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GENERAL GYNECOLOGY

Up-regulation of inhibitors of DNA binding/differentiation gene during alendronate-induced osteoblast differentiation

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Abstract
Purpose To investigate the effect of alendronate on the expression of *Id* genes in osteoblast differentiation.
Methods C2C12 cells were treated with alendronate for various concentrations and time periods. For evaluation of alendronate-induced osteoblast differentiation in C2C12 cells, alkaline phosphatase (ALP) activity was measured. The expression of osteoblast differentiation markers such as ALP, type-1 collagen (Col 1), and osteocalcin (OCN), and the expression of Id-1 and Id-2 were measured by RT-PCR. In order to understand the mechanism underlying the regulation of *Id* genes, the promoter region of the *Id-1* gene was identified. Database analysis of the promoter region for *Id-1* using known consensus sequences identified several putative response elements, including CCAAT/enhancer-binding protein beta (C/EBPβ).
Results Alendronate treatment significantly increased not only ALP activity but also the expression of ALP, Col 1, and OCN, Id-1 and Id-2. C/EBPβ and alendronate cooperatively increased the promoter activity and expression of Id-1.
Conclusions These results suggest that C/EBPβ-mediated *Id-1* transcriptional activation may regulate alendronate-induced osteoblast differentiation of C2C12 cells.

Keywords Alendronate · Osteoblasts · Osteoporosis

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Introduction

Bisphosphonates are commonly used in the treatment and prevention of excessive bone resorption diseases such as post-menopausal osteoporosis due to their inhibitory osteoclast activity [1]. However, there has been increasing evidence that a variety of bisphosphonates can also stimulate osteoblast proliferation, differentiation, and bone formation, as well as inhibit osteoblast apoptosis [2–5].

The process of osteoblast differentiation is under various central and local controls including bone morphogenetic proteins (BMP), Indian hedgehog, fibroblast growth factor-2 (FGF2), Wnt, parathyroid hormone, and leptin [6–9]. Further, studies have proposed several possible mechanisms governing bisphosphonate-mediated osteoblast differentiation [10, 11].

Inhibitors of DNA binding/differentiation *(Id),* which are inhibitory helix–loop–helix (HLH) transcription factors, have been reported to affect the balance between cell growth and differentiation of osteoblast [12, 13]. Further, it has been indicated that a balanced regulation of *Id* gene expression plays an important role in promoting proliferation at the early stage of osteoblast lineage-specific differentiation [12]. Bone morphogenetic proteins (BMPs) are known to convert the differentiation pathway of myoblastic cell lines into osteoblast lineages and stimulate osteoblast lineage-specific differentiation of mesenchymal stem cells by controlling expression of inhibitors of DNA binding/differentiation *(Ids)* [6, 12].

Alendronate, which is a well-known third-generation bisphosphonate, enhances the expression of BMP-2 and osteoblast maturation [4]. However, no studies to date have evaluated the possible role of Ids in alendronate-induced osteoblast differentiation. Therefore, the purpose of this study was to investigate the expression of *Ids* genes in alendronate-induced osteoblast differentiation using myoblastic C2C12 cells.

Materials and methods

Cell culture and alendronate treatment

C2C12 cells were maintained under 5% CO_2 at 37°C in growth medium, consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL) and 1% penicillin–streptomycin (PS; Gibco BRL). The medium was changed every 2 or 3 days, and the cells were cultured in serum-free DMEM with various concentrations of alendronate.

