Dose-dependent inhibitory effects of zoledronic acid on osteoblast viability and function *in vitro*

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Abstract. Zoledronic acid (ZA), which is one of the most potent and efficacious bisphosphonates, has been commonly used in clinical practice for the treatment of various bone disorders. The extensive use of ZA has been associated with increasing occurrence of jaw complications, now known as bisphosphonate-associated osteonecrosis of the jaw (BRONJ). However, the mechanism underlying BRONJ remains to be fully elucidated. The aim of the present study was to investigate the effects of different concentrations of ZA on the MC3T3-E1 murine preosteoblast cell line cells and examine the possible pathogenesis of BRONJ. In the present study, the effect of ZA on the viability, apoptosis, differentiation and maturation of MC3T3-E1 cells, as well as its relevant molecular mechanism, were examined The results of a Cell Counting Kit 8 assay, a flow cytometric Annexin-V/propidium iodide assay and western blot analysis demonstrated that ZA exhibited a significant inhibition of cell viability and induction of apoptosis at concentrations >10 μM. Subsequently, the effect of ZA on cell differentiation at concentrations <1 μM were investigated. In this condition, ZA inhibited bone nodule formation and decreased the activity of alkaline phosphatase. The results of reverse transcription-quantitative polymerase chain reaction and western blot analyses indicated that ZA downregulated the expression levels of the marker genes and proteins associated with osteogenic differentiation. Further investigation revealed that the suppression of differentiation by ZA was associated with decreased expression of bone morphogenetic protein-2 (BMP-2) and downregulation of the phosphorylation levels in the downstream extracellular signal-regulated kinase 1/2 and p38 pathways. These adverse effects of ZA were observed to be concentration-dependent. The results from the present study suggested that ZA at higher concentrations induces cytotoxicity towards osteoblasts, and ZA at lower concentrations suppresses osteoblast differentiation by downregulation of BMP-2. These results assist in further understanding the mechanisms of BRONJ.

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Abbreviations: BRONJ, bisphosphonate-associated osteonecrosis of the jaw; ZA, zoledronic acid; ALP, alkaline phosphatase; BMP-2, bone morphogenetic protein-2; ERK 1/2, extracellular signal-regulated kinase 1/2; BPs, bisphosphonates; TGF-β, transforming growth factor-β; Col I, collagen type I; OCN, osteocalcin; Runx2, runt-related transcription factor 2; GAPDH, glyceraldehyde phosphate dehydrogenase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

Key words: zoledronic acid, bisphosphonates, MC3T3-E1, bisphosphonate-associated osteonecrosis of the jaw, osteoblast, cell viability, cell function, differentiation, bone morphogenetic protein-2

Introduction

By inhibiting bone resorption, bisphosphonates (BPs) have been extensively used clinically for the treatment of osteoporosis, Paget's disease and malignant diseases, including multiple myeloma and metastasis to the bone (1,2). Based on their chemical structure, BPs can be classified as either nitrogen-containing or non-nitrogen-containing (3). As a bone metabolic regulator, nitrogen-containing bisphosphonates (N-BPs) predominantly act on osteoclasts. By inhibiting farnesyl diphosphate synthase, a key enzyme in the mevalonic acid pathway, N-BPs inhibit the prenylation of small GTPases, which maintain the functioning of osteoclasts. The small GTPases accumulate in the cells, which erroneously stimulates the downstream pathway, inhibiting the formation of osteoclasts and inducing apoptosis of osteoclasts. Thus, the bone resorption mediated by osteoclasts is reduced, lowering the bone turnover rate and eventually inhibiting the bone mass loss (4). Among the N-BPs, ZA exhibits the most potent pharmacological action and affinity to bones, particularly in sites of active bone metabolism (5).

In the last 30 years, with the continuing increase in the clinical application of N-BPs, there has been increasing awareness of the adverse reactions associated with their use, including gastrointestinal symptoms, which develop with oral administration, and severe esophagitis, vasculitis, pyrexia, hypocalcemia and hypophosphatemia, which may

be associated with intravenous administration (6,7). In 2003, bisphosphonate-associated osteonecrosis of the jaw (BRONJ) was reported for the first time, and relevant reports have been constantly emerging since (8,9). The American Association of Oral and Maxillofacial Surgeons defines BRONJ as necrotic bone exposed in the maxillofacial region, lasting for >8 weeks in patients treated with BPs who have not undergone head and neck radiation therapy (10). Notably, a 0.8-12% cumulative incidence of BRONJ in the USA was reported following the intravenous injection of N-BPs for malignant disease in 2009 (10). This side effect is predominantly observed with zoledronic acid (ZA) treatment due to its high capacity for bone adhesion (11). To date, while the etiology of BRONJ remains to be fully elucidated, a reduction in osteoblasts and inhibition of osteoblast function have been observed in *in vivo* studies (12,13), suggesting that the development of BRONJ may be directly associated with the impact of ZA on osteoblasts.

Bone morphogenetic proteins (BMPs), one of the important extracellular signaling molecules regulating the differentiation of osteoblast precursors into mature osteoblasts, are a member of the transforming growth factor-β (TGF-β) superfamily, which is a group of highly conservative functional proteins with similar structures (14). BMPs induce the formation of bones, cartilage and bone-associated connective tissues in organisms by osteoblasts in an autocrine and paracrine-manner (15). BMP-2 is one of the most investigated BMP family members, and has been identified as the most potent inducer of osteogenesis. It acts by regulating the Small mothers against decapentaplegic signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway and Runt-related transcription factor 2 (Runx2) signaling pathway (16). Previous studies have suggested that extracellular signal-regulated kinase (ERK) 1/2 activation stimulates osteoblast proliferation and differentiation, activation of P38 is vital in osteoblast differentiation and Runx2 is an essential transcription factor for osteoblast differentiation (17-21).

