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ORIGINAL ARTICLE

Bisphosphonates modulate the expression of OPG and M-CSF in hMSC-derived osteoblasts

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Abstract Bisphosphonates have been known to suppress osteoclast activity, survival, and recruitment. In this study, we tested effects of BPs on expression of two critical genes for osteoclastogenesis, M-CSF, and OPG in the process of osteoblast differentiation from hMSC. (1) The cells were cultured in osteogenic induction medium together with 0 (control group) and 10–8 M alendronate, pamidronate for up 2 and 3 weeks (for real-time PCR) and 3 and 4 weeks (for ELISA). (2) The real-time PCR protocol for M-CSF, OPG, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) consist of 40 cycles. (3) Enzyme-linked immunosorbent assay (ELISA): the amounts of M-CSF and OPG in the culture medium were determined using commercially available ELISA kits for M-CSF and OPG. Treatment of differentiating cells with alendronate or pamidronate, nitrogen-containing BPs increase the expression of OPG, which suppresses osteoclastogenesis, whereas it decreases the expression of M-CSF, which enhances preosteoclast formation. These results suggest a new mechanism by which BPs inhibit osteoclastogenesis. Results support hypothesis that progressive accumulation of bisphosphonate in jaws causes imbalance in osteogenesis and bone absorption and collateral osteoclast–osteoblast interaction. Bisphosphonate-related osteonecrosis of jaw (BPONJ) is one of the most serious complications of bisphosphonate (BP) therapy. However, the mechanism behind the this process of BPONJ is still unclear and there are so many hypotheses. Among many hypotheses, we focused on osteoclast–osteoblast interaction in this study. The findings of this study show new light on the present BPONJ occurrence theory based on the osteoclastic activity of BPs. Also, a more advanced and developed theory for BRONJ occurrence may be obtained by combining the osteoclast inhibition mechanism and the effects on osteoblastic differentiation by BPs.

Keywords Bisphosphonates(BPs) · BMSC · M-CSF · OPG · BPONJ

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Introduction

Although the rate of increase of bisphosphonate-related osteonecrosis of the jaws (BPONJ) is fast, the pathogenesis of BPONJ is not yet clear, and there are so many hypotheses [1]. Despite the many hypotheses focused on bone remodeling suppression, it can be summed up in two hypotheses. First, BPs tends to be highly concentrated in the jaw rather than other skeletal sites because of its high vascularity and rapid bone turnover. In addition, the force of masticatory function and periodontal ligament (PDL) can easily induce microfracture. Woven bone formation is not compromised in the presence of BPs, thus wound healing is delayed and this in turn develops into BPONJ [1–3]. Second, because of its toxic effect on osteoclast, BPs suppress osteoclast-mediated bone remodeling and bone

turnover [4]. After long-term administration of BPs, the inability of osteoclasts to resorb old bone causes osteoblasts and osteocytes to die leaving an acellular bone matrix. Inadvertent trauma to the thin oral mucosa can introduce oral microbes into avascular bone matrix leading to osteonecrosis. However, the two hypotheses mentioned above were not based on molecular level. Therefore, we planned to investigate molecular pathogenesis of BPONJ in the process of osteoclastogenesis [5].

In addition, there are a number of various different points of view on this issue. According to the hypothesis focusing on the relationship between BPONJ and vascularity, bisphosphonates have antiangiogenic effects, leading to speculation that this could contribute to the BPONJ pathogenesis [6]. Compromised angiogenesis would most likely be involved in post-intervention healing, although other aspects of the vasculature (e.g., blood flow) could contribute to BPONJ. In this hypothesis, the most intriguing role of altered angiogenesis with bisphosphonates may be related to wound healing [7, 8].

