Role of Hyperphosphatemia and 1,25-Dihydroxyvitamin D in Vascular Calcification and Mortality in Fibroblastic Growth Factor 23 Null Mice

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ABSTRACT
Fibroblastic growth factor 23 (FGF23) regulates renal phosphate reabsorption and 1α-hydroxylase activity. Ablation of FGF23 results in elevated serum phosphate, calcium, and 1,25-dihydroxyvitamin D3 [1,25(OH)2D] levels; vascular calcifications; and early death. For determination of the independent roles of hyperphosphatemia and excess vitamin D activity on the observed phenotypic abnormalities, FGF23 null mice were fed a phosphate- or vitamin D–deficient diet. The phosphate-deficient diet corrected the hyperphosphatemia, prevented vascular calcifications, and rescued the lethal phenotype in FGF23 null mice, despite persistent elevations of serum 1,25(OH)2D and calcium levels. This suggests that hyperphosphatemia, rather than excessive vitamin D activity, is the major stimulus for vascular calcifications and contributes to the increased mortality in the FGF23-null mouse model. In contrast, the vitamin D–deficient diet failed to correct either the hyperphosphatemia or the vascular calcifications in FGF23 null mice, indicating that FGF23 independently regulates renal phosphate excretion and that elevations in 1,25(OH)2D and calcium are not sufficient to induce vascular calcifications in the absence of hyperphosphatemia. The vitamin D–deficient diet also improved survival in FGF23 null mice in association with normalization of 1,25(OH)2D and calcium levels and despite persistent hyperphosphatemia and vascular calcifications, indicating that excessive vitamin D activity can also have adverse effects in the presence of hyperphosphatemia and absence of FGF23. Understanding the independent and context-dependent interactions between hyperphosphatemia and excessive vitamin D activity, as well as vascular calcifications and mortality in FGF23 null mice, may ultimately provide important insights into the management of clinical disorders of hyperphosphatemia and excess vitamin D activity.


Disorders of mineral metabolism are nontraditional risk factors that are associated with high mortality in ESRD.1,2 Epidemiologic studies have linked excessive cardiovascular mortality in ESRD with hyperphosphatemia, hypercalcemia, and elevated parathyroid hormone (PTH) levels.3,4 Vascular calcifications, which are highly prevalent and directly correlated with mortality in this population, are the likely mechanism whereby disordered mineral metabolism contributes to cardiovascular mortality in ESRD.3 Experimental models demonstrate that hyperphosphatemia is a major initiator of extracellular matrix mineralization and vascular calcification.4 In addition, clinical data in ESRD show a strong association between hyperphosphatemia and mortality, as well as between phosphate levels and excess calcium with vascular calcifications.5,6 The relationship among disordered mineral metabolism, the presence of vascular calcifications, and
increased mortality in ESRD has led to consensus Kidney Disease Outcomes Quality Initiative (K/DOQI) recommendations to control more stringently serum calcium and phosphate levels in ESRD in an effort to minimize vascular calcifications and improve survival.

Whereas there is general consensus regarding control of serum phosphate and calcium, there is less agreement regarding the optimal use of vitamin D analogues. On the one hand, the high prevalence of nutritional vitamin D deficiency and low circulating levels of 1,25-dihydroxyvitamin D3 [1,25(OH)\textsubscript{2}D] in ESRD, along with the many salutary effects of vitamin D on various tissues, strongly support the practice of administering active vitamin D analogues to patients with chronic kidney disease. Indeed, epidemiologic studies indicate that vitamin D deficiency is associated with an increased risk for cardiovascular mortality,15–17 which normalized the circulating 1,25(OH)\textsubscript{2}D levels without correcting the hyperphosphatemia, to determine the individual contributions of serum phosphorus and 1,25(OH)\textsubscript{2}D to the development of vascular calcifications and early mortality in this mouse model. We found hyperphosphatemia to be the major contributor to vascular calcifications in FGF23 null mice and that in vivo elevations in 1,25(OH)\textsubscript{2}D in the absence of hyperphosphatemia had no demonstrable toxicity in this model.

