Effects of alendronate on the Notch1-RBP-Jκ signaling pathway in the osteogenic differentiation and mineralization of vascular smooth muscle cells

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Abstract. Cardiovascular disease and osteoporosis are major causes of mortality in the elderly. Alendronate, a bisphosphonate, is widely used in the treatment of osteoporosis and may be used to inhibit vascular calcification. However, its mechanisms are not completely understood. The present study aimed to explore novel signaling mechanisms behind the action of alendronate in the osteoblastic differentiation of rat aortic vascular smooth muscle cells (VSMCs). The osteoblastic differentiation of VSMCs was induced by an osteogenic medium. Using von Kossa staining and direct cellular calcium content determination, mineralization was found to be significantly increased in VSMCs induced with osteogenic medium, consistent with an enhanced alkaline phosphatase activity. Osteoblastic differentiation in VSMCs was significantly reduced by the action of alendronate in a dose-dependent manner. In addition, the expression of Notch1 and RBP-Jk was significantly upregulated in VSMCs cultured with osteogenic medium at the mRNA and protein levels. The effects of Notch1-RBP-Jk were inhibited by treatment with alendronate in a dose-dependent manner. In summary, results of the current study indicate that alendronate inhibits vascular calcification through downregulation of the Notch1-RBP-Jĸ signaling pathway.

Introduction

Cardiovascular disease (CVD) is a major cause of premature mortality, accounting for approximately one-third of mortalities (1,2). Each year, 17 million individuals succumb to CVD (3-5). Osteoporosis is a serious public health problem with an estimated worldwide incidence of >200 million (4,6). CVD and osteoporosis are the main causes of mortality in the elderly (7,8). Vascular calcification, as an independent risk factor for CVD, was originally considered to be a passive and unregulated process; however, this process is now known to be an active and tightly regulated phenomenon, in which a variety of osteogenic regulatory factors are involved (9,10). Clinical studies have demonstrated that the majority of patients with osteoporosis have vascular calcification (11), and that vascular calcification is alleviated following treatment with bisphosphonates (3,12-14). These studies are indicative of a correlation between arterial and bone pathologies (15).

Alendronate, a bisphosphonate, is widely used as a treatment for osteoporosis and other pathological conditions associated with bone loss (16-18). In addition, a number of studies have shown that alendronate is not only suitable for the treatment of the aforementioned diseases, but also for vascular calcification (3). Analysis of the mechanism by which alendronate inhibits vascular calcification is likely to clarify the regulatory mechanisms involved in arterial and bone pathologies.

Notch1 signaling plays a crucial role in cell fate determination and various developmental processes, and translates cell-cell interactions into specific transcriptional programs (19-23). In the transcriptional process, cell-cell interactions cause cleavage of the Notch intracellular domain (NICD), which migrates into the nucleus and associates with RBP-JK, further activating the transcription of target genes (22,23). Msx2, the target gene activated by the Notch1-RBP-JK signaling pathway, is considered to represent a key regulator of vascular calcification and has been identified as a homeodomain transcription factor responsible for osteoblast differentiation and mineralization (9,22,24,25).

However, to date, a limited number of studies have focused on the effect of alendronate on the expression of the Notch1-RBP-J κ signaling pathway. In the present study, an *in vitro* rat model of vascular calcification was used to determine the effect of alendronate on expression of the Notch1-RBP-J κ signaling pathway, and the possible mechanisms behind the inhibition of artery calcification by alendronate were explored.

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Materials and methods

Cell culture. Primary vascular smooth muscle cells (VSMCs) were isolated from the thoracic aortas of Sprague-Dawley rats (4 weeks old; male; Animal Center, Tongji Medical College, Wuhan, China), maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin and incubated in 75-cm² tissue culture flasks at a density of 1x10⁴ cells/ml. VSMCs at passages 5 and 6 were used for subsequent experiments. The study was approved by the ethics committee of the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

Osteoblastic differentiation was induced by culturing cells in osteogenic medium containing 50 mg/ml ascorbate-2-phosphate and 10 mmol/l β-glycerol phosphate. DMEM was used as a control. N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenyl-glycinet-butyl ester (DAPT; 10 mmol/l), a potent y-secretase inhibitor, was used as a positive control for inhibition of the Notch1-RBP-Jk-dependent signaling pathway (26). VSMCs were also cultured in the presence of various concentrations of alendronate $(10^{-8}, 10^{-6}, 10^{-4} \text{ and } 10^{-3} \text{ mmol/l})$. After 14 days, cultured VSMCs were subjected to the following experimental conditions in 7 groups: the control (VSMCs cultured with DMEM); osteogenic (OS; VSMCs cultured with osteogenic medium); and DAPT groups (VSMCs cultured with OS and DAPT); and the alendronate groups (VSMCs cultured with OS and alendronate), which were divided into four subgroups based on varying concentrations of alendronate (10⁻⁸, 10⁻⁶, 10⁻⁴) and 10⁻³ mmol/l). The medium was replaced with fresh medium every 2 days. For time-course experiments, the first day of culture in calcification medium was defined as day 0.