Several studies have demonstrated that BPs can cause osteoclast apoptosis in vitro and in vivo [9–12]. On a cellular level, BPs are clearly targeting the osteoclasts and may inhibit their function in several ways [13–15]: (1) inhibition of osteoclast recruitment, (2) diminishing the osteoclast life span, and (3) inhibition of osteoclastic activity at the bone surface. At a molecular level, it has been postulated that BPs modulate osteoclast function by interacting with a cell surface receptor or an intracellular enzyme [15–17].

Because of the uncertainty regarding the exact mechanism of BPONJ development, investigations continue to be warranted to examine the effects of BPs on bone at the cellular and molecular level. The ASBMR task force specifically mentioned that the cellular and molecular mechanisms of BP action should be evaluated. The task force also mentioned the relationship between angiogenesis and bone resorption as well as “bone turnover”, as important areas for further research [18, 19].

Bone is continuously destroyed and reformed in vertebrates in a stringently regulated equilibrium between osteoblastic bone formation and osteoclastic resorption. During this process, osteoblasts stimulate not only bone formation but also mediate osteoclast differentiation and function via cell-to-cell contact with osteoclast precursors. Bone marrow stem cells (BMSCs) can differentiate into multiple cell types (e.g., pre-osteoblast, osteoblast). Because BMSCs express RANKL on their cell surface, they also indirectly modulate osteoblast–osteoclast balance [19].

Therefore, the interaction between osteoclast and osteoblasts is essential for bone remodeling. Macrophage colony-stimulating factor (M-CSF) and osteoprotegerin (OPG) are two essential factors produced by osteoblast/stromal cell for osteoclast–osteoblast interaction. Initiation of osteoclastogenesis depends on interaction between osteoclast precursors and cells in the osteoblast lineage. Osteoblasts produce M-CSF, which is required for survival of cells in the macrophage–osteoclast lineage and controls of cell migration and reorganization [20]. The role of OPG is largely associated with an initiation phase in which OPG counteracts RANKL osteoclastogenic activity. During bone formation, osteoclast differentiation is suppressed through OPG produced by osteoblasts. On this wise, osteoclast–osteoblast interaction leads to increased OPG production and reduced osteoclastogenesis [21].

The purpose of this study was to examine the effects of BPs (alendronate, pamidronate) on the expression of M-CSF and OPG during the process of osteogenic differentiation in normal human mesenchymal stem cells (hMSCs) into osteoblast.

Materials and methods

Cell culture

After written informed consent was obtained, normal human mesenchymal stem cell were gathered from iliac cancellous bone of healthy male in his twenties. [22]. Primary culture human BMSCs was cultured in osteogenic induction medium (10% fetal bovine serum (FBS, welGENE, Korea) with 1% antibiotics (10,000 U/ml penicillin+10 mg/ml streptomycin) in DMEM (welGENE, Korea), 0.1 μM dexamethasone, 10 mM β-glycerol phosphate, and 50 μg/ml L-ascorbic acid 2-phosphate) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. For experimental treatments, the cells were seeded onto each 100-mm tissue culture plates at a density of 2×10^4 cells/cm^2. Because of the time difference between gene expression and the consequential protein expression, we planned to make a time lag in cell culture. After overnight incubation, the cells were cultured in osteogenic induction medium together with 0 (control group) and 10^{-8} M alendronate, pamidronate for up 2 and 3 weeks (for real-time PCR) and 3 and 4 weeks (for ELISA).

Real-time PCR analysis

Total RNA was prepared by using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's specifications. The mRNA was converted into complementary DNA (cDNA) using RNA PCR kit (Fermentas, Switzerland), and resulting cDNA was diluted in 50 μl sterile distilled water. The PCR assays were performed on a Mini opticon (BIO-RAD, CA, USA). The PCR protocol for M-CSF, OPG, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) consist of 40 cycles (denaturation at 94°C for 30 s→annealing at 60°C for

30 s→extension at 72°C for 30 s). All the real-time PCR reactions were performed in triplicate, and the specificities of the PCR products were verified by melting curve analysis. The sequences of the primers used and the size of the PCR products are listed in Table 1.

