1α,25-Dihydroxyvitamin D₃ inhibits the differentiation and bone resorption by osteoclasts generated from Wistar rat bone marrow-derived macrophages

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Abstract. The steroid hormone 1α,25-dihydroxyvitamin D₃ [1α,25-(OH)₂D₃] plays an important role in maintaining a balance in calcium and bone metabolism. To study the effects of 1α,25-(OH)₂D₃ on osteoclast (OC) formation and bone resorption, OC differentiation was induced in bone marrow-derived mononuclear cells from Wistar rats with the addition of macrophage colony stimulating factor and receptor activator for nuclear factor-κB ligand *in vitro*. Cells were then treated with 1α,25-(OH)₂D₃ at 10^{-9}, 10^{-8} or 10^{-7} mol/l. OCs were identified using tartrate-resistant acid phosphatase staining and activity was monitored in the absorption lacunae by scanning electron microscopy. Expression levels of functional proteins associated with bone absorption, namely carbonic anhydrase II, cathepsin K and matrix metalloproteinase-9 were evaluated by western blot analysis. The results showed that 1α,25-(OH)₂D₃ inhibited the formation and activation of OCs in a dose-dependent manner and downregulated the expression levels of bone absorption-associated proteins.

Introduction

Bone tissue is constantly renewed. Bone homeostasis is maintained with bone formation by osteoblasts (OBs) and osteoclast (OC)-driven bone resorption. Dysregulation of this process can result in metabolic bone diseases, including osteoporosis and osteopetrosis (1). OCs, which are terminally differentiated multinuclear cells originating from hematopoietic stem cells, are the only cells with bone resorption activity and they play an important role in bone turnover. The occurrence of bone metabolic diseases is closely associated with the formation and activation of OCs.

As the main active metabolite of vitamin D, 1α,25-dihydroxyvitamin D₃ [1α,25-(OH)₂D₃] displays strong bioactivity, regulating calcium and phosphorus metabolism and assisting in the maintenance of calcium homeostasis (2). 1α,25-(OH)₂D₃ is involved in the regulation of bone metabolism, not only through a receptor on the small intestine and kidneys but also by acting on osteocytes directly (3). 1α,25-(OH)₂D₃ enhances expression of the receptor activator for nuclear factor-κB ligand (RANKL), which can regulate and control bone resorption and the formation of OCs indirectly by acting on the vitamin D receptor (VDR) on the surface of OBs. The VDR has been identified on OC precursor cells, and Kogawa *et al* (4) reported that 1α,25-(OH)₂D₃ induces OC precursor cells to differentiate into mature OCs that display bone absorption activity. In contrast to these findings, Uchiyama *et al* (5) have demonstrated that pharmacological doses of active vitamin D₃ suppress bone resorption. This discrepancy in the effects of vitamin D on bones requires further exploration.

In the present study, OC differentiation was induced *in vitro* by the treatment of Wistar rat bone marrow-derived macrophage cells with 25 µg/l macrophage-colony stimulating factor (M-CSF) and 45 µg/l RANKL. OCs were treated with 1α,25-(OH)₂D₃ at concentrations of 0, 10^{-9}, 10^{-8} or 10^{-7} mol/l, or with ethyl alcohol as solvent control. To confirm OC differentiation, cells were stained with tartrate resistant acid phosphatase (TRAP), and examination of the absorption lacuna was conducted to study the effects of 1α,25-(OH)₂D₃ on the bone absorption activity of OCs. Additionally, the expression levels of proteins involved in bone absorption were measured by western blotting.

Materials and methods

Experimental animals. Three-week-old male Wistar rats of clean grade were provided by the Comparative Medical Center of Yangzhou University (Yangzhou, China). This study was

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conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. The animal care and use committee of Yangzhou University approved all experiments and procedures conducted on the animals (approval ID: SYXK (Su) 2007-0005).

