Physiological Regulation and Disorders of Phosphate Metabolism —Pivotal Role of Fibroblast Growth Factor 23—

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Abstract

Fibroblast growth factor (FGF) 23 has been identified as the last member of FGF family. FGF23 reduces serum phosphate level by suppressing proximal tubular phosphate reabsorption and intestinal phosphate absorption. FGF23 is produced by bone and acts on the kidney through a specific receptor system which is composed of Klotho and certain subtypes of FGF receptors. Excess actions of FGF23 cause several hypophosphatemic diseases characterized by impaired renal phosphate reabsorption and rickets/osteomalacia. In contrast, deficient actions of FGF23 result in hyperphosphatemic tumoral calcinosis with enhanced renal phosphate reabsorption. These results indicate that FGF23 works as a hormone to regulate the serum phosphate level.

Key words: hypophosphatemia, hyperphosphatemia, fibroblast growth factor, Klotho, hormone

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Introduction

Inorganic phosphate is an essential and the most abundant anion in our body. Phosphate is one of the major components of bone as hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, a constituent of the cell membrane and works in various cellular functions as numerous phosphorylated metabolites. In order to accomplish these functions, it seems to be necessary that the circulatory phosphate level is regulated to within a narrow range. While the regulatory mechanisms of the serum calcium level have been extensively studied and explained by actions of two calcium-regulating hormones, parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D $[1,25(\text{OH})_2\text{D}]$, it has been largely unknown how the serum phosphate level is maintained. Recent identification of fibroblast growth factor (FGF) 23 indicated that the serum phosphate level is regulated by a humoral mechanism and FGF23 plays an essential role in the maintenance of the serum phosphate level. In addition, several disorders of phosphate metabolism have been shown to be caused by deranged actions of FGF23.

Regulation of the Serum Phosphate Level

The serum phosphate level is regulated by intestinal phosphate absorption, renal phosphate reabsorption and dynamic equilibrium between extracellular phosphate and intracellular phosphate or phosphate in bone (Fig. 1). Of these, renal phosphate reabsorption is believed to be the main determinant of serum phosphate level at least in a chronic state, while acute changes of serum phosphate are usually caused by movement of extracellular phosphate into intracellular pool and bone, or vice versa. Filtered phosphate from glomeruli is largely reabsorbed in proximal tubules. Therefore, the molecule that mediates proximal tubular phosphate reabsorption has an indispensable role in the regulation of serum phosphate level.

Several sodium-dependent phosphate cotransporters have been identified to date. Type IIa and IIc sodium-phosphate cotransporters are expressed in proximal tubules and mediate physiological phosphate reabsorption in proximal tubules. Actually, knockout mice for type IIa sodium-phosphate cotransporter show hypophosphatemia by impaired reabsorp-

tion of phosphate (1). In addition, mutations in a gene coding for type IIc sodium-phosphate cotransporter were shown to result in a genetic disease called hereditary hypophosphatemic rickets with hypercalciuria (HHRH) (2, 3). HHRH is characterized by hypophosphatemia due to impaired phosphate reabsorption. Because hypophosphatemia increases serum $1,25(\text{OH})_2\text{D}$ level and $1,25(\text{OH})_2\text{D}$ stimulates intestinal calcium absorption, patients with HHRH show high $1,25(\text{OH})_2\text{D}$ and hypercalciuria.

Causes of Rickets/Osteomalacia

Rickets and osteomalacia are bone diseases characterized by impaired mineralization of bone matrix. Because of this disordered mineralization, softening of bone is observed. Rickets is a disease of childhood which causes growth retardation and bowing of legs. On the contrary, muscle weakness and bone pain are major symptoms of osteomalacia observed in adults. Although there are various etiologies for rickets/osteomalacia, chronic hypophosphatemia is one of the most important and frequent causes of these diseases. There are several diseases causing hypophosphatemic rickets/osteomalacia which have very similar clinical features, these are three genetic diseases called autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR), autosomal recessive hypophosphatemic rickets/osteomalacia (ARHR),

X-linked hypophosphatemic rickets/osteomalacia (XLH), one paraneoplastic syndrome named tumor-induced rickets/osteomalacia (TIO) and hypophosphatemic rickets/osteomalacia associated with McCune-Albright syndrome/fibrous dysplasia of bone. Hypophosphatemia due to impaired renal phosphate reabsorption is a typical feature of these diseases as seen in HHRH. However, serum $1,25(\text{OH})_2\text{D}$ level is not high, and remains low normal or low in patients with these diseases in contrast to high $1,25(\text{HO})_2\text{D}$ in HHRH. Therefore, these patients show inappropriately low $1,25(\text{OH})_2\text{D}$ level for hypophosphatemia indicating that both phosphate reabsorption and vitamin D metabolism are deranged in these diseases.

