Bone Morphogenetic Protein-7 Inhibits Vascular Calcification Induced by High Vitamin D in Mice

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Vascular calcification refers to the deposition of calcium phosphate in cardiovascular tissues, including arteries and myocardium. Vascular calcification is frequently associated with cardiovascular disease. Recently, bone morphogenetic protein-7 (BMP-7) has been proposed to play an inhibitory role in vascular calcification, but its inhibitory effect has not been fully elucidated. We therefore tested the hypothesis that BMP-7 inhibits vascular calcification by using two conditions, high levels of vitamin D and phosphate, each of which could enhance vascular calcification. C57BL/6 mice were treated for 3 days with high vitamin D (500,000 IU/kg/day) in the presence or absence of recombinant human BMP-7 (rhBMP-7). Expression levels of osteopontin and osteocalcin, markers of the osteoblastic phenotype, were assessed by immunohistochemical staining or Western blotting analysis. Vitamin D increased calcium staining in thoracic aortas and hearts and the expression levels of osteopontin and osteocalcin in mice. Importantly, pretreatment for 7 days and subsequent treatment for 3 days with rhBMP-7 (10 μg/kg/day) abolished the vitamin D-mediated increases in the above parameters. In addition, human aortic smooth muscle cells (HASMCs) were cultured with high β-glycerophosphate, a phosphate donor, for 2 weeks in the presence or absence of rhBMP-7. High β-glycerophosphate increased expression levels of osteopontin and osteocalcin as well as calcium staining in HASMCs, but these changes were attenuated by treatment with BMP-7. Thus, BMP-7 inhibits vascular calcification associated with high levels of vitamin D or phosphate. We propose that BMP-7 treatment may be helpful in reducing the risks of cardiovascular disease related to vascular calcification.

Keywords: BMP-7; vascular calcification; vitamin D; phosphate; vascular smooth muscle cell

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and is associated with the loss of SMC markers and onset of osteoblastic gene expression, including alkaline phosphatase, osteopontin, and osteocalcin, while BMP-7 has been shown to play an inhibitory role in vascular calcification (Hruska et al. 2005; Tobin and Celeste 2006). Several factors, such as high levels of phosphate, parathyroid hormone, vitamin D, reactive oxygen species, and glucocorticoids have been reported to induce vascular calcification (Vattikuti and Towler 2004; Johnson et al. 2006).

With regard to the effect of BMP-7 on vascular calcification, a previous study showed that BMP-7 can prevent vascular calcification stimulated by chronic kidney disease in low density lipoprotein receptor-deficient (LDLR −/−) mice fed high-fat/cholesterol diets, a commonly used model of atherosclerosis and vascular calcification (Davies et al. 2003). In another study, BMP-7 was shown to preserve smooth muscle cell phenotype and reverse progression towards an osteoblastic phenotype in vascular smooth muscle cells in vitro (Dorai et al. 2000). However, the inhibitory effects of BMP-7 on vascular calcification were not fully studied in wild-type mice.

In the present study, we examined the effect of recombinant human BMP-7 (rhBMP-7) on vascular calcification of mice treated with high vitamin D levels in vivo and calcium deposition in human aortic smooth muscle cells (HASMCs) induced by high phosphate levels in vitro.

Materials and Methods

Reagents

Vitamin D (cholecalciferol) was purchased from Sigma (St. Louis, MO, USA). For injections, vitamin D solution (1.32 × 10⁷ IU) was prepared as described below. Vitamin D (33 mg) in 200 ul of absolute ethanol was mixed with 1.4 ml of emulsophor (alkamuls EL-620, Sigma) for 15 minutes at room temperature (RT), and this solution was then mixed with 18.4 ml sterilized water containing 750 mg of dextrose for an additional 15 minutes at RT. The alkaline phosphatase (ALP) staining kit, silver nitrate, ascorbic acid and β-glycerophosphate were purchased from Sigma. Sodium pyruvate, insulin and fetal bovine serum (FBS) were purchased from Join Bio-Innovation (Amersham, Little Chalfon, UK). The membrane was blocked in Tris-buffered saline containing 5% skim milk and 1% PBS-Tween-20 (Amersham, Little Chalfon, UK). The membrane was blocked in Tris-buffered saline containing 5% skim milk and 1% PBS-Tween-20 for one hour, and then incubated overnight at 4°C with antibodies to mouse normal diet (Super Bead Co., Korea) containing 0.4% phosphate were purchased from Sigma. Sodium pyruvate, insulin and fetal bovine serum (FBS) were purchased from Join Bio-Innovation (Amersham, Little Chalfon, UK). The membrane was blocked in Tris-buffered saline containing 5% skim milk and 1% PBS-Tween-20 for one hour, and then incubated overnight at 4°C with antibodies to mouse normal diet (Super Bead Co., Korea) containing 0.4%}

In Vivo Methods

Animals and Diets

C57BL/6J mice (8-weeks-old, male, approximately 20 g) were purchased from Hyochang Company (Daegu, Korea) and fed experimental mouse normal diet (Super Bead Co., Korea) containing 0.4% phosphorous and 0.6% calcium by weight. In all experiments, mice were sacrificed by inhalation of CO₂ gas. Permission to perform these animal experiments was obtained from the Bioethical Committee of Pusan National University.