Reagents and instruments. α Minimum essential medium (α-MEM) was obtained from Gibco Life Technologies (Carlsbad, CA, USA). Penicillin and streptomycin were purchased from Shandong Lukang Pharmaceutical Ltd. (Shandong, China). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), and M-CSF & RANKL from PeproTech Inc. (Rocky Hill, NJ, USA). 1α,25-(OH)₂D₃ and the TRAP staining kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). Carbonic anhydrase II (CA II; ab6621), cathepsin K (CK; ab19027) and matrix metalloproteinase-9 (MMP-9; ab137867) antibodies were purchased from Abcam (Cambridge, UK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; FL-335) antibodies were purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA), while bovine cortical slices (50 μm thick; 1,600 cryo-cut microtomes) were obtained from Leica Microsystems GmbH (Wetzlar, Germany). Other reagents used in this study were of analytical grade and made in China.

Instruments used in this study included an inverted phase contrast microscope (DMI-3000B; Leica Microsystems GmbH), an environmental scanning electron microscope (XL-ESEM; Philips, Amsterdam, Netherlands) and an ultrasonic wave cleaner (KQ-250; Kunshan Ultrasonic Instruments Co., Ltd., Kunshan, China).

Osteoclastogenesis in vitro. For osteoclastogenesis from rat bone-marrow-derived precursor cells, 3-week-old male Wistar rats were used. Animal experimental protocols were approved by the Committee on the Care and Use of Animals in Research at Yangzhou University. A total of 3 rats were used per experiment. Whole bone marrow cells, isolated by flushing the marrow space of femurs and humerus and centrifuging the resulting suspension at 500 x g for 5 min, were resuspended in α-MEM containing 100 U/ml penicillin and 100 μg/ml streptomycin. Following the removal of erythrocytes by lysing in buffer (0.15 M NH₄Cl, 1 mM KHCO₃ and 0.1 mM ethylene diamine tetraacetic acid, pH 7.2), the bone-marrow cells were plated on 100-mm culture dishes and cultured in α-MEM supplemented with 10% FBS for 24 h in 5% CO₂ at 37°C. Non-adherent cells were collected, plated on 100-mm bacterial dishes and cultured for 3 days in the presence of 25 ng/ml M-CSF. The adherent cells were considered to be bone marrow-derived macrophages and were used as OC precursor cells. The macrophages were seeded on 48-well plates at 4.0x10⁵ cells/ml and cultured with 25 μg/l M-CSF and 45 μg/l RANKL at 37°C in 5% CO₂ for 3 days. Fresh medium was then added and subsequently changed every 2 days.

TRAP staining and OC counting. Bone marrow-derived macrophage cells were incubated in 48-well plates at a density of 4.0x10⁵ cells/ml (0.5 ml/well) with 25 μg/l M-CSF and 45 μg/l RANKL and different five groups were created as follows: Group A, control; group B, 10⁻⁹ mol/l 1α,25-(OH)₂D₃; group C,

10⁻⁸ mol/l 1α,25-(OH)₂D₃; group D, 10⁻⁷ mol/l 1α,25-(OH)₂D₃; group E, 0.1% ethyl alcohol (solvent control). Following 8 days of culture, the medium was removed, the plates were rinsed with phosphate-buffered saline (PBS) and the cells fixed with 4% paraformaldehyde for 20 min prior to staining with the TRAP staining kit according to the manufacturer's instructions. Ten random fields were selected for examination with an inverted microscope and the red-stained multinucleated cells containing ≥3 nuclei were counted.

F-actin staining. Cells used for F-actin staining were prepared as previously described (6), with the exception that the density of plated cells was increased to 4.0x10⁵ cells/ml (1 ml/well). After 8 days of culture, the plates were rinsed three times with PBS. Cells were fixed with 4% paraformaldehyde for 20 min then rinsed three times with PBS and incubated with Triton X-100 (0.5% v/v) for 20 min. They were again rinsed three times with PBS and treated with 5% bovine serum albumin for 30 min. Finally, 20 μmol/l phalloidin-tetramethylrhodamine isothiocyanate (TRITC; Invitrogen Life Technologies, Carlsbad, CA, USA) was added to the plates according to the manufacturer's instructions and incubated for 120 min in the dark. The OCs were observed by fluorescence microscopy (DMI3000B; Leica, Germany) and images captured under the red channel.

