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- Development of a New Animal Model -

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Role of Osteoprotegerin in Arterial Calcification
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Objectives—Enhanced osteoclastogenesis, increased bone resorption, and osteoporosis have been reported in osteoprotegerin-deficient (OPG (−/−)) mice. OPG (−/−) mice available in Japan usually do not show vascular calcification. We have found that arterial calcification can be quickly induced by a simple procedure in OPG (−/−) mice.

Methods and Results—Male OPG (−/−), OPG (+/−), and OPG (+/+) mice were fed a high phosphate diet from 6 to 10 weeks after birth, and then 1α,25-dihydroxyvitamin D3 (calcitriol) was injected for 3 days. We found that severe calcification developed in the media of the aorta in OPG (−/−) mice. Under electron microscopy, calcium deposits were observed in the cytoplasm and extracellular matrix of vascular smooth muscle cells (VSMCs). Neither apoptosis of VSMCs nor infiltration of macrophages was observed. Alkaline phosphatase (ALP) activity of aortic tissue correlated with the calcified lesion area. Mouse aorta and bone extracts revealed an identical pattern by ALP electrophoresis.

Conclusions—Our results demonstrated that OPG had anticalcification activity in the aorta, probably through the downregulation of ALP activity. Because the time course of arterial calcification after the injection of calcitriol is accurate and reproducible, this mouse model will be useful for further investigation of vascular calcification. (***Arterioscler Thromb Vasc Biol.* 2007;27:2058-2064.**)

Key Words: osteoprotegerin ■ alkaline phosphate ■ vascular smooth muscle cells ■ calcium deposits

Vascular calcification, which is frequently observed in patients with end-stage renal disease, diabetes, aging, and osteoporosis, can also lead to cardiovascular diseases and even sudden death.[1–3] Until recently, vascular calcification was considered to be a passive process that occurred as a nonspecific response to tissue injury or necrosis. Now it is becoming increasingly clear that vascular calcification is an actively regulated process that resembles bone metabolism and involves alkaline phosphatase (ALP) and other bone-related proteins.[4–7]

Osteoprotegerin (OPG) is abundantly produced by osteoblasts at the bone surface and inhibits osteoclast activity, working as a key regulator of bone homeostasis.[8,9] Since it has been reported that OPG (−/−) mice exhibit severe osteoporosis attributable to enhanced osteoclastogenesis, OPG is considered to be a protective factor in bone metabolism.[10,11] In the vasculature, the function of OPG is unknown because it is unclear whether vascular calcification takes place in OPG (−/−) mice or not.[10,11] Moreover, it was reported that the serum OPG level is associated with the presence and severity of coronary artery disease (CAD).[12] It remains to be clarified whether OPG is involved in the progression of CAD or whether the upregulation of serum OPG concentration is a compensatory mechanism. ALP is a crucial enzyme for initiating mineralization in bone and is present in systemic arteries, arterioles, and some capillaries.[13] It is possible that this enzyme plays a role in arterial calcification by the same mechanism of action as that in bone.[14] Activation of ALP in the arterial wall may result in enhanced vascular calcification.

It is well known that either an elevated serum phosphate level or treatment with high doses of vitamin D induces vascular calcification in animal models as well as in humans.[15,16] In the present study, using OPG (−/−) mice, we established a mouse model in which arterial calcification can be quickly induced by treatment with a high phosphate diet plus 1α,25-dihydroxyvitamin D3 (calcitriol) injection, and this model allowed us to perform detailed pathological and biochemical examinations at desired time points.

Materials and Methods

Male OPG (−/−), OPG (+/−), and OPG (+/+) mice, 6 weeks of age were used in this study. We divided the mice with 3 different