FGF23 was identified almost simultaneously by three groups. *FGF23* was first cloned by homology to *FGF15* in mice (4). In addition, positional cloning identified *FGF23* as a responsible gene for ADHR (5). Furthermore, FGF23 was cloned as a causative factor for TIO (6). These results indicate that FGF23 is involved in the development of several hypophosphatemic diseases.

Structure of FGF23

Sequence of cDNA for *FGF23* indicates that FGF23 is a protein with 251 amino acids (Fig. 2). There is a signal peptide with 24 amino acids in the N-terminal portion of FGF23 protein. Expression of recombinant FGF23 protein and detection of FGF23 protein by Western blotting indicated that a part of FGF23 protein is proteolytically cleaved between Arg¹⁷⁹ and Ser¹⁸⁰ by proprotein convertase, furin (6, 7). FGF23 has a FGF homology region in the N-terminal portion of this processing site.

FGF23 belongs to FGF family. FGF family members are defined as humoral factors which have a FGF homology region and show affinity for FGF receptors (FGFRs) (8). There are 22 FGF family members in humans and these family members are subdivided into several subfamilies. FGF23 belongs to FGF19 subfamily together with FGF19

Figure 1. Phosphate balance. Serum phosphate level is regulated by intestinal phosphate absorption, renal phosphate reabsorption and dynamic equilibrium between extracellular phosphate and intracellular phosphate or phosphate in bone.

Figure 2. Structure and function of FGF23. FGF23 is a protein with 251 amino acids. There is a signal peptide with 24 amino acids in the N-terminal portion of FGF23 protein. A part of FGF23 is cleaved between Arg¹⁷⁹ and Ser¹⁸⁰ by furin recognizing Arg¹⁷⁶-X-X-Arg¹⁷⁹ motif. FGF23 has a FGF homology region in the N-terminal portion of this processing site. FGF23 reduces serum phosphate by suppressing proximal tubular phosphate reabsorption and intestinal phosphate absorption.

and FGF21. Four FGF11 subfamily members, FGF11 to FGF14, are also called FGF homologous factors (9). Because FGF11 subfamily members show no affinity for FGFRs, some researchers exclude these members from the FGF family.

Actions of FGF23

The biological activity of FGF23 was examined using recombinant FGF23. A single injection of FGF23 resulted in reduced serum phosphate and 1,25(OH)₂D levels (10). FGF23 reduces renal phosphate reabsorption by suppressing expression of type 2a and 2c sodium-phosphate cotransporter in brush border membrane of proximal tubules. Prior to the reduction of serum phosphate level, FGF23 suppresses the expression of 25-hydroxyvitamin D-1 α -hydroxylase and conversely enhances the expression of 25-hydroxyvitamin D-24-hydroxylase in kidney. 25-hydroxyvitamin D-1 α -hydroxylase is an enzyme that mediates the production of 1,25(OH)₂D and 25-hydroxyvitamin D-24-hydroxylase converts 1,25(OH)₂D into more hydrophilic metabolites with less activity. Therefore, FGF23 reduces serum 1,25(OH)₂D by modifying expression levels of these vitamin D-metabolizing enzymes. Because 1,25(OH)₂D stimulates intestinal phosphate absorption, FGF23 reduces serum phosphate by suppressing proximal tubular phosphate reabsorption and intestinal phosphate absorption (Fig. 2).

This biological activity of FGF23 to reduce serum phosphate level was observed using uncleaved FGF23. In contrast, processed N-terminal and C-terminal fragments of FGF23 did not reduce the serum phosphate level (11). These results indicate that the processing of FGF23 protein between Arg¹⁷⁹ and Ser¹⁸⁰ abolishes its effect to reduce serum phosphate level (Fig. 2).