Lacunar absorption analysis. The OC precursor cells were collected and incubated following a procedure similar to that described in previous sections with the exception that sterilized bovine cortical slices were placed at the bottom of 48-well plates prior to incubation. To remove the cells attached to the osteocomma and absorption lacuna, bovine cortical slices were removed at day 9 and rinsed three times with PBS. Bone slices were treated with an ultrasonic wave cleaner in 0.25 mol/l ammonium hydroxide three times (5 min each time). Following dehydration in a graded ethanol series of 40, 70, 80, 95 and 100% (v/v), the bone slices were air-dried and gilded using an ion-plating apparatus (SCD500 Sputter Coater; Leica Microsystems GmbH) which was followed by observation with an XL30-ESEM scanning electron microscope. The area of lacunar absorption was measured using a JD801 image analysis system (Nanjing University, Nanjing, China).

Western blot analysis. Western blot analysis was carried out as described previously with minimum alterations (7). The OC precursor cells were collected and incubated in 100-mm cell culture plates. After 8 days of culture, cells were collected and total protein extracted using the Cell Total Protein Extraction kit (Applygen Technologies, Inc., Beijing, China). Protein concentration was determined using a Bicinchoninic Acid Protein kit and samples adjusted to a similar concentration. Samples were heated for 10 min at 100°C for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A total of 60 μg protein was loaded in each lane for 10-15% SDS-PAGE and run with a constant voltage of 120 V for 120 min. Proteins were transferred to nitrocellulose membranes with a constant voltage of 120 V for 90 min. The membrane was blocked at room temperature for 2 h with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 (TBST), and then incubated for 12 h at 4°C
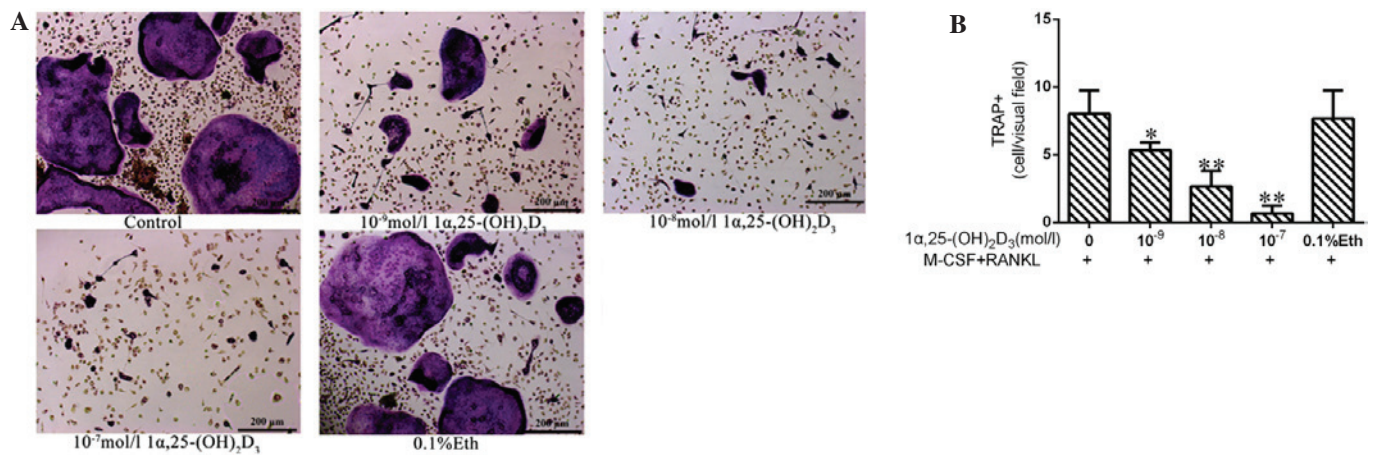
Figure 1. 1α,25-(OH)₂D₃ inhibits the differentiation of bone marrow-derived mononuclear cells to osteoclasts *in vitro*. (A) Red-stained multinucleated cells following treatment with different concentrations of 1α,25-(OH)₂D₃. Scale bars, 200 µm. (B) Numbers of osteoclasts with ≥3 nuclei from 10 random fields of view were counted. Results are expressed as mean ± standard deviation. *P<0.05, **P<0.01 vs. the control. 1α,25-(OH)₂D₃, 1α,25-dihydroxyvitamin D3; TRAP, tartrate-resistant acid phosphatase; M-CSF, macrophage-colony stimulating factor; RANKL, receptor activator for nuclear factor-κB ligand; Eth, ethanol.
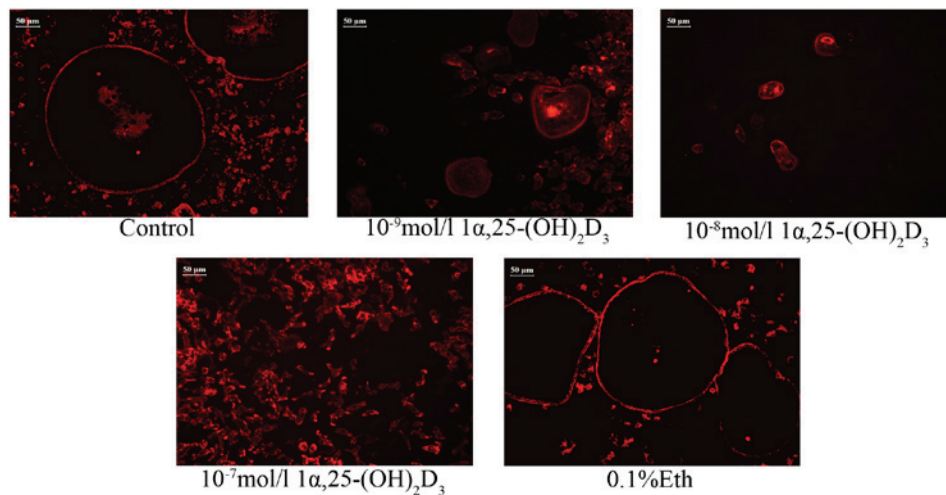