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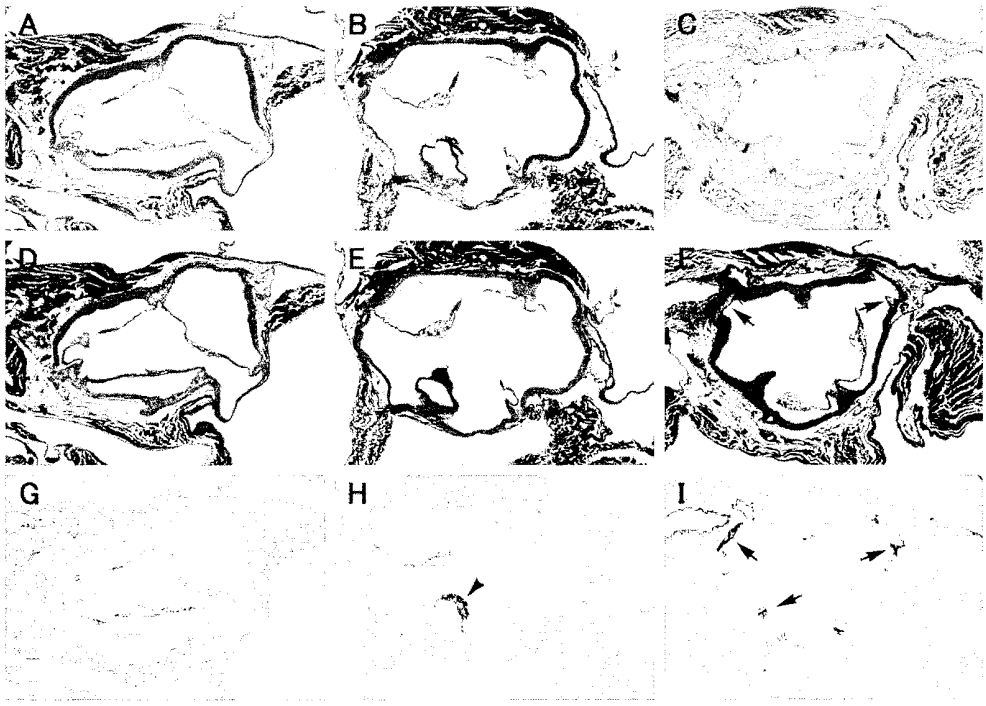

Figure 1. Representative serial sections of aortic sinus from mice given high-phosphate diet plus calcitriol injection. Sections were obtained from OPG (+/+) mice (A, D, G), OPG (+/−) mice (B, E, H), and OPG (−/−) mice (C, F, I). Hematoxylin and eosin stain (A-C), Azan stain (D-F), and von Kossa stain (G-I) were performed. Calcification of the aortic sinus is more obvious in OPG (−/−) mice (I) than in OPG (+/+) (G) and OPG (+/−) mice (H). Arrow, calcified lesion. Arrowhead, hemosiderin deposit.

genotypes into 3 different load groups; standard diet plus saline injection, high phosphate diet plus saline injection, and high phosphate diet plus calcitriol injection. The 9 groups of mice were fed a standard diet until 6 weeks. The mice were fed either a standard or high (1.5%) phosphate diet from 6 weeks to 10 weeks and water ad libitum throughout the study. At 9 weeks of age, a subcutaneous injection of saline or 5 μg/kg body weight of calcitriol was given for 3 days. The mice were euthanized at 10 weeks of age, and histopathologic and histochemical analyses were performed. For details, please see supplemental data, available online at http://atvb.ahajournals.org.

Results

Establishment of Vascular Calcification Model

We found that a combination of a lower dose of a high phosphate diet, containing 1.5% phosphorus, and injection of calcitriol (5 μg/kg, for 3 days) resulted in significant calcification in the arterial wall of OPG (−/−) mice (Figure 1). The mortality rate of mice treated with a high phosphate diet plus calcitriol was 0%, 17%, and 25% in OPG (+/+), OPG (+/−), and OPG (−/−) mice, respectively. Most of the deaths took place on day 4 or 5, and hemorrhage into the thoracic cavity was frequently observed, probably attributable to aortic dissection that occurred during vascular calcification. After this critical time point, most of the mice could survive.

Light Microscopic Analysis

Sections of the aortic sinus in OPG (+/+), OPG (+/−), and OPG (−/−) mice fed a high-phosphate diet plus calcitriol injection were observed by light microscopy (Figure 1). In OPG (+/+) mice, there was no visible calcification (Figure 1G). On the other hand, in OPG (−/−) mice, calcification was detected by von Kossa staining, shown by dark brown in the arterial media (Figure 1I, arrow). Azan staining showed reduced blue staining in the same lesion, indicating decreased elastic fibers (Figure 1F, arrow). These lesions were associated with a reduction in the thickness of the vascular smooth muscle layer (Figure 1C). In immunohistochemical analysis, expression of α-SM actin in these mice was not decreased in the calcified arterial lesions. In the arterial wall from all groups, there were no F4/80-positive cells (data not shown).

