Osteopontin Deficiency Suppresses High Phosphate Load-Induced Bone Loss via Specific Modulation of Osteoclasts

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Phosphate (Pi) plays a critical role in the maintenance of mineralized tissues and signaling in the intracellular environment. Although extracellular phosphate concentration is maintained at fixed levels, physiological machineries involved in phosphate homeostasis in bone, which is the largest phosphate storage site, have not yet been fully elucidated. Here we examined the role of osteopontin (OPN) in a high-Pi diet load-induced bone loss. A high-Pi diet significantly reduced bone mineral density as well as bone mass in wild type. In contrast, OPN deficiency totally prevented reduction in bone mineral density and bone mass. Analyses of bone turnover-related components revealed that bone formation parameters (bone formation rate and mineral apposition rate) were enhanced by high-Pi diet load similarly in wild-type and OPN-deficient mice. In sharp contrast, bone resorption parameters (osteoclast number and osteoclast surface) were enhanced by high-Pi diet load in wild type but not at all in OPN-deficient mice. Bone marrow cell cultures revealed no major effects of OPN deficiency on high-Pi diet modulation of mineralized nodule formation in culture. On the other hand, tartrate-resistant acid phosphatase-positive multinucleated cell development in cultures were enhanced by high-Pi diet load in wild-type cells, but such effects of high Pi-diet were totally abolished in the absence of OPN. These data indicated that OPN is needed for osteoclastic activity to resorb bone on high phosphate loading. (Endocrinology 147: 3040–3049, 2006)
anism of high Pi load-induced enhancement in bone turnover has not yet been fully understood. We suspected that OPN would be induced by high extracellular Pi in osteoblasts might play a role of regulating bone remodeling. Therefore, we examined the role of OPN in Pi load-induced high bone turnover.

**Materials and Methods**

**Animals**

Productions of OPN-deficient mice were described previously (8). The OPN-deficient mice were back-crossed to 129 background, and the progenies from original homozygous crosses were maintained as separate colonies. Twelve-week-old female mice with either OPN-deficient or wild-type genotypes were used. The mice were housed under controlled conditions on a 12-h light, 12-h dark cycle and fed with standard laboratory chow containing normal calcium and phosphate and given tap water unless otherwise described. All animal experiments were approved by the Animal Welfare Committee of Tokyo Medical Dental University.

**Experimental protocol**

Two types of diets were used in the present study; a normal (control) Pi diet (AIN93G diet, Oriental Yeast Co. Ltd., Tokyo, Japan), containing 0.5% (500 mg per 100 g) calcium and 0.16% (160 mg per 100 g) phosphorus, and a high-Pi diet (modified-AIN93G diet, Oriental Yeast Co.) containing 0.5% (500 mg per 100 g) calcium and 0.6% (600 mg per 100 g) phosphorus. The mice were fed for 4 wk. To evaluate dynamic bone formation parameters, the mice were sc injected with 4 mg/kg calcein phosphorus. The mice were fed for 4 wk. To evaluate dynamic bone formation parameters, the mice were sc injected with 4 mg/kg calcein

**Measurement of bone mineral density (BMD)**

BMD of the whole femora and tibiae was measured based on dual-energy x-ray absorptiometry (DEXA) using PIXI apparatus (GE Lunar, Madison, WI).

**Histomorphometric analysis of bone**

Samples of right femora were prepared by fixing the bone in 70% ethanol, pretained with Villanueva osteochrome (bone stain), and embedded in methacrylate to prepare undecalciﬁed sections. Serial 5-μm-thick sections in a sagittal plane were made using a microtome. Undecalciﬁed sagittal sections were subjected to the analyses of cancellous (ﬁrst and second calcine) bone formation [bone formation rate (BFR) and mineral apposition rate (MAR)] in a square area of 1.6 mm² (0.8 x 2.0 mm), which was 0.2 mm away from the growth plate. The histomorphometric analysis was carried out at the magniﬁcation of ×200. Osteocalst number was examined using decalciﬁed 5-μm-thick decalciﬁed sagittal sections in the metaphysis of the right tibia, which were ﬁxed in 4% paraformaldehyde in PBS, decalciﬁed in EDTA, embedded in parafﬁn, and sectioned, followed by staining for tartrate-resistant acid phosphatase (TRAP) activity. TRAP-positive multinucleated cells attached to bone within a square area of 1.6 mm² (0.8 x 2.0 mm) located 0.2 mm away from the growth plate of the proximal end of tibia were scored as osteoclasts to obtain osteoclast number per bone surface and osteoclast surface per bone surface (Oc.S/BS).

