Intermedin inhibits vascular calcification by increasing the level of matrix γ-carboxyglutamatic acid protein

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Aims
Vascular calcification (VC) is highly associated with increased morbidity and mortality in patients with advanced chronic kidney disease. Paracrine/autocrine factors such as vasoactive peptides are involved in VC development. Here, we investigated the expression of the novel peptide intermedin (IMD) in the vasculature, tested its ability to prevent VC in vivo and in vitro, and examined the mechanism involved.

Methods and results
Rat VC was induced by administration of vitamin D3 plus nicotine (VDN). IMD (100 ng kg−1 h−1) was systemically administered by a mini-osmotic pump. VDN-treated rat aortas showed lower IMD content and increased expression of its receptors, along with increased vascular calcium deposition and alkaline phosphatase (ALP) activity. Low IMD levels were accompanied by increased calcium deposition in human atherosclerotic plaques. In vivo administration of IDM greatly reduced vascular calcium deposition and ALP activity in VDN-treated rats when compared with vehicle treatment, which was further confirmed in cultured vascular smooth muscle cells. Concurrently, the loss of smooth-muscle lineage markers and matrix γ-carboxyglutamic acid (Gla) protein (cMGP) in aortas was ameliorated by administering IMD to rats with VC, and the increased phosphor-Smad1/5/8 and core binding factor α-1 levels in calcified vasculature were also reduced. However, the inhibitory effects of IMD on VC were eliminated upon pre-treatment with warfarin or small interfering RNA to reduce cMGP.

Conclusion
Reduced endogenous IMD levels are associated with increased mineralization in vivo, and administration of IMD inhibits VC development by increasing cMGP levels. IMD may be an endogenous vasoprotective factor for VC.

Keywords
Vascular calcification • Peptides • Matrix γ-carboxyglutamatic (Gla) protein • Phenotype transition

1. Introduction
Atherosclerosis and vascular calcification (VC) are major complications of advanced chronic kidney disease; in particular, cardiovascular complications are promoted in patients with parathyroid hormone oversuppressed by excessive calcium or vitamin D3, and this is associated with a 20- to 30-fold increase in cardiovascular mortality.2–4 VC is an active, cell-regulated process, with many similarities to bone formation.4–6 Recent studies have shown that vascular smooth muscle cells (VSMCs) can lose the expression of smooth-muscle lineage markers and begin to express osteogenic markers and deposit a mineralized bone-like matrix.4–9 Once the osteogenic phenotype is induced, cells of the vascular wall show a distinctive molecular fingerprint, as indicated by increased levels of bone morphogenetic protein 2 (BMP2) and core binding factor α-1 (Cbfα1);9–11 alternatively, loss of endogenous inhibitors of mineralization, such as γ-carboxyglutamic acid (Gla) protein (cMGP), also contributes to VC.11,12
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2. Methods

2.1 Tissue specimens

Human atherosclerotic tissue samples were obtained from patients by directional atherectomy, according to protocols approved by the Medical Ethical Committee of Peking University that comply with the principles outlined in the Declaration of Helsinki. The specimens were fixed in 4% formaldehyde in phosphate-buffered saline and were paraffin embedded. Specimens were cut into 6 µm sections, then underwent haematoxylin and eosin (H and E), von Kossa, and immunohistochemistry staining.

2.2 Rat model of VC

All animals received humane care in compliance with the Institutional Authority for Laboratory Animal Care of Peking University that comply with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The rat VC model was created in our laboratory as described previously, briefly, rats were randomly assigned to VC group (Cal), IMD treatment group (Cal + IMD), or vehicle control group (Ctrl). Seventeen rats were given vitamin D3 (300,000 IU kg⁻¹ in arachis oil, intramuscularly) plus nicotine (25 mg kg⁻¹ in 5 mL peanut oil, intragastrically) (Sigma, St Louis, MO, USA) at 9 am on day 1. Nicotine was re-administered at 7 pm. Seven of these rats were treated with IMD (100 ng kg⁻¹ h⁻¹) (Phoenix Pharmaceuticals, Belmont, CA, USA) 24 h later. IMD was administered subcutaneously in pure water through an Alzet Mini-osmotic Pump (Alzet® model 2004, DURECT Corp., Cupertino, CA, USA). The remaining rats were the calcification control. An additional 10 rats were given corresponding solvents as a vehicle control.