Measurement of Calcified Lesion Area

The calcified lesion area in the aortic sinus was carefully determined in 72 mice, and individual data points are plotted by genotype, diet, and calcitriol injection in Figure 2. In OPG (−/−) mice, aortic sinus calcification was significantly augmented by a high-phosphate diet plus calcitriol injection. Among the 3 genotypes with this treatment, there was a significant difference in the calcified lesion area, which was approximately 2.5 times higher in OPG (+/−) mice, and 17.7 times higher in OPG (−/−) mice than in OPG (+/+) mice (Figure 3).

Electron Microscopy

To clarify the time course of aortic calcification induced by a high phosphate diet plus calcitriol treatment, we obtained the ascending aortas from the 3 genotypes at 2, 4, and 7 days after the initiation of saline or calcitriol injection. On day 2, there were no abnormal findings in the 3 genotypes (data not shown). However, on day 4, treatment with a high phosphate diet plus calcitriol injection induced calcification, ranging

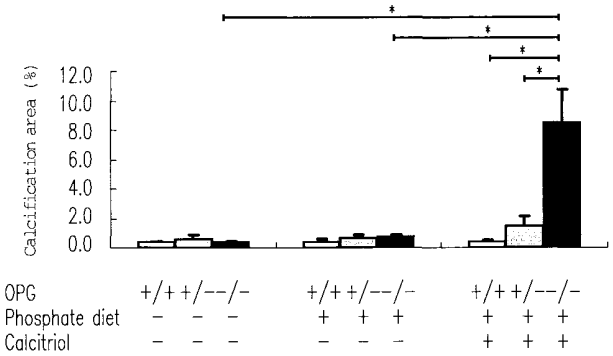

Figure 2. Effect of high-phosphate diet plus calcitriol injection on calcified area of aortic sinus in 3 genotypes. Calcified area was calculated in OPG (+/+) (white bar), OPG (+/−) (gray bar), and OPG (−/−) mice (black bar). OPG (−/−) mice given a high-phosphate diet plus calcitriol injection showed significant arterial calcification. Values are expressed as mean±SEM (n=8). *$P<0.0001$.

from minimal to severe depending on the genotype. In OPG (+/+) mice treated with a high phosphate diet plus calcitriol, localized dense deposits in the extracellular matrix of vascular smooth muscle cells (VSMCs) and granular deposits in the cytoplasm of VSMCs were occasionally seen (Figure 3B). On the other hand, in OPG (−/−) mice treated with a high phosphate diet plus calcitriol, extensive diffuse calcification in the cytoplasm and extracellular matrix of VSMC was observed (Figure 3D through 3F). On day 7, the same results as those on day 4 were observed (data not shown). On intensive examination of specimens from the three genotypes (day 2, 4, and 7), we could not detect any apoptotic smooth muscle cells or infiltrating cells, such as macrophages.

Serum and Aortic Tissue ALP Activity

In the standard diet plus saline injection group at 10 weeks of age, OPG (−/−) mice showed significantly elevated serum ALP activity compared with that of OPG (+/+) mice and OPG (+/−) mice (Figure 4A). In the high phosphate diet plus saline injection groups, there was no difference in serum ALP activity compared with the standard diet plus saline injection groups in each genotype. On the other hand, in each genotype, calcitriol injection increased the serum ALP activity. In the high phosphate diet plus calcitriol injection groups, OPG (−/−) mice showed significantly elevated aortic tissue ALP activity compared with that of OPG (+/+) and OPG (+/−) mice (Figure 4B).

ALP Isozymes

Representative ALP electrophoretic membranes of serum and organs from OPG (+/+) and OPG (−/−) mice given a standard diet plus saline injection or a high-phosphate diet plus calcitriol injection are shown in Figure 5. As mentioned