MTT (3-dimethylthiazol-2,5-diphenyltetrazolium bromide) assay

C2C12 cells were plated at a density of 2×10^4 cells in 24-well plates. After overnight incubation, alendronate was added to final concentrations ranging from 10^{-3} to 10^{-9} M for 24, 48, and 72 h. At the time points indicated, the cells were washed with PBS, and 100 µl of MTT stock solution (5 mg/ml, Sigma, St. Louis, MO, USA) was added to each culture medium and continued for 1 h at 37°C. This time period permitted the cellular conversion of MTT to an insoluble form. Then, the cells were lysed, and the formazan crystals were dissolved in DMSO at room temperature for 5 min, after which 100 µl of supernatant was transferred to the wells of a 96-well microplate. Colorimetric changes were subsequently quantified using a microplate reader at a wavelength of 540 nm (Spectra MAX 250, Molecular Devices Co., USA).

Alkaline phosphatase activity assay

To mediate the differentiation of C2C12 cells to osteoblasts, C2C12 cells were first plated at a density of 2×10^4 cells in 24-well plates. After overnight incubation, the cells were cultured in serum-free DMEM with or without alendronate at concentrations ranging from 10^{-4} to 10^{-9} M for 24, 48, and 72 h. At the time points indicated, the cells were washed with ice-cold phosphate-buffered saline (PBS), lysed in 1% Triton X-100 (Sigma), and subjected to three freeze–thaw cycles. After centrifugation (4,000*g*) of the lysates, the cellular debris were removed and supernatants were collected. The collected supernatants were then mixed with a colorless *p*-nitrophenyl phosphate (Sigma) according to the manufacturer's protocol, and the conversion of colored *p*-nitrophenol was measured using a microplate reader at a wavelength of 405 nm.

RNA preparation and RT-PCR

Quantitative RT-PCR conditions were set for analysis of three osteoblast differentiation markers, namely, alkaline phosphatase activity (ALP), type-1 collagen (Col 1) and osteocaclin (OCN). Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Complementary DNA was synthesized from 5 µg of total RNA with AMV Reverse Transcriptase (Promega, Madison, WI, USA) using random hexamers (Bioneer, Daejon, Korea) at 42°C for 1 h. Template cDNA was subjected to PCR amplification using gene-specific sense and antisense primers (Table 1). The cDNAs were amplified by PCR under the following conditions: 28–35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s in a thermal cycler. PCR products were visualized by electrophoresis on 1.2% agarose gels. The PCR bands were quantified and normalized relative to the control band with Image J, version 1.35d (National Institutes of Health Image software).

Transient transfection of C2C12 cells and luciferase activity assay

C2C12 cells were plated in 24-well plates 1 day before transfection. The cells were transiently transfected with a reporter vector and β-galactosidase expression plasmid, along with each indicated expression plasmids using Jetpei (polyplus-transfection, Illkirch, France); addition of pcDNA3.1/HisC plasmid DNA was added to maintain equal amounts of DNA per transfection. After 48 h post-transfection, the cells

Table 1 Primers sequences used for PCR amplification

Gene	Primer sequence
Alkaline phosphatase (ALP)	
Forward	5′- TCATGTTCCTGGGAGATTGGGTATG -3′
Reverse	5′- GCATTAGCTGATAGGCGATGTCC -3′
Type I Collagen (Col 1)	
Forward	5′- CAAGGGTGAGACAGGGCAAC -3′
Reverse	5′- CTCGAACTGGAATCCATCGGT -3′
Osteocalcin (OCN)	
Forward	5′- CTGAGTCTGACAAAGCCTTC -3′
Reverse	5′- GCTGCTGTGACATCCATACTTGC -3′
Cathepsin K (CTSK)	
Forward	5′- GGGCCAGGATGAAAGTTGTA -3′
Reverse	5′- CCGAGCCAAGAGAGCATATC - 3′
Inhibitor of differentiation-1 (Id1)	
Forward	5′- CTGCTCTACGACATGAACGGCTG -3′
Reverse	5′- CGGATTCCGAGTTCAGCTCCAAC - 3′
Inhibitor of differentiation-2 (Id2)	
Forward	5′- GGGCCAGGATGAAAGTTGTA -3′
Reverse	5′- CCGAGCCAAGAGAGCATATC -3′
β-Actin	
Forward	5′- GACTACCTCATGAAGATC -3′
Reverse	5′- GATCCACATCTGCTGGAA -3′

were rinsed with ice-cold PBS and lysed with 1 × Cell Culture Lysis Buffer (Promega). Luciferase activity was determined using an analytical-luminescence luminometer according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency according to the corresponding β-galactosidase activity.