von Kossa staining. Cells in culture flasks were washed 3 times with PBS, followed by a fixation with 4% paraformaldehyde for 15 min. Next, cells were washed 3 times with distilled water, incubated with 5% silver nitrate solution and exposed to bright sunlight for 30 min, then washed with distilled water for 5 min and treated with 5% sodium thiosulfate for 2 min. Calcium particles were observed by microscope in visual fields (magnification, x40).

Measurement of calcium content. Cultures were decalcified with 0.6 mol/l HCl for 24 h. The calcium content of the HCl supernatant was determined by the o-cresolphthalein complexone method (calcium kit; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Following decalcification, the cells were washed with PBS and solubilized with 0.1 mol/l NaOH-0.1% SDS. The cells were dissolved in HNO₃ and then dried in an oven and redissolved with blank solution (27 nmol/l KCl and 27 μ mol/l LaCl₃ in deionized water). Calcium content was measured using an atomic absorption spectrophotometer (SpectrAA-240 FS; Agilent Technologies, Santa Clara, CA, USA) at 422.7 nm.

Alkaline phosphatase (ALP) assay. ALP activity of various cells was measured using a Lab Assay ALP kit (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions.

Real-time quantitative PCR. Total RNA was isolated from the VSMCs using the TRIzol chloroform method, according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA), and reverse transcribed into cDNA with a Toyoba reverse transcription kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Real-time quantitative PCR was performed using the ABI PRISM 7900 sequence detector system (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. β -actin was used as an endogenous control. The PCR mixture contained SYBR Green/Fluorescein qPCR Master Mix (2X; Thermo Fisher Scientific Inc.), cDNA and the primers. Relative gene expression levels (the amount of target, normalized against an endogenous control gene) were calculated using the comparative Ct method formula, $2^{-\Delta\Delta Ct}$. The following primer sequences were used for real-time quantitative PCR: rat Notch1 forward, 5'-GAGGCTTGAGATGCTCCCAG-3' and reverse, 5'-ATTCTTACATGGTGTGCTGAGG-3'; rat RBP-Jk forward, 5'-GAGCCATTCTCAGAGCCAAC-3' and reverse, 5'-TCCCCAAGAAACCACAAAAG-3'; rat msx2 forward, 5'-AAGGCAAAAAGACTGCAGGA-3' and reverse, 5'-GGATGGGAAGCACAGGTCTA-3'; and β-actin forward, 5'-CACGATGGAGGGGCCGGACTCATC-3' and reverse, 5'-TAAAGACCTCTATGCCAACACAGT-3'

Western blot analysis. Total protein was extracted from cultured VSMCs in radio immunoprecipitation assay buffer containing 50 mmol/l Tris, 150 mmol/l NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton X-100 in the presence of aprotinin, PMSF, okadaic acid and leupeptin. Total protein (50 µg/sample) was loaded onto 12% SDS-polyacrylamide gels and separated at 100 V, followed by transfer to PVDF membranes at 200 mA and 4°C for 70 and 110 min for Notch1 and Msx2, and RBP-Jk signals, respectively. Membranes were blocked in 5% non-fat milk in 0.1 mol/l PBS (pH 7.4) at room temperature for 2 h and then incubated with the following primary antibodies: goat anti-Notch1 polyclonal (1:400), rabbit anti-RBP-Jk polyclonal (1:300) or goat anti-Msx2 polyclonal (1:400; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following washing, membranes were incubated in HRP-conjugated rabbit anti-goat or goat anti-rabbit secondary antibodies (1:40,000; Wuhan Boster Biological Technology Ltd., Wuhan, Hubei, China) for 2 h at room temperature, followed by washing and 5 min incubation with enhanced chemiluminescence reagents. The membranes were stripped and equal protein loading was determined by GAPDH expression using a mouse monoclonal antibody (1:75,000; GoodHere Biological Technology, Ltd., Hangzhou, China).

Statistical analysis. Data are presented as the mean \pm SEM. Significant differences were estimated by ANOVA followed by Student-Newman-Keuls multiple comparison tests. P \leq 0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA).