Although the *in vivo* and *in vitro* actions of ZA on osteoclasts have been well-described, its role in osteoblast function remains to be fully elucidated and remains controversial at present. While Scheper *et al* claimed that ZA concentrations of 0.4-4.5 μM were detected in the bone tissues of patients with BRONJ (22), no well-recognized data are available on the concentration of ZA to which osteoblasts are exposed in organisms. In the past several decades, certain studies have used specific concentrations of ZA to stimulate experimental systems consisting of different types of osteoblasts, resulting in varying conclusions (23-25). In addition, the impact of different concentrations of ZA on the expression of BMP-2 e in osteoblasts remains to be fully elucidated.

Therefore, the present study investigated the effects of different concentrations of ZA on the viability and functions of MC3T3-E1 cells, in order to reevaluate the effects of ZA on osteoblasts *in vitro* and to examine the possible etiopathogenesis of BRONJ.

Materials and methods

Cell culture. MC3T3-E1 cells, which are a well described as a model for the osteoblastic phenotype (26), were obtained from the Cell Center of the Chinese Academy of Medical Sciences (Beijing, China) and were seeded at a density of $1x10^4$ cells/cm^2 for culture in regular growth culture media containing α-minimum essential medium (α-MEM; GE Healthcare Life Sciences, Logan, UT, USA) in a humidified atmosphere of 5% CO_2 at 37˚C. The medium was supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Grand Island, NY, USA), 100 U/l penicillin and 100 mg/l streptomycin (Gibco Life Technologies) in a humidified atmosphere of 5% CO_2 at 37˚C. At 80% confluence, the cells were cultured in osteoinductive medium, which was comprised of α-MEM containing 10% FBS, 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 50 μg/ml L-ascorbic acid (Sigma-Aldrich) and 100 nM dexamethasone (Sigma-Aldrich). The cells were then incubated with ZA (Sigma-Aldrich) at various concentrations in a humidified atmosphere of 5% CO_2 at 37˚C. Cells in the control group were cultured in osteoinductive medium without ZA. The medium was replaced every 3 days.

Cell Counting Kit (CCK)-8 assay. Cell viability was measured by the conversion of Dojindo's highly water-soluble tetrazolium salt, WST-8, to a yellow colored water-soluble formazan. The quantity of formazan dye generated by the activity of mitochondrial dehydrogenases in the cells is directly proportional to the cell viability. For the assay, the MC3T3-E1 cells ($1x10^4$ cells/well) were incubated in 96-well plates with osteoinductive medium in the presence of various concentrations of ZA (0-100 μM) for 1, 3, 5 and 7 days in a humidified atmosphere of 5% CO_2 at 37˚C. Following treatment, 10 μl CCK-8 solution (Wuhan Boster Biological Technology, Ltd., Wuhan, China) was added to each well and incubated at 37˚C for 2 h. The optical density of each well was measured using a microculture plate reader (Epoch; BioTek, Winooski, VT, USA) at a wavelength of 450 nm.

Flow cytometric Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay. To assess apoptosis, an Annexin V/PI apoptosis kit (MultiSciences Biotech, Co., Ltd., Hangzhou, China) was used, according to the manufacturer's instructions. Briefly, following incubation in 6-well plates with osteoinductive medium in the presence of various concentrations of ZA (0, 0.01, 0.1, 1, 10 or 100 μM) for 1, 4 and 7 days, the cultured MC3T3-E1 cells were gently resuspended in binding buffer and incubated for 5 min at room temperature in the dark with 5 μl Annexin V-FITC and 10 μl PI. The AnnexinV-FITC and PI-labelled cells were analyzed using a flow cytometer (FACSort; BD Biosciences, Burlington, MA, USA). Using flow cytometry, dot plots of Annexin V-FITC, on the X-axis, against PI, on the Y-axis, were used to distinguish viable cells, which are negative for PI and Annexin V-FITC, early apoptotic cells (Annexin V-positive/PI-negative) and late apoptotic or necrotic cells (AnnexinV-FITC-positive/PI-positive staining). The resultant data was analyzed using CellQuest software version 3.1 (BD Biosciences, San Jose, CA, USA).

Alizarin Red S staining. To visualize mineralization of the extracellular matrix as a marker of terminal differentiation, Alizarin Red S staining was performed. The MC3T3-E1 cells were seeded into 24-well plates at a density of $1x10^4$ cells/cm^2

and incubated in the presence of various concentrations (0, 0.01, 0.1 or 1 μM) of ZA for 21 days in a humidified atmosphere of 5% CO_2 at 37˚C. The cultured cells were washed with phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde (Wuhan Boster Biological Technology, Ltd.) for 15 min. The fixed cells were then stained at room temperature for 5 min with Alizarin Red S solution (Sigma-Aldrich). Following removal of the dye, the cells were washed with distilled water and images were captured with a digital camera (EOS 60D, Canon, Inc., Tokyo, Japan). Calcified nodules appear bright red following Alizarin Red S staining.

Measurement of ALP activity. The MC3T3-E1 cells were cultured in 24-well plates at a density of $1x10^4$ cells/cm^2 with osteoinductive medium in the presence or absence of ZA (0, 0.01, 0.1 or 1 μM) for 7 and 14 days in a humidified atmosphere of 5% CO_2 at 37˚C. The medium was removed, and the cell monolayer was gently washed with ice-cold PBS three times and lysed with radioimmunoprecipitation assay buffer (RIPA; Beyotime Institute of Biotechnology, Haimen, China). The lysate was centrifuged at 12,000 x g for 15 min, and the clear supernatant was used for the measurement of ALP activity using an alkaline phosphatase activity kit (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China). The ALP activity of each sample was normalized to the total protein concentration.

ALP staining. The activity of ALP in the cells was measured using ALP staining, to confirm the ALP activity in the ECM on cell layer in the MC3T3-E1 cells. Following culture in 24-well plates at a density of $1x10^4$ cells/cm^2 with osteoinductive medium in the presence or absence of ZA (0, 0.01, 0.1 or 1 μM) for 10 days in a humidified atmosphere of 5% CO_2 at 37˚C, the MC3T3-E1 cells were rinsed with PBS three times and fixed in 4% paraformaldehyde for 15 min. The cells were then stained using a Leukocyte Alkaline Phosphatase kit (Sigma-Aldrich) for 30 min at 37˚C. Following washing with PBS, images of the cells were captured using the digital camera.

