Original Article

Treatment with Vitamin K₂ Combined with Bisphosphonates Synergistically Inhibits Calcification in Cultured Smooth Muscle Cells

Eiji Saito, Hiroshi Wachi, Fumiaki Sato, Hideki Sugitani, and Yoshiyuki Seyama

Department of Clinical Chemistry, Hoshi University School of Pharmacy and Pharmaceutical Sciences, Tokyo, Japan.

Aim: Vascular calcification is a common feature in patients with advanced atherosclerosis, postmenopausal women and patients with renal failure, which results in reduced elasticity of arteries. Pamidronate, a bisphosphonate, is used as a therapeutic agent for anti-osteoporosity, although there are adverse side effects, such as renal damage and aortic inflamed plaque rupture. In the present study, we demonstrated the effects of vitamin K₂ alone or in combination with pamidronate in an arterial calcification model induced using inorganic phosphate in cultured bovine aortic smooth muscle cells (BASMCs).

Methods: Calcification was induced by the addition of Pi (3 mM) in BASMCs. Calcium deposition was determined by Calcium C-test Wako and von Kossa staining. mRNA expression was assessed by semi-quantitative reverse transcription-polymerase chain reaction.

Results: Calcium deposition assay and von Kossa staining showed that calcification could be inhibited in a dose-dependent manner by treatment with vitamin K₂ alone, and that its inhibitory effect was enhanced when combined with pamidronate. It was found that the expression of tropoelastin mRNA was synergistically enhanced by combined treatment with vitamin K₂ and pamidronate, and the expression matrix Gla protein mRNA and osteopontin mRNA expression were also enhanced and suppressed, respectively, by treatment with vitamin K₂ or pamidronate. Moreover, our data showed that the suppression of TE expression by siRNA significantly increased Pi-induced vascular calcification.

Conclusion: Taken together, our study suggests that vitamin K₂ in combination with pamidronate synergistically inhibits arterial calcification via the increased expression of tropoelastin, which would be a useful marker for developing effective therapeutic or prophylactic agents for arterial calcification.

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Key words; Vitamin K₂, Calcification, Tropoelastin, Drug synergism

Introduction

Arterial calcification is a common event in the pathogenesis of arteriosclerosis in dialysis patients. Vascular calcification has been positively correlated with an increased risk of myocardial infarction¹⁾. The pathological mineralization of the vascular system, kidneys and heart, unlike bone mineralization, is called ectopic calcification, which is promoted by menopause or

aging, and such calcification is frequently observed in patients with diabetes or uremia¹⁾. The development of calcification impairs organizational flexibility²⁾ and, of special note, vascular calcification causes the functional disturbance of organs with increased risk of cardiac infarction³⁾. It has been thought that the release of calcium or phosphate from bone caused by endocrine hypofunction is an etiological factor of ectopic calcification.

It is well known that bisphosphonates prevent experimentally induced calcification of many soft tissues⁴⁾, including the arteries, kidneys and skin, when given parenterally or orally. Etidronate, a type of bisphosphonate, is also able to impair the mineralization of normally calcified tissues such as bone and cartilage⁵⁾. However, bisphosphonates which bind strongly to hy-

Address for correspondence: Hiroshi Wachi, Department of Clinical Chemistry, Hoshi University School of Pharmacy and Pharmaceutical Sciences, 2-4-41 Ebara, Shinagawa-ku Tokyo, 142-8501, Japan.

E-mail: wchrs_1107@hoshi.ac.jp

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droxyapatite cause inflamed plaque rupture associated with mural thrombus⁶) and are contraindicated in patients with serious renal damage⁷.

Targeted deletion of the matrix gamma-carboxy glutamate (Gla) protein (MGP) gene causes rapid calcification of the elastic lamellae of arterial media. Calcification begins at birth and is sufficiently extensive by 3-6 weeks of age so that the arteries become rigid tubes that fracture, causing death by exsanguination in most affected animals by 6 weeks of age^{8,9}. MGP is a vitamin K-dependent, 14 kDa protein found most abundantly in bone and cartilage¹⁰. The relevant function of vitamin K is that it serves as a cofactor for the endoplasmic enzyme gamma-glutamyl carboxylase, where it promotes the post-translational conversion of selective protein-bound glutamate residues into Gla. We have previously shown that vitamin K₂ inhibits vitamin D-induced calcification in rat soft tissues such as the arteries and kidneys¹¹.