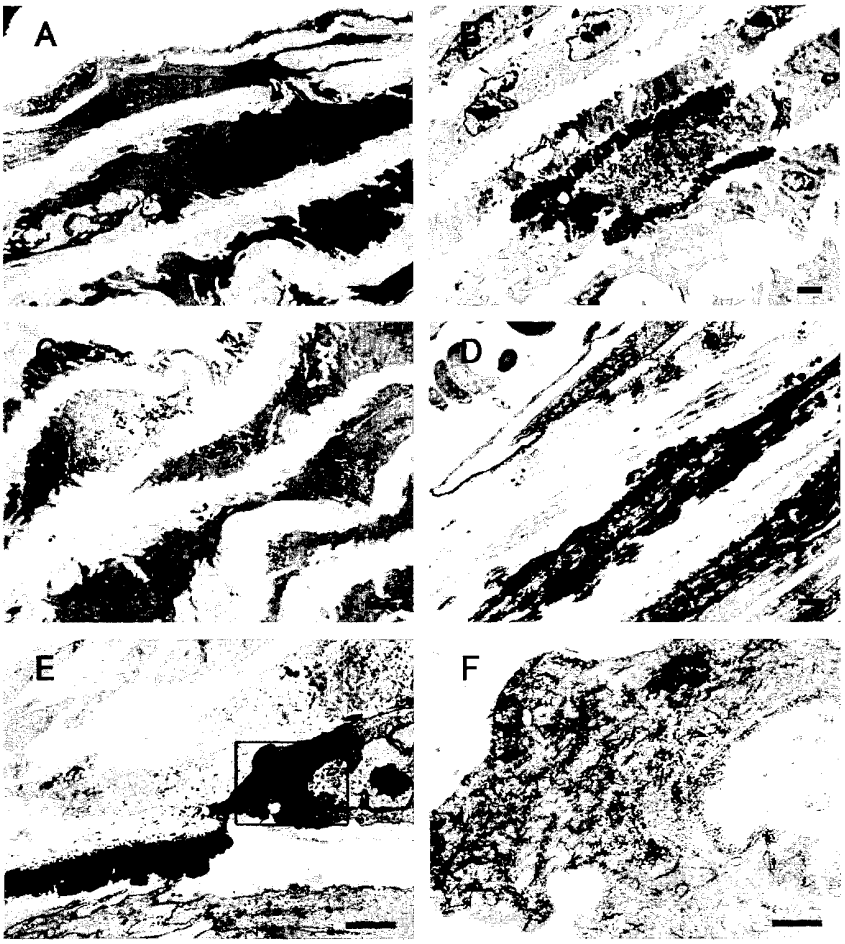

Figure 3. Electron micrographs of ascending aorta 4 days after initiation of saline or calcitriol injection. Samples were obtained from OPG (+/+) (A, B) and OPG (−/−) mice (C-F). A and C, Ascending aortic wall of mice treated with standard diet plus saline injection showed normal vascular smooth muscle cells, regardless of genotype. B, In OPG (+/+) mice treated with a high-phosphate diet plus calcitriol injection, dense deposits in the extracellular matrix and granular deposits in the cytoplasm of VSMC were occasionally seen. D through F, In OPG (−/−) mice treated with a high phosphate diet plus calcitriol injection, extensive diffuse calcification was observed in the cytoplasm and extracellular matrix of VSMC. E, In the panel, dense deposits in the extracellular matrix and needle-like crystals in the cytoplasm of VSMCs were observed. F, Higher magnification of panel E. In A-E, bar=2 μm; F, bar=500 nm.

