Role of Osteoprotegerin in Arterial Calcification
Development of New Animal Model
Yuichi Orita, Hideya Yamamoto, Nobuoki Kohno, Masaaki Sugihara, Hiroaki Honda, Seiichi Kawamata, Shinji Mito, Nwe Nwe Soe, Masao Yoshizumi

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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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/H9262 g/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 (+/+), 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...
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 in the figure, OPG (−−−−) mice showed significant arterial calcification.
show any distinct bands, probably because of weak ALP activity (Figure 4B).

**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\textsubscript{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\textsubscript{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. These 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.5,7,21,22 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 (+/+). 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. 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. 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.

References


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MATERIAL AND METHODS

Experimental model

All experimental procedures were approved and carried out in accordance with the guidelines of the Committee of Animal Experimentation, Hiroshima University. OPG (-/-) mice of C57BL/6J strain were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mizuno et al. did not report whether or not vascular calcification occurred in their OPG (-/-) mice. Bucay et al. reported that approximately two-thirds of OPG (-/-) mice developed arterial calcification in the first several weeks after birth. According to these papers, both mice seemed to be derived from the same embryonic stem cells (129SV), and the neo cassette was inserted into the Pst I site in exon 2 to disrupt the OPG reading frame. Mizuno et al. backcrossed the mouse to the C57BL/6 strain. On the other hand, Bucay et al. backcrossed the mouse to the C57BL/6 strain or Swiss black mice. Although it was not certain that how many times both mice were backcrossed, the genetic backgrounds of the two mice might be very similar. Heterozygous (OPG (+/-)) mice and control wild-type (OPG (+/+)) mice were obtained by intercrossing OPG (+/-) mice. All animals were housed in a room where lighting was controlled (12 hours on, 12 hours off) and the temperature was kept at 25°C. All newborns were genotyped by polymerase chain reaction methods using DNA from the distal tail. The following primers were used for detecting target genotypes: OPG (forward, 5'-TCC TGG CAC CTA CCT AAA ACA GCA C-3'; reverse, 5'-GCT AAC GCC CTT CCT CAC ACT CAC-3') and neo (forward, 5'-GCT GCA TAC GCT TGA TCC GGC TAC-3'; reverse, 5'-TAA AGC ACG AGG AAG CGG TCA GCC-3'). The pups were weaned at 4 weeks of age and then maintained on a standard diet (MF; Oriental Yeast Co., Tokyo, Japan). Adult male OPG (-/-), OPG (+/-), and OPG (+/+), and OPG (+/-) mice, 6 weeks of age with body weight ranging from 21 to 35 g (27.8 ± 1.0 g, 28.3 ± 0.6 g, and 27.6 ± 0.6 g, n.s.), were used in this study. We divided the mice with three different genotypes into three different
load groups; standard diet plus saline injection, high phosphate diet plus saline injection and high phosphate diet plus vitamin D3 injection. The nine groups of mice were fed a standard diet until 6 weeks. Each of the OPG (-/-), OPG (+/-), and OPG (+/+) 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. The standard diet contained 0.83% phosphorus and 1.11% calcium by weight, while the high phosphate diet contained 1.5% phosphorus by adding 2.64% potassium di-hydrogen phosphate (KH$_2$PO$_4$) to the standard diet. At 9 week of age, a subcutaneous injection of saline or 5 µg/kg body weight of 1α,25-dihydroxyvitamin D3 (calcitriol) (Kirin Co., Tokyo, Japan) was given into the back for three days. Systolic blood pressure was measured with a programmable sphygmomanometer (BP-98A, Softron, Tokyo, Japan) by the tail-cuff method.

**Tissue preparation and histopathological study**

The mice were sacrificed at 10 weeks of age (7 days after the initiation of injection). They were anesthetized with diethyl ether, and an incision was made through the ribs to expose the heart. A 23-gauge needle was inserted through the apex of the heart into the left ventricle, and blood was collected. Physiological pressure-perfusion-fixation (100 mmHg) was performed with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 30 seconds. After the procedures, the heart with the ascending aorta and femurs were harvested. For histological investigation, the tissues were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 4 hours, and the femurs were decalcified in 10% EDTA-2Na in Tris buffer for two weeks and then embedded in paraffin. We also harvested aortic tissue from the thoracic aorta to the iliac bifurcation, small intestine, liver and femurs, for measurement of ALP activity. Paraffin sections were prepared as previously reported with modification. Serial sections (5 µm thick) at the level where the three aortic valves were distinctly visible were evaluated by light microscopy. The sections were stained with hematoxylin
and eosin, Azan stain for fibrous tissue, and von Kossa stain for mineral salts and calcified tissue. Quantification of the calcified lesion area was performed by the following methods. The von Kossa-stained sections were evaluated under a microscope (Olympus Provis AX80, Tokyo, Japan) and digitized using appropriate software (Lumina Vision version 2.2, Mitani Co., Fukui, Japan). For histological analysis of bone, the femurs were stained with hematoxylin and eosin, and the porous area and cortical area were measured using the same methods. Immunohistochemical staining was performed by streptavidin-biotin-peroxidase technique. These sections were stained using an anti-α-smooth muscle (α-SM) actin monoclonal antibody (clone 1A4, Dako, dilution 1:200) for vascular smooth muscle cells, an and anti-macrophage F4/80 antibody (clone C1:A3-1, Cosmo Bio. Co., Ltd., dilution 1:200) for macrophages and osteoclast precursor cells. All sections were immunostained by the indirect immunoperoxidase method, and peroxidase activity was detected using diaminobenzidine tetrahydrochloride as a chromogen. Slides were then counterstained with hematoxylin. Negative controls were obtained by either omitting the primary antibody or by substitution with an irrelevant antibody. For electron microscopic study, the mice were sacrificed at 2, 4, and 7 days after the start of administration of saline or calcitriol. The mice were perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) using a syringe. The heart and ascending aorta were removed. The ascending aorta was cut transversely and fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2-10 hours. Samples were postfixed in 1% OsO₄ in 0.1M phosphate buffer (pH 7.4) for 2-3 hours at 4°C, rinsed in 10% saccharose 3 times (10 min each), stained en bloc in 3% aqueous uranyl acetate for 1 hour at room temperature, dehydrated in an ascending series of ethanol and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate and observed using a JEM-1200 electron microscope (JEOL Ltd., Tokyo, Japan).