An *in vitro* vascular calcification model was developed as a useful method for analyzing the cellular mechanisms of calcification^{12,13}, in which the addition of inorganic phosphate (Pi) or β -glycerophosphate is used to induce calcium deposition. The present study was designed to examine the synergistic effects of vitamin K₂ when combined with bisphosphonate pamidronate on Pi-induced vascular calcification in that model.

Materials and Methods

Materials

Bovine aortic smooth muscle cells (BASMCs) were purchased from Coriell Cell Repositories (Ref. No. AG08504). Vitamin K₂ was obtained from Eisai Co. (Tokyo, Japan). Pamidronate was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, non-essential amino acids and sodium pyruvate were purchased from Invitrogen (Carlsbad, CA, USA).

Cell Culture and Induction of *in vitro* Calcification

BASMCs were cultured in DMEM containing 4.5 g/L glucose supplemented with 10% FBS, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 unit/mL penicillin and 100 μ g/mL streptomycin and were grown to confluence. *In vitro* calcification was induced as described previously¹³⁻¹⁶, with slight modifications. Briefly, confluent cells were incubated in DMEM containing 4.5 g/L glucose supplemented with 10% FBS in the presence of Pi (3 mM). In experiments using drugs, BASMCs were cultured in medium con-

taining vitamin K₂ (10^{-10} - 10^{-6} M) and/or pamidronate (10^{-10} - 10^{-6} M) in the presence of Pi (3 mM). The initial day of culture in Pi-containing media was defined as day 0. Media were changed every 2 days.

Calcium Deposition Assay

The cells were washed two times with Tris-buffered saline (pH 7.4) and were treated with 0.6 N HCl for 24 h. Calcium concentrations in HCl supernatants were determined by Calcium C-test Wako (Wako Pure Chemical Industries, Osaka, Japan) as previously described^{12,13}. Each cell layer was washed with Tris-buffered saline (pH 7.4) and solubilized with 0.1 N NaOH containing 0.1% sodium dodecyl sulfate (SDS). Protein concentrations in solubilized cell extracts were measured with a MicroBCA protein assay kit (Pierce, Rockford, IL, USA). The calcium content of each cell layer was normalized against the protein content. Mineral deposition was assessed by von Kossa staining. Cells were fixed with 3.5% formaldehyde solution, and were then treated with 5% silver nitrate solution for 30 min under ultraviolet irradiation to detect phosphate deposition.

RNA Isolation and Semi-quantitative RT-PCR

RNA isolation and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) were performed as described previously^{12,13}. RT-PCR was carried out using primers 5'-CACTCTCCATCTCTAGGGGAAGTTG-3' (forward) and 5'-GGAGAAGTGCTAATGAAGTACAGTG-3' (reverse) to amplify nucleotide positions 2536-3176 in the tropoelastin (TE) gene; 5'-primers ATGAAGAGCCTGCTCCTTCTC-3' (forward) and 5'-TTTGGCCCCCTCGGCGCTGCCG-3' (reverse) to amplify nucleotide positions 6-314 in the MGP gene; and primers 5'-GATTTGCTTCTGCCTCTTGG-3' (forward) and 5'-ACACTATCACCTCGGCCATC-3' (reverse) to amplify nucleotide positions 78-530 in the osteopontin (OPN) gene. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and primer extension at 72°C for 30 s; this was performed for 20 (OPN), 21 (TE) or 26 (MGP) cycles, with a final extension step at 72°C for 10 min. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control; the same amplification protocol (20 cycles) and the upstream primer 5'-ATCAATGACCCCTTCATTGACC-3' and downstream primer 5'-ATACTTGGCAGGTTTCTC-CAGG-3' (61-729; AJ000039, Gene Bank) were used. The resulting PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. Band intensities were evaluated using Kodak 1D.