Enzyme-linked immunosorbent assay (ELISA) for the detection of secreted BMP-2. A BMP-2 ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) was used to detect the levels of BMP-2. Briefly, the MC3T3-E1 cells were seeded into 24-well plates at a density of $1x10^4$ cells/cm^2 were treated with osteoinductive medium containing various concentrations (0, 0.01, 0.1 or 1 μM) of ZA. Following 7 days of incubation in a humidified atmosphere of 5% CO_2 at 37˚C, the culture medium from each well was transferred to individual microcentrifuge tubes and centrifuged for 20 min at 1,000 x g and 4˚C. The resulting supernatant was then stored at -20˚C until measurement using the ELISA kit. The assay was performed, according to the manufacturer's instructions.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from the harvested cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Extracted total RNA (2-5μg) was used to synthesize cDNA with the SuperScript II cDNA synthesis kit

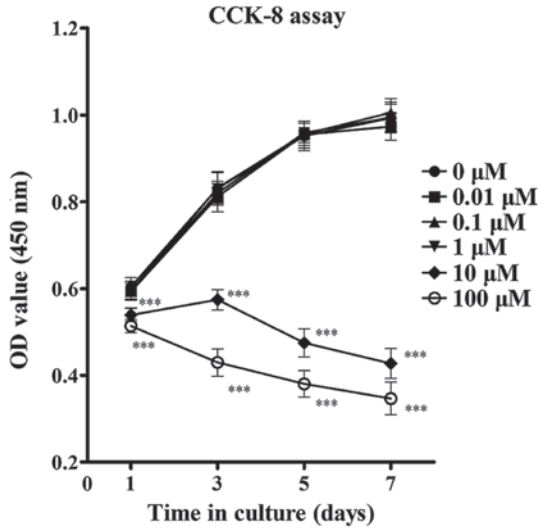

Figure 1. Effect of ZA on the viability of MC3T3-E1 cells. Cell viability was analyzed using a CCK-8 assay. The MC3T3-E1 cells were incubated with osteoinductive medium in the presence of various concentrations of ZA (0-100 μM) for 1, 3, 5 and 7 days. The results are expressed as the mean ± standard error of the mean (n=6 for each group). ***P<0.001, compared with the 0 μM group. ZA, zoledronic acid; CCK-8, Cell Counting Kit-8; OD, optical density.

(Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Gene expression analysis was performed using qPCR (iQ5 system; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized against 18S RNA. The PCR reactions were run in a total volume of 20 μl, containing 10 μl TransStart® Top Green qPCR SuperMix (concentration, 2X; Beijing Transgen Biotech Co., Ltd., Beijing, China) and 0.2 μM of each primer. Subsequently, 1 μl template was added to the reaction mix. The cycling conditions were as follows: 30 sec of polymerase activation at 94˚C, followed by 45 cycles at 94˚C for 5 sec and 60˚C for 30 sec. In the present study, the expression levels of collagen type I (Col I), ALP, osteocalcin (OCN), runt-related transcription factor 2 (Runx2), BMP-2 in the MC3T3-E1 cells were detected. The primer sequences were as follows: Forward, 5'-TTCGAACGTCTGCCCTATCAA-3' and reverse, 5'-ATGGTAGGCACGGGGACTA-3' for 18S RNA; forward, 5'-ACGTCCTGGTGAAGTTG-3' and reverse, 5'-CAGGGAAGCCTCTTTCTCCT-3' for Col I; forward, 5'-GCCTTACCAACTCTTTTGTGCC-3' and reverse, 5'-GCTTGCTGTCGCCAGTAAC-3' for ALP; forward, 5'-CTGACCTCACAGATCCCAAGC-3' and reverse, 5'-TGGTCTGATAGCTCGTCACAAG-3' for OCN; forward, 5'-GACTGTGGTTACCGTCATGGC-3' and reverse, 5'-ACTTGGTTTTTCATAACAGCGGA-3' for Runx2; and forward, 5'-GGGACCCGCTGTCTTCTAGT-3' and reverse, 5'-TCAACTCAAATTCGCTGAGGAC-3' for BMP-2. The $2^{-\Delta\Delta CT}$ method was used to quantify the mRNA levels comparatively (27).

Western blot analysis. The MC3T3-E1 cells were seeded into 6-well dishes at a density of $1x10^4$ cells/cm^2. Following incubation with osteoinductive medium containing different concentrations of ZA (0, 0.01, 0.1 or 1 μM) for 7 days, the cells were washed with PBS three times and lysed for 30 min at 4˚C