Statistical analysis

All experiments were performed at least five times, and the data are expressed as the mean ± SD. The statistical significance of differences between the experiment and control groups were evaluated by student's *t* test and one-way *ANOVA*. Values of $P < 0.05$ were considered to be statistically significant.

Results

Effect of alendronate on cell viability and ALP activity in C2C12 cells

There were no significant changes in the percentage of viable cells at concentrations of alendronate ranging from 10^{-4} to 10^{-9} M up to 72 h. However, cell viability was significantly decreased with a higher dose (10^{-3} M) of alendronate (Fig. 1). Based on the results of this viability

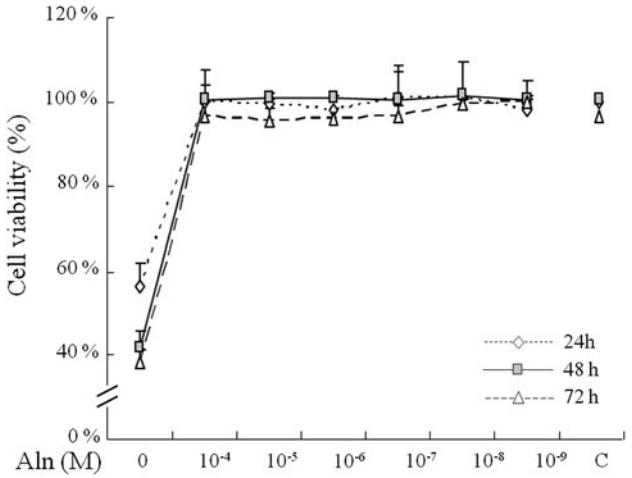

Fig. 1 Effect of alendronate on cell viability. C2C12 cells were treated with alendronate using concentrations ranging from 10^{-3} to 10^{-9} M for 24, 48, and 72 h. Cell viability was determined using MTT assay (mean ± SD, $n = 5$)

assay, we assessed osteoblast differentiation by measuring ALP activity at different concentrations of alendronate ranging from 10^{-4} to 10^{-9} M. Alendronate-induced osteoblast differentiation of C2C12 cells increased nearly twofold as determined by ALP activity in response to all concentrations of alendronate after 24 h of treatment compared to the control group. Furthermore, elevated ALP activity was also observed at alendronate concentrations from 10^{-6} to 10^{-8} M for 48 h (Fig. 2). Together, these results suggested that alendronate might play a role in the early stage of C2C12 cell differentiation into osteoblasts.

Effect of alendronate on osteoblast-specific gene expression

To clarify the potential role of alendronate in osteoblast differentiation of C2C12 cells, we next examined the expression of three osteoblast-specific genes, namely, ALP, Col 1, and OCN. For these experiments, C2C12 cells were treated with alendronate at concentrations ranging from 10^{-6} to 10^{-8} M for 48 h based on the ALP activity assays. Treatment with alendronate treatment resulted in a significant increase in expression of ALP, Col 1, and OCN compared to controls. Further, the expression levels of ALP and Col 1 peaked at an alendronate concentration of 10^{-8} M (Fig. 3a, b).