RESULTS

High Mortality, Disordered Mineral Metabolism, and Vascular Calcifications in FGF23 Null Mice

Compared with wild-type mice, FGF23 null mice on a control diet displayed an age-dependent increase in mortality beginning at 5 wk of age. No FGF23 null mice that received a regular diet survived beyond 14 wk of age (Figure 1A). Also, FGF23 deficiency resulted in severe growth retardation as evidenced by a significant reduction in body weight (Figure 1B). The shortened survival in FGF23 null mice was associated with hyperphosphatemia, hypercalcaemia, and elevated circulating 1,25(OH)\textsubscript{2}D levels (Table 1), consistent with the known actions of FGF23 to inhibit renal phosphate reabsorption and 1α-hydroxylase.

The FGF23 null mice on a regular diet also displayed extensive soft tissue and vascular calcifications that were apparent by 6 wk of age (Figure 2 through 4). Alizarin red staining of tissues from the FGF23 null mice revealed the presence of widespread calcifications in various organs, including the aorta and kidneys (Figure 2) as well as lung (data not shown). Histologic analyses of these tissues demonstrated calcifications of the elastic lamina in medial layer of the aorta (Figure 2C) along with marked calcifications of the small- and medium-sized vessels of the kidney (Figure 3A).
Microcomputed tomography (μCT) analysis of the kidneys revealed extensive cortical calcium deposition that correlated with histologic evidence of calcifications of small- to medium-size arteries (Figure 3, A and B). In addition, Fourier transform infrared (FTIR) microspectroscopic imaging analysis demonstrated focal mineral deposition distributed in a manner similar to that exhibited by Von Kossa staining (Figure 4A). In the spectra of the mineral deposits, there is a broad peak at 1082 cm⁻¹ that is associated with the P-O vibration modes (Figure 4B). The broad, relatively featureless contour that characterizes this peak indicates that the mineral spots contain amorphous calcium phosphate but not highly crystallized apatite.

Figure 1. Survival and growth curves of wild-type (WT) and fibroblast growth factor 23 (FGF23) null mice after dietary manipulation of phosphate and vitamin D. (A) On a control diet, FGF23 null mice exhibited 0% survival (n = 9) compared with 100% survival of WT mice (n = 6) at 14 wk of age. A significant improvement in survival of FGF23 null mice was observed on both the phosphate-deficient (PD; n = 10) and vitamin D–deficient (DD; n = 9) diets, with 90% of the mice surviving to 14 wk of age. WT mice on both the PD (n = 6) and DD (n = 6) diets had 100% survival rate at 14 wk of age. (B) WT mice that were fed a PD diet had severe (P < 0.01) and mild (P < 0.05) growth retardation, respectively. FGF23 null mice had severe growth retardation on a control diet compared with WT mice (P < 0.01). This growth retardation was not worsened by feeding a PD diet and was improved with a DD diet (P < 0.05).

Low-Phosphate Diet in FGF23 Null Mice Prolongs Survival in Association with Correction of Hyperphosphatemia, Prevention of Soft Tissue Calcifications, and Persistently Elevated 1,25(OH)₂D Levels

Phosphate restriction of wild-type mice resulted in severe growth retardation as assessed by a 50% reduction in body weight (Table 1) but had no adverse effect on survival (Figure 1). In addition, phosphate restriction in wild-type mice resulted in a marked decrease in serum phosphate levels and a five- to eight-fold increase in serum 1,25(OH)₂D₂ levels, attaining values similar to FGF23 null mice. The increase in serum 1,25(OH)₂D₂ is consistent with the known effects of hypophosphatemia to 1α-hydroxylase activity. Phosphate restriction also increased calcium levels and suppressed serum PTH levels compared with wild-type controls that were fed a regular diet (Table 1).

