Original Article
Zoledronic acid regulates osteoblast differentiation via the mTORC1 signaling pathway

Feng Gao1,2, Yuanhang Liu3, Mingfa Liu3, Lixin Zhu1

1Department of Orthopedic Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, P.R. China; 2Department of Orthopedic Surgery, Inner Mongolia Autonomous Region Hospital of Traditional Chinese Medicine, Hohhot, P.R. China; 3Department of Emergency Medicine, Inner Mongolia Hohhot No. 1 Hospital, Hohhot, P.R. China

Received August 6, 2017; Accepted February 14, 2018; Epub May 15, 2018; Published May 30, 2018

Abstract: Zoledronic acid (ZA) exerts an important role in regulating bone metabolism. The purpose of this study was to investigate mechanisms of ZA in terms of their effect on bone formation in vitro. Human osteoblasts (MG63 cell line) were treated with serial concentrations of ZA (0, 10⁻³, 10⁻⁶, 10⁻⁹ and 10⁻¹² M). Cell viability, cell apoptosis, alkaline phosphatase (ALP) activity, and expression levels of the marker genes and proteins associated with osteogenic differentiation were evaluated. Signaling from mechanistic target of rapamycin complex 1 (mTORC1) of MG63 cells was detected after ZA treatment, combined with or without rapamycin (mTORC1 inhibitor). Data were statistically analyzed by one-way analysis, with a significance level of 5%. An MTT assay and a flow cytometric Annexin-V/propidium iodide assay revealed that exposure of MG63 cells to ZA exhibited a significant inhibition of cell viability and induction of apoptosis at concentrations of 10⁻³ and 10⁻⁶ M, but no changes at concentrations of 10⁻⁹ and 10⁻¹² M (short-time treatment), compared to no drug. In addition, ZA caused an increased expression level of marker genes and proteins associated with osteogenic differentiation and ALP activity at concentrations of 10⁻⁶ and 10⁻¹² M, but inhibited osteogenic differentiation at concentrations of 10⁻³ and 10⁻⁶ M, compared to no drug. Furthermore, research into the mechanism responsible showed that low concentrations of ZA induced osteogenic differentiation through preventing mTORC1 activity. In contrast, high concentrations of ZA reduced osteogenic differentiation through promoting mTORC1 activity. Rapamycin, an mTORC1-specific inhibitor, could rescue the defect of osteogenic differentiation caused by exposure to high concentrations of ZA. This in vitro study indicates that ZA-mediated osteoblastic differentiation is mTORC1-dependent. Low concentrations of ZA are better for regulating bone formation via inhibiting mTORC1 activity.

Keywords: Zoledronic acid, bone disease, osteoblast, mTOR

Introduction

Bisphosphonates (BPs) have been largely used clinically for the treatment of metabolic bone diseases like osteoporosis, Paget’s disease, osteolytic disease, and malignant diseases [1, 2]. Treatment with BPs increases bone mineral density by preventing osteoclast proliferation and recruitment of osteoclast precursors [3]. Zoledronic acid (ZA), as a third-generation BP, has been proven to inhibit osteoclast function and cause apoptosis [4, 5]. ZA is available for treating a variety of bone diseases by inhibiting the maturation and activity of osteoclasts, which leads to a decreased resorption mediated by osteoclasts and eventually lowers the bone mass loss [6]. In addition, increased evidence supports the view that ZA could target osteoblasts for the cure of osteogenesis imperfecta (OI), which is characterized by low bone mass and increased bone fragility [7, 8]. These studies suggest that ZA is a potent regulator for bone formation through regulating the balance between osteoblasts and osteoclasts. However, by far, the exact notion about the effect on osteoblasts exhibited by ZA is unclear.