Following the treatment with 10^{-8} M of alendronate, the concentration of which resulted in peak levels of ALP and Col 1, C2C12 cells were treated with alendronate of 10^{-8} M for 24, 48, and 72 h to evaluate the effect of alendronate according to treatment time. The time course study indicated that the expression of ALP and Col 1 was significantly increased until 48 h, but decreased thereafter. Likewise, the expression of OCN was significantly increased from 72 h (Fig. 3c, d). These results indicate the

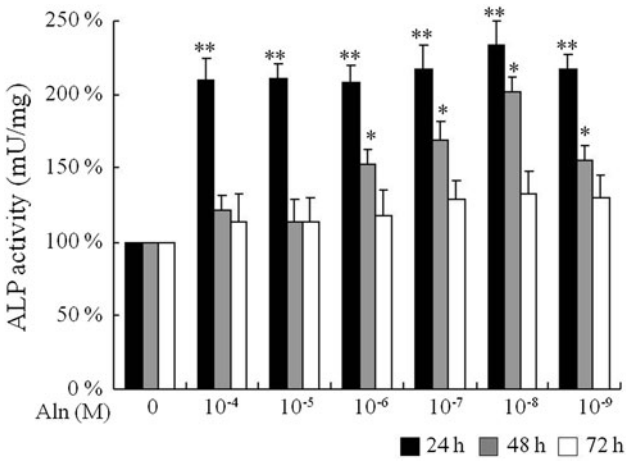

Fig. 2 Effect of alendronate-induced osteoblast differentiation of C2C12 cells. Cells were treated with alendronate at different concentration and time periods as indicated. The ALP activity was measured at 405 nm. The experiment was repeated five times and performed with triple samples. $*P < 0.05$ and $**P < 0.01$ compared (vs. control)

presence of different expression patterns for each osteoblast-specific marker according to the concentration and duration of alendronate treatment in alendronate-induced osteoblast differentiation.

Expression of Id-1 and Id-2 during alendronate-induced osteoblast differentiation of C2C12 cells

Next, we carried out expression analysis of *Id* genes in alendronate-induced osteoblast differentiated C2C12 cells. Alendronate treatment significantly stimulated Id-1 and Id-2 mRNA expression at all treated doses compared to controls (Fig. 4a, b).

We further examined the expression of Id-1 and Id-2 at 24, 48, and 72 h after treatment with alendronate at concentration of 10^{-8} M based on above data. Alendronate treatment exhibited a significant increase in the expression of both *Id-1 and Id-2* genes compared to controls. The expression of Id-1 was significantly increased after alendronate treatment at all time-periods, although levels peaked at 48 h. Similarly, Id-2 expression was significantly up-regulated until 48 h, but was undetectable thereafter (Fig. 4c, d). These results indicated that *Id-1 and Id-2* might be involved in alendronate-induced early stage of osteoblast differentiation in C2C12 cells.

Effect of alendronate on C/EBPβ-mediated Id-1 transcriptional activity

In order to investigate the transcriptional mechanism by which alendronate regulates the expression of Id-1, we examined the promoter region of the *Id-1* gene using the GeneBank database to search for known consensus sequences in the *Id-1* promoter. Several putative response elements, such as Nuclear factor-kappaB (NF-kB), acute myelogenous leukemia1/runt-related transcription factor1 (AML1/RUNX1), CDX, cAMP response element binding (CREB), and CCAAT/enhancer-binding protein beta (C/EBPβ) were detected in the *Id-1* promoter region. C2C12 cells were transiently co-transfected with *Id-1*-luciferase reporter vector along with expression vector encoding these response elements or empty vector. After transfection, *Id-1* promoter activity was significantly increased in CREB or C/EBPβ overexpressing cells compared to controls. Especially, C/EBPβ induced approximately 4.3-fold increase in *Id-1* promoter activity (Fig. 5a). To investigate whether C/EBPβ regulates the expression of Id-1 stimulated by alendronate, C2C12 cells were co-transfected with the *Id-1* luciferase reporter plasmid along with C/EBPβ-expression vector, followed by alendronate treatment. Overexpression of C/EBPβ and alendronate treatment synergistically increased *Id-1* promoter activity (Fig. 5b).