Measurements of calcium, inorganic phosphate, ALP activity, and parathyroid
hormone-related protein (PTHrP)

Serum was prepared by centrifugation at 3000 rpm for 20 min and frozen at -80°C for later analysis. Serum calcium was measured by OCPC method, and serum inorganic phosphate was measured using an enzymatic method. Serum ALP activity was assayed using 4-nitrophenyl phosphate as a substrate. These tests were performed with an auto analyzer system (HITACHI 7180, Hitachi Co., Tokyo, Japan). The aorta was washed with PBS and homogenized by ultrasound with 0.1% Triton X-100 in 0.9% saline in ice, and centrifuged at 15,000 rpm for 15 minutes at 4°C. The supernatant was extracted and ALP activity was measured as described above. ALP activity was normalized to total protein measured with a DC protein assay kit (Bio-Rad, Tokyo, Japan). PTHrP level was measured using a PTHrP IRMA kit (Mitsubishi Kagaku Latron, Inc., Tokyo, Japan) and was normalized to total protein level. Samples of bone, liver, and intestine were obtained in the same way as for the aorta.

Measurement of ALP isozyme

ALP isozymes were separated by cellulose acetate membrane electrophoresis (Taitan 3 kit, Helena Ltd., Saitama, Japan) using an automated analyzer (Epalyzer II, Helena Ltd.). As a marker for the mobility of ALP isozymes in the membrane, we utilized a marker for human studies that is available for regular clinical laboratory use. This marker is a mixture of human serum and tissue extracts from bovine liver and intestine. For example, mouse intestinal extract shows thick two bars located far from the ALP5 band (human intestine) of the marker. Similarly, mouse liver extract shows different bands from the marker (ALP1 and ALP2; bovine liver).

Statistical analysis

Data were expressed as mean ± SEM, and analyzed using one-factor ANOVA. If a significant
effect was found, Bonferroni / Dunn test was performed to isolate the difference between the groups. Statistical analysis was performed with StatView 5.0 software. A value of $P < 0.05$ was regarded as statistically significant.
FIGURE LEGENDS

Figure I. Effect of high phosphate diet plus calcitriol injection on PTHrP level of aortic tissue in two genotypes. PTHrP level of aortic tissue was measured and normalized by tissue protein content and expressed as mean ± SEM (n = 10). In the standard diet plus saline injection groups, OPG (-/-) mice showed a significantly lower aortic tissue PTHrP level of 0.95 pmol/g compared to that of 1.50 pmol/g in OPG (+/+) mice. Similarly, in the high phosphate diet plus calcitriol injection group, OPG (-/-) mice showed a lower aortic tissue PTHrP level of 0.95 pmol/g than that of 1.50 pmol/g in OPG (+/+) mice. *P < 0.05.

Figure II. Effect of high phosphate diet plus calcitriol injection on morphology of femoral bone in two genotypes. The ratio of porous area / cortical area in femoral cortical bone is expressed as mean ± SEM (n = 6). In the standard diet plus saline injection group, OPG (-/-) mice showed a significantly higher ratio of porous area / cortical area; 27.8 % compared to 5.8 % in OPG (+/+) mice. Similarly, in the high phosphate diet plus calcitriol injection group, OPG (-/-) mice showed a higher ratio of porous area / cortical area; 25.0 % compared to 5.1 % in OPG (+/+) mice. *P < 0.0001.

Figure III. Effect of high phosphate diet plus calcitriol injection on serum calcium and inorganic phosphate levels in three genotypes. Each group of mice is the same as those shown in Figure 2. (A) In the high phosphate diet plus calcitriol injection group, OPG (-/-) mice showed a significantly elevated serum calcium level of 15.1 ± 0.5 mg/dL compared to that of 10.3 ± 0.2 mg/dL and 10.8 ± 0.6 mg/dL in OPG (+/+) mice and OPG (+/-) mice, respectively. Values are expressed as mean ± SEM (n = 14). *P < 0.0001. (B) There was no significant difference in inorganic phosphate level
among the experimental models. Values are expressed as mean ± SEM (n = 14).
REFERENCE


5. van de Wijngaert FP, Tas MC, Burger EH. Characteristics of osteoclast precursor-like cells grown from mouse bone marrow. Bone Miner. 1987;3:111-123.
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