Enzyme-linked immunosorbent assay

The amounts of macrophage colony-stimulating factors (M-CSF) and osteoprotegrin (OPG) in the culture medium were determined using commercially available Quantikine ELISA kits for M-CSF (R&D system, CA, USA) and OPG (Apotech, Switzerland), according to the manufacturer's instructions. This assay employs the quantitative sandwich immunoassay technique. A monoclonal antibody specific for M-CSF and and OPG has been pre-coated onto a microplate.

Statistical analysis

All experiments were performed in triplicate. Each value represents the mean±S.D. The significant of differences was determined using the Bonferroni–Dunn posthoc test of two-way ANOVA. Differences with p values <0.05 were considered significant.

Results

Real-time PCR test

In this study, we calculated the gene expression level as follows: the calculation of gene-fold increase=2 (control Ct AVG−GAPDH Ct AVG) /2 (control Ct AVG−gene Ct AVG). That is, if the value is below 1, the gene expression was down-regulated, and if the value is above 1, the gene expression was up-regulated.

(1) M-CSF: In the alendronate- or pamidronate-treated group, the mRNA levels of M-CSF were lower than those in the control group (Figs. 1 and 2). Two or 3 weeks treatment with alendronate- or pamidronate leaded to the down-regulation of M-CSF mRNA expression, but 3 weeks treatment down-regulated its mRNA level to a lesser degree than the 2 weeks treatment (Figs. 1 and 2).

(2) OPG: In the alendronate- or pamidronate-treated group, the mRNA levels of the 2-week group was lower than those in the control group, but the mRNA levels of the 3-week group was higher than those in the control group (Figs. 3 and 4). Two weeks treatment with alendronate or pamidronate leaded to the down-regulation of OPG expression, but 3 weeks treatment up-regulated its mRNA level to a lesser degree than 2 weeks (Figs. 3 and 4).

ELISA test

The mean of optical density (OD) of all groups continue to increase as time passed (3→4 weeks).

(1) M-CSF: In the alendronate- or pamidronate-treated group, M-CSF protein levels were lower than those in the control group (Fig. 5). Three or 4 weeks treatment with alendronate- or pamidronate leaded to down-regulation of M-CSF protein expression, but 3 weeks treatment down-regulated its protein level to a lesser degree than 4 weeks, and the degree of alendronate-treated group was higher than pamidronate-treated group (Fig. 5).

In comparison among bisphosphonates, time, and all groups, each group was mutually statistically significant ($p<0.05$), but there was no significant difference between alendronate and pamidronate group within the 3-week treatment group.

(2) OPG: In pamidronate-treated group, the level of expression of OPG at 3-weeks culture was lower than that in the control group. In alendronate-treated group, the protein expression level at 3 weeks was lower than that in the control group, but interestingly, in the 4 weeks treatment of alendronate, the protein level of OPG was higher than that of the control group (Fig. 6).

In comparison between the bisphosphonate groups, each of the group was mutually statistically significant ($p<0.05$). Within respective bisphosphonate groups, the results were mutually statistically significant based on the time ($p<0.05$).

Table 1 Primers used for real-time PCR experiments

Gene name	Forward	Reverse	Size (bp)
M-CSF	CTC CAG AGA GAG GAG CCT GA	AGT ATA GAC ACT CGT CAC TGG TG	151
OPG	GCG CTC GTG TTT CTG GAC A	AGT ATA GAC ACT CGT CAC TGG TG	226