In order to determine further the effect of alendronate-induced osteoblast differentiation on C/EBPβ-mediated Id-1 expression, we transiently transfected the C/EBPβ expression vector or empty vector as a control in the absence or presence of alendronate. Consistent with the results of the reporter assay, expression of Id-1 was slightly increased in the presence of C/EBPβ. Overexpression of C/EBPβ and alendronate markedly increased ALP expression in C2C12 cells (Fig. 5c, d). These results indicated that alendronate increased ALP expression, a marker of early osteoblast differentiation, and this increased expression might be associated with increased *Id-1* expression through transcriptional regulation of C/EBPβ.

Discussion

The present study demonstrated that alendronate induced osteoblast differentiation of the C2C12 myoblastic cell line. This study also revealed an interesting finding whereby alendronate stimulated the expression of *Id* genes, which was accompanied by up-regulation of C/EBPβ-mediated *Id-1* expression.

The expression of *Id* genes was significantly increased in the early stage of BMP stimulated-osteoblast differentiation [12, 14]. Especially, BMP-2 stimulates not only various osteoblast-specific differentiation markers, but also converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage [6, 8, 12]. Im et al [4] reported that alendronate enhances the expression of BMP-2 in osteoblasts. Such previous results imply that alendronate might stimulate osteoblast differentiation by regulation of