FGF23 knockout mice were created to examine whether FGF23 has physiological functions (12, 13). These mice show hyperphosphatemia, enhanced renal phosphate reabsorption and high 1,25(OH)₂D level. These results indicate that FGF23 is physiologically working to reduce serum phosphate and 1,25(OH)₂D levels. Replacement of *FGF23* gene by lacZ indicated that FGF23 is produced by bone (13). Other study shows that FGF23 is produced by osteocytes (14). Therefore, FGF23 seems to be produced by bone and regulate serum phosphate and 1,25(OH)₂D levels acting on kidney. Thus, there should be a specific receptor for FGF23 in kidney. These features are clearly different from those of "classical" FGF family members. Classical FGF family members have been shown to bind to FGFRs and thought to work as local factors.

In order to identify the specific receptor for FGF23 in kidney, binding proteins to FGF23 in renal homogenate was analyzed and the major binding protein was found to be Klotho (15). *Klotho* mice were created by transgenic method and these mice show severely reduced expression of Klotho (16). *Klotho* mice have been regarded as a model animal for senescence. However, it has been also shown that *Klotho*

mice have hyperphosphatemia and high 1,25(OH)₂D levels like *FGF23* knockout mice suggesting the involvement of Klotho in FGF23 signaling (16). Subsequent *in vitro* studies indicated that FGF23 can provoke intracellular signaling such as phosphorylation of extracellular signal-regulated kinase and induction of *early growth response-1* only in cells expressing Klotho (15). However, Klotho alone was not sufficient for FGF23 signaling. There are four genes for FGFRs and alternate splicing from these genes produces several subtypes of FGFRs. Klotho was shown to work as a coreceptor for FGF23 together with certain subtypes of FGFRs including FGFR1c (15, 17). Thus, FGF23 is produced by bone, works on kidney through a specific receptor for FGF23 and physiologically regulates serum phosphate and 1,25(OH)₂D levels. These features indicate that FGF23 is a hormone rather than a local cytokine like other classical members of FGF family (18).

Involvement of FGF23 in the Development of Hypophosphatemic Rickets/Osteomalacia

As mentioned before, ADHR, ARHR, XLH, TIO and hypophosphatemic rickets/osteomalacia associated with McCune-Albright syndrome/fibrous dysplasia are characterized by hypophosphatemia and rather low 1,25(OH)₂D levels. These biochemical features are those expected to be caused by excess actions of FGF23. Actually, after the cloning of *FGF23*, several enzyme-linked immunosorbent assays for FGF23 have been established. These assays confirmed that FGF23 levels are basically high in patients with these hypophosphatemic diseases (19, 20). However, the mechanisms of excess actions of FGF23 in these diseases are variable (Table 1).

FGF23 was cloned from a tumor responsible for TIO (6). This disease has a profound effect on the quality of life of the patients. Because of severe muscle weakness and bone pain, patients with TIO sometimes become completely bedridden. However, this disease is cured by complete resection of responsible tumors. Circulatory FGF23 level is elevated in virtually all patients with TIO, and rapidly decreases into or below the reference range after removal of the responsible tumors (20-22). In addition, it has been shown that tumors causing TIO strongly express FGF23 (6, 23). Therefore, TIO is considered to be caused by overexpression of FGF23 in responsible tumors. Most tumors causing TIO are now pathologically classified as phosphaturic mesenchymal tumor (mixed connective tissue variant) (PMTMCT) (24). However, it is unknown why some PMTMCTs overexpress FGF23.

FGF23 was also cloned as a responsible gene for ADHR and several missense mutations in *FGF23* gene were reported in patients with FGF23 (5). These mutations are found in codons coding for either Arg¹⁷⁶ or Arg¹⁷⁹ just before the processing site between Arg¹⁷⁹ and Ser¹⁸⁰ (Fig. 2). The amino acid sequence before the processing site, Arg¹⁷⁶-X-X-Arg¹⁷⁹, is the consensus site recognized by furin. Therefore,

Table 1. Hypophosphatemic Diseases Caused by Excess Actions of FGF23 and Causes of Excess FGF23 Action