with RIPA buffer, according to the manufacturer's instructions. Following centrifugation at 10,000 x g for 15 min, the soluble fraction was used to perform western blotting. The total protein concentrations for each sample were determined using a Bicinchoninic Acid Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA), and bovine serum albumin (BSA) was used as a standard. Equal quantities of the proteins (30 μg) were loaded onto 8-10% SDS-PAGE gels (Wuhan Boster Biological Technology, Ltd.), separated and transferred onto nitrocellulose membranes (Immobilon-P; EMD Millipore, Billerica, MA, USA). The membranes were blocked at room temperature for 1 h with 5% BSA and were then incubated overnight at 4˚C with primary antibodies in blocking solution. Primary antibodies against the following targets were used: Monoclonal rabbit anti-p38 antibody (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 8690), monoclonal rabbit anti-phosphorylated (p)-p38 antibody (1:1,000; Cell Signaling Technology, Inc.; cat. no. 4511), monoclonal rabbit anti-ERK 1/2 antibody (1:1,000; Cell Signaling Technology, Inc.; cat. no. 4695), monoclonal rabbit anti-p ERK 1/2 antibody (1:1,000; Cell Signaling Technology, Inc.; cat. no. 4370), polyclonal rabbit anti-inactive caspase-3 antibody (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. SC-7148), polyclonal rabbit anti-OCN antibody (1:500; Santa Cruz Biotechnology, Inc.; cat. no. SC-30045), polyclonal rabbit anti-active caspase-3 antibody (1:200; Abcam, Cambridge, UK; cat. no. ab2302), monoclonal rabbit anti-ALP antibody (1:20,000; Abcam; cat. no. ab108337), polyclonal rabbit anti-BMP-2 antibody (1:1,000; Abgent Biotech Co., Ltd., Suzhou, China; cat. no. AP13858c), polyclonal rabbit anti-Runx2 antibody (1:400; Wuhan Boster Biological Technology, Ltd.; cat. no. BA3613-2), monoclonal mouse anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody (1:400; Wuhan Boster Biological Technology, Ltd.; cat. no. BM1623), monoclonal mouse anti-β actin antibody (1:400; Wuhan Boster Biological Technology, Ltd.; cat. no. BM0627). Subsequently, the membranes were washed thee times for 10 min each with tris-buffered saline (TBS) containing 0.1% Tween-20 (Wuhan Boster Biological Technology, Ltd.), and incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Wuhan Boster Biological Technology, Ltd.) for 1 h at room temperature. Finally, the membranes were washed three times with TBS buffer, and immunoreactive bands were detected using a BeyoECL Plus Western Blotting detection system (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. For the blot densitometry assay, images of the bands were captured using a Bio-Rad Gel Doc XR documentation system (Bio-Rad Laboratories, Inc,) and the band density was determined using Image Lab software version 5.1 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical analyses were performed using one-way analysis of variance to compare groups and experiments were repeated three times. All statistical analyses were performed using SPSS 20.0 (IBM SPSS, Armonk, NY, USA) and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of ZA on the viability of MC3T3-E1 cells. The present study first determined the effect of ZA on the viability of the MC3T3-E1 cells by performing a CCK-8 assay. The results revealed that exposure of MC3T3-E1 cells to ZA at a concentration between 0.01 and 1 μM for 1, 3, 5 and 7 days did not affect cell viability significantly. However, significant inhibition of cell viability was observed following treatment with ZA at concentrations >10 μM after 1 day, which was enhanced after 3, 5 and 7 days (Fig. 1). As the number and viability of MC3T3-E1 cells markedly reduced at concentrations ≥10 μM, concentrations of ZA in the range between 0.01 and 1 μM were used in the subsequent assays to observe the differentiation and mineralization of osteoblasts.

Effects of ZA on the apoptosis of MC3T3-E1 cells. To determine whether the inhibition of cell viability following ZA treatment was ascribed to apoptosis, Annexin V/PI flow cytometric analyses were performed, to distinguish among healthy cells, early apoptotic cells and late apoptotic or necrotic cells. The results demonstrated that, in MC3T3-E1 cells treated with 0.01, 0.1 and 1 μM ZA for 1, 4 and 7 days, the percentage of viable cells was not significantly different to that of the control group (0 μM ZA). By contrast, a dose-dependent and time-dependent increase in the number of early apoptotic cells and late apoptotic or necrotic cells were observed following culture with higher concentrations of ZA. In the cells cultured with 10 and 100 μM for 1 day, the percentage of viable cells decreased, and the percentage of early apoptotic cells and late apoptotic or necrotic cells increased marginally. Following 4 days of incubation with 10 and 100 μM ZA, the early apoptotic cells increased to 11.4±3.6 and 35.3±2.4%, respectively, and the late apoptotic or necrotic cells increased to 8.0±0.7 and 9.7±1.5%, respectively. Following 7 days of incubation with 10 or 100 μM ZA, the early apoptotic cells increased to 30.5±7.9 and 43.1±12.8%, and the late apoptotic or necrotic cells increased to 22.6±10.7 and 30.5±7.9%, respectively. These data were in accordance with the trend of cell viability (Fig. 2). Caspase-3 is a protease involved in the initiation of the apoptotic pathway, at which the endogenous and exogenous apoptotic pathways converge. The activation of caspase-3 ultimately causes apoptosis (28). In order to assess the effects of ZA on caspase-3 activation, total protein was extracted from the MC3T3-E1 cells cultured with or without ZA (0.01-100 μM) for 3 days. Western blotting was then performed to detect the inactive caspase-3 and active caspase-3, as indicators of the activation of the apoptotic pathways. The protein level of inactive caspase-3 was downregulated and the protein level of active caspase-3 was upregulated at ZA concentrations of 10 and 100 μM, compared with the other concentrations (Fig. 3). These results demonstrated that the inhibition of cell viability by ZA was due to the induction of apoptosis.

Effects of ZA on the differentiation and maturation of MC3T3-E1 cells. The formation of calcified nodule is one of the markers of osteoblastic maturation (29). Although, ZA had no significant effect on cell growth or apoptosis at concentrations of ≤1 μM in the present study, the formation of mineralized nodules was significantly suppressed by ZA