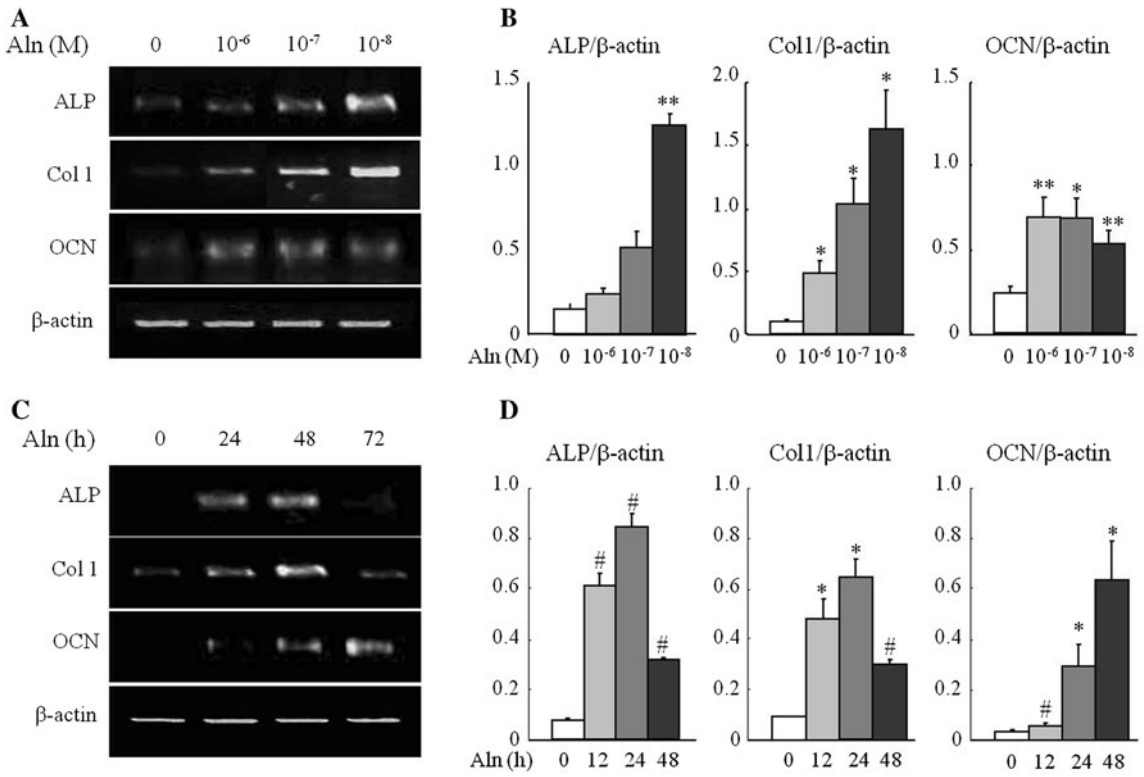

Fig. 3 Effect of alendronate on the expression of osteoblast differentiation markers. Osteoblast differentiation was determined by RT-PCR analysis for the expression of ALP, and Col 1. C2C12 cells were treated with alendronate at concentrations ranged from 10^{-6} to 10^{-8} M for 48 h (**a**) and at 10^{-8} M concentration for different time periods (24, 48, and 72 h) (**c**). Data are from a representative experiment. **b**, **d** The amount of each mRNA was normalized to that of β-actin mRNA. Quantitative data are means ± SD from six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{\#}P < 0.005$ (vs. control)

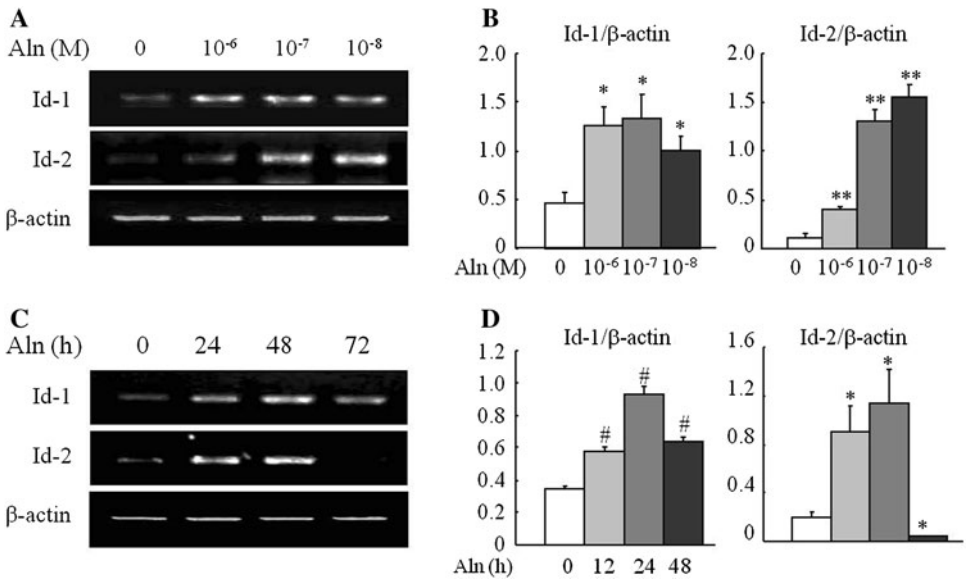

Fig. 4 Expression of *Id-1* and *Id-2* genes during alendronate-induced osteoblast differentiation. C2C12 cells were treated with alendronate at concentrations ranging from 10^{-6} to 10^{-8} M for 48 h (**a**) and at 10^{-8} M concentration for different time periods (24, 48, and 72 h) (**c**). At the indicated time after alendronate treatment, the expression of *Id-1* and *Id-2* mRNA was analyzed by RT-PCR. Data are from a representative experiment. **b, d** The amount of Id-1 and Id-2 mRNA was normalized btoy that of β-actin mRNA. Quantitative data are mean ± SD from six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{\#}P < 0.005$ (vs. control)