Disease	Cause of excess FGF23 action
Autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR)	Dysregulated expression of FGF23 by mutations in <i>FGF23</i> gene
Autosomal recessive hypophosphatemic rickets/osteomalacia (ARHR)	Overexpression of FGF23 in bone by mutations in <i>DMP1</i> gene
X-linked hypophosphatemic rickets/osteomalacia (XLH)	Overexpression of FGF23 in bone by mutations in <i>PEHX</i> gene
McCune-Albright syndrome/Fibrous dysplasia	Overexpression of FGF23 in bone
Tumor-induced rickets/osteomalacia	Overexpression of FGF23 in responsible tumors

PEHX: phosphate-regulating gene with homologies to endopeptidases on the X chromosome

DMP1: dentin matrix protein 1

mutations in Arg¹⁷⁶ or Arg¹⁷⁹ destroy this consensus sequence and were considered to result in impaired processing of FGF23 protein. Actually, mutant FGF23 proteins observed in patients with ADHR are resistant to the processing between Arg¹⁷⁹ and Ser¹⁸⁰ (11, 25). This resistance to the processing was expected to cause increased amount of biologically active full-length FGF23 and enhanced activity of FGF23. However, FGF23 level is not always high in patients with ADHR. FGF23 level fluctuates in these patients and it was reported that high FGF23 level is associated with hypophosphatemia (26). These results suggest that mutations in *FGF23* gene somehow impair the regulatory mechanism of FGF23 production and dysregulated FGF23 production underlie hypophosphatemia in patients with ADHR.

Responsible genes for XLH and ARHR are *phosphate-regulating gene with homologies to endopeptidases on the X chromosome* (*PHEX*) (27) and *dentin matrix protein 1* (*DMP1*) (28, 29), respectively. Although the precise functions of *PHEX* and *DMP1* proteins are not yet elucidated, enhanced expression of FGF23 in bone has been reported in model mice for XLH and *DMP1*-null mice (28, 30). Together with elevated FGF23 levels in patients with these diseases (19-21, 28), it is suggested that *PHEX* and *DMP1* somehow suppress FGF23 expression in bone. FGF23 has been also shown to be overexpressed in bone including regions affected by fibrous dysplasia in patients with McCune-Albright syndrome/fibrous dysplasia (31). While somatic activating mutations in *GNAS1* gene are responsible for this disease, it is unclear whether or not increased cyclic AMP level causes enhanced expression of FGF23. Regulatory mechanisms of FGF23 expression in normal and abnormal bone tissues are one of important remaining questions to be clarified.

FGF23 and Hyperphosphatemic Diseases

In contrast to several hypophosphatemic diseases caused by excess actions of FGF23, patients with hyperphosphatemic tumoral calcinosis show hyperphosphatemia, en-

hanced renal tubular phosphate reabsorption and a high 1,25(OH)₂D level (32). These features are also observed in *FGF23* knockout mice and *Klotho* mice. Thus, it was postulated that hyperphosphatemic tumoral calcinosis is caused by deficient actions of FGF23. Therefore, it was a surprise that *GALNT3* encoding UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 3 (ppGaNTase-T3) was first reported to be responsible for hyperphosphatemic tumoral calcinosis (33). ppGaNTase-T3 is an enzyme that transfers N-acetylgalactosamine from UDP-N-acetyl-alpha-D-galactosamine to serine or threonine residue as an initial sugar of mucin-type O-linked glycosylation. However, it was unclear how mutations in *GALNT3* gene cause hyperphosphatemia. There are several assay methods for FGF23. Intact assay uses two monoclonal antibodies that recognize N-terminal and C-terminal portion of the processing site of FGF23 and detects only full-length uncleaved FGF23 (20). On the other hand, C-terminal assay uses two kinds of polyclonal antibodies to the C-terminal region of the processing site and measures both full-length and cleaved C-terminal fragment of FGF23 (19). FGF23 levels evaluated by these two assays correlate very well in patients with XLH (21). However, when FGF23 levels in patients with *GALNT3* mutations were analyzed by these two assays, it was found that FGF23 was quite high by C-terminal assay, but low-normal in intact assay (34) (Table 2). These results suggested that there is only a little full-length FGF23, but large amount of processed C-terminal fragment of FGF23 in these patients. Actually, *in vitro* study indicated that FGF23 has three O-linked glycans (34) and ppGaNTase-T3 is specifically involved in the synthesis of O-glycan attaching to Thr¹⁷⁸ just before the processing site between Arg¹⁷⁹ and Ser¹⁸⁰ (35). In addition, silencing ppGaNTase-T3 expression enhanced the processing of FGF23 protein (34). Therefore, the clinical features of patients with mutations in *GALNT3* gene could be explained as shown below. Mutations in *GALNT3* gene result in impaired glycosylation of FGF23 proteins at Thr¹⁷⁸ and cause enhanced processing of FGF23. Hyperphosphatemia and high 1,25(OH)₂D derive from defi-