Treatments and induction of vascular calcification

The mice were divided into control, vitamin D-treated and vita-

min D + BMP-7 treated groups (6 per group). The mice in the vita-

min D-treated and vitamin D + BMP-7 treated groups were injected with a high dose of vitamin D (500,000 IU/kg/day) subcutaneously for 3 days at 0, 24 and 48 hours (Price et al. 2001b). In the control group, a 0.9% sodium chloride solution was administered instead of vitamin D. In the vitamin D + BMP-7 treated group, the mice were injected subcutaneously with rhBMP-7 (10 µg/kg/day) beginning at 7 days before the first injection of vitamin D and continuing for the duration of vitamin D treatment. They were monitored every 12h and sacrificed at 4 days after the third injection of vitamin D. Thoracic aortas and hearts were removed for histological analysis. Calcium levels in serum were determined colorimetrically with cresolphthalein complexone.

Histological Analysis

Thoracic aortas and hearts were placed in 4% buffered formaldehyde at pH 7 and fixed for 24h at RT. Tissues were then embedded, sectioned (4 µm) and stained with alizarin red or von Kossa stain. Immunohistochemical (IHC) staining using osteopontin and osteocalcin antibodies was also performed. Tissue analysis was performed by an experienced pathologist.

In Vitro Methods

Cell culture

Human aortic smooth muscle cells were purchased from Clonetics (San Diego, CA, USA) and grown in growth medium provided by the same company. Cells from passages between 4 and 9 were used for all experiments. All cells were placed in serum-reduced medium (0.1% FBS) for 24 hours to induce quiescence prior to treatment. All experiments were repeated several times with cells from different passages. The data presented here are the results obtained in a single representative experiment.

Induction of calcification

To examine the effect of rhBMP-7 on phosphate induced calcification, HASMCs were cultured until confluent and then placed in calcification medium (DMEM containing 10 mmol/L sodium pyruvate, 10⁻⁶ mol/L insulin, 50 µg/ml ascorbic acid, 100 U/mL penicillin, 100 µg/mL streptomycin and 12 mmol/L β-glycerophosphate) for 14 days. The medium was replaced with fresh medium every two to three days and the first day of culture in calcification medium was defined as day 0 (Shioi et al. 1995; Chen et al. 2002). In some experiments, HASMCs were also treated with rhBMP-7 (200 ng/mL).

Western blot analyses

Cells were washed with cold phosphate-buffered saline (PBS) and incubated in ice-cold lysis buffer containing 5 mmol/L HEPES (pH 7.9), 150 mmol/L NaCl, 26% (vol/vol) glycerol, 1.5 mmol/L MgCl₂, 1.0 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mmol/L dithiothreitol (DTT), and 1.0 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 µmol/L pepstatin A, 1 µmol/L leupeptin, 0.1 µmol/L apro-

lin. Whole cell lysates (30 µg) were mixed with an equal volume of 4×Laemmli sample buffer. The lysates and pre-stained molecular weight markers were boiled for five minutes and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to a nitrocellulose membrane (Amersham, Little Chalfon, UK). The membrane was blocked in Tris-buffered saline containing 5% skim milk and 1% PBS-Tween-20 for one hour, and then incubated overnight at 4°C with antibodies to mouse normal diet (Super Bead Co., Korea) containing 0.4%}

β-glycerophosphate were purchased from Sigma. Sodium pyruvate, insulin and fetal bovine serum (FBS) were purchased from Join Bio-Innovation (Amersham, Little Chalfon, UK). The membrane was blocked in Tris-buffered saline containing 5% skim milk and 1% PBS-Tween-20 for one hour, and then incubated overnight at 4°C with antibodies to mouse normal diet (Super Bead Co., Korea) containing 0.4%}
against osteopontin and osteocalcin (goat polyclonal antibody, Santa Cruz, CA, USA). The membrane was washed with PBS-Tween and then incubated with a peroxidase-labeled secondary antibody against goat IgG or mouse IgG diluted 1:3,000 in 1% PBS-Tween. The membrane was washed again four times, and protein bands were visualized by enhanced chemiluminescence (LAS3000, Fuji, Japan). The band intensity was analyzed by scanning densitometry (UVITEC, Cambridge, UK).