A recent in vivo study shows that trabecular bone is significantly improved after ZA therapy due to an increase in trabecular bone volume, trabecular number, and connectivity [9]. Thus, bisphosphonate administration does not adversely affect skeletal growth [10]. Numerous reports have described the role of BPs or ZA
and their action on osteoblasts in terms of its effect on the proliferation, differentiation, and mineralization of osteoblasts [5, 11-16]. However, these results concerning the influence of ZA on osteoblasts differ, and the relevant mechanism of ZA-mediated osteoblastic development is still less clear. ZA affects the proliferation of primary osteoblasts in a dose-dependent manner. Low concentrations of ZA may be beneficial for osteoblast proliferation and differentiation and help facilitate bone formation. Adversely, high concentrations of ZA are toxic to osteoblasts and prohibit their proliferation and differentiation [12]. In an ectopic ossicle model, ZA administration led to an anti-proliferative effect on implanted BMSCs in early osseous development but enhanced osteoblastic differentiation, eventually resulting in an increase in total bone volumes [17]. Similarly, treatment with ZA shows an anti-proliferative effect on immortalized human fetal osteoblasts and promotes their differentiation and bone-forming activities [11].

The mechanism of the affect of ZA on osteoblastic development is unclear. The mechanistic target of rapamycin (mTOR) is the sensitive target of rapamycin, it can integrate both intracellular and extracellular signals to regulate cell metabolism, growth, proliferation and survival, and mTOR functions are encompassed in two complexes, mTORC1 and mTORC2 [18]. mTORC1 signal has been proven to be involved in regulating osteoblastic expansion and differentiation, and inactivation of mTORC1 is beneficial to bone formation [19]. In addition, ZA has been widely reported to display anti-tumor effects in breast cancer treatment [20] and osteosarcoma treatment [21] through inhibiting the mTOR pathway. Thus, we hypothesized that ZA might exhibit a pivotal role in regulating bone osteoblastic expansion and differentiation via regulating the mTOR pathway.

In the present study, we demonstrate that low concentrations of ZA are promising for promoting bone formation. Further study of the mechanism showed mTORC1-dependence in the process of ZA-mediated osteoblastic differentiation.

Materials and methods

Cell culture

A human osteoblast MG63 cell line (ATCC, USA) was cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA) with 10% fetal bovine serum (FBS; Gibco), and 100 IU/mL, 100 μg/mL and 2 mM of penicillin, streptomycin, and glutamine respectively. The cells were incubated in a cell culture incubator (37°C with 5% CO₂).

As previously reported [14], cells were seeded (3×10⁴ cells/cm²) in wells of 12-well plates in complete DMEM containing β-glycerol phosphate and ascorbic acid (Sigma-Aldrich, USA). After a 72-hour incubation period, the complete DMEM was replaced by new DMEM without FBS, and the cells were incubated for an additional 24 h. Several different concentrations of ZA (Novartis Pharma AG, Switzerland; 0, 10⁻³, 10⁻⁶, 10⁻⁹ and 10⁻¹² M) were then added to DMEM without FBS and the cells were incubated in contact with these ZA solutions for 7, 14, or 21 days. During these periods, the ZA solutions were replaced every 72 h. DMEM without ZA (0 M) served as the control group. Several experiments were performed as described below.

Cell viability: MTT assay

Cell viability was determined by MTT assay as previously described [12]. Briefly, cells were cultured for 24 h in 96-well culture plates at a density of 2×10⁴ cells/well and then growth medium was replaced. Cells were treated with ZA at concentrations of 0, 10⁻³, 10⁻⁶, 10⁻⁹ and 10⁻¹² M for 7, 14 or 21 days. For this assay, a 900 μL quantity of DMEM without FBS and a 100-μL quantity of MTT solution (5 mg/mL of MTT salt; Sigma-Aldrich) was added to the samples and incubated for 4 h. The MTT solution was then aspirated and the formazan crystals were dissolved with 600 μL of acidified isopropanol (0.04 N of HCl). Cell viability was determined by absorbance of the purple solution obtained from crystal dissolution in a spectrophotometer at 570 nm (Thermo Plate, China).

Apoptosis assay: flow cytometry

Flow cytometric Annexin-V(propidium iodide (PI)) assay was used to detect cell apoptosis, to distinguish among live cells, early apoptotic cells and late apoptotic cells. An Annexin V/PI apoptosis kit (MultiSciences Biotech, China) was used, according to the manufacturer’s protocol. Cells were prepared as above, then were gently re-suspended in binding buffer and incubated for 5 min at room temperature in the dark with 5 μL Annexin V-FITC and 10 μL PI.
Regulation of zoledronic acid in osteoblasts

Annexin V-FITC and PI-labelled cells were analyzed using a flow cytometer (FacsCalibur; BD Biosciences, USA), and data analysis was performed using FlowJo V10 (FlowJo LLC).