Preparation of Small Interfering RNA Targeting BTE and Transfection

Small interfering RNA (siRNA) was designed to target bovine TE (BTE; accession no. J02717) obtained from TaKaRa Bio Co., LTD (Otsu, Shiga, Japan). The sequences for BTE were 5'-UUGGAGGCAUCCCC-ACAUTT and 5'-AAUGUGGGAAUGCCUCCAA-TT. Twenty-four hours after BASMC seeding onto 24-well plates, cells were cultured in DMEM with 10% FBS for 24 hours, and then transfected with BTE (20 pmol/ μ L) siRNA using GenomONE transfection reagent (Ishihara Sangyo Kaisha, Ltd., Osaka, Japan). To evaluate the effect of BTE siRNA on Ca deposition, siRNA was transfected when BASMC reached 60% to 70% confluence. We then applied the siRNA-transfected culture to the *in vitro* calcification model. The loss of BTE by transfection of siRNA was validated by immunoblotting with bovine tropoelastin-specific monoclonal anti-elastin antibody BA4 (Sigma, St. Louis, MO, USA) for BTE protein in culture medium 24 hours after siRNA transfection.

Immunoblot Assay for Analyzing BTE Expression

Before collecting the culture media from treated cultures for protein level analysis, cells were washed with PBS, and then the media were replaced with fresh. After 24 h, the culture medium was precipitated with ammonium sulfate (176 mg/mL) in the presence of protease inhibitor: 1 mM ethylenediaminetetraacetic acid, N-ethylmaleimide, and phenylmethyl sulfonyl fluoride. Proteins from the cultured medium were resolved on 7.5% SDS-PAGE under reduction with 1 mM DTT. The proteins were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, CA, USA). Membranes were blocked with Tris-buffered saline supplemented with 0.05% Tween 20 (T-TBS) containing 5% skim milk, and then were treated with BA4 anti-TE monoclonal antibody. After membranes were washed with T-TBS, they were treated with anti-mouse-IgG or anti-rabbit-IgG antibody conjugated horseradish peroxidase (Amersham, Buckinghamshire, UK). Membranes were thoroughly washed, and treated with ECL reagent (Amersham, Buckinghamshire, UK); each membrane was exposed to X-ray film (Kodak, Rochester, NY, USA) for fluorography.

Statistical Analysis

Data were analyzed for statistical significance by ANOVA with post-hoc Bonferroni/Dunn's analysis using StatView version 5.0. All examinations were repeated three times and were reproducible.

Results

Pamidronate and Vitamin K₂ Inhibit the Calcification of BASMCs

We examined the effects of vitamin K₂ and pamidronate using the Pi-induced *in vitro* calcification model as previously described^{13, 16}. The addition of vitamin K₂ significantly inhibited calcium deposition in Pi-induced calcification at 6 days in a dose-dependent manner. Calcium contents were reduced 62.5% by the addition of 10⁻⁶ M of vitamin K₂ (**Fig. 1**). Treatment with pamidronate also significantly inhibited calcium deposition in Pi-induced calcification at 6 days in a dose-dependent manner (**Fig. 2**). Calcium contents were reduced 87.5% by the addition of 10⁻⁶ M pamidronate. Moreover, our data showed that combined treatment with vitamin K₂ and pamidronate significantly and synergistically inhibited calcium deposition in Pi-induced calcification at 6 days (**Fig. 3**). We also confirmed the inhibitory effect using von Kossa staining (**Fig. 4**). These results suggest that vitamin K₂ and pamidronate synergistically suppress vascular calcification.

Expression of mRNA

We previously reported that the expression of TE, a soluble precursor of elastin, is down-regulated by treatment with Pi or β -glycerophosphate in cultured BASMCs. It is also known that the phenotype of calcified smooth muscle cells transforms to osteoblast-like cells. Therefore, the expression of mRNAs encoding TE, MGP, and OPN following treatment with vitamin K₂ and/or pamidronate in Pi-induced BASMC calcification was determined by RT-PCR. In order to estimate the extent of the expression of those mRNA, the ratio of the band intensity of each mRNA to that of GAPDH was calculated as previously reported¹³. The expression of OPN mRNA induced by Pi or the expression of TE and MGP mRNA suppressed by Pi was inhibited by treatment with vitamin K₂ and/or pamidronate (**Fig. 5A, B**). Of special note, the expression of TE mRNA was synergistically enhanced by combined treatment with vitamin K₂ and pamidronate.

Suppression of Tropoelastin mRNA by siRNA Accelerated the Calcification of BASMCs

To assess the impact of endogenous TE mRNA on calcification, we demonstrated the levels of calcium deposition in Pi-induced calcification using siRNA of TE. The suppression of TE at the protein level was determined by Western blot analysis (**Fig. 6A**). Our data also revealed that calcification levels were accelerated about 3 times by the suppression of TE (**Fig. 6B**). This

Fig. 1. Vitamin K₂ inhibits Pi-induced calcium deposition.

