture. However, ALN mediated ALP and BSP gene expression levels were negligible. BSP gene of op-MSCs from #60 patient was effectively expressed by all bisphosphonates tested at 7 days after culture. hMSCs from healthy groups generally showed low expression level of ALP and BSP genes under most culture conditions. However, hp-MSCs from #67 showed up-regulation of ALP and BSP genes under all bisphosphonate treatment conditions and PC condition at 7 days after culture. During osteoblastic differentiation, BSP gene is known to be expressed slower than ALP gene. Accordingly, BSP gene can be seen as a late osteoblastic differentiation marker. In this study, although expression level was dependent upon individual donor, BSP gene expression level in op-MSCs from osteoporosis patients was significant increased compare to that in hp-MSCs at

7 days after culture. These results suggest that osteoporosis patient derived hMSCs have the property to differentiate to osteoblastic lineage with bisphosphonate treatment faster than hp-MSCs.

3.4 Statistical analysis for osteogenic differentiation of hBMSCs isolated from osteoporosis patients and healthy patients

In this study, osteogenic differentiation and cell response of hMSCs from clinical sample to each bisphosphonate tested showed a donor-specific pattern. However, there were no significant differences in osteogenic differentiation or cell response among alendronate, ibandronate, and zoledronate treatments (Fig. 5, Tables S1–S3). In other words, the observed effect was not dependent on the type of bisphosphonate. These results have significant implications for

Fig. 4 Effect of bisphosphonate treatment on osteogenic gene expression in differentiating hBMSCs. **A** At 7 days after treatment. **B** At 21 days after treatment. Although ALP and BSP gene expression levels were dependent on individual donor, the expression

level of BSP gene, a late osteoblastic differentiation marker, in op-MSCs was increased significant earlier compare to hp-MSCs. *PC* positive control, *NC* negative control, *ALN* alendronate, *IBN* ibandronate, *ZON* zoledronate

Fig. 5 Comparative statistical effect of bisphosphonate treatment in op-MSCs and hp-MSCs. **A** ALP activity. **B** Calcium concentration. PC positive control, NC negative control, ALN alendronate, IBN ibandronate, ZON zoledronate

treatment of metabolic bone disorders such as osteoporosis with bisphosphonates. Although zoledronate and alendronate are effective for non-vertebral fractures and hip fracture prevention, ibandronate is known to be ineffective in preventing these types of fractures. Thus, bisphosphonates should be prescribed based on the efficacy and side effects of each drug for osteoporosis patients. In addition, alendronate and zoledronate are recommended to have a drug holiday after 3–5 years of treatment due to the risk of osteonecrosis of jaw or atypical femoral fracture. However, ibandronate has fewer restrictions on drug holiday due to its less side effects. Therefore, patient-specific differences in responses to bisphosphonates must be considered for osteoporosis treatment.

As shown in Fig. 5, effects on ALP activity and calcium contents in op-MSCs groups was significantly higher than those in hp-MSCs groups under osteogenic conditions (positive control and bisphosphonate treatment condition). However, these showed almost similar levels under negative control condition. Osteogenic activity of op-MSCs suppressed by osteoporosis might have been ameliorated under osteogenic condition while they lost their pluripotent capacity. Thus, op-MSCs may differentiate into osteoblasts along the osteoblastic pathway in the early phase. On the other hand, statistical significance in ALP activity or calcium content was not found between the positive control group and bisphosphonate treatment group (Tables S1, S2,

and S3). These results are different from those reported in a previous study where bisphosphonate increases osteoblastic differentiation of MSCs [22–24]. Such discrepancy in results might be due to cell type and concentration of bisphosphonate used. It has been shown that pro-osteoblastogenesis or inhibitory effect can be induced depending on bisphosphonate concentrations used for treatment [25–27]. Additionally, since hMSCs from individual clinical patients were tested, unlike previous studies. Thus, highly variable osteogenic potential was expected.

In vivo bone turnover is determined by a delicate balance between osteoclastic bone resorption and osteoblastic bone formation. This study suggests that bisphosphonates can impact both sides of this balance. They can inhibit osteoclastic activity. At the same time, they have anabolic effect on osteoblasts. Whether osteogenic effects of bisphosphonates *in vitro* would result in greater mineral content and stronger bone structure *in vivo* remains unclear.

In this study, we evaluated effects of three FDA-approved and clinically utilized bisphosphonates (alendronate, ibandronate, and zoledronate) on osteogenic differentiation of hMSCs from clinical patients. Bisphosphonate treatment had a positive influence with a patient-specific pattern on osteogenic differentiation of op-MSCs and hp-MSCs. Statistical analysis showed that osteogenesis of op-MSCs was higher than those of hp-MSCs in the osteogenic condition. However, there were no comparative effect among alendronate, ibandronate, and zoledronate. Our results have very important clinical implications for patient-specific prescription to treat metabolic bone disorders with bisphosphonates. Patient-specific responses to bisphosphonates should be considered rather than bisphosphonate type prior to prescription.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement Institutional review board approval and patient consenting was obtained for this study (IRB No. 06-2010-142).

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