Figure 2. 1α,25-(OH)₂D₃ inhibits cytoskeleton formation. Fluorescent microscopy reveals that 1α,25-(OH)₂D₃ inhibits the formation of the cytoskeleton in osteoclasts differentiated from bone marrow mononuclear cells. Scale bar, 50 µm. 1α,25-(OH)₂D₃, 1α,25-dihydroxyvitamin D3; Eth, ethanol.

with the primary antibodies: Rabbit anti-mouse CA II antibody (1:3,000); rabbit anti-mouse MMP-9 (1:1,000); rabbit anti-mouse CK (1:500) and anti-GAPDH as an internal reference. The membranes were rinsed six times with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G fraction polyclonal antibody (1:5,000) at room temperature for 2 h. Following additional washes, the membranes were visualized using an ECL detection kit (Merck Millipore, Billerica, MA, USA) according to the manufacturer's instructions, and exposed to X-ray film. Analyses were performed to determine the intensity of western blot bands using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. Data were analyzed with SPSS t-test software (version 19.0; IBM SPSS, Armonk, NY, USA) for significance analysis and are expressed as mean ± standard deviation. P-values <0.05 and <0.01 were considered to indicate results that were statistically significant and highly statistically significant, respectively.

Results

Effects of 1α,25-(OH)₂D₃ on OC formation. In the groups treated with M-CSF and RANKL, TRAP-positive multinucleated cells were observed by day 8 (Fig. 1). The solvent (ethyl alcohol)-treated group (group E; 7.7±2.08 cells/field) exhibited no apparent difference in cells/field as compared with group A (8.0±1.3 cells/field). 1α, 25-(OH)₂D₃ inhibited the formation of OCs significantly. The numbers of OCs in groups B (10⁻⁹ mol/l 1α,25-(OH)₂D₃), C (10⁻⁸ mol/l 1α,25-(OH)₂D₃) and D (10⁻⁷ mol/l 1α,25-(OH)₂D₃) were 5.3±0.58, 2.7±1.15 and 0.7±0.56 cells/field, respectively (*P<0.05 or **P<0.01). The results indicated that 1α,25-(OH)₂D₃ had inhibitory effects on OC formation.

Effects of 1α,25-(OH)₂D₃ on OC F-actin-based cytoskeletons. OCs isolated from rat bone marrow-derived macrophage cells and incubated with M-CSF and RANKL had a regular, clear, red and rich F-actin-based cytoskeleton (Fig. 2). OCs in the solvent-treated group (group E) had intense and highly aligned F-actin, which exhibited no apparent difference compared with
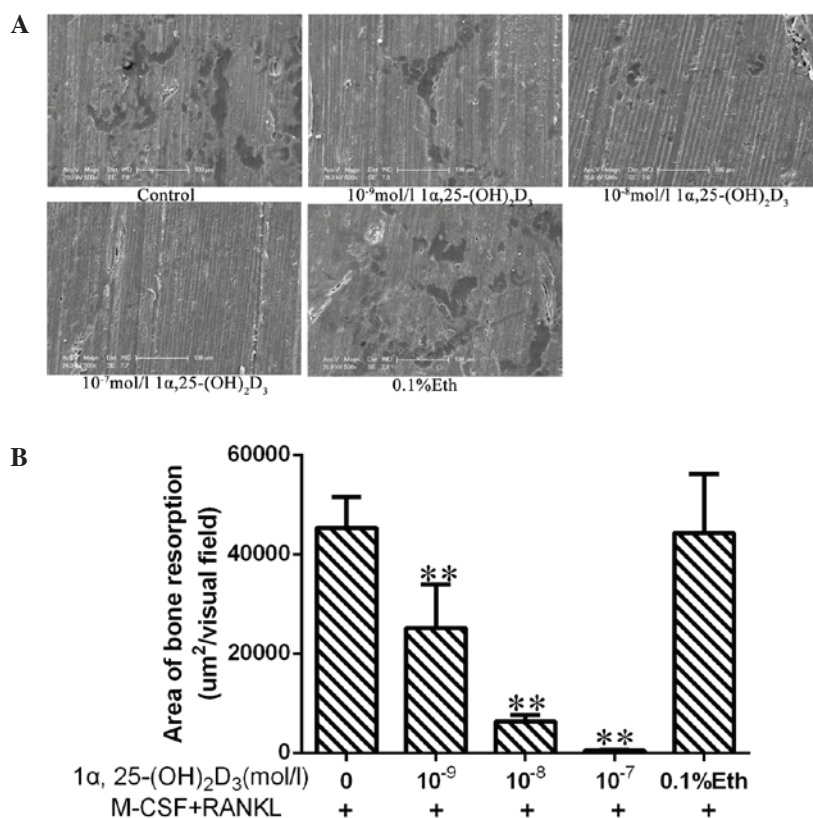
A

B

Figure 3. 1α,25-(OH)$_2$D$_3$ inhibits the bone resorption activity of osteoclasts. (A) Bone absorption lacunae visualized by scanning electron microscopy (scale bars, 100 μm). (B) Five visual fields from each group were randomly selected and the area of bone absorption lacunae measured by image analysis. The results are expressed as mean ± standard deviation. **P<0.01 vs. control. 1α,25-(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D3; M-CSF, macrophage-colony stimulating factor; RANKL, receptor activator for nuclear factor-κB ligand; Eth, ethanol.