2.3 Physiologic measurement

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Physiologic measurement involved use of a 2.0 F micromanometer conductance catheter. The catheter was placed in the right carotid artery and advanced into the left ventricle. Blood pressure (BP) and left intraventricular pressure were recorded by polygraphy (NEC San-ei Instruments, Japan).

2.4 Measurement of IMD content

After treatment for 28 days, rats were anesthetized and killed. The aortas were excised and immediately acidified with 1.0 M acetic acid, then heated at 100°C for 10 min to inactivate proteases, and tissue was applied to a Sep-Pak® C18 cartridge (Millipore-Waters, Milford, MA, USA). The plasma samples were prepared and pre-treated with aproatin (500 KIU L⁻¹). Radioimmunoassay was performed to measure plasma and tissue IMD content by use of a commercial radio-immunoassay kit (Phoenix Pharmaceuticals).

2.5 VSMC calcification model

VSMCs were isolated from the thoracic aortic arteries of Sprague–Dawley rats (150–180 g) and grown to passages 5 to 8. Cells at 100% confluence were treated with calcification medium containing β-glycerophosphate (10 mM) or warfarin (10 µM) 72 h before CaCl₂ (3.6 mM) as described previously with or without human IMD.

2.6 MGP small interfering RNA (siRNA) transfection

MGP siRNA was designed by use of Bicok-iT™ RNAi Designer and chemically modified by the manufacturer (Invitrogen, Carlsbad, CA, USA). Sequences corresponding to rat MGP siRNA (5′-GGA AGG GAC UGA CUU CAU AGG AUU C-3′) were sense, UGA AGG GAC UGA CUU CAU AGG AUU C, and antisense, GAA UCC UAU GAA GUC AGU CCC UUC A. Transfection of rat VSMCs with siRNA involved use of Oligofectamine (Invitrogen). A scrambled siRNA (Invitrogen) served as a negative control.

2.7 Detection of vascular calcification

Von Kossa staining and alizarin red staining were performed as described previously. Calcium content was measured by atomic
absorption spectrometry at 422.7 nm (Jena, novAA 300),26,27 and results were normalized by dry tissue weight or total protein.

2.8 ALP activity assay
Plasma, aortic tissue homogenate, and VSMC lysates were prepared as described by our laboratory.26,27 ALP activity was measured by use of an ALP assay kit (Jiancheng Bioengineering Co., Nanjing, China). Results were normalized to levels of total protein.

2.9 Real-time PCR analysis
Total RNA from aortic tissue or VSMCs was isolated and reverse transcribed by use of a reverse transcription system (Promega, Madison, WI, USA). One microtiter of the reaction mixture underwent real-time PCR. The amount of PCR product formed in each cycle was evaluated by SYBR Green I fluorescence. The forward and reverse PCR primers (rat) were IMD, 5'-CTA CGG TTC ATC TGC CTC AGG-3' and 5'-ACA GGC GAT GGC TGA TAT CTC-3'; osteopontin (OPN), 5'-AGA CCA GCC ATG AGT CAA GTC A-3' and 5'-TGA AAC TCG TGG CTC TGA TGT T-3'; BMP2, 5'-TCA AGG CAA ACA CAA ACA AGC GAC-3' and 5'-TGA GCT AAG CTC AGT GGG-3'; MGP, 5'-AAT CTC AGC AAA GCA TGG AAT C-3' and 5'-GCA GGC TTG TTG AGT TCC C-3'; Cbfα1, 5'-GCC AGG TTC AAC GAT GTG CGT AG-3' and 5'-GAG GCG TTC AGT AAC AGC GCC GGG-3'; b-actin, 5'-GAG ACC TTC AAC ACC CCA GCC CC-3' and 5'-TCG GGG CAT CGG AAC CGC TCA-3'. All amplification reactions involved use of the Mx3000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA).