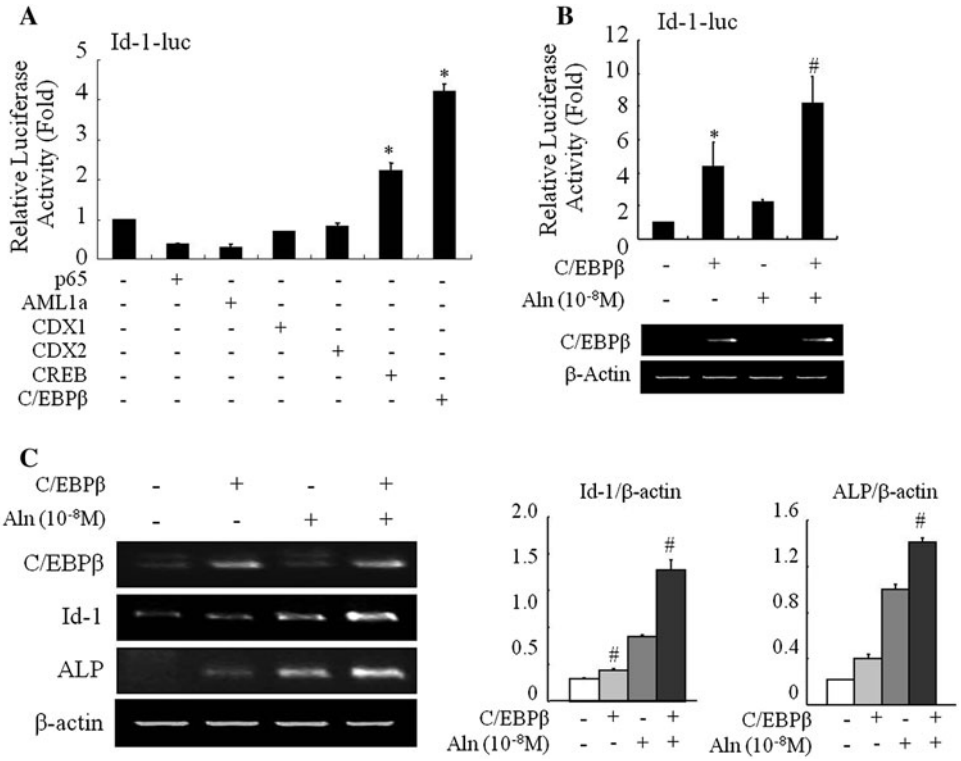

Fig. 5 Effect of alendronate on *Id-1* transactivation. **a** Determination of response elements in the *Id-1* promoter region. C2C12 cells were transiently transfected with *Id-1*-luciferase reporter vector along with expression vector encoding p65, AML1a, CDX1, CDX2, CREB or C/EBPβ, or empty vector as control. After 48 h of transfection, cell lysates were assayed for luciferase activity. Results are the average of three independent experiments, with fold induction referring to the level observed divided by the reporter activity alone. * <0.05 (vs. empty vector). **b** The effect of alendronate on C/EBPβ-mediated *Id-1* transactivation. C2C12 cells were transiently transfected with *Id-1*-luciferase reporter plasmid along with an expression vector encoding for C/EBPβ or an empty vector and then treated with 10^{-8} M of alendronate. After 48 h of transfection, cell lysates were assayed for luciferase activity (*upper panel*) and subjected for expression of the indicated transcripts by RT-PCR (*lower panel*). *Bars* represent the mean ± SD of six independent experiments with duplication.* <0.05 (vs. empty vector) and # <0.005 (vs. C/EBPβ alone). **c** C2C12 cells were transiently transfected with expression vector pcDNA3 alone (empty vector) or containing C/EBPβ expression vector and then treated with 10^{-8} M of alendronate. The Id-1 and ALP mRNA levels were detected via RT–PCR (*left panel*). The amount of each mRNA was normalized to that of β-actin mRNA (*right panel*). Experiments were performed at least five times; representative experiments are shown. # < 0.005 (vs. control)

Id gene expression. In our study, the expression of Id-1 and Id-2 peaked within 48 h of alendronate-induced osteoblast differentiation of C2C12 cells. In this respect, our results suggest the possibility that alendronate might be associated with the BMP-2 signaling pathway to induce osteoblast differentiation. However, further study is needed to evaluate this hypothesis.