In contrast, FGF23 null mice that were fed a low-phosphate diet displayed marked improvement in survival. In this regard, a phosphate-depleted diet resulted in 90% survival of FGF23 null mice at the end of the 14-wk observation period (Figure 1) compared with no survivors in FGF23 null mice that were fed a regular diet. Phosphate restriction had no effect on the already low body weight in FGF23 null mice. Dietary phosphate restriction, however, significantly reduced serum phosphate levels in FGF23 null mice (Table 1). 1,25(OH)₂D₂ levels remained six- to 10-fold elevated, and hypercalcemia and suppressed PTH persisted in FGF23 null mice that were fed a phosphate-depleted diet (Table 1).

The soft tissue calcifications associated with the absence of FGF23 were completely corrected by the phosphate-deficient diet (Figures 2 and 3). In this regard, there were no demonstrable calcifications by alizarin red staining, μCT, or histologic analysis of any tissue, including heart, aorta, and kidney, from FGF23 null mice on a low-phosphate diet, despite hypercalcaemia and elevated 1,25(OH)₂D₂ levels.

Vitamin D–Deficient Diet in FGF23 Null Mice Prolongs Survival in Association with Normalization of Serum 1,25(OH)₂D Levels but Fails to Correct the Hyperphosphatemia or Soft Tissue Calcifications

Wild-type mice that were fed a vitamin D–deficient diet had a slightly lower body weight and 100% survival. By 14 wk of age, wild-type mice that were fed a vitamin D–deficient diet exhibited elevation of serum PTH, consistent with the induction of secondary hyperparathyroidism. Serum calcium and 1,25(OH)₂D₂ were maintained in the normal range, but serum phosphate was decreased in 14-wk-old wild-type mice that were fed a vitamin D–deficient diet.

A vitamin D–deficient diet also resulted in improved survival of FGF23 null mice, with 90% of the mice surviving at the end of the 14-wk observation period (Figure 1). The improved survival was not associated with correction of either the hyperphosphatemia or vascular calcifications (Table 1, vide infra). A growth advantage was also noted in the FGF23 null mice on the
Table 1. Serum biochemistries in 6- and 14-wk-old WT and FGF23 null mice on control, phosphate-deficient, and vitamin D–deficient diets