2.10 Western blot analysis
Rat tissue extracts containing equal amounts of total protein were resolved by SDS–PAGE and then transferred to a nitrocellulose membrane. The membranes were incubated with primary antibody and IRDye™700 or 800-conjugated secondary antibody (dilution 1:20000, Rockland Inc., Gilbertsville, PA, USA). The fluorescence signal was then detected by use of the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). Primary antibodies for b-actin, receptor activity-modifying protein 1 (RAMP1), 2, and 3 and calcitonin receptor-like receptor (CRLR) were purchased from Santa Cruz Biotechnology (dilution 1:200; Santa Cruz, CA, USA); anti-calcitonin receptor-like receptor (CRLR) and anti-RAMP1, 2, and 3 were from Alexis Biochemicals (1:1000, Lausen, Switzerland); and anti-a-SMA antibody (dilution 1:1000) was from Sigma.

2.11 Immunohistochemistry
Frozen sections (6 μm) of aortas were incubated with primary anti-IMD (dilution 1:50, Phoenix Pharmaceuticals) or anti-a-actin (dilution 1:400) antibody, horseradish peroxidase-conjugated secondary antibody, and 3,3-diaminobenzidine successively; corresponding normal IgG (Santa Cruz Biotechnology) served as a negative control. Sections were then counterstained with H&E.

2.12 Statistical analysis
Data are expressed in mean ± SEM. Repeated measurements of calcified nodules were averaged before further analysis. Comparisons between two groups involved Student’s t-test, and that between more than two groups involved ANOVA, then Tukey–Kramer post hoc testing. A P < 0.05 was considered statistically significant.

3. Results

3.1 IMD and its receptor levels in vascular calcification
First, we investigated the expression of IMD in human atherosclerotic lesions obtained by directional atherectomy. H&E staining showed typical atherosclerotic lesions (Figure 1Aa). Von Kossa staining showed calcium-phosphate salt deposition in the tunica intima under the lesion and part of the fibrous cap (Figure 1A and B), and immunohistochemical staining showed significantly lower level of IMD than that in von Kossa-negative areas (Figure 1A–C).

Next, we investigated the levels of IMD in VC rat aortas and plasma. VDN-treated rats showed typical VC features, which show several similarities with calcification occurring in human athero- and arteriosclerosis,34 by increased vascular wall calcium deposition (Figure 2A and B) accompanied by systolic hypertension and low body weight (see Supplementary material online, Table S1). Plasma and aortic-tissue IMD contents were lower, by ~30 and ~56% (both P < 0.01), respectively, than in the control group (Figure 1B and C). Immunostaining revealed significantly lower IMD accumulation in the tunica media of calcified aortas than in the control group (see Supplementary material online, Figure S1), although the level of IMD mRNA in vascular tissue did not differ from that in controls (Figure 1D). Western blot analysis revealed the protein levels of CRLR and RAMP1, 2, and 3 increased by ~74, ~40, ~94, and ~80% (all P < 0.01), respectively (Figure 1E). Thus, the level of IMD was decreased but that of its receptors was increased in calcified vasculature.

3.2 IMD treatment attenuated VC
To investigate the functional contribution of IMD to VC, we treated VC rats with human IMD1–5. Von Kossa staining revealed dispersed calcified nodules among the elastic fibres in calcified aortas but not in control or IMD treatment rats (Figure 2A). The decreased calcium-phosphate salt deposition in IMD-treated calcified aortas was further confirmed by the aortic calcium content in IMD treatment group being lower, by ~83% (P < 0.01), than that in the vehicle group (Figure 2B). Furthermore, increased ALP activity, the functional phenotypic marker of osteoblasts, was decreased in both plasma (Figure 2C) and aorta homogenates with IMD treatment (Figure 2D).