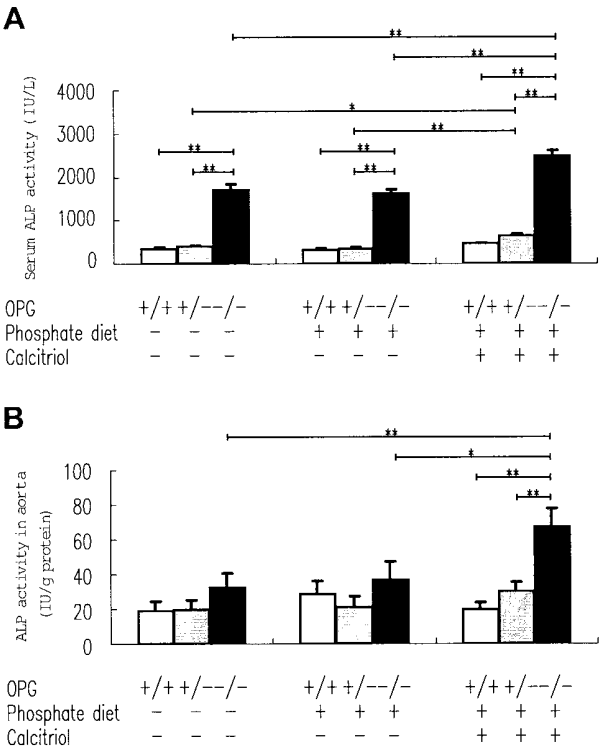

Figure 4. A, Effect of high-phosphate diet plus calcitriol injection on serum ALP activity in 3 genotypes. Values are expressed as mean±SEM (n=14). *P<0.05, **P<0.01. B, Effect of high-phosphate diet plus calcitriol injection on aortic tissue ALP activity in 3 genotypes. Aortic tissue ALP activity was measured and normalized by tissue protein content and expressed as mean±SEM (n=9). Aortic tissue ALP activity in OPG (−/−) mice was significantly upregulated by administration of calcitriol compared with the other genotypes. *P<0.01, **P<0.001.

in the methods, the running patterns of mouse samples are totally different from those of bovine and human samples. In OPG (−/−) mice given a high phosphate diet plus calcitriol injection, aortic and bone extracts revealed an identical pattern; however, mouse serum showed a completely different mobility pattern. Aortic extract in other groups did not show any distinct bands, probably because of weak ALP activity (Figure 4B).

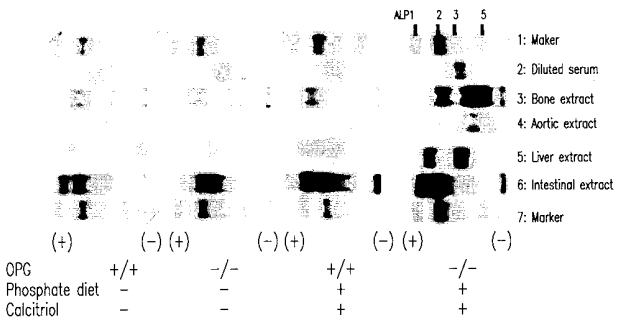

Figure 5. Representative ALP electrophoretic membranes of serum and organs from OPG (+/+) and OPG (−/−) mice given standard diet plus saline injection or high phosphate diet plus calcitriol injection. Samples were run from the application point (−) to the front line (+). For the aortic extract, 2 aortas were combined. Serum was diluted 5-fold with saline. Bone extract was a homogenate of femur containing bone marrow. For loading, roughly 0.2, 0.2, 1.0, 5.0, and 0.4 mg protein was applied in lanes 2, 3, 4, 5, and 6, respectively.

PTHrP Level in Aortic Tissue

In the standard diet plus saline injection groups, aortic PTHrP level in OPG (−/−) mice was lower than that in OPG (+/+) mice (supplemental Figure I). Administration of a high phosphate diet plus calcitriol injection did not change PTHrP level in each genotype. There were no significant differences in the plasma level of PTHrP among the experimental groups (data not shown).

Histological Analysis of Femur

In the standard diet plus saline injection groups, the ratio of porous area/cortical area in OPG (−/−) mice was higher than that in OPG (+/+) mice (supplemental Figure II). Administration of a high-phosphate diet plus calcitriol injection did not change the ratio of porous area/cortical area in each genotype.

Serum Calcium and Inorganic Phosphate (P_i) Levels

Serum calcium level was significantly elevated by subcutaneous administration of calcitriol in OPG (−/−) mice (supplemental Figure IIIA). There was no significant difference in P_i level among the experimental models (supplemental Figure III B).

Blood Pressure

While receiving a standard diet, systolic blood pressure was similar in the 3 genotypes (OPG (−/−) 109±7 mm Hg, OPG (+/−) 120±3 mm Hg, OPG (+/+) 118±4 mm Hg). A high-phosphate diet for 3 weeks before injection did not change the systolic blood pressure in any group. Under a high phosphate diet, 7 days after the initiation of calcitriol injection, systolic blood pressure in OPG (−/−) mice was significantly lower than that in OPG (+/−) and OPG (+/+) mice (95±15, 112±4, and 110±6 mm Hg, respectively, P<0.05).

Discussion

The present study shows that we have established a mouse model in which arterial calcification can be quickly induced by a simple procedure. This model provides both an accurate time course and high reproducibility for the development of vascular calcification, and will be useful to clarify the mechanism of arterial calcification. There are 2 well-known knockout mice—matrix Gla protein (MGP)-deficient mice and Klotho-deficient mice, which are reported to develop extensive vascular calcification and other organ disorders from a few weeks after birth.[17,18] Their average lifespan is about 2 months, therefore these mice are not suitable for vascular calcification research. For these reasons, we used OPG (−/−) mice to establish a mouse model to clarify the mechanism of arterial calcification. We could produce arterial calcification by a combination of a high (1.5%)-phosphate diet and calcitriol administration.