ALP activity assay

Alkaline phosphatase (ALP) activity was detected according to the previously reported method [5]. Cells were prepared as above. Cell lysates were then obtained for ALP activity analysis using an alkaline phosphatase activity kit (Nanjing Jiancheng Biological Engineering Institute, China). The ALP activity of each sample was normalized to the total protein concentration.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

To assess the mineralization capacity of MG63 osteoblasts, gene expression of alkaline phosphatase (ALP, a marker of early osteoblasts) and osteocalcin (OCN, a marker of mature osteoblasts) was evaluated. Total RNA was extracted from cells and trabecular bone using the RNeasy kit (TaKaRa). Equal amounts of total RNA from each sample were subjected to oligo(dT)-primed cDNA synthesis using SuperScript II (Invitrogen). qPCR was performed using the following specific forward and reverse primers: ALP: 5'-GACAAGAAGCCCTTCACTGC-3' and 5'-AGACTGCGCCTTCATGTTG-3'; OCN: 5'-GGCGCCTTCATGTTG-3' and 5'-TCAGGCAACTCGTCACAGTC-3'; The relative expression of each gene was obtained after normalization to RPL13: 5'-CGCTCTGGACCTCATA3-3' and 5'-CCTGTACTCCAGCCAACCT-3'. The 2-ΔΔCT method was used to quantify the mRNA levels comparatively [14].

Western blot assay

Total cell extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was then analyzed using specific antibodies. Osteoblastic differentiation markers were analyzed. The following primary antibodies were used: monoclonal rabbit anti-ALP antibody (1:5,000; Abcam); polyclonal rabbit anti-OCN antibody (1:500; Santa Cruz Biotechnology); monoclonal mouse anti-p-S6 (Ser235/236) (1:1000; Cell Signaling Technology); polyclonal mouse anti-S6 (1:1000; Santa Cruz); monoclonal mouse anti-β-Actin antibody (1:1000; Santa Cruz Biotechnology). For the blot densitometry, images of the bands were captured using a Bio-Rad Gel Doc XR documentation system (Bio-Rad Laboratories, Inc.) and the band densities were determined using Image J software (National Institutes of Health, USA).

Rapamycin treatment

The mTORC1 inhibitor, rapamycin, was used to detect whether mTOR signaling participates in ZA-mediated MG63 osteoblast differentiation and maturation. As previously reported [19], MG63 osteoblasts exposed to ZA were simultaneously treated with 0.1 nM rapamycin. qRT-PCR and Western blot assays were used to evaluate ALP and OCN expression levels.

Statistical analysis

Data were analyzed using one-way analysis of variance to compare groups and experiments were repeated at least three times and all values are expressed as mean ± SEM. All statistical analyses were performed using SPSS 20.0 (IBM SPSS, USA) and P < 0.05 was considered to indicate a statistically significant difference.

Results

Effects of ZA on the viability of MG63 osteoblasts

The results of cell viability by MTT assay of MG63 osteoblasts treated with ZA for 7, 14 and 21 days showed that ZA at concentrations

Figure 1. Effect of ZA on the viability of MG63 osteoblasts. Cell viability was analyzed using an MTT assay. The MG63 osteoblasts were treated with ZA at 0, 10^{-12}, 10^{-9}, 10^{-6} and 10^{-3} M for 7, 14 and 21 days. The results are expressed as the mean ± standard error of the mean (n=3 for each group). *p < 0.05, **p < 0.01, ***p < 0.001, compared with 0 M group at each time. ZA, zoledronic acid.
Regulation of zoledronic acid in osteoblasts

Table 1. Cell viability of MG63 osteoblasts treated with ZA at different concentrations and for different times.