Results

von Kossa staining for VSMC calcification. To determine the calcification of VSMCs, von Kossa staining was performed



Figure 1. von Kossa stain of VSMCs. Cells were cultured in various media for 14 days. (A) Control cultured with DMEM medium; (B) osteogenic cultured with osteogenic medium; (C-F) alendronate groups treated with various concentrations of alendronate, (C) 10⁻⁸, (D) 10⁻⁶, (E) 10⁻⁴ and (F) 10⁻³ mmol/l; and (G) DAPT group treated with DAPT. von Kossa staining was negative when VMSCs were cultured in the control group, and was positive when VSMCs were cultured in the osteogenic group. von Kossa staining was weakly positive when VSMCs were treated with DAPT and various concentrations of alendronate (magnification, x40). VSMC, vascular smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenyl-glycinet-butyl ester.



Figure 2. ALP activity and calcium content in VSMCs. (A) ALP activity and (B) calcium content were significantly increased in VSMCs treated with osteogenic media compared with the control group, confirming an osteogenic conversion in the VSMCs. Enhanced ALP activity and calcium content induced by osteogenic medium were repressed significantly following the addition of DAPT, and were also significantly reduced by alendronate in a dose-dependent manner, reaching a minimum at 10^{-4} mmol/l alendronate. *P<0.05 vs. control; #P<0.05 vs. osteogenic medium; *P<0.05 vs. 10⁻⁴ mmol/l alendronate (n=3). ALP, alkaline phosphatase; VSMC, vascular smooth muscle cells; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenyl-glycinet-butyl ester.

and the results showed that no calcification was detected in the control group (Fig. 1A), while a marked positive staining (black) was detected in VSMCs cultured in osteogenic medium (Fig. 1B), indicating increased calcification in VSMCs. Increased calcification was eradicated significantly by DAPT treatment (Fig. 1G). The calcification of VSMCs was markedly reduced in the presence of alendronate in a dose-dependent manner (Fig. 1C-F) compared with those in osteogenic medium only (Fig. 1B), indicating that alendronate and DAPT repressed the calcification of VSMCs induced by osteogenic media.

ALP activity and calcium content of VSMCs. To further determine the calcification of VSMCs, ALP activity and calcium content were analyzed. The results showed that ALP activity (Fig. 2A) and calcium content (Fig. 2B) were significantly increased in VSMCs treated with osteogenic media (P<0.05 vs. control). Again, the osteogenic conversion of VSMCs was repressed by DAPT and reduced in the presence of alendronate in a dose-dependent manner (P<0.01 vs. the osteogenic group). ALP activity and calcium content reached minimum levels at 10^{-4} mmol/l alendronate (P<0.05 vs. the osteogenic group; Fig. 2).

Alendronate downregulates the Notch1-RBP-J κ -dependent signaling pathway. mRNA and protein levels of Notch1 (Fig. 3A and B) and RBP-J κ (Fig. 3C and D) in VSMCs were measured by real-time RT-PCR and western blot analysis. As shown in Fig. 3, Notch1 and RBP-J κ were significantly increased at the mRNA and protein levels in the osteogenic group compared with controls (P<0.05), indicating that the Notch1-RBP-J κ signaling pathway was activated in the osteogenic conversion of VSMCs.

To determine the effects of alendronate on expression of the Notch1-RBP-J κ signaling pathway *in vitro*, four concentrations (10⁻⁸, 10⁻⁶, 10⁻⁴ and 10⁻³ mmol/l) of alendronate were added to osteogenic VSMCs and DAPT was added as a positive control for inhibition of the Notch1-RBP-J κ signaling pathway. The results showed that alendronate reduced the expression of Notch1 and RBP-J κ compared with the osteogenic media group in a dose-dependent manner, and the expression of Notch1 and RBP-J κ reached minimum levels at a dose of



Figure 3. Expression of Notch1 and RBP-J κ in VSMCs. mRNA levels of (A) Notch1 and (C) RBP-J κ were measured by real-time RT-PCR in VSMCs cultured in various media. Protein levels of (B) Notch and (D) RBP-J κ were determined by western blot analysis in VSMCs cultured in various media. Notch1 and RBP-J κ levels significantly increased in VSMCs cultured in osteogenic medium compared with the control group at the mRNA and protein levels. DAPT reduced the expression of Notch1 and RBP-J κ in VSMCs at the mRNA and protein levels compared with that in the osteogenic group. Similarly, alendronate reduced the expression of Notch1 and RBP-J κ compared with VSMCs in the osteogenic medium in a dose-dependent manner, and reached the minimum at 10⁻⁴ mmol/l alendronate. *P<0.05 vs. control; #P<0.05 vs. osteogenic medium; \$P<0.05 vs. alendronate 10⁻⁴ mmol/l (n=3). VSMC, vascular smooth muscle cells; DAPT, N-[N-(3,5-diffuorophenacetyl-L-alanyl)]-S-phenyl-glycinet-butyl ester.