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal WT</th>
<th>FGF23 Null WT</th>
<th>Phosphate-Deficient Diet WT</th>
<th>FGF23 Null WT</th>
<th>Vitamin D-Deficient Diet WT</th>
<th>FGF23 Null WT</th>
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<tr>
<td>6 wk old</td>
<td></td>
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<tr>
<td>serum phosphorus (mg/dl)</td>
<td>8.4 ± 0.3 (n = 7)</td>
<td>14.0 ± 0.5&lt;sup&gt;h&lt;/sup&gt; (n = 5)</td>
<td>3.2 ± 0.5&lt;sup&gt;i&lt;/sup&gt; (n = 8)</td>
<td>2.5 ± 0.3&lt;sup&gt;f&lt;/sup&gt; (n = 5)</td>
<td>7.8 ± 0.3 (n = 7)</td>
<td>12.6 ± 0.6&lt;sup&gt;b&lt;/sup&gt; (n = 5)</td>
</tr>
<tr>
<td>serum calcium (mg/dl)</td>
<td>10.1 ± 0.4 (n = 7)</td>
<td>11.7 ± 0.3&lt;sup&gt;e&lt;/sup&gt; (n = 5)</td>
<td>13.7 ± 0.6&lt;sup&gt;e&lt;/sup&gt; (n = 8)</td>
<td>14.4 ± 0.8 (n = 5)</td>
<td>9.0 ± 0.2 (n = 7)</td>
<td>10.4 ± 0.3&lt;sup&gt;b&lt;/sup&gt; (n = 5)</td>
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<tr>
<td>serum 1,25(OH)&lt;sub&gt;2&lt;/sub&gt;D (pm)</td>
<td>148 ± 32 (n = 7)</td>
<td>1356 ± 286&lt;sup&gt;h&lt;/sup&gt; (n = 5)</td>
<td>1241 ± 176&lt;sup&gt;e&lt;/sup&gt; (n = 8)</td>
<td>875 ± 80 (n = 5)</td>
<td>177 ± 33 (n = 7)</td>
<td>128 ± 16&lt;sup&gt;e&lt;/sup&gt; (n = 5)</td>
</tr>
<tr>
<td>serum PTH (pg/ml)</td>
<td>15.6 ± 1.3 (n = 7)</td>
<td>5.9 ± 0.4&lt;sup&gt;e&lt;/sup&gt; (n = 5)</td>
<td>6.2 ± 0.5&lt;sup&gt;e&lt;/sup&gt; (n = 8)</td>
<td>6.1 ± 0.6 (n = 7)</td>
<td>29.0 ± 11.2 (n = 7)</td>
<td>10.7 ± 2.0&lt;sup&gt;a&lt;/sup&gt; (n = 4)</td>
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<tr>
<td>14 wk old</td>
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<tr>
<td>serum phosphorus (mg/dl)</td>
<td>7.1 ± 0.4 (n = 6)</td>
<td>N/A</td>
<td>3.5 ± 0.5&lt;sup&gt;i&lt;/sup&gt; (n = 5)</td>
<td>3.2 ± 0.2 (n = 7)</td>
<td>6.9 ± 0.3 (n = 9)</td>
<td>10.3 ± 0.9&lt;sup&gt;b&lt;/sup&gt; (n = 9)</td>
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<tr>
<td>serum calcium (mg/dl)</td>
<td>8.5 ± 0.2 (n = 7)</td>
<td>N/A</td>
<td>13.4 ± 0.7&lt;sup&gt;e&lt;/sup&gt; (n = 6)</td>
<td>14.2 ± 0.7 (n = 6)</td>
<td>8.9 ± 0.1 (n = 9)</td>
<td>8.9 ± 0.2 (n = 6)</td>
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<tr>
<td>serum 1,25(OH)&lt;sub&gt;2&lt;/sub&gt;D (pm)</td>
<td>200 ± 33 (n = 6)</td>
<td>N/A</td>
<td>793 ± 85&lt;sup&gt;e&lt;/sup&gt; (n = 6)</td>
<td>1453 ± 229&lt;sup&gt;e&lt;/sup&gt; (n = 7)</td>
<td>44 ± 14&lt;sup&gt;e&lt;/sup&gt; (n = 7)</td>
<td>128 ± 36 (n = 9)</td>
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<tr>
<td>serum PTH (pg/ml)</td>
<td>18.5 ± 3.4 (n = 7)</td>
<td>N/A</td>
<td>4.3 ± 1.2&lt;sup&gt;c&lt;/sup&gt; (n = 4)</td>
<td>5.6 ± 0.9 (n = 5)</td>
<td>41.2 ± 8.6&lt;sup&gt;b&lt;/sup&gt; (n = 8)</td>
<td>116 ± 53.9 (n = 7)</td>
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<sup>a</sup>Data are means ± SEM from 6- and 14-wk-old mice. 1,25(OH)<sub>2</sub>D, 1,25-dihydroxyvitamin D; FGF23, fibroblast growth factor 23; WT, wild-type.

<sup>b</sup>P < 0.01 between WT and FGF23 null mice within each diet group, t test.

<sup>c</sup>P < 0.001 versus mice of the same genotype on a normal diet, t test.

<sup>d</sup>P < 0.05 versus mice of the same genotype on a normal diet, t test.

<sup>e</sup>P < 0.005 between WT and FGF23 null mice within each diet group, t test.
DISCUSSION

In this study, we used dietary manipulation of phosphate and vitamin D to assess separately the contributions of hyperphosphatemia and elevated 1,25(OH)2D on vascular calcification and mortality in FGF23 null mice. Similar to other reports, we found that the FGF23 null mouse develops a mineral profile and phenotype similar to patients who have ESRD and are treated with high-dosage vitamin D analogues, namely, elevated serum calcium, phosphate, and 1,25(OH)2D levels along with vascular calcifications and decreased survival (Table 1, Figures 1 and 2). By restricting phosphate intake in the FGF23 null mice, we were able to lower selectively serum phosphate levels while maintaining markedly elevated serum levels of 1,25(OH)2D and calcium (Table 1). In contrast, feeding FGF23 null mice a vitamin D–deficient diet resulted in normalization of circulating 1,25(OH)2D levels while maintaining a state of hyperphosphatemia (Table 1).