Vascular calcification is an actively regulated process that is associated with bone-related proteins bone morphogenetic protein-2, MGP, osteopontin (OPN), and osteocalcin (OC), as

well as several transcription factors, including osteoblast transcription factors Runx2/Cbfa1, Msx2, and chondrocyte transcription factors, such as Sox9.[4-6,19,20] Various vascular cells in the arterial wall participate in the process of calcification. Especially, it is reported that VSMCs derived from the aorta have calcifying capacity and express MGP, OPN, and OC.[4,7,21,22] OPG is produced in various tissues including main components of the human vasculature, such as VSMCs and endothelial cells.[8,23]

OPG has been isolated by 2 laboratories independently.[8,9] It is a secreted protein of the tumor necrosis factor (TNF) family that regulates bone mass by inhibiting osteoclast differentiation and activation. OPG exerts its inhibitory effects on osteoclasts by binding to receptor activator of nuclear factor κB (RANK) ligand, thereby inhibiting the interaction between RANK and RANK ligand on osteoclasts and their precursors.[24] In mice, targeted deletion of the OPG gene resulted in an overall decrease in total bone density and a high incidence of bone fractures.[10,11] The osteoporosis with early onset observed in these mice was characterized by an increased number and activity of osteoclasts. In human, osteoporotic patients have a higher prevalence of arterial calcification.[25,26] Osteoporosis and vascular calcification frequently occur together and share many of the same risk factors.[3,25-27]

OPG (−/−) mice reported by Mizuno et al are available in Japan.[10] Mizuno et al did not report whether or not vascular calcification occurred in their OPG (−/−) mice.[10] Bucay et al reported that approximately two-thirds of OPG (−/−) mice developed arterial calcification in the first several weeks after birth.[11] However, it was reported that the calcified lesions were extremely limited.[28] Unfortunately, we could not obtain the OPG (−/−) mice that Bucay et al developed and used in their study. According to the papers, the genetic backgrounds of the 2 mice might be very similar.[10,11] This discrepancy in phenotype between these reports and our results might be attributable to differences in environment such as diet and drinking water, especially in their mineral content.

Here, we focused on 2 molecules, 1α,25-dihydroxyvitamin D3 (calcitriol) and phosphate, which have been implicated in the induction of vascular calcification. Vitamin D3 is critically important for the development, growth, and maintenance of a healthy skeleton from birth to death. However, its function is very complex. A physiological dose of vitamin D3 has the effect of promoting both bone resorption and formation. On the other hand, high-dose vitamin D3 increases bone resorption by activating osteoclastogenesis and inhibiting the expression of OPG.[29] In our experiments, the bone morphology in OPG (−/−) mice showed an osteoporotic phenotype including increased porous area and decreased bone volume of femoral cortex, consistent with earlier reports (supplemental Figure II). Administration of a high-phosphate diet plus calcitriol injection did not influence the bone morphology in both OPG (−/−) and OPG (+/+) mice. One possibility is that the effect of OPG deficiency was far more potent than the effects of a high phosphate diet plus calcitriol injection. The other possibility is that the time course after calcitriol treatment was only 1 week, which may be too short to affect bone density.

In the vasculature, it has been reported that high-dose vitamin D3 induces vascular calcification in animal studies, but the precise mechanism is not clearly understood.[15,30] It was reported that matrix metalloproteinase (MMP) is involved in aortic calcification, and inhibiting MMP activity could reduce calcium accumulation in the arterial wall.[31] It was also reported that excess vitamin D3 can increase calcium uptake into smooth muscle cells.[32,33] In VSMCs, vitamin D3 increases the expression of bone-related proteins such as OPN and ALP, which may be responsible for vascular calcification.[7,34] In our experiments, administration of calcitriol in OPG (−/−) mice given a high phosphate diet increased their aortic tissue ALP activity, and serum ALP and calcium level. It is thought that active osteoclastogenesis in OPG (−/−) mice was further enhanced by treatment with calcitriol, and resulted in elevation of serum calcium level.