We observed the in vivo effects of IMD on reducing VC in cultured VSMCs. IMD treatment significantly decreased calcium-phosphate salt deposition in cell calcification induced by b-glycerophosphate (Figures 2E and F and 6E) or CaCl2 (Figure 6C) assessed by alizarin-red staining and calcium content assay. As well, ALP activity was increased when VSMCs were cultured in calcification medium but was significantly decreased on treatment with IMD (Figures 2G and 6D and F).

3.3 IMD prevented loss of its lineage markers in VSMCs
Because the phenotype transition of smooth muscle cells is associated with VC in vitro and in vivo,7 we further investigated the expression of smooth-muscle lineage markers. Compared with
media of normal vessels, that of VC arteries showed significantly decreased level of SM α-actin on immunostaining and western blot analysis; however, IMD administration prevented the reduction in SM α-actin level (Figure 3A and B). Next, we investigated whether the levels of other smooth-muscle lineage markers, SM-22α and calponin, were rescued by IMD treatment during the mineralization process. Indeed, the level of SM-22α and calponin was reduced in parallel in VC aortas, and levels of both were rescued by IMD treatment (Figure 3B).

The in vivo effects of IMD on preventing smooth muscle from loss of its lineage markers were further confirmed in cultured VSMCs. The levels of the smooth-muscle lineage markers SM-22α, SM α-actin, and calponin were strikingly decreased in calcified VSMC cultures by day 7, but the decrease was greatly ameliorated with IMD treatment (Figure 4A).

### 3.4 IMD prevented the development of an osteogenic phenotype in VSMCs

Bone-associated molecules are present during mineralization of vasculature. We examined the expression of the osteogenic genes BMP2, Cbfa1, and OPN by real-time PCR. All three genes had a tendency to increase in level in calcified rat aortas (Figure 3C–E), but only the enhanced expression of Cbfa1 mRNA was fully prevented with IMD treatment (Figure 3C). The inhibitory effect of IMD on Cbfa1 expression was further confirmed by western blot analysis (Figure 5C).

These results were further confirmed by in vitro experiments showing the expression of BMP2 and Cbfa1 increased in calcified VSMCs at day 7; however, the levels of these two genes were strikingly decreased with IMD treatment (Figure 4B and C).
3.5 IMD inhibited the Smad1/5/8 signalling pathway in vivo and in vitro

BMP-dependent Smad1/5/8 signalling is a critical co-factor for Cbfα1-dependent osteogenic differentiation. cMGP is an inhibitor of tissue calcification and can antagonize the BMP signal. VC rat aortas and control aortas did not differ in MGP mRNA expression, but the cMGP protein level was strikingly decreased, by 84%, in VC aortas (P < 0.05, densitometric analysis data in see Supplementary material online, Figure S2). However, IMD treatment significantly increased the levels of MGP mRNA and cMGP protein (Figure 5A and C).

Correspondingly, phosphor-Smad1/5/8 and Cbfα1 protein levels were increased in calcified rat aortas, which were reversed by IMD treatment (Figure 5C). We treated live VSMCs with rhodamin-labelled IMD, and found rhodamin fluorescence was colocalized with lysosome, which was blocked by IMD17–47 (a blocking peptide of IMD receptors) (see Supplementary material online, Figure S4A). Thus, similar results were obtained in cultured VSMCs (Figure 5B and D), and IMD treatment increased the MGP mRNA expression as early as 6 h, which was completely blocked by cAMP-PKA inhibitor H-89 (see Supplementary material online, Figure S4B).
3.6 IMD inhibited VSMC calcification by increasing cMGP level

We next aimed to demonstrate whether cMGP is necessary for IMD preventing VC. First, VSMCs treated with warfarin, an inhibitor of MGP carboxylation, for 72 h showed significantly decreased cMGP levels (Figure 6A). Second, siRNA targeting rat MGP (siMGP) was used to knockdown MGP expression. The levels of MGP mRNA (see Supplementary material online, Figure S3) and cMGP protein (Figure 6B) were decreased on transfection with siMGP. Correspondingly, the inhibitory effects of IMD on VSMC calcification, as assessed by calcium content and ALP activity, were eliminated in the presence of warfarin (Figure 6C and D) and siMGP (Figure 6E and F). Thus, preventing the reduction in cMGP protein level is essential for IMD inhibiting VC.