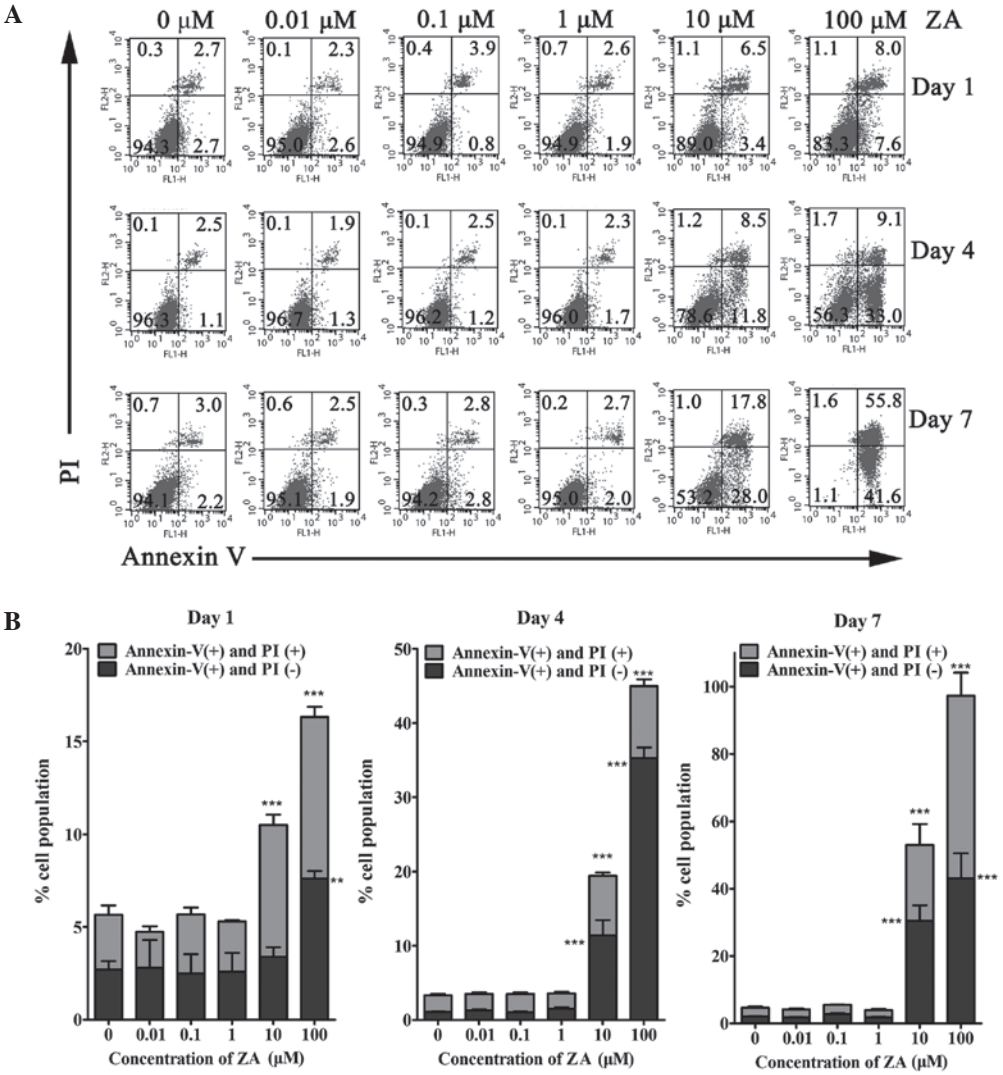

Figure 2. Effect of ZA on apoptosis of the MC3T3-E1 cells, measured using a flow cytometric Annexin-V/PI assay. The MC3T3-E1 cells were incubated with osteoinductive medium in the presence of various concentrations of ZA (0-100 μM) for 1, 4 and 7 days. (A) Representative dot plots of flow cytometric analysis. The numbers in the quadrants of each plot indicate the percentage of positive cells. The lower left quadrant indicates the viable cells, which are Annexin-V (-)/PI (-). 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 Annexin-V (+)/PI (-) cells in each group, presented as bar graphs. The results are expressed as the mean ± standard error of the mean (n=3 for each group). **P<0.01 and ***P<0.001, compared with the 0 μM group. ZA, zoledronic acid; PI, propidium iodide.

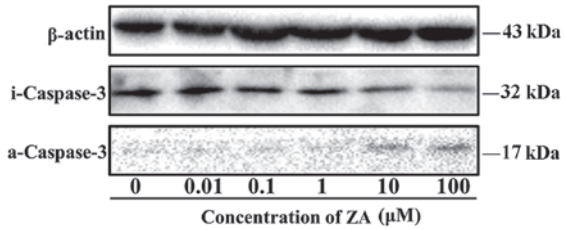

Figure 3. Effect of ZA on activation of Caspase-3 in MC3T3 cells. Protein level of inactive Caspase-3 and active Caspase-3 detected by western blot analysis normalized to β-actin. MC3T3-E1 cells were incubated in 6-well plates with osteoinductive medium in the presence of various concentrations of ZA (0-100 μM) for 4 days. i-Caspase-3, inactive Caspase-3; a-Caspase-3, active Caspase-3; ZA, zoledronic acid.

in a dose-dependent manner (Fig. 4A). ALP is a phenotypic marker for the early differentiation of osteoblasts. ALP activity was examined using the alkaline phosphatase activity kit and microplate reader, then confirmed with ALP staining to assess the effect of ZA on the differentiation of MC3T3 E1 cells. Following 7 and 14 days of ZA treatment, ZA decreased the ALP activity of the MC3T3-E1 cells at concentrations between 0.01 and 1 μM, compared with the control, in a dose-dependent manner (Fig. 4B and C). The effect of ZA on the expression levels of the critical genes associated with osteogenic differentiation, Col I, ALP, OCN and Runx2, were also examined. At day 7, the cells treated with 0.01, 0.1 and 1 μM ZA exhibited downregulation in the expression levels of the marker gene, compared with the control (0 μM), and this downregulation was also concentration-dependent (Fig. 4D). The results of the western blot analysis revealed that ZA had decreased the protein levels of ALP, OCN and Runx2 in the MC3T3-E1 cells at day 7 of differentiation. These results are consistent with those of the gene expression levels (Fig. 4D and E). Taken together, the data obtained suggested that ZA at concentrations <1 μM