control group A (M-CSF and RANKL only). Following treatment with 1α,25-(OH)$_2$D$_3$, the distribution of F-actin changed and the levels of expression decreased. The changes were particularly apparent in group D (10^{-7} mol/l 1α,25-(OH)$_2$D$_3$), in which the cytoskeleton could barely be observed. The results show that 1α,25-(OH)$_2$D$_3$) inhibits the formation of the cytoskeleton in OCs.

Effects of 1α,25-(OH)$_2$D$_3$ on OC bone resorption. Bone marrow-derived macrophage cells incubated with M-CSF and RANKL differentiated into OCs, which exhibited bone absorption activity. Absorption lacunae were observed on bovine cortical slices following 8 days of culture (Fig. 3A). The lacunae in bone in the solvent-treated group (group E) were no different to those in control group A. By contrast, 1α,25-(OH)$_2$D$_3$ inhibited the formation of lacunae.

The area of the lacunae in the groups treated with 1α,25-(OH)$_2$D$_3$ was smaller than those in the control group, with the areas of the lacunae in groups B, C and D measured as 25,174±8,754, 6,336±1,346 and 514±169 μm^2/visual field, respectively (Fig. 3B). The results showed that 1α,25-(OH)$_2$D$_3$ reduced the area of lacunae in a dose-dependent manner and inhibited the bone resorption activity of OCs.

1α,25-(OH)$_2$D$_3$ regulates the expression of proteins associated with bone absorption. OCs that had differentiated from bone marrow-derived macrophage cells after treatment with M-CSF and RANKL expressed CA II, CK and MMP-9, as

shown by western blotting (Fig. 4). The expression levels of these proteins in group E (solvent control) and group A were not significantly different. By contrast, the expression levels of CA II, CK and MMP-9 decreased significantly in the groups treated with 1α,25-(OH)$_2$D$_3$ (*P<0.05 or **P<0.01).

Discussion

OCs are terminally differentiated multinuclear cells. As the only type of cells displaying bone absorption activity, OCs play an important role in bone turnover (8). As it is demanding to isolate, purify and culture OCs *in vitro*, and subcultures cannot be established, the study of OCs is challenging. Currently, RAW264.7 cells, which are OC precursors (9), are the main cells used to obtain OCs through differentiation. The RAW264.7 cell line was established from a tumor induced by Abelson murine leukemia virus; therefore, biological discrepancies may exist and results obtained using this cell line may be controversial (10). In the present study, OCs obtained from bone marrow-derived mononuclear cells isolated from Wistar rats and differentiated by M-CSF and RANKL treatment displayed typical characteristics of OCs, such as TRAP-positive staining and bone absorption activity. The biological features of OCs obtained in this study are similar to those of OCs *in vivo*, indicating that the method can be used to obtain results that are accurate and relevant.