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>21D</th>
<th>14D</th>
<th>7D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 M</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10^{-9}</td>
<td>97</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>95</td>
<td>96</td>
<td>97</td>
</tr>
<tr>
<td>10^{-3}</td>
<td>88</td>
<td>90</td>
<td>91</td>
</tr>
</tbody>
</table>

of $10^{-3}$ M and $10^{-6}$ M intensely inhibited cell viability of MG63 osteoblasts at 7, 14 and 21 days compared with that in their respective control groups. ZA at concentrations of $10^{-9}$ and $10^{-12}$ M did not change MG63 osteoblast viability at 7 and 14 days compared with that in their respective control groups. However, a significant decrease of MG63 osteoblast viability was observed in the 21-day group (Figure 1). Thus, the effect of ZA on the viability of MG63 cells worked in a concentration- and time-dependent manner.

Figure 2. Effect of ZA on apoptosis of MG63 osteoblasts. Cell apoptosis was analyzed using a flow cytometric Annexin-V/PI assay. MG63 osteoblasts were treated with ZA at 0, $10^{-12}$, $10^{-9}$, $10^{-6}$ and $10^{-3}$ M for 7, 14 and 21 days. (A) Representative dot plots of flow cytometric analysis. The numbers in the quadrants of each plot indicate the percentage of positive cells. The lower right quadrant indicates early apoptotic cells, which are Annexin-V (+)/PI (-). The upper right quadrant indicates the late apoptotic or necrotic cells, which are Annexin-V (+)/PI (+). (B) Percentages of Annexin-V (+)/PI (-) and (C) Annexin-V (+)/PI (+) cells in each group are presented as bar graphs. The results are expressed as the mean ± standard error of the mean (n=3 for each group). $^* p < 0.05$, $^{**} p < 0.01$, $^{***} p < 0.001$, compared with 0 M group at each time. ZA, zoledronic acid; PI, propidium iodide.
Regulation of zoledronic acid in osteoblasts

Effects of ZA on the apoptosis of MG63 osteoblasts

To determine whether apoptosis resulted in the inhibition of cell viability following ZA treatment, Annexin V/PI flow cytometry assay was done. Our results revealed that, in MG63 osteoblasts treated with ZA (10⁻⁶ and 10⁻³ M) for 7, 14 and 21 days, the percentages of early apoptotic cells and late apoptotic or necrotic cells were significantly increased compared to that in their respective control groups. In the cells cultured with ZA (10⁻¹² and 10⁻⁹ M) for 7 and 14 days, no changes of the percentages of apoptotic cells were observed, but slight increases of the percentages of early apoptotic cells and late apoptotic or necrotic cells were found in the cells cultured with ZA (10⁻¹² and 10⁻⁹ M) for 21 days (Figure 2A-C). The findings were consistent with the results shown in Figure 1. Thus, the apoptosis caused by ZA treatment might be related to the inhibition of MG63 osteoblast viability.

Effects of ZA on ALP activity of MG63 osteoblasts

ALP activity, a well-known early-stage marker of osteoblast differentiation [22], was measured after ZA treatment for 7, 14 and 21 days. We found that the low concentrations of ZA (10⁻⁹ and 10⁻¹² M) significantly promoted ALP activity after 7 days of treatment compared to that in the control group, although unchanged ALP activity in both 14- and 21-day periods was observed. The drastic reductions of ALP activity beginning with 7-day ZA treatment were observed at high concentrations of ZA (10⁻³ and 10⁻⁶ M) compared to those in the respective control groups (Figure 3). Thus, high concentrations of ZA treatment might be negative for MG63 osteoblast ALP activity and short treatment time with low-doses of ZA was better for MG63 osteoblast ALP activity.

Effects of ZA on differentiation of MG63 osteoblasts

Bone formation is tightly related to osteoblast differentiation by osteoblast-specific gene expression, for example, ALP (an early osteoblast-specific gene) and osteocalcin (OCN, a mature osteoblast-specific gene) [23]. Examination of osteoblast-specific gene expression in MG63 osteoblasts after 7, 14 and 21 days of ZA treatment revealed a dramatic increase of OCN gene expression in ZA (10⁻⁹ and 10⁻¹² M) groups compared to that in their respective control groups, but an inhibition of OCN gene expression was found in ZA (10⁻³ and 10⁻⁶ M) groups compared to that in their respective control groups (Figure 4A). After 7 days of ZA treatment, ALP gene expression was significantly increased in ZA (10⁻⁹ and 10⁻¹² M) groups compared to that in control group. However, no changes of ALP gene expression were observed compared to that in their respective control groups after both 14 and 21 days of ZA treatment (10⁻⁹ and 10⁻¹² M). Significant reductions of ALP gene expression in ZA treatment (10⁻³ and 10⁻⁶ M) groups were found compared to that in their respective control groups at all experimental intervals (Figure 4B). The results of ALP mRNA levels were consistent with the results shown in Figure 3. As a result, low concentrations of ZA treatment (10⁻⁹ and 10⁻¹² M) might be beneficial for MG63 osteoblast differentiation and maturation.