Lowering serum phosphate levels by dietary phosphate restriction resulted in a complete resolution of the soft tissue and vascular calcifications and near normalization of survival in FGF23 null mice (Figures 1 and 2). The rescue of vascular calcifications and the lethal phenotype of FGF23 null mice, despite elevated 1,25(OH)2D and serum calcium levels, suggests that serum phosphorus is the main determinant of vascular calcifications and a significant contributor to the early mortality in this mutant mouse model. The predominant role of phosphate in vascular calcifications is consistent with recent in vitro and in vivo findings showing that phosphate rather than calcium is the primary factor necessary for mineralization of extracellular matrix. Although applying these results to human populations is somewhat speculative, the apparent association between hyperphosphatemia and vascular calcifications in this model suggest that the clinical observational data linking hyperphosphatemia, vascular calcifications, and mortality in patients with ESRD may be causal associations.

The related finding that markedly elevated 1,25(OH)2D levels in FGF23 null mice on a phosphate-deficient diet had no apparent adverse effects, including failure to stimulate vascular calcifications, suggests that this potential toxicity of high concentrations of active vitamin D is context dependent, being modified by FGF23 deficiency, vascular calcifications, and hyperphosphatemia or other factors. Several clinical studies have also failed to find a relationship between circulating active vitamin D levels and calcifications. In addition, these find-
ings support the interpretation that the lower mortality associated with the use of less potent vitamin D analogues, such as paricalcitol and doxercalciferol, could be explained by less hyperphosphatemia. Regardless, these studies are the first to show that restriction of dietary phosphate and lowering of the serum phosphate prevent vascular calcifications and improve survival in a hyperphosphatemic model. As such, these animal studies provide further support for the recommendation to treat aggressively hyperphosphatemia as a potential means of improving survival in patients with ESRD.

We also observed context-dependent adverse effects of excessive 1,25(OH)2D levels in FGF23 null mice. In this regard, normalization of 1,25(OH)2D levels by dietary vitamin D restriction neither fully corrected elevated serum phosphorus levels nor rescued the calcification phenotype in FGF23 null mice but paradoxically improved survival, similar to that of phosphate restriction alone.12 Similar to our studies, abolition of vitamin D–dependent activities in FGF23 null mice by creating compound mutant mice that lacked either the 1α-hydroxylase or VDR gene prevented vascular calcifications and prolonged survival in FGF23 null mice.22,29 In these previous studies, however, the 1α-hydroxylase and VDR null mice resulted in correction of both the hyperphosphatemia and excess activation of vitamin D–dependent signaling in FGF23 null mice, thereby precluding the separate assessment of the contribution of hyperphosphatemia and excess vitamin D.

The ability of decrements in circulating 1,25(OH)2D concentrations to have a positive impact on survival without reducing vascular calcifications is opposite to the purported salutary effect of vitamin D treatment in patients with ESRD.9 The potential toxicity of high circulating concentrations of active vitamin D analogues, however, is consistent with the observation that the survival benefit of calcitriol therapy in patients with ESRD is attenuated at high dosages.12 In any event, this study raises new questions regarding the necessity and potential toxicity of supraphysiologic concentrations of circulating 1,25(OH)2D, as well as the potential interactions between hyperphosphatemia and elevated 1,25(OH)2D levels.

Previous results from superimposed 1α-hydroxylase or VDR deficiency in FGF23 null mice that completely corrected the hyperphosphatemia suggested a minor function of FGF23 to regulate phosphate transport across the proximal tu-