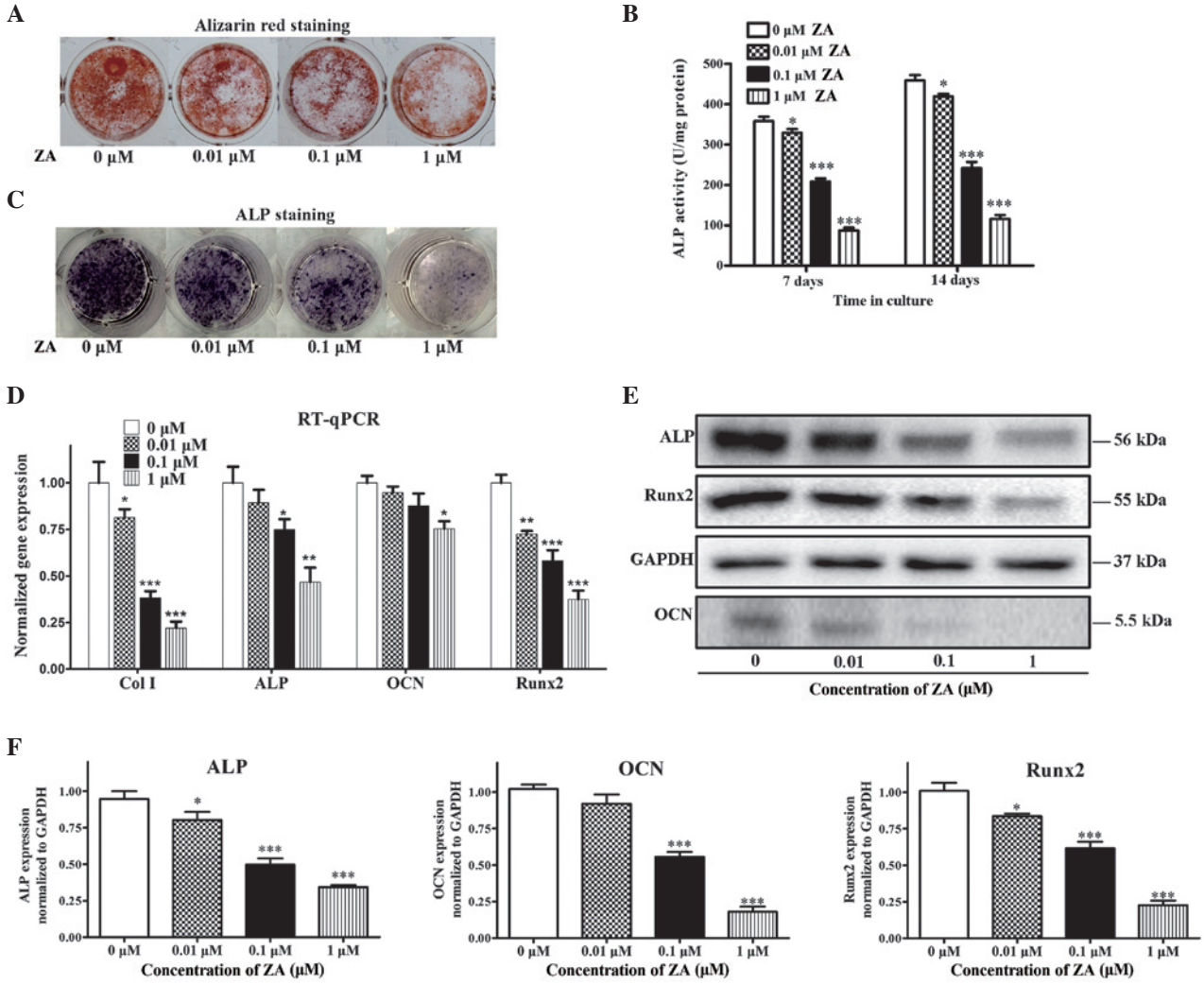

Figure 4. Effects of ZA on the differentiation and maturation of MC3T3-E1 cells. The MC3T3-E1 cells were treated with various concentrations of ZA (0-1 μM). (A) Alizarin red S staining following 21 days of culture. (B) ALP activity on days 7 and 14. (C) ALP staining on day 7. (D) Gene expression levels of Col I, ALP, OCN and Runx2 on day 7, determined using RT-qPCR analysis normalized to 18S. (E) Protein levels of ALP, OCN and Runx2 on day 7, determined using western blot analysis normalized to GAPDH. (F) Quantitative analysis of the blots for the ALP, OCN and Runx2 proteins. The results are expressed as the mean ± standard error of the mean (n=3 for each group). *P<0.05, **P<0.01 and ***P<0.001, compared with the 0 μM group. ZA, zoledronic acid; Col I, collagen type I; ALP, alkaline phosphatase; OCN, osteocalcin; Runx2, runt-related transcription factor 2; GAPDH, glyceraldehyde phosphate dehydrogenase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

exert inhibitory effects on the differentiation and maturation of MC3T3-E1 cells.

Effects of ZA on the expression of BMP-2 and phosphorylation of the ERK 1/2 and p38 pathways in MC3T3-E1 cells. Given the importance of BMP-2 in osteoblastic differentiation, the present study investigated whether ZA mediated the alteration of osteoblast differentiation through regulation of the expression of BMP-2. To confirm whether the expression levels of BMP-2 were affected by the presence of ZA, a BMP-2 ELISA kit was used. The results indicated that ZA (0.01, 0.1 and 1 μM) significantly decreased the protein levels of BMP-2, in a concentration-dependent manner, following 7 days of treatment (Fig. 5A). For RT-qPCR and western blot analyses, the cell extracts were collected 7 days following treatment of the MC3T3-E1 cells with vehicle or various concentrations of ZA. Dose-dependent decreases in the gene and protein expression levels of BMP-2 were detected at concentrations <1 μM, which were consistent with the results from the ELISA described above (Fig. 5B-D). Binding of BMP-2 to the BMP receptor induces receptor heterodimeric complexes and subsequently activates MAPKs by phosphorylation (14). The present study evaluated the activation of p38 and ERK1/2 in ZA-treated cells. Treatment with ZA did not affect the expression levels of unphosphorylated p38 or ERK 1/2, however decreases in levels of p-p38 and p-ERK 1/2 were observed following 7 days exposure of the MC3T3-E1 cells to ZA (Fig. 5C and D). Taken together, these results indicated that ZA suppressed cell maturation and differentiation of the MC3T3 cells in a BMP-2-dependent manner.