The sequences of the primers are shown with the corresponding product sizes

M-CSF Macrophage colony-stimulating factors, *OPG* osteoprotegerin

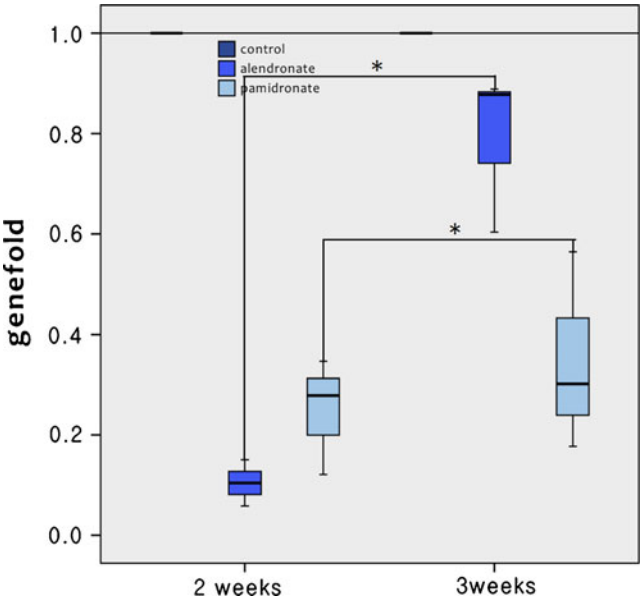

Fig. 1 Distribution of M-CSF gene fold. The gene expression level is below the control level on both alendronate and pamidronate groups. The *box* means distribution of gene fold and the *whiskers* mean standard deviation.. The *asterisk* indicates significant difference between groups based on Bonferroni–Dunn posthoc test ($p<0.05$)

Discussion

The effects of BPs on osteoclasts are well understood, and this effect of osteoclastic toxicity of BPs is thought to be one of the reasons in the occurrence of BRONJ. Besides the inhibition of osteoclasts, many complicated events may be related in the occurrence of BRONJ, and the interaction among bone cells must also be considered as a whole [23]. However, studies on the effect of BPs on osteoblasts are

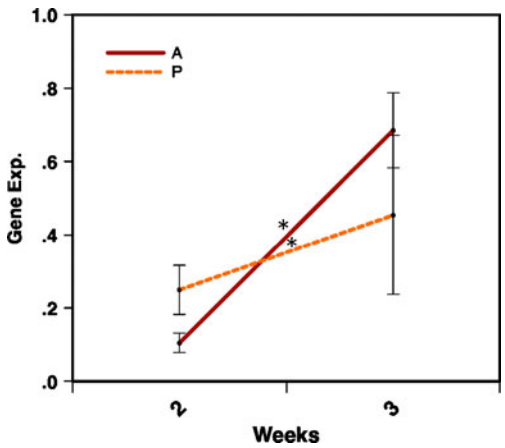

Fig. 2 Expression of M-CSF gene according to the type of bisphosphonates and time. Two or 3 weeks treatment with alendronate or pamidronate leaded to down-regulation of M-CSF mRNA expression, but 3 weeks treatment down-regulated its mRNA level to a lesser degree than 2 weeks. The *asterisk* indicates significant difference between groups based on Bonferroni–Dunn posthoc test ($p<0.05$)

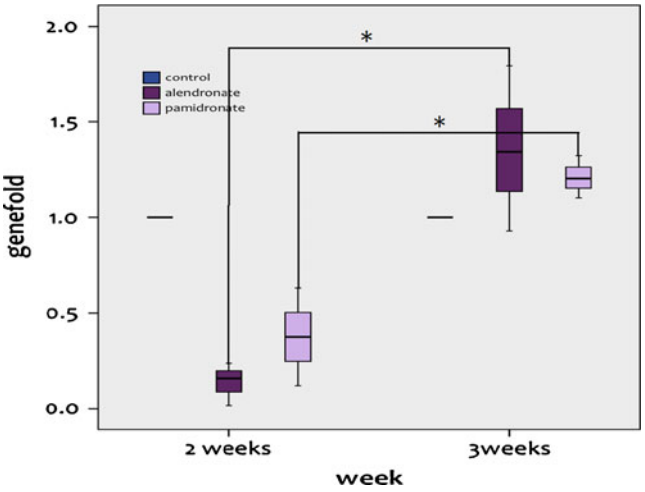

Fig. 3 Distribution of OPG gene fold. The gene expression level is below the control level on the 2-week pamidronate groups, but on the 3-week group, the gene was up-regulated on alendronate and pamidronate treatment group. The *box* means distribution of gene fold and the *whiskers* mean standard deviation. The *asterisk* indicates significant difference between groups based on Bonferroni–Dunn posthoc test ($p<0.05$)

under debate; moreover, the effects of BPs on osteoblastic actions have been sparsely investigated in terms of the BRONJ development [24]. In this research, the effect of BPs on the osteoblastic differentiation of hMSCs was studied.