Quantification of calcium deposition
HASMCs were decalcified with 0.6 N HCl for 24 hours. The calcium content of the HCl supernatants was determined colorimetrically by the o-cresolphthalein complexone method (QuantiChrom Calcium Assay Kit, BioAssay Systems, Hayward, CA, USA) (Shioi et al. 1995; Chen et al. 2002). After decalcification, the cells were washed three times with PBS and solubilized with 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS). The protein content was measured with a Bradford Protein Assay kit (Bio-Rad, Richmond, CA, USA). The calcium content of the cell layer was normalized to protein content.

Determination of calcium deposition by calcium stainings
For qualitative staining, HASMCs were grown to subconfluence, then placed in serum-reduced medium and treated as described above in 6 well dishes. Cells were rinsed with water, plates were drained, and 2% alizarin red solution (pH 6.0) was added. After 30-second incubation at room temperature, the plates were rinsed three times with distilled water (dH2O).

For detection of mineralization, von Kossa staining was performed as described by Murshed et al. (Murshed et al. 2004). In brief, HASMCs cultured in calcified medium for 2 or 3 weeks were fixed with 0.1% glutaraldehyde (Sigma) for 15 minutes at RT and washed with dH2O twice, then incubated with 5% silver nitrate (von Kossa) for 30 minutes at RT. The cells were exposed to sunlight for 2 hours until color development was complete. The silver nitrate solution was removed and the cells were rinsed twice with dH2O. After washing, the cells were then rinsed again with dH2O and processed by scanning with an EXPRESSION 1680/Pro flatbed scanner (EPSON, Korea).

Statistical analyses
All data are presented as the mean ± SEM or percentage of control. Data were analyzed for statistical significance by ANOVA with post hoc Tukey’s analysis. Analyses were performed using Prism 4 for windows (GraphPad Software Inc., San Diego, CA, USA). A value of P < 0.05 was considered statistically significant.

Fig. 1. Calcium staining in sections of aortas from mice in the control group, the vitamin D-treated group and the vitamin D + BMP-7 treated group. (a) hematoxylin-eosin stain (dark purple), (b) Alizarin red stain (dark red), (c) von Kossa stain (dark brown). BMP-7 attenuated arterial calcification induced by high doses of vitamin D. Magnification, × 20 in all panels. Arrows indicated the calcification areas in the vessels.
Results

Effect of BMP-7 on vascular calcification induced by high vitamin D

Microscopic examination of alizarin red and von Kossa stained sections of thoracic aortas revealed extensive calcification of the intima and media of arteries in the vitamin D-treated mice compared with those of the control group. However, staining was attenuated in the arteries of mice in the vitamin D + BMP-7 treated group (Fig. 1). In sections from hearts of the vitamin D-treated mice, there was increased calcium stainings in the walls of the coronary vessels and myocardium, which was attenuated by treatment of BMP-7 (Fig. 2). Immunoreactivity of osteopontin and osteocalcin antibodies was increased in the aortas by vitamin D pretreatment, but this immunoreactivity was absent in those of vitamin D + BMP-7 treated group (Fig. 3). Vitamin D-treated mice also showed increased serum calcium levels compared with those of the control group (4.71 ± 0.30 mg/dL vs. 1.87 ± 0.10 mg/dL, respectively), while there was no significant difference in serum calcium levels between mice in the vitamin D treated group and those in the vitamin D + BMP-7 treated group (5.14 ± 0.41 mg/dL) (Fig. 4).

Effect of BMP-7 on high phosphate-induced calcium deposition of HASMCs

To induce HASMC calcification, cells were incubated in calcification medium with a high concentration of phosphate for 14 days. In order to explore the effect of BMP-7 on phosphate-induced calcification, HASMCs were treated with rhBMP-7 (200 ng/mL) in the presence of 12 mmol/L β-glycerophosphate. Cell lysates were extracted, and Western blotting revealed that the expression of osteopontin and osteocalcin was significantly increased in cells incubated in calcification medium alone. However, in the presence of rhBMP-7, this increased expression was significantly attenuated (Fig. 5). To further characterize the role of BMP-7 in calcium deposition in HASMCs, mineralization of HASMCs treated with high phosphate in the presence or absence of rhBMP-7 was qualitatively evaluated using alizarin red and von Kossa stainings. After 2 weeks, the inten-