To confirm this, protein extracts from MG63 osteoblasts after 14 days of ZA treatment were measured using western blot assay. The results revealed that expression levels of both ALP and OCN in ZA (10⁻³ and 10⁻⁶ M) groups were lower than that in the control group, but obvious increase of OCN expression levels in ZA (10⁻⁹ and 10⁻¹² M) groups was found (Figure 4C-E).
Regulation of zoledronic acid in osteoblasts

Taken together, low concentrations of ZA could promote MG63 osteoblast differentiation and maturation.

*mTORC1 signaling pathway involvement in ZA-mediated maturation of MG63 osteoblasts*

mTORC1 signaling has been described as a critical regulator of bone formation [19, 24]. Here, protein extracts from MG63 osteoblasts treated with ZA for a 14-day period were collected. We found that mTORC1 in MG63 osteoblasts was activated (indicated by P-S6 (S235/236)) by high concentrations of ZA (10^{-3} and 10^{-6} M) treatment, but a lower level of mTORC1 activity was observed while being exposed to low concentrations of ZA (10^{-9} and 10^{-12} M) compared to that in the control group (Figure 5A and 5B).

Next, we investigated the relationship between ZA and mTORC1 activity in regulating MG63 osteoblast differentiation and maturation. The mTORC1 inhibitor, rapamycin, has been proven to influence the differentiation of various osteoblastic lineage cells *in vitro* [19, 25, 26]. In the present study, MG63 osteoblasts were simultaneously treated with ZA and rapamycin for 14 days. The results revealed that rapamycin significantly reduced P-S6 (S235/236) expression, suggesting that highly active mTORC1 caused by ZA (10^{-3} and 10^{-6} M) treatment was
Regulation of zoledronic acid in osteoblasts

Reversed by rapamycin (Figure 5C and 5D). Next, the osteogenetic gene products (ALP and OCN) were detected by Western blot. We found that expression levels of both ALP and OCN in rapamycin plus ZA (10⁻³ and 10⁻⁶ M) treatment groups were increased compared to those in ZA only treatment groups (10⁻³ and 10⁻⁶ M). Also, expression of OCN in rapamycin plus ZA (10⁻³ and 10⁻⁶ M) was increased compared to that in the control group (Figure 5C, 5E and 5F). It could be considered that rapamycin might rescue the defects of MG63 osteoblast maturation resulting from high concentrations of ZA treatment through lowering mTORC1 activity.

Discussion

Therapies for bone diseases and bone metastasis with bisphosphonate ZA have been widely discussed [22, 27]. ZA, a known regulator of bone remodeling, effectively maintains the balance of bone homeostasis in favor of bone formation by inhibiting osteoclastic functions [28]. The study in vivo indicates that ZA treatment in mice leads to a significant increase in bone volume fraction, number, connectivity and apparent density of trabecular bone, although cortical bone is unaffected [9]. However, research into ZA-induced adverse side effects, for example, osteonecrosis caused by long-term ZA
Regulation of zoledronic acid in osteoblasts