BASMCs were treated with 3 mM inorganic phosphate (Pi) and vitamin K₂, where noted, for 6 days. Extracellular calcium deposition was measured using the o-cresolphthalein complexone method and was normalized to the corresponding cellular protein. Values are expressed as the means $\pm$ SE ($n=4$). Differences compared with Pi alone (Pi=3 mM) for each concentration were statistically significant (** $p < 0.01$, *** $p < 0.001$, Bonferroni/Dunn's t -test).

Fig. 2. Pamidronate inhibits Pi-induced calcium deposition.

BASMCs were treated with 3 mM inorganic phosphate (Pi) and pamidronate where noted for 6 days. Extracellular calcium deposition was measured using the o-cresolphthalein complexone method and was normalized to the corresponding cellular protein. Values are expressed as the means $\pm$ SE ($n=4$). Differences compared with Pi alone (Pi=3 mM) for each concentration were statistically significant (** $p < 0.01$, *** $p < 0.001$, Bonferroni/Dunn's t -test).

Fig. 3. BASMCs were treated with 3 mM Pi for 6 days in the absence or presence of vitamin K₂ and/or pamidronate.

Extracellular calcium deposition was measured using the o-cresolphthalein complexone method and was normalized to corresponding cellular protein. Inorganic phosphate (Pi)-induced calcification of BASMCs was suppressed drastically according to the synergistic effects of vitamin K₂ and pamidronate. Values are expressed as the means $\pm$ SE ($n=4$). Differences compared with Pi alone (Pi=3 mM) for each concentration were statistically significant (** $p < 0.01$, *** $p < 0.001$, Bonferroni/Dunn's t -test).

Fig. 4. Photomicrographs of *in vitro* calcification of BASMCs.

BASMCs were cultured for 6 days with 3 mM inorganic phosphate (Pi) in the presence or absence of vitamin K₂ and/or pamidronate: control cells (A), Pi (3 mM)-treated cells (B), Pi (3 mM) + Vitamin K₂ (10⁻⁶ M)-treated cells (C), Pi (3 mM) + Pamidronate (10⁻⁶ M)-treated cells (D), and Pi (3 mM) + Vitamin K₂ (10⁻⁶ M) + Pamidronate (10⁻⁶ M)-treated cells (E). Calcified cells were stained using the von Kossa staining method (Magnification: $\times 100$).

Fig. 5. Expression levels of mRNAs encoding TE, MGP and OPN.

BASMCs were treated with 3 mM inorganic phosphate (Pi) for 6 days in the presence of vitamin K₂ (10⁻⁶ M) and/or pamidronate (10⁻⁶ M), and total RNA was then isolated by extraction in phenol/chloroform. RT-PCR was carried out using specific primers for bovine TE, MGP, OPN and GAPDH, which were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining (A). Results show densitometric analysis of intensities of bands. TE, MGP and OPN were normalized to GAPDH (B). Values are expressed as the means $\pm$ SE ($n=3$). Differences compared with Pi alone (Pi=3 mM) for each concentration were statistically significant (** $p<0.01$, *** $p<0.001$, Bonferroni/Dunn's t -test).

Fig. 6. Suppression of TE mRNA by siRNA accelerated the calcification of BASMCs.

A, Tropoelastin (TE) specific short-interfering RNA (siRNA) was transfected into bovine smooth muscle cells (BASMCs), and immunoblot assay was carried out at 24 hours after transfection. B, Calcium deposition on cultured BASMCs transfected with BTE siRNA (20 pmol/ μ L) and treated with inorganic phosphate (3 mM) for 6 days. Extracellular calcium deposition was measured using the o-cresolphthalein complexone method and was normalized to the corresponding cellular protein. Values are expressed as the means $\pm$ SE ($n=4$). Differences compared with Pi alone (-siRNA, Pi=3 mM) for each concentration were statistically significant (** $p<0.001$, Bonferroni/Dunn's t -test).

result suggests that TE plays a critical role in the suppression of arterial calcification.