Following this purpose, the gene and protein level of M-CSF and OPG expression were investigated [25]. Interestingly, alendronate and pamidronate seemed to suppress M-CSF and increase OPG when compared to the control group. These results may display the effects of BPs on M-CSF and OPG during osteoblastic differentiation, relating the significance of BPs on bone. Bone

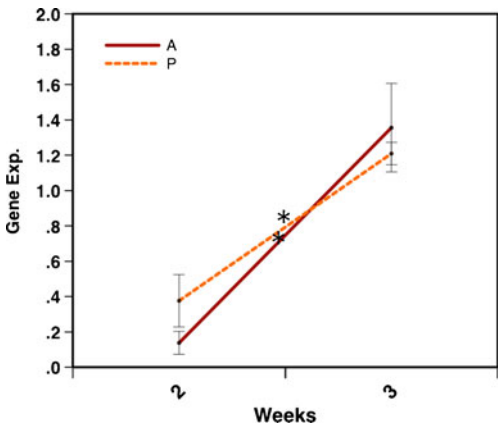

Fig. 4 Expression of OPG gene according to the type of bisphosphonates and time. Two weeks treatment with alendronate or pamidronate leaded to down-regulation of OPG mRNA expression, but 3 weeks treatment leaded to up-regulated its mRNA level to a lesser degree than 2 weeks. The *asterisk* indicates significant difference between groups based on Bonferroni–Dunn posthoc test ($p<0.05$)