Discussion

Although several hypotheses with persuasive data have been put forward (30-32), the mechanism underlying BRONJ remains to be fully elucidated. Inhibition of osteoclasts, reduced angiogenesis and local infection may be involved, at least in part, in BRONJ, but cannot entirely explain the etiology (33).

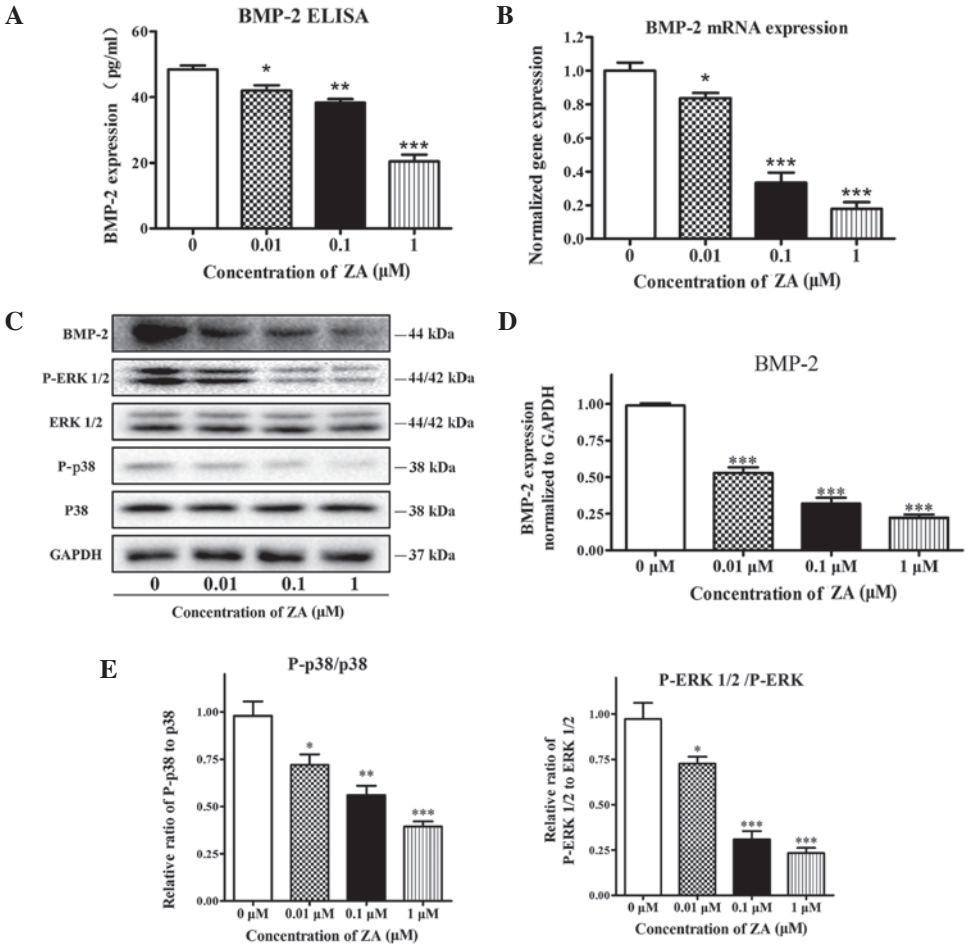

Figure 5. Effect of ZA on the expression of BMP-2 and phosphorylation of the ERK 1/2 and p38 pathways in MC3T3-E1 cells. The MC3T3-E1 cells were incubated with various concentrations of ZA (0-1 μM) for 7 days. (A) Levels of secreted BMP-2, measured using an ELISA. (B) mRNA expression levels of BMP-2, determined using reverse transcription-quantitative polymerase chain reaction analysis. (C) Protein levels of BMP-2, p38, p-p38, ERK 1/2 and p-ERK 1/2, detected using western blot analysis and normalized to GAPDH. (D) Quantitative analysis of the blots for BMP-2. (E) Relative expression of p-ERK, normalized to ERK, and relative expression of p-p38 normalized to p38. The results are expressed as the mean ± standard error of the mean (n=3 for each group). *P<0.05, **P<0.01 and ***P<0.001, compared with the 0 μM group. BMP-2, bone morphogenetic protein 2; ERK 1/2, extracellular signal-regulated kinase 1/2; p-, phosphorylated; ELISA, enzyme-linked immunosorbent assay.

Trauma of the jaw bone is considered to be the most common risk factor for BRONJ, and the majority of the BRONJ cases reported have occurred following trigger events, including dental extractions and dentoalveolar surgery (34). For this risk factor, a possible explanation is that the BPs, which are accumulated on the bones, are locally released due to surgery or other trauma, which directly affects the surrounding cells and leads to the development of BRONJ. Previous studies have suggested that patients receiving treatment with N-BPs may be at a higher risk of BRONJ, compared with those treated with non-N-BPs (11). Considering that ZA is the N-BP with the most potent pharmacological action, a high affinity for bone, and is the most commonly used BP for malignant diseases, ZA-associated BRONJ has attracted increasing attention. Raje *et al* (35) observed that, in multiple myeloma patients with BRONJ, intravenous injection of ZA resulted in inhibition of the osteogenic markers at the gene and protein levels. Compared with healthy subjects, downregulation of the genes involved in osteoblast differentiation was observed, regardless of the presence or absence of BRONJ, and was more marked in patients with BRONJ (35). Recker *et al* (36) reported that annual ZA injections may lead to inhibition of osteogenic markers. It can, therefore, be hypothesized that the direct effect of ZA on osteoblasts contributes to the development of BRONJ. This hypothesis is supported by the results of the present study, as higher concentrations of ZA caused cytotoxicity to osteoblasts and induced their apoptosis, and lower concentrations of ZA suppressed osteoblast differentiation by downregulating the level of BMP-2.