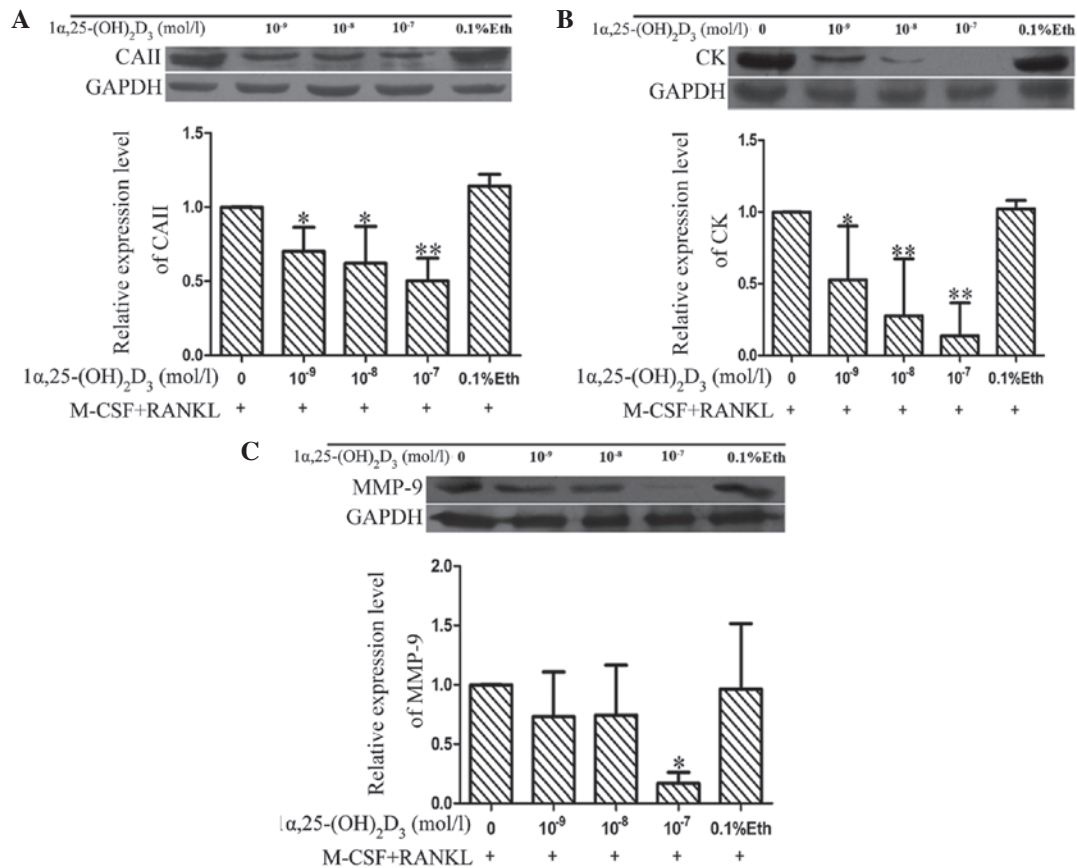Vitamin D is important in calcium homeostasis and bone metabolism, although differences in the regulation of OCs

Figure 4. 1α,25-(OH)$_2$D$_3$ reduces the expression of (A) CA II, (B) cathepsin K and (C) MMP-9. Results are expressed as mean ± standard deviation. *P<0.05, **P<0.01 vs. the control. 1α,25-(OH)$_2$D$_3$, 1α,25-dihydroxyvitamin D3; CA II, carbonic anhydrase II; CK, cathepsin K; MMP, matrix metalloproteinase; M-CSF, macrophage-colony stimulating factor; RANKL, receptor activator for nuclear factor-κB ligand; Eth, ethanol.

cultured *in vitro* or *in vivo* have been reported (10). Certain studies have shown that 1α,25-(OH)$_2$D$_3$ inhibits the formation of OCs from mouse monocyte-macrophage cells (11,12), while Medhora *et al* (13) reported enhanced OC formation and increases in the expression of integrin β3 when 1α,25-(OH)$_2$D$_3$ was added in an early study of an OC culture model *in vitro* (14). Vitamin D was initially identified as being useful for the treatment of rickets. A lack of vitamin D can lead to the onset of rickets in younger animals and chondropathy in adult animals (15). The present study showed that different concentrations of 1α,25-(OH)$_2$D$_3$ may inhibit the formation and activity of OCs, as well as downregulating the expression levels of CA II, CK and MMP-9. These results are similar to those reported by Arai *et al* (16) who found that colony-stimulating factor-1 (c-Fms) was the key signaling molecule controlling RANK expression and 1α,25-(OH)$_2$D$_3$ inhibited OC formation by downregulating the expression of c-Fms to reduce RANK expression.