Figure 4. Expression of Msx2 in VSMCs. (A) mRNA levels of msx2 were measured by real-time RT-PCR and (B) protein levels of Msx2 were determined by western blot analysis in VSMCs cultured in various media. Expression of Msx2 was significantly increased in the osteogenic group compared with the controls at the mRNA and protein levels. Activation of Msx2 by osteogenic media was eradicated by the addition of DAPT, and the addition of alendronate significantly reduced the expression of Msx2 in a dose-dependent manner and reached the minimum at 10⁻⁴ mmol/l alendronate. *P<0.05 vs. control; #P<0.05 vs. osteogenic mediau; *P<0.05 vs. 10⁻⁴ mmol/l alendronate (n=3). VSMC, vascular smooth muscle cells; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenyl-glycinet-butyl ester.

 10^{-4} mmol/l (P<0.05 vs. the osteogenic and 10^{-4} mmol/l alendronate groups).

Alendronate downregulates the expression of Msx2. The expression of Msx2 was measured by real-time RT-PCR and western blot analysis. The mRNA and protein levels of Msx2 were significantly increased in the osteogenic group compared

with the control group (P<0.05), indicating that VSMCs were transformed to osteoblast-like cells. Results in the alendronate group showed that the transformation regulated by Msx2 was inhibited in an alendronate dose-dependent manner compared with the osteogenic media group (P<0.05). The expression of Msx2 in the DAPT group, as the negative control, was inhibited, which indicated that inhibition of the Notch1-RBP-J κ

signaling pathway decreased the expression of Msx2 (P<0.05 vs. the osteogenic group; Fig. 4).

Discussion

Osteoporosis and vascular calcification share a number of common risk factors and pathogenetic mechanisms, involving proteins, hormones, elements, lipids and vitamins (27). Previous studies have indicated that bone morphogenetic proteins, the RANKL/RANK/OPG and Wnt signaling pathways, matrix Gla protein, vitamins K and D, osteopontin and the renin-angiotensin-aldosterone system are involved in this process (2,23). In addition, the Notch signaling pathway plays a key role in the development and homeostasis of the cardiovascular system and bone (19,23,29-32).

VSMC fate decisions, including cell growth, migration and apoptosis, are fundamental features in the pathogenesis of vascular disease. The signaling pathways that regulate VSMC growth, death, differentiation and migration are the focus of regulation and control targets, and recent studies have attempted to clarify the pathway for osteoblastic transformation of VSMCs, including the Wnt signaling pathway (28,33-35). The Notch signaling pathway is also important and the Notch1 signaling pathway has been reported to induce the osteogenic differentiation and mineralization of VSMCs (22). The Notch1 signaling pathway promotes the osteogenic differentiation and mineralization of VSMCs by direct activation of Msx2 gene transcription via RBP-Jk (22,36,37). RBP-Jk, a major mediator of Notch1 signaling, binds NICDs and forms a complex that further activates the transcription of target genes from their cognate DNA binding sequence in the nucleus (21,22,37).

Msx2, the downstream target gene of the Notch1-RBP-J κ signaling pathway, is a key regulatory osteogenic factor of vascular calcification. Decreased levels of Msx2 imply that the osteogenic differentiation of VSMCs is inhibited (22,38). In the present study, the expression of Notch1 and RBP-J κ was increased in osteogenic VSMCs, consistent with the increased intracellular calcium deposition (P<0.05). The osteogenic effects on VSMCs, including increased Msx2, enhanced ALP activity and the deposition of calcium in VSMCs, were eradicated by DAPT, an inhibitor of the Notch1 signaling pathway (26), indicating that the Notch1-RBP-J κ signaling pathway contributes to the calcification of VSMCs (P<0.05).

In clinical medicine, alendronate has been successfully developed as a treatment for osteoporosis and other pathological conditions associated with bone loss (6,13,29). Clinical studies have shown that alendronate may also be used for treating vascular calcification (3,4,25). In addition, alendronate inhibits the calcification of arteries and valves without affecting serum levels of calcium or phosphate (12). Results of the present study demonstrated that alendronate represses VSMC calcification via downregulation of the Notch1-RBP-J κ signaling pathway *in vitro* in a dose-dependent manner. The expression of Msx2, the downstream target of the Notch1-RBP-J κ signaling pathway, was repressed in a dose-dependent manner. These observations indicate that osteogenic conversion of VSMCs may be repressed by alendronate through downregulation of the Notch1-RBP-J κ signaling pathway.

In summary, results of this study indicate that VSMCs differentiate into osteoblast-like cells by upregulating the

Notch1-RBP-J κ signaling pathway, and osteogenic transformation leads to vascular calcification. Alendronate was found to inhibit osteogenic transformation regulated by this pathway to protect arteries. Alendronate, a drug that has been found to protect the arteries and bones, is likely to be important for the prevention of CVD and osteoporosis to preserve the quality of life of elderly individuals.

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