In the present study, it was observed that when ZA concentrations were >10 μM, cell viability decreased significantly and cell apoptosis increased significantly. At concentrations <1 μM, ZA appeared to have no effect on cell viability or apoptosis. This is consistent with the previous observations of Pozzi *et al* (13), Peter *et al* (37) and Orriss *et al* (38). However, other studies reported opposite conclusions. Bellido and Plotkin reported that, by promoting the expression of connexin 43, BPs, including ZA, preserve the viability of osteoblasts and osteocytes and inhibit their apoptosis (39). Von Knoch *et al* reported that 10 nM ZA stimulates human bone marrow stromal cell proliferation and viability (23). In a study by Im *et al* on alendronic acid, which is also a type of N-BP, it was found that alendronic acid promotes osteoblasts proliferation at concentrations <0.1 μM (40).

Col I, a major protein constituting the bone matrix, is excreted by osteoblasts and provides the backbone for the maturation and mineralization of the bone matrix (41). ALP is an early stage indicator for bone differentiation as it is vital in the calcification of bone matrix and is not expressed in undifferentiated precursor cells (42). OCN is an intermediate-late stage indicator of osteogenic differentiation, as it is excreted from the cells during mineralization of bone matrix, being involved in the formation of calcium hydroxyapatite (43). Runx2 is a transcription factor, which is essential for osteoblast differentiation and regulates the expression of bone matrix proteins, including OCN, Col I, osteopontin and bone sialoprotein (21). Runx2 gene mutation in mice results in dysosteogenesis of the clavicle and skull and significant defects in bone formation (44). Calcium deposition occurs in the late stage of osteogenic differentiation, and the mineralization potentiality can be evaluated by Alizarin Red S staining. In the present study, when the ZA concentration was ≥10 μM, the number and viability of osteoblasts were markedly reduced, making it not possible to observe the differentiation and mineralization potentiality of osteoblasts. In the present study, when the concentration of ZA was ≤1 μM, the expression levels of Col I, ALP, OCN and Runx2 were downregulated with increasing ZA concentrations, the ALP activity was suppressed, and the formation of calcium nodules was inhibited. It was concluded that ZA inhibits various levels of the cell differentiation process between the early and terminal stages, to inhibit the maturation and differentiation of MC3T3-E1 cells in a dose-dependent manner. These results are supported by the studies of Pozzi *et al* (13), Schindeler and Little (25), Orriss *et al* (38), and Idris *et al* (45). By contrast, Reinholz *et al* (46) suggested that, despite inhibiting osteoblast proliferation, ZA may promote their differentiation. Kellinsalmi *et al* (47) observed that ZA reduces calcium deposition in a dose-dependent manner, without interfering with osteoblast differentiation. Pan *et al* (24) reported that high concentrations (5-25 μM) of ZA increase mineral deposition of human bone-derived osteoblast-like cells, despite reductions in cell numbers due to cytotoxicity. These contradictions on the effects on cell viability and functions may be attributed to the experimental systems comprising different cell types and culture conditions.

BMP-2 is one of the most important extracellular signaling molecules stimulating bone formation and inducing osteoblasts differentiation. By stimulating osteoblasts differentiation, BMP-2 is important role in bone formation and bone remolding (48,49). BMPs exert their biological effects by binding to BMP receptors on the surface of cellular membrane. Among transgenic mice, in which the expression of BMP receptors in bone tissues was inhibited, the mice exhibited disorders of physical development, short figure, skeletal maldevelopment and reduced bone density. Bone morphometric investigation revealed rarefaction of trabecular bone and mineralization disorder in these mice (50). ERK 1/2 is important in the proliferation and differentiation of osteoblasts. Previous studies have suggested that ERK 1/2 is an important mediator in inducing osteoblast differentiation, and that inhibiting the activation of the ERK pathway may lead to the downregulation of osteogenic markers (51,52). In the differentiation process of osteoblasts, P38 contributes to the BMP-2-associated gene expression of Col I and OCN, and the regulation of ALP activity. It has been reported that BMP-2 may increase the activation and activity of P38, and inhibition of P38 may attenuate the role of BMP2 in stimulating osteogenic differentiation (17,18). BMP2 controls the activity of Runx2 through the ERK 1/2 and P38 pathways (53). In the present study, as ZA concentration increased, the expression of BMP-2 gradually reduced at the gene and protein levels and in the exocrine culture. In addition, decreased phosphorylation of the downstream ERK 1/2 and p38 pathways, and lower expression levels of the key transcription factor, Runx2 were observed. These results suggested that the dose-dependent inhibition of the expression of BMP2 may be important in the process of ZA inhibiting osteoblast differentiation.

The results of the present study led to the hypothesis regarding the possible pathogenesis of BRONJ that, following administration *in vivo*, ZA accumulates rapidly within the bone and the cell viability and differentiation of osteoblasts in the jaw are inhibited due to the continuous exposure. The dead osteocytes fail to be replaced by fully functioning osteoblasts, leading to impaired matrix mineralization and bone formation, which eventually leads to sequestrum with empty lacuna. The present study also hypothesized that, in the event of dental surgery or other trauma, ZA adhering to the hydroxyapatite is released either directly or due to enhanced bone resorption, which increases the concentration of ZA that the osteoblasts are exposed to. This change may aggravate suppression of osteoblasts activities and increase the incidence of BRONJ. It is noteworthy that the results of this *in vitro* investigation with ZA requires careful interpretation, as no consensus has been reached on the concentration at which ZA binds to bone matrix or the concentration of ZA to which bone cells are exposed.

In conclusion, the investigations performed in the present *in vitro* study demonstrated that ZA at higher concentrations induced cytotoxicity towards osteoblasts, and ZA at lower concentrations suppressed osteoblast differentiation by downregulating the expression of BMP-2. These negative effects of ZA on osteoblast activities may, at least partly, contribute clinically to the development and evolution of BRONJ.

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