**Bone marrow cell cultures**

Bone marrow was flushed out from the left femora. The number of total bone marrow cells was counted and the cells were plated at a density of 5 x 10⁶ cells/well in 24-well plates (2 cm² per well). TRAP-positive osteoclast-like multinucleated cells were formed after the culture in αMEM supplemented with 10% fetal bovine serum, 100 μg/ml antibiotics-antimycotics mixture, 10 mM 1α, 25 hydroxyvitamin D₃, and 100 nM dexamethasone. Osteoclastogenesis was evaluated after 7 d in culture. Other sets of bone marrow cells from the right tibiae were cultured in αMEM supplemented with 10% fetal bovine serum, 100 μg/ml antibiotics-antimycotics mixture, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate to examine mineralized nodule formation. After 21 d, the cells were rinsed with PBS and ﬁxed in 95% EtOH for 10 min. The cells were stained for 10 min in a saturated solution of alizarin red, rinsed with water, and dried in air. The area of mineralized nodules per total culture area was quantiﬁed based on image analyses using Luxez-F automated image system (Nireco).

**Blood and urine biochemistry**

Serum were analyzed on d 28 of the experiments. Serum calcium and Pi were measured colorimetrically. Serum PTH was measured using a rat intact PTH ELISA kit (Immutopics, San Clemente, CA).

**Statistical analysis**

The results were presented as mean values ± SD. The statistical analysis consisted of 2 x 2 factorial ANOVA with the factors of genotype (wild type or OPN−/−) and diet (control or high Pi). Statistical signiﬁcance of the main effect of genotypes and diets and their interaction was calculated and the P values for interaction were presented. In all tests, P < 0.05 was considered to be statistically signiﬁcant.

**Results**

To examine the role of OPN in phosphate-induced alterations in bone metabolism, we subjected mice to high-Pi diet load for 4 wk. Body weight was not altered signiﬁcantly due to high Pi diet in all of our experiments (Table 1). All mice survived during the course of experiments.

At the end of 4 wk of the experiments, the femoral bones from wild-type and OPN-deﬁcient mice, which were fed with normal Pi diet or high-Pi diet, were subjected to the analyses of BMD, using a DEXA apparatus. A 2 x 2 factorial ANOVA indicated signiﬁcant interaction (P < 0.05) between genotypes and diets. High-Pi diet suppressed the levels of BMD by about 15% in wild-type mice (Fig. 1). In contrast to wild-type mice, high-Pi diet load did not suppress the levels of BMD in OPN-deﬁcient mice (Fig. 1).

We then went on to examine the effects of OPN deﬁciency on the high-Pi diet load-induced changes in the patterns of trabecular bones. 2D micro-CT analysis indicated trabecular bone patterns in the distal end of the femur became sparse in wild-type mice in the group of high-Pi diet loading, com-

**TABLE 1. Body weights (g)**

<table>
<thead>
<tr>
<th>Day</th>
<th>CTL WT</th>
<th>High P</th>
<th>CTL OPN−/−</th>
<th>High P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>23.22 ± 2.65</td>
<td>23.18 ± 2.94</td>
<td>24.50 ± 1.99</td>
<td>24.85 ± 3.38</td>
</tr>
<tr>
<td>28</td>
<td>24.07 ± 2.32</td>
<td>24.62 ± 2.89</td>
<td>25.50 ± 2.36</td>
<td>25.55 ± 3.22</td>
</tr>
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The body weights of all the mice during the course of experiments were not altered by high Pi diet within the 4-wk period in both wild-type (WT) and OPN−/− mice. CTL: Control; P: phosphate loaded.
pared with normal diet (Fig. 2, A and B). In the case of OPN-deficient mice fed with normal Pi diet, trabecular bone patterns were similar to those in wild-type mice fed with normal Pi diet. In contrast to wild-type mice, high-Pi diet did not alter the trabecular bone patterns in OPN-deficient mice (Fig. 2, C and D).

Quantification of the trabecular BV/TV was conducted based on the image analyses of the 2D micro-CT pictures. The $2 \times 2$ factorial ANOVA indicated significant interaction ($P < 0.05$) between genotypes and diets. The BV/TV values indicated about 50% suppression in the wild-type mice on the high-Pi diet load (Fig. 2E). In sharp contrast to such suppression in the BV/TV levels in wild-type mice on high-Pi diet load, high-Pi diet did not induce any suppression in the levels of trabecular BV/TV levels in OPN-deficient mice (Fig. 2E). Thus, OPN deficiency protects bone against cancellous bone loss due to high-Pi diet load.