Calcium-regulating hormones such as PTHrP may modulate atherosclerotic calcification. It was reported that PTHrP inhibits BVSMC calcification through depression of ALP activity, and that PTHrP secreted from BVSMCs acts as an endogenous inhibitor of vascular calcification, suggesting that VSMCs may be equipped with an autocrine or paracrine system that regulates calcium metabolism.[35] In our experiment, deficiency of the OPG gene decreased the PTHrP level in aortic tissue, suggesting that vascular calcification might be partly PTHrP-dependent. However, the load of a high phosphate diet plus calcitriol injection did not significantly change the PTHrP level in aortic tissue, but strongly enhanced vascular calcification. It is suggested that the enhancement may be PTHrP-independent.

Hyperphosphatemia is a frequent complication in patients with end-stage renal failure, who have severe calcification of vessel walls and high mortality from cardiovascular disease. In vitro, elevation of the phosphate level stimulates VSMC phenotypic transition and mineralization via the activity of a sodium-dependent phosphate cotransporter.[36-38] In spite of a high (1.5%)-phosphate diet, serum phosphate level was not changed in all genotypes, probably as a result of renal compensation. However, the serum calcium level of OPG (−/−) mice with high-phosphate diet plus calcitriol injection was relatively high. Interestingly, it was reported that in human VSMCs, calcium was a more potent inducer of vesicle-mediated calcification than phosphate.[39]

In our light microscopic analysis, increased calcium deposition was seen in the medial layer of the aortic sinus in OPG (−/−) mice with a high-phosphate diet plus calcitriol treatment. However, OPG (−/−) mice did not show atherosclerotic lesions with lipid accumulation and inflammation, but these calcified lesions were confined to the media of arterial wall. In electron microscopic analysis, we observed a needle-like calcium matrix in the cytoplasm of VSMCs and calcium deposits around VSMCs in OPG (−/−) mice with a high-phosphate diet and calcitriol treatment. However, we could not detect any apoptotic cells or infiltrating macrophages in the arterial wall showing calcific changes. Endothelial cell morphology was normal in all groups. Our findings suggest that the mechanism of calcification in this mouse model is not related to apoptosis, but rather is derived from the cellular activity of VSMCs.

ALP are highly ubiquitous enzymes present in most species from bacteria to man. Although their wide distribution in nature indicates that these enzymes perform important biological functions, their detailed roles and natural substrate are not known. In humans, there are at least 4 ALP genes: liver/bone/kidney (non-tissue–specific), intestinal, placental, and placental-like. The non–tissue-specific form is located on chromosome 1, whereas the latter 3 are located together on chromosome 2. In human serum, there are 2 major circulating ALP isozymes—bone-type and liver-type. They are derived from a single gene and differ only in posttranslational glycosylation. Bone-type ALP is crucial for initiating mineralization of bone. Min et al reported that serum ALP activity in OPG (−/−) mice was elevated compared with that in wild-type mice, probably because of increased osteoclast activity in OPG (−/−) mice.[40] After administration of recombinant OPG in OPG (−/−) mice, serum ALP activity decreased to that in wild-type mice, probably because of normalization of osteoclast activity. However, they did not examine ALP activity in the aorta. In the present study, ALP activity was measured in both serum and aorta. Serum ALP activity was elevated regardless of a high phosphate diet plus calcitriol treatment; however, aortic tissue ALP activity was significantly upregulated only in OPG (−/−) mice treated with a high phosphate diet plus calcitriol. Shioi et al reported that cultured human VSMCs could express bone-type ALP derived from the non–tissue-specific ALP gene under certain circumstances.[7] Our electrophoretic data showed that the pattern in aortic extract resembled that in bone extract and did not resemble that in serum, liver extract, or intestinal extract. These results suggest that in OPG (−/−) mice treated with a high-phosphate diet and calcitriol, bone-type ALP activity in the aorta is increased and may contribute to aortic calcification.

Limitation

As is the case with animal experiments with serious histological changes in the arterial system, the mortality rate of our model is relatively high. We could not clarify the cause of death in OPG (−/−) mice when there was no hemorrhage into thoracic cavity. In such cases, renal failure may be responsible for a cause of death.

In conclusion, we have established an arterial calcification model with high reproducibility using OPG-deficient mice with a high (1.5%)-phosphate diet plus calcitriol treatment. In one sense, treatment with a high-phosphate diet plus calcitriol injection highlighted the role of OPG in the pathogenesis of vascular calcification. This OPG (−/−) mouse model will be useful for further investigation of vascular calcification.

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Disclosures

None.

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