C/EBPs are critical for normal cellular differentiation and metabolic functions in various tissues. Especially, C/EBPβ is expressed in osteoblastic cells and up-regulated during osteoblast differentiation [15]. This result led us to the hypothesis that increased expression of Id-1 by alendronate might be mediated via a C/EBPβ-binding element contained within the *Id-1* promoter. The present study showed that overexpression of C/EBPβ and alendronate treatment synergistically increased the promoter activity and expression of Id-1. To the best of our knowledge, this is the first study to report a potential role of Id-1 and C/EBPβ in alendronate-induced differentiation of C2C12 cells into osteoblasts.

We demonstrated the presence of differential patterns of increased expression of Id-1 and Id-2 expression by alendronate. This finding was not unexpected, as *Id-1* is known to be a direct target gene for BMPs that strongly activate its promoter [16–19]. Although the exact mechanism is unclear, induction of Id-2 in alendronate-induced osteoblast differentiation of C2C12 cells might involve indirect targeting of BMP-signaling.

In their study of alendronate localization in rat bones, Sato et al. [20] reported that alendronate was accumulated in the resorption space at a maximum concentration of 10^{-3} M after alendronate injection. Consistent with the results of our study, Garcia-Moreno et al. [2] showed that no viable cells were detected with alendronate concentrations of 10^{-3} M or higher, while at lower concentrations of alendronate, there were no significant effects compared to

controls. The effects of alendronate have been shown to be greatly dose-dependent in a rat model of arthritis; high doses of alendronate have an adverse effect on osteoblast Function [20]. This dose dependent effect of alendronate was also supported by previous studies showing that bisphosphonates increase bone marrow-derived preosteoblastic cell proliferation and inhibit the apoptosis of osteocytes and osteoblasts at low concentrations [3, 21]. This dose-dependent effect was also observed in our present study, which showed that low concentrations of alendronate stimulated early signs of osteoblast differentiation such as increased ALP activity. Thus, it can be concluded that low doses of alendronate may stimulate osteoblast differentiation of C2C12 cells, whereas a higher dose may inhibit osteoblast function.

The present study shows that the expression pattern of each osteoblast marker differed according to the time periods of alendronate treatment; ALP and Col1 expression increased up to 48 h, but decreased thereafter, whereas OCN expression was increased only after 48 h. This result may be attributable to the fact that each osteoblast differentiation marker reflects different stages of differentiation and thus different osteoblastic activity; ALP and Col 1 are early markers of osteoblast differentiation, while OCN appears late, concomitant with mineralization [6, 22].

In this present study, gene expression at the mRNA level was evaluated but expression at the protein level was not. This would be the limitation of this study. Despite the limitations of our study, the presented data may contribute to the understanding of the mechanism of alendronate-induced osteoblast differentiation, suggesting that alendronate might initially promote the gene expression of C/EBPβ-mediated Id-1 and trigger the sequential activation of osteoblast-specific genes such as ALP, Col 1, and OCN.

Differentiation processes are associated with morphological changes. Nakashima et al. [23] reported that the transformed cell (Wnt3a-C2C12) exhibited a distinct morphological change along with osteoblast gene expression. However, our present study could not observe morphological changes of the C2C12 cells during the 3 days of culture. This may be due to short culture duration. Nakashima et al. cultured transformed (Wnt3a-C2C12) cells for 3–9 days, and morphological changes were found at the 6th day of culture. Therefore, to warrant our data, further studies on the observation of morphological changes at the protein level are needed, using stable Id-1 transformed C2C12 cells for long-term culture.

In conclusion, the present study shows that the expression of *Id-1* and *Id-2* genes was stimulated in alendronate-induced differentiation of C2C12 cells myoblasts into osteoblast lineage. In addition, this study suggests that the increased expression of *Id-1* in alendronate-induced osteoblast differentiation may be regulated by C/EBPβ.

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Conflict of interest None.

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