We also conducted analysis on the cortical bone envelope. 2D micro-CT analysis on the cross-section in the middiaphyses of the femur indicated that the thinner of the cortex in the wild-type mice with high-Pi diet loading, compared with normal diet (Fig. 3, A and B). In the case of OPN-deficient mice fed with normal Pi diet, cortical bone patterns were similar to those in wild-type mice fed with normal Pi diet. As observed in the case of trabecular bone envelope, in contrast to wild-type, high-Pi diet did not alter cortical bone appearance of OPN-deficient mice (Fig 3, C and D). We quantified following parameters, cortical bone area (Fig. 3E), total cross-section (Fig. 3F), periosteal circumference (Fig. 3G), and cortical thickness (Fig. 3H). The $2 \times 2$ factorial ANOVA indicated significant interaction ($P < 0.05$) between genotypes and diets (Fig. 3, E, F, G, and H). Values of these parameters indicated that high-Pi diet loading decreased each parameters in wild-type mice. In contrast, OPN-deficient mice did not review the reduction in response to high-Pi diet loading (Fig. 3, E, F, G, and H).

The bone volume is determined by the balance between bone formation and resorption. First, bone formation parameters were examined based on histomorphometry. MAR, which indicates activities of osteoblasts, was enhanced by high-Pi diet load in wild-type mice as reported previously (16, 17) (Fig. 4, A–E). In the case of OPN-deficient mice fed with control Pi diet, basal levels of MAR were similar to those in wild-type mice fed with normal Pi diet (Fig. 4, A, C, and E). In OPN-deficient mice, MAR levels were enhanced by high-Pi diet load similarly to the enhancement observed in the levels in wild-type fed with high-Pi diet (Fig. 4E). Mineralizing surface (MS) levels were also examined to estimate the overall bone forming surface. In wild-type mice, high-Pi diet load enhanced MS, and again OPN-deficient mice exhibited similarly high-Pi diet-induced enhancement in the.
levels of MS, compared with wild-type. (Fig. 4F). Secondary parameters such as BFR based on mineral apposition rate and mineralizing surface showed the similar increase due to high Pi diet load in both wild-type (A and B) or OPN-deficient mice (C and D). E, Cortical bone area; ANOVA effects: interaction, $P = 0.041$. F, Total cross-section area of femora; ANOVA effects: interaction, $P = 0.041$. G, Periosteal circumference. ANOVA effects: interaction, $P = 0.049$. Interaction between the effects introduced by genotypes and diets was tested by $2 \times 2$ factorial ANOVA. Data are expressed as means and SD for bones from seven mice from each of the groups. Cont, Control; P, Pi.

Fig. 3. OPN deficiency prevents high-Pi diet load-induced reduction in cortical bone volume. 2D micro-CT pictures were obtained in the midshaft planes to the long axis of the femora after 4 wk of normal Pi diet or high-Pi diet feeding in wild-type (A and B) or OPN-deficient mice (C and D). E, Cortical bone area; ANOVA effects: interaction, $P = 0.041$. F, Total cross-section area of femora; ANOVA effects: interaction, $P = 0.041$. G, Periosteal circumference. ANOVA effects: interaction, $P = 0.049$. Interaction between the effects introduced by genotypes and diets was tested by $2 \times 2$ factorial ANOVA. Data are expressed as means and SD for bones from seven mice from each of the groups. Cont, Control; P, Pi.
FIG. 4. OPN deficiency does not affect high-Pi diet-induced enhancement in bone formation parameters. Calcein-labeled surfaces in the cancellous bones in the distal regions of the femora after 4 wk of normal Pi diet (A and C) or high-Pi diet feeding (B and D) in wild-type (A and B) or OPN-deficient (C and D) mice. Original magnification, ×1100. Arrows (A–D, right panels) indicate the lines of calcein labeling (light green), which was injected 3 and 1 d before the animals were killed. Original magnification, ×400. E, MAR. ANOVA effects: genotype, interaction, NS (P = 0.554). F, MS/BS; ANOVA effects: interaction, NS (P = 0.661). G, BFR. ANOVA effects: interaction, NS (P = 0.610). NS, Not significant. Interaction between the effects introduced by genotypes and diets was tested by 2 × 2 factorial ANOVA. Data are expressed as means and SD for bones from six mice from each group. Cont, Control; P, Pi.
5F). Therefore, OPN deficiency blocks high-Pi diet load-induced reduction in bone mass and BMD not through the alteration in bone formation side but specifically through the prevention of enhanced osteoclastic activities due to high-Pi diet.