Fig. 2. Calcium staining in sections of hearts from mice in the control group, the vitamin D-treated group and the vitamin D + BMP-7 treated group. (a) hematoxylin-eosin stain, (b) Alizarin red stain, (c) von Kossa stain. BMP-7 attenuated arterial calcification in the walls of the coronary vessels and in the adjacent myocardium induced by high doses of vitamin D. Magnification, × 20 in all panels. Arrows indicate the calcification areas in the coronary vessels and adjacent myocardium.
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The intensity of calcium staining was increased, and this phosphate-induced mineralization was attenuated in the presence of BMP-7. These findings were also consistent with the results obtained by alkaline phosphatase (ALP) staining (Fig. 6). After 3 weeks, the intensity of calcium staining was even more prominent, and this effect was also effectively attenuated by BMP-7 treatment (Fig. 7). In addition, the mineralization of HASMCs treated with phosphate in the presence or absence of BMP-7 was evaluated quantitatively by measuring the total calcium deposition by HCl extraction. The total amount of calcium deposition was significantly higher in HASMCs exposed to calcification medium than in control cells ($7.39 \pm 0.56$ vs. $3.21 \pm 0.43 \mu\text{mol/mg protein}$, $p < 0.01$). There was a small decrease in calcium deposition in cells treated with BMP-7 ($5.58 \pm 0.82 \mu\text{mol/mg protein}$), although it was not statistically significant (Fig. 8). There was a nonsignificant effect of BMP-7 on calcium deposition and expression of osteopontin and osteocalcin in control group.

**Discussion**

BMP-7 [or osteogenic protein-1 (OP-1)] is widely expressed during embryonic growth (Helder et al. 1995) and is an essential morphogen in renal, skeletal, and ocular development (Luo et al. 1995; Dudley et al. 1995; Jena et al. 1997). Many previous studies have demonstrated the efficacy of BMP-7 as a therapeutic agent for various renal disorders including chronic kidney disease (Vukicevic et al. 1998; Hruska et al. 2000; Zeisberg et al. 2003; Wang et al. 2003; Li et al. 2004). Several studies have also reported that BMP-7 reduced ischemic injury and improved behavioral outcome following ischemic stroke resulting from middle cerebral artery occlusion (Lin et al. 1999; Chang et al. 2003). On the other hand, the effect of BMP-7 on vascular calcification elicited by various pathologic conditions...
has not been fully elucidated. The present study evaluated the effect of BMP-7 on vascular calcification both in vivo, in mice treated with high doses of vitamin D, and in vivo, in HASMCs incubated in calcification medium with high phosphate. Our results indicate that BMP-7 may inhibit the progression of vascular calcification induced by conditions such as high levels of vitamin D or phosphate.

In our study, BMP-7 inhibited calcification in the thoracic aortas and hearts of mice treated with vitamin D. BMP-7 has been shown to ameliorate renal injury and preserve renal function in various animal models of chronic kidney disease, including diabetic nephropathy (Dudley et al. 1995; Vukicevic et al. 1998; Hruska et al. 2000; Zeisberg et al. 2003; Wang et al. 2003; Li et al. 2004). Previous studies investigating the roles of BMP7 in renal osteodystrophy have revealed that BMP-7 affects osteoblast morphology and number, eliminates peritubular fibrosis, decreases bone resorption, increases bone formation in secondary hyperparathyroidism and restores normal rates of bone formation in adynamic bone disorder (Bellusci et al. 1996; Li et al. 2004; Lund et al. 2004). Vitamin D is known to be a potent stimulator of bone resorption and one possible mechanism by which BMP-7 could inhibit arterial calcification induced by high doses of vitamin D is through the inhibition of bone resorption. In our study, there was no significant change in serum calcium levels between mice in the vitamin D treated group and those in the vitamin D + BMP-7 treated group, indicating that inhibition of arterial calcification was not due simply to decreased serum calcium levels. Although we did not check the serum phosphate level, Lund et al. (2004) showed previously that BMP-7 reduced plasma phosphorus by increasing extrusion from the exchangeable phosphate pool into the skeleton. This effect may be the reason for the reduction of arterial calcification observed with BMP-7 (Lund et al. 2004). Price and colleagues also showed that arterial calcification induced by treatment with warfarin and vitamin D was inhibited by osteoprotegerin (Price et al. 2001b) and bisphosphonates such as alendronate and ibandronate (Price et al. 2001a), all of which are potent inhibitors of bone resorption, and suggested that arterial calcification is linked to bone resorption. We observed marked immunohistochemical staining of osteopontin and osteocalcin, markers of an osteoblastic phenotype, in sections of thoracic aortas from mice treated with vitamin D, and this staining was attenuated by treatment with BMP-7. This finding is consistent with the results of previous in vitro studies that demonstrated that vascular smooth muscle cells undergo a dramatic phenotype; they become osteoblast-like and begin actively mineralizing their extracellular matrix. BMP-7 may effectively prevent vascular smooth muscle cells from undergoing osteoblastic changes (Tintut et al. 1998; Dorai et al. 2000; Jono et al. 2000).