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Figure 4. Fourier transform infrared (FTIR) microspectroscopic imaging analysis of the kidney sections from WT and FGF23 null mice. (A) FTIR microspectroscopic imaging analysis of the kidney. Several mineralized areas were distributed in the cortical zone of the kidney in FGF23 null but not in WT mice. (B) Spectra of the mineralized and nonmineralized areas. In the FGF23 null kidneys on a regular diet, there is a broad peak at 1075 cm⁻¹, which is associated to the P-O vibration modes. This broad and relatively featureless contour of this peak indicates that the mineral deposits contain amorphous calcium phosphate (apatite). An example of the typical analysis for a highly crystallized area of apatite (insert) is shown for comparison.
The finding of this study, namely that hyperphosphatemia was present in FGF23 null mice (Table 1) in the setting of normal circulating 1,25(OH)\(_2\)D levels, however, is consistent with an important direct role of FGF23 deficiency to increase renal phosphate reabsorption by the kidney independent of its effects to regulate 1α-hydroxylase activity. The inability to observe the antiphosphaturic effects in FGF23 null mice with ablated 1α-hydroxylase and VDR may be due to the masking of the renal phenotype by decreased gastrointestinal absorption of phosphate.

Finally, the relevance of our findings in FGF23 null mice to the potential toxicity of phosphate and excessive activation of the vitamin D axis in hyperphosphatemic clinical disorders, such as tumoral calcinosis,\(^30\)–\(^34\) and hyperphosphatemic patients who have renal failure and are treated with calcitriol analogues, requires further investigation given the limitations of this study. In this regard, the nonuremic FGF23 null mouse model does not address the impact of renal failure, which could contribute additional factors that lead to vascular calcifications and mortality. In addition, although our study clearly demonstrated a survival benefit for both vitamin D and phosphorus restriction in the FGF23 null mice, we are unable to describe the mechanisms behind this improved survival. Last, FGF23 levels are markedly increased in the setting of renal failure,\(^25\)–\(^27\) rather than decreased as in the FGF23 null mice. The function of FGF23 in ESRD is not known. It is not clear whether the biologic effects of FGF23 are increased in ESRD or decreased as a result of a loss of kidney function. Because the kidney seems to function as the main target organ for FGF23, it is possible that the loss of a necessary kidney-derived constituent, such as Klotho, is promoting the exponential elevations of serum FGF23 in the setting of ESRD.\(^39\) Regardless, these findings suggest that effective lowering of serum phosphate will have important effects to prevent vascular calcifications. Prospective clinical trials are needed to examine the separate roles of phosphate control and intensity of therapy with active vitamin D analogues on vascular calcifications and mortality in patients with chronic kidney diseases.

**CONCLUSION**

The results of this study implicate hyperphosphatemia in the pathogenesis of soft tissue and vascular calcifications in FGF23 null mice and support a context-dependent toxic role of excess circulating 1,25(OH)\(_2\)D. These observations suggest important interactions between hyperphosphatemia and excess 1,25(OH)\(_2\)D and support the underlying premise of the K/DOQI clinical recommendations to limit the dosage of active vitamin D analogues in the setting of hyperphosphatemia and to strive to achieve lower serum phosphate concentrations.\(^7\) In addition, a better understanding of the interactions between hyperphosphatemia and excessive vitamin D activity in the FGF23 null mouse model may provide insights into the management of chronic kidney disease, where hyperphosphatemia and treatment with vitamin D analogues often coexist.
scans per pixel. Image sizes were approximately $300 \times 300 \mu m$ with 6.25-$\mu m$ pixel resolution. An atmosphere correction was applied to the raw image to subtract the contribution of atmosphere absorbance (water vapor and carbon dioxide). Images were created using Spectrum Spotlight software.

**µCT Analysis**

The kidneys from 6-wk-old mice were collected and fixed in 70% ethanol. High-resolution µCT was used to evaluate mineralization of kidney samples (µCT40; Scanco Medical AG, Basserdorf, Switzerland). A 100-$\mu m$-thick area from the middle of each kidney was evaluated for three-dimensional image and quantitative evaluation of the mineralized kidney volume fraction.

**Statistical Analyses**

We evaluated differences between groups by one-way ANOVA for multiple group comparison and $t$ test for two-group comparison. All values are expressed as means ± SEM. $P < 0.05$ was considered statistically significant. All computations were performed using the GraphPad Prism 4 software (GraphPad Software, San Diego, CA).

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**DISCLOSURES**

None.

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