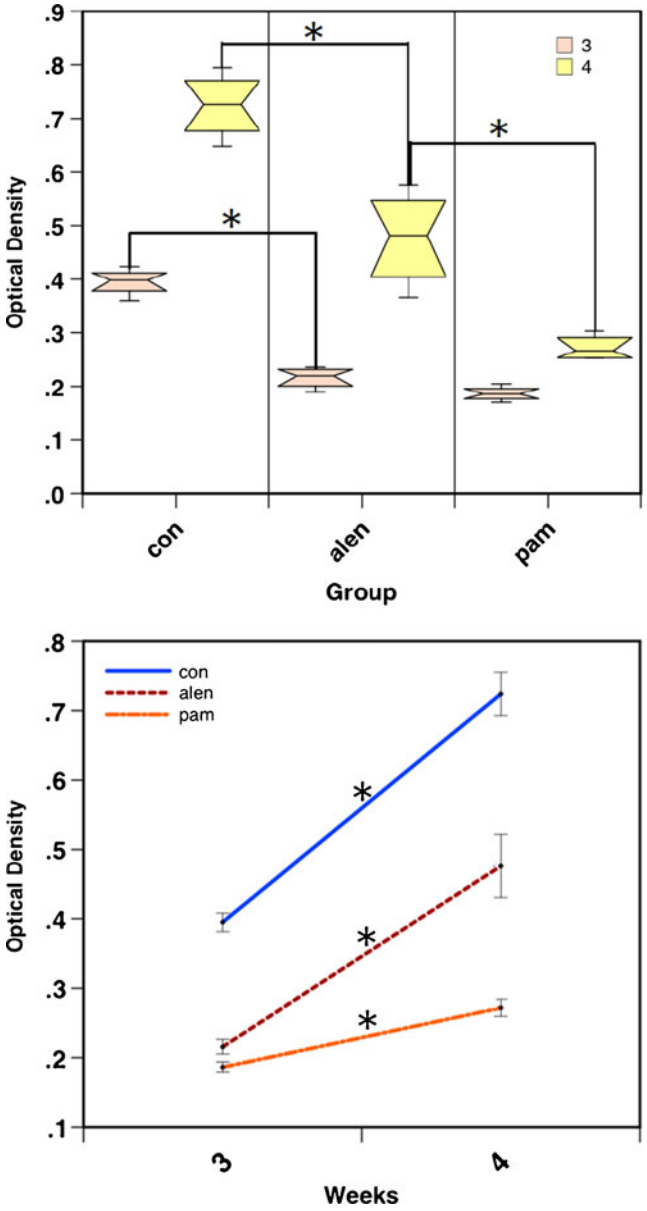

Fig. 5 Expression of M-CSF protein according to the type of bisphosphonates and time. Three or 4 weeks treatment with alendronate or pamidronate leaded to down-regulation of M-CSF protein expression, but 3 weeks treatment down-regulated its protein level to a lesser degree than 4 weeks. And the degree of alendronate-treated group was higher than pamidronate-treated group. The *asterisk* indicates significant difference between groups based on Bonferroni–Dunn posthoc test ($p<0.05$)

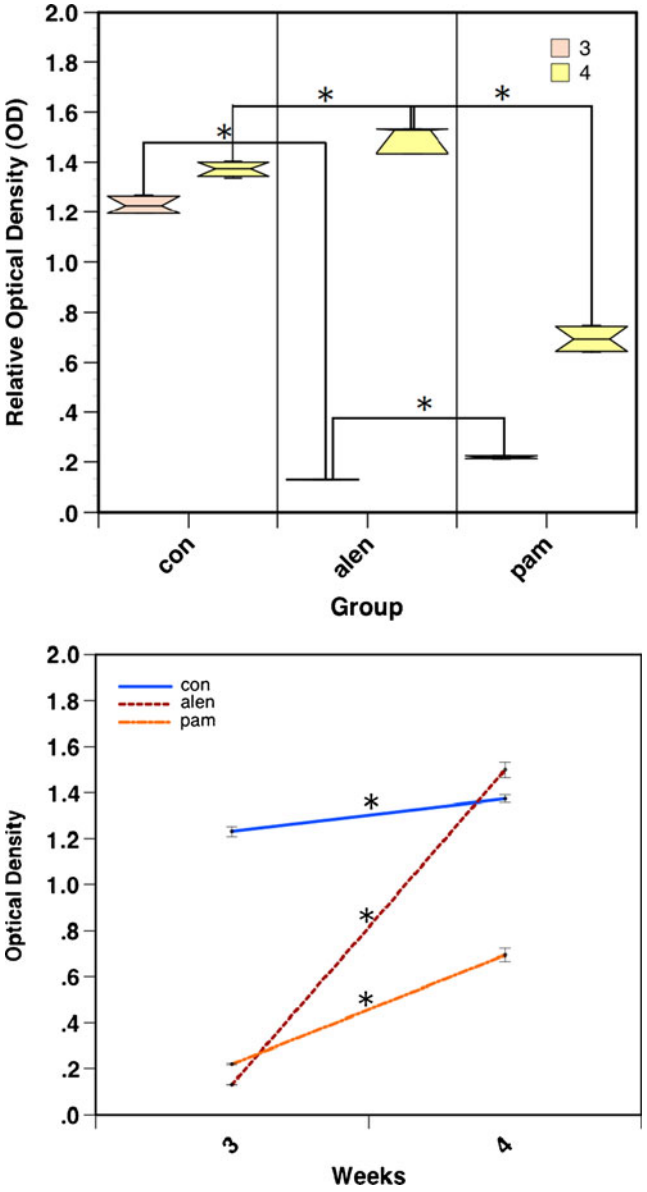

Fig. 6 Expression of OPG protein according to the type of bisphosphonates and time. Expression of OPG protein according to the type of bisphosphonates and time. Three or 4 weeks treatment with alendronate or pamidronate leaded to down-regulation of M-CSF protein expression, but 3 weeks treatment down-regulated its protein level to a lesser degree than 4 weeks, and the degree of alendronate-treated group was higher than pamidronate-treated group. The *asterisk* indicates significant difference between groups based on Bonferroni–Dunn posthoc test ($p<0.05$)