Because these animal studies were conducted based on the whole-body analyses, it is possible that OPN deficiency may affect organs other than bone first, and this may in turn block the high-Pi diet-induced bone loss indirectly. Therefore, to obtain insights into direct effects of OPN deficiency on bone cells, we conducted bone marrow cell cultures. In the case of bone formation, bone marrow cells were cultured in the presence of 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid for 21 d, and the levels of nodule formation were examined after the cell cultures were stained with alizarin red. The 2 × 2 factorial ANOVA did not indicate significant interaction between genotypes and diets. These experiments indicated that high-Pi diet load enhanced the nodule formation in the cultures of bone marrow cells obtained from the wild-type mice and similarly in the cells from OPN-deficient mice (Fig. 6, A–E). Therefore, the effects of OPN deficiency on high-Pi diet were not apparent at the level of osteoblastic side in the bone marrow cell cultures.

We then conducted osteoclastogenesis experiments in vitro to examine the effects of high-Pi diet load by using bone marrow cells taken from wild-type and OPN-deficient mice after normal or high-Pi diet feeding. When bone marrow cells were cultured in the presence of 100 nM dexamethasone and
Fig. 6. Mineralized nodule formation in cultures of bone marrow cells from both wild-type and OPN-deficient mice fed with either normal Pi diet or high-Pi diet. Mineralized nodule formation was examined in the cultures of bone marrow cells obtained from wild-type (A and B) or OPN-deficient mice (C and D) after 4 wk of normal Pi diet (Cont) (A and C) or high-Pi diet (P) feeding (B and D). E, Quantification of the mineralized nodules shown in A–D. Interaction between the effects introduced by genotypes and diets was tested by 2 × 2 factorial ANOVA (P = 0.240; not significant). Data are expressed as means and SD for the bones from six mice from each group.

10 nm 1α,25(OH)2D3 for 7 d, TRAP-positive cells were formed in culture. The 2 × 2 factorial ANOVA indicated significant interaction (P < 0.05) between genotypes and diets. The levels of the TRAP-positive cell number were increased in bone marrow cells taken from wild-type mice fed with a high-Pi diet, compared with normal diet group (Fig. 7, A, B, and E). Basal levels of the multinucleated TRAP-positive cells in the cultures of bone marrow cells from OPN-deficient mice fed with normal diet were similar to those in wild-type mice fed with normal diet. In contrast to wild-type mice, multinucleated TRAP-positive cell formation in the OPN-deficient bone marrow cells taken from the mice fed with high and Pi diet was not enhanced (Fig. 7, C, D, and E). These observations indicated that the suppression by OPN deficiency of high-Pi diet-induced increase in bone resorption parameters observed in vivo based on histomorphometry as well as OPN deficiency-dependent blockage of the reduction of bone mass and BMD would be due to the direct effect of OPN deficiency on osteoclastogenesis (Fig 7, A–E).

Our data indicated that high-Pi diet-induced bone loss was dependent on the presence of OPN in vivo, and this was, at least in part due to a direct effect on osteoclastogenesis as seen in vitro. However, high-Pi diet may also affect systemic mineral metabolism, which may be influenced by the extraskelatal organs being involved in calcium homeostasis. We therefore examined the levels of systemic parameters. The 2 × 2 factorial ANOVA did not indicate significant interaction between genotypes and diets. Basal serum phosphate levels, serum calcium levels, and serum alkaline phosphatase (ALP) levels were similar between wild-type and OPN-deficient mice, which were both fed with normal Pi diet (Fig. 8, A, B, and C). High-Pi diet feeding did not significantly alter the levels of serum phosphate in wild-type (Fig. 8A). Similarly, OPN-deficient mice fed with high-Pi diet did not significantly change the levels of serum phosphate (Fig. 8A). With regard to serum calcium in wild-type and OPN-deficient mice, baseline levels were similar and high-Pi diet load did not significantly alter serum calcium levels in wild-type as well as OPN-deficient mice (Fig. 8B). As known previously (16, 17), serum PTH levels were significantly enhanced by high-Pi diet load in wild-type mice. Similarly, OPN-deficient mice indicated elevation of serum PTH after the treatment with high-Pi diet (Fig. 8D). Thus, OPN deficiency did not affect these serum data.