4. Discussion

VC, frequently observed in patients with end-stage renal disease, is an important risk factor for cardiovascular events, especially for patients with over suppression of parathyroid hormone by excessive calcium or vitamin D3, which promotes cardiovascular complications. In this study, we used high doses of vitamin D3 to promote VC in rats and found that IMD prevented VC. A substantial decrease in IMD level was associated with increased calcium deposition in the arterial wall in both human atherosclerotic lesions and in rat aortas with VC. Using a gain-of-function approach, we demonstrated that exogenously administered IMD inhibited rat VC, increased the expression of smooth-muscle lineage markers, and decreased Smad1/5/8-Cbfα1 signalling, which led to attenuating the osteogenic transition of contractile

Figure 3 IMD depressed smooth muscle cell phenotypic transition in rat aortas. (A) Rat aortas in the control (Ctrl), calcification (Cal), and IMD-treated (Cal+IMD) groups were stained with anti-α-smooth muscle (SM) α-actin antibody. Results are from one representative experiment of six. (B) Protein samples from aortic tissue were prepared for western blot analysis; smooth-muscle lineage markers (calponin, SM-22α, and SM α-actin) were detected; β-tubulin from the same blot were controls for protein loading. (C–E) Quantitative real-time PCR of mRNA levels of osteogenic markers [core binding factor α-1 (Cbfα1), bone morphogenetic protein (BMP2), osteopontin (OPN)] in VC. n = 5–10 rats, *p < 0.05 vs. Ctrl. #p < 0.05 vs. Cal.
VSMCs. The molecular basis underlying this protective effect is the increased level of cMGP protein by IMD treatment, which was supported by the fact that IMD treatment increased the cMGP level, and the vasoprotective properties disappeared when cMGP level was blocked with warfarin or siRNA treatment.

In this study, rats treated with high doses of vitamin D plus nicotine showed typical VC, which has several similarities with calcification occurring in human athero- and arteriosclerosis. Rat aortas showed increased calcium content, by a 13-fold, along with systolic hypertension and low body weight, which is in line with results of several previous studies by our laboratory and others. However, unlike results of ADM in the rat model or patients with arteriosclerosis, we found a marked reduction in IMD protein level in VC rat aortas. And IMD treatment significantly alleviated these complications (see Supplementary material online, Table S1), especially the increased systolic BP in VC rats was significantly reduced by subcutaneously treated with IMD (100 ng kg\(^{-1}\) h\(^{-1}\)) through an Alzet Mini-osmotic Pump for 28 days, but it was not lower than the control rat, which may differ from the direct hypotensive effect by bolus intravenous injection of higher dose of IMD (\(5000–50000\) ng kg\(^{-1}\)). Hence, it may indicate that the effect of IMD on lowering BP in the present study is a result of reduced VC. Therefore, consumed endogenous IMD may confer more sensitivity of the vasculature to calcification conditions, and with the addition of exogenous IMD, the system is reactivated and protects the vasculature against VC.

Ample evidence has suggested that increasing ALP activity and promoting osteogenic transition of contractile VSMCs are related to vitamin D-induced VC. Thus, arterial calcification is considered associated with a loss of smooth-muscle lineage markers and a gain of an osteogenic phenotype as indicated by increased expression of BMP2 and Cbf1. In the present study, we found strikingly decreased levels of SM-22\(\alpha\), SM\(\alpha\)-actin and calponin, enhanced ALP activity, and increased expression of BMP2 and Cbf1 in calcified vascular cells in vivo and in vitro. These features were reversed on IMD treatment. Therefore, IMD attenuated the osteogenic transition of contractile VSMCs, thus reducing vascular calcium deposition and ALP activity.