Furthermore, we examined the effect of BMP-7 on calcium deposition induced by high phosphate in HASMCs. Some previous reports have demonstrated that VSMC calcification could be induced in vitro with high doses of β-glycerophosphate (β-GP) in the absence or presence of rhBMP7 (200 ng/mL) for 14 days. In cells with rhBMP-7, increased expression of osteopontin and osteocalcin was significantly attenuated. Data are shown as the mean ± SEM from three experiments.

Fig. 5. The effect of rhBMP-7 on expression of osteopontin and osteocalcin in HASMCs stimulated high levels of phosphate. HASMCs were incubated in calcification medium containing 12 mmol/L β-glycerophosphate (β-GP) in the absence or presence of rhBMP7 (200 ng/mL) for 14 days. In cells with rhBMP-7, increased expression of osteopontin and osteocalcin was significantly attenuated. Data are shown as the mean ± SEM from three experiments.

*, p < 0.05 vs. β-GP + BMP-7

**, p < 0.01 vs. β-GP + BMP-7
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erophosphate (Shioi et al. 1995; Watson et al. 1998; Chen et al. 2002), which stimulated VSMCs to produce alkaline phosphate and non-collagenous proteins such as osteopontin and osteocalcin. In our study, when HASMCs were incubated in calcification medium for 14 days, expression of osteopontin and osteocalcin was significantly increased in western blot analyses and this increased expression was significantly attenuated by treatment with rhBMP-7. BMP-7 also attenuated the increased intensity of alizarin red and von Kossa staining in HASMCs maintained in calcification medium for 2 to 3 weeks, and this effect was more pronounced as the stimulation time increased. Staining for alkaline phosphatase, commonly considered a marker of early differentiation to osteoblasts, was also attenuated in the presence of BMP-7. Total calcium deposition in HASMCs in calcification medium was significantly increased compared to control, and although it was not statistically significant, the amount of calcium deposited was decreased in the presence of rhBMP-7. One possible reason that the observed decrease was not statistically significant is that the deposition of bone-associated proteins precedes overt calcification, suggesting that the expression of these proteins is an early event during the change of VSMCs to osteoblast-like cells and that these proteins may regulate subsequent calcification. Hence, any decrease in the rate of total calcium deposition in HASMCs in the presence of BMP-7 might be relatively small over a period of 2 weeks.

It has been reported that the effect of BMP-7 in preventing trans-differentiation of VSMCs into the osteoblastic phenotype is primarily due to the induction of p21 and the upregulation of inhibitory smads 6 and 7 (Dorai et al. 2000; Li et al. 2004; Hruska et al. 2005). Subsequently, this may result in a decrease in vascular calcium deposition by BMP-7.

According to a previous study, increased expression of osteopontin induced by β-glycerophosphate is dependent on

Fig. 6. Qualitative calcium stainings of HASMCs after 2 weeks. HASMCs were incubated in calcification medium containing 12 mmol/L β-glycerophosphate (β-GP) in the absence or presence of rhBMP7 (200 ng/mL) for 2 weeks. Calcium staining was more intense in HASMCs in calcification media alone compared to control and BMP-7 attenuated phosphate-induced mineralization in HASMCs. Results are representative of three independent experiments with similar results. (a) alkaline phosphatase (ALP) stain, (b) von Kossa stain, (c) Alizarin red stain
alkaline phosphatase activity and Na/Pi co-transport (Chen et al. 2002). Three types of Na/Pi co-transporters have been identified thus far. Among these, the type III Na/Pi co-transporter, so called pit-1, was found to be expressed in human VSMCs (Lund et al. 2004; Li et al. 2004; Li et al. 2006). Therefore, another mechanism through which BMP-7 may suppress vascular calcium deposition is by inhibiting alkaline phosphatase activity and type III Na/Pi co-transporters in HASMCs.

**Conclusion**

The present study demonstrates that BMP-7 inhibits vascular calcification in the condition of high vitamin D or phosphate levels, each condition of which is encountered in chronic kidney disease and other pathologic conditions. Further studies are needed to identify the underlying mechanism, by which BMP-7 affects signaling pathways that are involved in vascular calcification process.

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