Due to the ease of use, TRAP staining was used to identify OCs in the present study. TRAP has the highest expression levels in normal bone tissue, and is not only regarded as a marker of OCs, but also participates in bone absorption and the maturation of OCs (17). In the present study, TRAP staining demonstrated that bone marrow-derived mononuclear cells from Wistar rats may be induced to differentiate into OCs with M-CSF and RANKL treatment. The OCs had typical features, including red particles in the cytoplasm and >3 nuclei,

following staining with TRAP. Compared with the control group, different concentrations of 1α,25-(OH)$_2$D$_3$ inhibited OC formation in a dose-dependent manner. OCs displayed F-actin-based cytoskeletons, which appeared red with clear-cut edges following staining with phalloidin-TRITC. Different concentrations of 1α,25-(OH)$_2$D$_3$ inhibited the formation of the F-actin-based cytoskeleton, findings that are similar to those from Kim *et al* (18). The results of bone resorption lacunae analysis showed that the area of lacuna decreased following treatment with 1α,25-(OH)$_2$D$_3$ in a dose-dependent manner. Together, the results show that 1α,25-(OH)$_2$D$_3$ reduces the formation of OCs from Wistar rat bone marrow-derived mononuclear cells *in vitro*, and inhibits the activity of bone absorption.

It is well known that CA II, CK and MMP-9 proteins are involved in the OC bone resorption process (19). CA II, a metalloenzyme whose active site contains Zn^{2+}, participates in numerous important physiological changes in the body. It has a critical regulatory function in bone resorption: Not only does it control the secretion of H$^+$, but it can also affect OC differentiation and movement, and plays a role in changing intracellular Ca^{2+} concentration and pH in absorption lacunae (20). The main function of CA II in OC resorption is to catalyze the reaction of CO$_2$ and H$_2$O to form H$^+$; H$^+$ secretion into the resorption cavity is important for OC activity in bone resorption. The acidic environment dissolves bone hydroxypropyl phosphate and OCs can further degrade bone matrix

components, mainly through the secretion of cathepsins and MMPs, a process in which CK and MMP-9 play significant roles (21). CK, abundantly expressed in OC, is a lysosomal cysteine protease that can degrade protein components of the demineralized bone matrix, including degradation of bone matrix of type I collagen, osteopontin and osteonectin. Type I collagen protein is the main component of the bone matrix and can be degraded by CK at multiple sites (22). MMP-9 is a type of zinc-dependent protease which has a highly conserved structure and is important in the early stages of OC differentiation and bone resorption (23). In the present study, OCs were obtained from bone marrow-derived mononuclear cells of rats by inducing differentiation via treatment with M-CSF and RANKL. OCs express high levels of CA II, CK and MMP-9. Treatment with 1α,25-(OH)$_2$D$_3$ inhibited the expression levels of CA II and CK proteins significantly. There was no significant effect on the expression levels of MMP-9 when treated with 10^{-9} or 10^{-8} mol/l 1α,25-(OH)$_2$D$_3$; however, at 10^{-7} mol/l 1α,25-(OH)$_2$D$_3$ inhibited the expression of MMP-9 significantly. The results demonstrated that 1α,25-(OH)$_2$D$_3$ is able to downregulate the expression of CA II, which reduces calcium salt-induced dissolution of bone tissue by decreasing the concentration of H$^+$ in the sealing zone and inhibiting the secretion of CK and MMP-9 proteins to prevent the degradation of bone matrix proteins during bone resorption. Ultimately, this inhibits the bone resorption activity of OCs.

In conclusion, within the 10^{-9} to 10^{-7} mol/l concentration range, 1α,25-(OH)$_2$D$_3$ inhibits OC formation from rat bone marrow-derived mononuclear cells, and inhibits bone resorption activity by downregulating expression levels of CA II, CK and MMP-9. These results indicated that 1α,25-(OH)$_2$D$_3$ can directly regulate bone metabolism by inhibiting the formation and maturation of osteoclasts.

Acknowledgements

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