Inhibitors such as pyrophosphate, OPN, osteoprotegerin, and MGP are necessary to prevent soft-tissue calcification. MGP is thought to be an inhibitor of VC because of its Ca\(^{2+}\)-binding \(\gamma\)-carboxyglutamic acid (Gla) motif, inhibition of cartilage calcification and binding to and antagonizing BMP2 to prevent osteogenic transition of VSMCs. Vitamin K-dependent \(\gamma\)-carboxylation is essential for the full function of MGP, by binding calcium, forming a MGP–BMP2 complex to prevent BMP receptor signalling and blocking the osteogenic transition of VSMCs. Our study provided direct evidence that decreased Gla modification of MGP was associated with vitamin D\(\alpha\) and nicotine-induced VC, which is also frequently found in patients undergoing haemodialysis and calciphylaxis. Interestingly, IMD treatment significantly increased MGP level and augmented its

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**Figure 4** IMD depressed smooth-muscle-cell phenotypic transition in calcified VSMCs. (A) Cultured rat VSMCs were treated with \(\beta\)-glycerophosphate for 7 days with or without IMD (10\(-7\) M). Western blot analysis of smooth-muscle lineage markers (calponin, SM-22\(\alpha\) and SM\(\alpha\)-actin); \(\beta\)-actin, and \(\beta\)-tubulin were controls for protein loading. (B and C) Quantitative real-time PCR analysed mRNA levels of osteogenic markers (Cbf1, BMP2) in calcified VSMCs. Results are relative to \(\beta\)-actin expression. \(n = 5–10\) experiments, *\(P < 0.05\) vs. Ctrl. #\(P < 0.05\) vs. Cal.
carboxylation in calcified vasculature. Because IMD could not prevent VSMC calcification when γ-carboxylation of MGP was blocked by warfarin or knocked down by siRNA, the calcification-inhibiting effects of IMD would be mediated at least in part by increased MGP expression and carboxylation, thus antagonizing BMP and altering cell differentiation. As Miller et al. reported, warfarin was widespread used in patients on haemodialysis. As well, animal models have well demonstrated that extensive VC occurs when γ-carboxylation of MGP is inhibited by warfarin blocking the vitamin K epoxide reductase cycle. Together with the present study, warfarin may promote VC in patients on haemodialysis by blocking the vitamin K epoxide reductase cycle and inhibiting the protection of endogenous active peptide on VC.

Under physiological conditions, IMD and its receptors are richly expressed in vessels, and cAMP accumulation by activating these receptors is thought to be associated with reduced VC. And MGP transcription is upregulated by cAMP. Increased cAMP accumulation may play a part in IMD-induced MGP expression. We found rhodamine-red labelled IMD internalized into lysosomes in live VSMCs, and its induction was blocked by the receptor blocking peptide IMD17–47 (see Supplementary material online, Figure S4A). As well, the increased expression of MGP was fully blocked by the cAMP-PKA inhibitor H-89 (see Supplementary material online, Figure S4A).
These results may indicate that IMD works mainly at the receptor level to reduce VC. The underlying molecular basis of IMD-induced increased expression and carboxylation of MGP should be further explored.

From our results, the novel vasoactive peptide IMD might be a new paracrine/autocrine factor that inhibits VC by preventing the osteogenic transition of contractile VSMCs through increasing cMGP level. IMD could be an endogenous cardiovascular protective peptide in a new therapeutic strategy for cardiovascular diseases and prevent VC in patients with end-stage renal disease.

The limitation of the present study is that we did not measure the levels of IMD in IMD-treated animals, and more pharmacological experiments about IMD shall be further explored.

Supplementary Material

Supplementary Material is available at Cardiovascular Research online.

Conflict of interest: none declared.
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