Discussion

We investigated whether vitamin K₂ in combination with pamidronate synergistically inhibits arterial calcification. Vitamin K₂ and pamidronate are known as treatments for osteoporosis. It has been revealed that anti-osteoporotic drugs, such as bisphosphonates or estrogens, have therapeutic effects for arterial calcification. In the therapeutic use of vitamin K₂ and pamidronate for osteoporosis, serum maximum concentrations when administering vitamin K₂ and pamidronate are 5.7×10^{-7} M (vitamin K₂)¹⁷ and 2.6×10^{-6} M (pamidronate)¹⁸. These drug concentrations for clinical use almost matched those used in this study, but there have been few reports about vitamin K₂ and/or pamidronate as drug therapy for arterial calcification. The inhibition of arterial calcification by bisphospho-

nate is caused by decreased blood levels of Pi due to the suppression of bone resorption⁴⁾. In the present study, we found that vitamin K₂ and pamidronate directly affect vascular calcification *in vitro*.

Vitamin K₂ is known as a coenzyme of γ -carboxylation of matrix Gla protein (MGP), and induces MGP. It is known that MGP acts as an agent of sodium phosphate binding to bone and extracellular matrix¹⁹⁻²¹⁾. It was also clarified that MGP is an important inhibitory factor in arterial calcification using MGP-deficient mice⁸⁾. Our present data indicated that the expression of MGP mRNA closely related to aortic calcification. Recently, it was reported that vitamin K₂ binds the steroid and xenobiotic receptor (SXR, PXR, NR1I2) in the nucleus²²⁾. SXR plays a central role in the transcriptional regulation of xenobiotic detoxifying enzymes and transporters such as CYP3A4 and MDR²³⁻²⁷⁾. Vitamin K₂ also regulates the expression of osteoprotegerin or MGP²⁸⁾. Thus, vitamin K₂ might inhibit the arterial calcification found in this study by binding to SXR. Vitamin K₂ also relates to growth arrest-specific gene 6 (Gas6), γ -carboxylated and vitamin K-dependent protein²⁹⁾. Gas6 is decreased in arterial calcification and suppresses arterial calcification via the inhibition of apoptosis on SMCs³⁰⁾. Apoptosis is known as a phenomenon of accelerating arterial calcification³¹⁾. Vitamin K₂ may inhibit arterial calcification via the Gas6-antiapoptotic pathway.

Extracellular matrix components are closely related to arterial calcification. For example, it has been revealed that fibronectin and collagen I promote arterial calcification³²⁾ and that elastin, or laminin, inhibits such calcification^{33, 34)}. We have previously reported that elastic fiber-related proteins, TE, fibrillin-1, and Lysyl oxidase, are suppressed by the induction of vascular calcification in cultured BASMCs¹²⁾ and the addition of recombinant tropoelastin significantly inhibits arterial calcification via 67 kDa elastin-binding protein (EBP)¹⁵⁾. It is believed that the hydrophobic sequence of TE, VGVAPG (Val-Gly-Val-Ala-Pro-Gly), binds to EBP^{35, 36)}. Our present data also showed that treatment with vitamin K₂ in combination with pamidronate inhibits down-regulated TE by Pi-induced calcification at the mRNA level, and the suppression of TE by siRNA significantly increased calcium deposition. These observations suggest that the expression of TE is correlated with the development of vascular calcification. High and local expression of TE would be useful for the inhibition of vascular calcification; however, we did not demonstrate the expression mechanisms of TE mRNA by the induction of calcification or treatment in combination with vitamin K₂ and pamidronate in this study. It has been reported that

the stimulation or suppression of TE mRNA by treatment with TGF- β 1³⁷⁾ or vitamin D₃³⁸⁾, respectively, is attributable to the stability of TE mRNA but not TE promoter activity; therefore, we considered that these alterations result from the stability of TE mRNA.

OPN is a bone matrix component, also expressed on SMCs, and it has been reported that OPN protein has an inhibitory effect on arterial calcification³⁹⁾. In this study, the induced expression of OPN mRNA by Pi treatment was suppressed by treatment with vitamin K₂ and pamidronate. This result suggests that the increased expression of OPN mRNA is an inverse relation marker to aortic calcification.

In conclusion, our present study suggests that the combination of vitamin K₂ and bisphosphonates, which inhibit phenotype transition from SMC to osteogenic cells, would be an effective therapeutic agent for patients with serious renal damage, and that the expression of TE mRNA would be a useful marker for developing therapeutic agents for arterial calcification.

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