remodeling is achieved by a delicate balance between osteoblasts and osteoclasts, and an effect on either part can cause a problem in bone remodeling. The suppression of M-CSF expression may cause problems in proliferation, differentiation, and surival of monocytes, macrophages, and bone marrow progenitor cells. And the increase of OPG expression may cause problems in the production of osteoclasts by inhibiting the differentiation of osteoclast precursor. As a result, the change of the two genes consequentially lead to an imbalance of osteoclast–osteoblast interaction. BPs interfere with bone remodeling processes that are controlled by mediators such as M-CSF, RANKL, RANK, and OPG. Therefore, the effect of BPs on osteoblasts will tip the balance between the osteoblast and osteoclast interaction, leading to bone remodeling failure and thus the occurrence of BRONJ. The findings of

this study show a new light on the present BRONJ occurrence theory based on the osteoclastic activity of BPs. Also, a more advanced and developed theory for BRONJ occurrence may be obtained by combining the osteoclast inhibition mechanism and the effects on osteoblastic differentiation by BPs.

We could carefully predict that different drugs had variable effect on gene and protein expression. Pamidronate acted more specifically on M-CSF in the experiment with expression of M-SCF. Pamidronate down-regulated its mRNA level of M-CSF to a lesser degree than alendronate did. The result of the ELISA test corroborated this finding.

When it comes to the mRNA expression of OPG, alendronate acted more specifically on OPG. In the 2-week group, the alendronate group showed less mRNA expression than that of the pamidronate group but the alendronate group showed significantly more mRNA expression of OPG at 3 weeks of the culture. The results were supported in the protein expression experiment. Unlike M-CSF which contributes mainly to the early stage of osteoclastogenesis, we may infer that this preferential effect of BPs comes from the point that OPG contributes to osteoclastogenesis throughout its overall process [26–28].

The above results may suggest that the nitrogen-containing group, pamidronate and alendronate of bisphosphonate drugs, inhibits the formation of osteoclasts by disturbing the feedback mechanism between osteoblasts and osteoclasts during differentiation from hMSCs to osteoblasts. That is, suppression of M-CSF and increased expression of OPG acts as a signal for apoptosis or inhibition of osteoclastogenesis by osteoclast–osteoblast interaction.

As mentioned earlier, mechanism of BRONJ formation is unclear, and many hypotheses were suggested. Not staying in the well-known effect of BPs on osteoclasts, this study was not limited to well-known direct adverse effect of bisphosphonate on osteoclast but focused on collateral communication between osteoclast and osteoblast from a molecular biological point of view.

Conclusion

Focusing on collateral communication between osteoclast–osteoblast during bone remodeling, bisphosphonate had an effect on M-CSF and OPG expression during stem cell differentiation to osteoblast and reached the following conclusion in our experiment.

(1) Both alendronate and pamidronate suppressed gene and protein expression of M-CSF.
(2) Alendronate accelerated gene and protein expression of OPG.
(3) In aspects of the effect of bisphosphonate on M-CSF, pamidronate have much specific effect than alendronate.
(4) In aspects of the effect of bisphosphonate on OPG, alendronate have much specific effect than pamidronate.

Results support previous hypothesis that progressive accumulation of bisphosphonate in jaws causes imbalance in osteogenesis and bone absorption and collateral communication between osteoclast and osteoblast. This study will help identify mechanism of BPONJ formation. However, this study was conducted in vitro, hence cannot reenact all conditions in vivo state, and there are many other factors which influence osteoclast formation. Further study will be needed to determine the effect of bisphosphonate on osteoblast and osteoclast–osteoblast interaction.

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

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