Because OPN is expressed in kidney (18), renal function could be a candidate target of OPN actions on Pi metabolism. Therefore, we examined the levels of mineral excretion in urine. The 2 × 2 factorial ANOVA did not indicate significant interaction (P < 0.05) between genotypes and diets. Urine analyses revealed that high-Pi diet significantly increased the excretion of Pi into the urine in both wild-type and OPN-deficient mice to a similar degree (Fig. 9A). In terms of the calcium excretion into urine, wild-type mice showed suppression of the calcium excretion into urine on the feeding with high-Pi diet, and similar basal levels as well as the Pi diet-induced suppression of the calcium excretion levels in urine were observed in OPN-deficient mice (Fig. 9B). These data indicated that OPN deficiency did not affect mineral levels in serum and urine as well as serum PTH levels.

Discussion

Alteration in bone metabolism induced by high-Pi loading is one of the pathological situations that lead to osteoporosis. However, how the bone loss occurs on Pi loading has not been fully understood. Our data indicated that high-Pi diet load-induced bone loss, which is under the influence of secondary hyperparathyroidism, requires OPN. OPN-deficient mice were resistant to bone loss due to high-Pi diet loading, and this was based on the resistance to high-Pi diet load-induced osteoclastic activities as shown by the analysis on bone resorption parameters in vivo. In vitro data also supported this notion that OPN is required for high-Pi diet-induced bone loss thorough its action on osteoclast develop-
Bone marrow cells may have a memory of being exposed to high-Pi diet to remain affected after 7 d in culture. High-Pi diet-induced enhancement in multinucleated TRAP-positive cell development in bone marrow cells in culture was totally blocked in the absence of OPN. Our data indicated that OPN could be one of the key molecules in pathological bone loss due to secondary hyperparathyroidism associated with high Pi levels.

For many decades the mechanism for the rise in blood calcium after PTH has been a subject of discussion. Pi loading causes hypocalcemia in the absence of PTH. One school of thought would argue that bone resorption was needed to supply the calcium to prevent the decrease of plasma calcium levels. Another school of thought, championed for many years by William Neuman, argued that osteoclastic resorption was inadequate to explain the large mobilization of calcium evoked by PTH. Our present data in this report would argue for the second case. Osteoclastic activation is not needed for PTH to maintain normal plasma calcium levels after high-Pi challenge.

Although OPN is expressed in kidney, its function in this organ has not been known (18). Both wild-type and OPN-deficient mice excreted Pi in urine at similar levels after high-Pi diet load. Therefore, kidney function to excrete high

![Image](https://example.com/image.png)
levels of Pi into urine feeding with on high-Pi diet was not impaired by the absence of OPN. Thus, OPN appears to be dispensable in the machinery regulating the urinary system including excretion of Pi from the glomerulus as well as its reabsorption from the renal tubules. These data suggest that OPN-deficiency effects observed in bone in this paper were not influenced by any alteration in kidney function. Rather, they were direct actions of this molecule in bone tissue.

Previous reports indicated that high-Pi concentration in medium could induce OPN gene expression in osteoblastic cells (3). Because high-Pi levels in vitro were shown to increase OPN, one of the possibilities was that loading with high levels of Pi may induce OPN expression in osteoblasts. To our surprise, OPN deficiency did not affect high-Pi diet-induced increase in bone formation parameters including MAR, MS, and BFR in vivo. In vitro cell culture experiments also supported the notion that OPN deficiency does not alter osteoblastic function in mice challenged with high-Pi diet load. Therefore, although osteoblasts are the cells that induced OPN expression in response to phosphate in vitro, OPN does not have any significant influence on bone formation side in response to Pi load at least in vivo.

It is intriguing that the absence of OPN blocked the loss of bone while maintaining calcium and phosphate homeostasis in the presence of high levels of PTH. This fact indicated that if OPN actions could be inhibited by certain drugs or biological reagents, it could provide a new dimension of the thought to contemplate treatment to block bone loss due to...
secondary hyperparathyroidism. Renal failure is accompanied by secondary hyperparathyroidism and such situation leads to renal osteodystrophy. Thus, blockage of OPN function may be beneficial in the case of the osteodystrophy. Our data indicated that OPN could be one of the key molecules in pathological bone loss due to secondary hyperparathyroidism in patients with high-Pi levels. Our observations proposed that OPN would be at least one of the possible targets for the treatment of patients with bone disease caused by high-Pi levels.

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