treatment has generated much interest [29, 30]. Thus, the dose of ZA therapy must be considered. The effect of ZA on osteoblast expansion and differentiation in vitro is dose-dependent. Concentrations of ZA between 0.01 μM and 1 μM exert inhibitory effects on the differentiation and maturation of mouse MC3T3-E1 osteoblasts [5]. ZA decreases human osteoblast expansion at concentrations below 10^{-6} M. However, ZA promotes human osteoblast expansion at concentrations greater than 10^{-10} M. Lower concentrations of ZA may be beneficial to osteoblast differentiation and maturation, and ZA greater than 10^{-8} M significantly inhibits osteoblastic mineralized matrix formation [12]. The in vitro study suggests that ZA at concentrations tested (both 1- and 5-μM) acutely inhibits the metabolism of human osteoblasts, cell viability reduction and osteogenic differentiation inhibition being observed after ZA treatment [14]. ZA at a concentration of 10^{-4} M has a cytotoxic effect on porcine osteoblast-like cells, but cell expansion and differentiation is still promoted at lower concentrations of 10^{-6} M and 10^{-8} M [13]. In contrast to this, ZA at a concentration of 10^{-6} M displays a cytotoxic influence on rat calvarial osteoblasts, but the different cytotoxic doses of ZA may be related to species-dependence [31]. In the present study, we found that the effects of different concentrations of ZA on human MG63 osteoblast development differed. Lower concentrations of ZA could increase the expression levels of OCN, an intermediate-late stage indicator of osteogenic differentiation, which is involved in bone formation [5]. Adversely, the inhibition of osteogenic maturation was observed after treating with higher concentrations of ZA. The results are in accordance with previous observations [12], ZA strongly inhibited osteoblast ALP activity and blocked bone formation at concentrations greater than 10^{-7} M. Our results also showed an inhibitory effect of ZA on bone formation at concentrations of 10^{-3} M and 10^{-6} M, indicating the higher concentration of ZA exerted a negative regulation on human MG63 osteoblast differentiation and maturation. Additionally, MTT assay demonstrated that higher concentrations of ZA more likely leaned to a cytotoxic effect on human MG63 osteoblasts. Furthermore, the negative effect caused by higher concentrations of ZA might be closely tied to the increase of cell apoptosis. This is evidenced by several studies, which prove that continuous exposure of osteoblasts to high-doses of ZA can result in apoptosis and thus inhibit osteoblast viability [5, 32-34].

mTORC1 is ubiquitously expressed in all types of cells to regulate growth and metabolism [35]. The role of mTORC1 in bone formation has been largely identified. One recent study views that low mTORC1 activity is crucial for osteogenesis, whereas hyperactive mTORC1 is not required for differentiation of preosteoblasts and rapamycin promotes preosteoblast differentiation by blocking mTORC1 [19]. It has been proven that suppression of the mTOR signaling pathway by rapamycin promotes BMSC differentiation into osteoblasts [36]. Thus, whether ZA regulates osteoblast differentiation via the mTOR signaling pathway should be discussed. In the present study, as ZA concentration increased, the expression level of P-S6 (S235/236) (indicator of mTORC1 activity [35]) increased, suggesting high-doses of ZA promoted mTORC1 activity. Accompanying results showed that lower expression levels of OCN were observed. Only lower-doses of ZA (10^{-9} M and 10^{-12} M) could down-regulate mTORC1 activity and promote ALP activity and OCN expression. These results suggest that the dose-dependent inhibition of mTORC1 activity may be important in the process of ZA facilitating human MG63 osteoblast differentiation. To identify the exact role of mTORC1 in ZA-mediated MG63 osteoblast differentiation, MG63 osteoblasts were treated with rapamycin (mTORC1-specific inhibitor [19]). The results show that rapamycin reversed the defect of MG63 osteoblast differentiation caused by higher doses of ZA (10^{-3} M and 10^{-6} M). As a result, ZA might regulate MG63 osteoblast differentiation via the mTORC1 signal pathway. Understanding the underlying molecular mechanism of ZA-mediated osteoblast differentiation and maturation may be helpful for determining solutions to high-dose ZA-induced adverse side effects in vivo and in vitro.

Acknowledgements

We acknowledge the work of researchers in the Central Laboratory, Southern Medical University.

Disclosure of conflict of interest

None.
Regulation of zoledronic acid in osteoblasts

Address correspondence to: Lixin Zhu, Department of Orthopaedic Surgery, Zhujiang Hospital, Southern Medical University, Guangzhou 510282, Guangdong, P.R. China. Tel: 86-20-62782578; E-mail: zhulixin1966@163.com

References

Regulation of zoledronic acid in osteoblasts