Table 2. Hyperphosphatemic Tumoral Calcinosis and Circulatory FGF23 Levels

Causative gene	Mechanism of impaired FGF23 action	FGF23 levels	
		Intact assay	C-terminal assay
<i>FGF23</i>	Enhanced processing of FGF23	Low – low normal	High
<i>GALNT3</i>	Enhanced processing of FGF23	Low – low normal	High
<i>Klotho</i>	Resistance to FGF23	High	High

cient actions of full-length FGF23. Although the precise mechanism of FGF23 production is not clear at the moment, hyperphosphatemia, high 1,25(OH)₂D or other metabolic changes stimulate FGF23 production. Together with enhanced processing of FGF23 protein, overexpression of FGF23 results in high C-terminal fragment of FGF23.

Subsequently, mutations in *FGF23* gene were also shown to cause hyperphosphatemic tumoral calcinosis (36-38). FGF23 levels in patients with mutations in *FGF23* are essentially the same as those with mutations in *GALNT3*, extremely high by C-terminal assay and low by full-length assay (36) (Table 2). Several missense mutations of *FGF23* gene were reported and these mutant FGF23 proteins are somehow susceptible to the processing. The mechanism of this susceptibility to the processing is unclear at the moment.

Recent analysis indicated that hyperphosphatemic tumoral calcinosis is also caused by a mutation in *Klotho* gene again indicating the involvement of *Klotho* in FGF23 signaling (39). FGF23 level in this patient is very high both by intact and C-terminal assays (Table 2). These results indicate that insensitivity to FGF23 is the cause of hyperphosphatemia in this patient in contrast to enhanced processing of FGF23 protein in patients with mutations in *GALNT3* or *FGF23*. Thus, hyperphosphatemic tumoral calcinosis by a mutation in *Klotho* gene is another example of hormone resistance.

FGF23 was reported to be high in hyperphosphatemic patients by chronic renal failure (40-43) or hypoparathyroidism (44). Considering the biological activities of FGF23, this high FGF23 seems to be the result of hyperphosphatemia again suggesting that hyperphosphatemia enhances FGF23 expression. However, FGF23 is extremely high in some patients with chronic renal failure undergoing dialysis in contrast to a slight increase of FGF23 in patients with hypoparathyroidism. These results suggest that some metabolic changes in end-stage renal disease stimulate FGF23 production although the precise mechanism of this high FGF23 is unknown.

Summary and Future Perspectives

Studies in the past several years clarified the essential role of FGF23 as a hormone to regulate serum phosphate and 1,25(OH)₂D levels. In addition, several hypophosphatemic and hyperphosphatemic diseases were shown to be caused by deranged actions of FGF23. The identification of *Klotho* as a coreceptor for FGF23 showed the novel means of interaction between FGF family members and FGFRs, and indicated the possibility of similar ligand-receptor coupling. Actually, FGF19 produced by the intestine was shown to inhibit bile acid synthesis in the liver through FGFR4 and β Klotho, a homologous protein to *Klotho* (45-48). Likewise, FGF21 mainly produced by the liver acts on adipose tissue through FGFR1c and β Klotho (47, 49-51). Based on these results, FGF19 family members, FGF19, FGF21 and FGF23, are now called endocrine FGFs.

Despite these findings, several important questions in addition to those already mentioned remain. First, while several results suggest that the serum phosphate level is at least one of the regulators of FGF23 production and circulatory FGF23 level, it is unknown how and where changes of serum phosphate are sensed in our body and induce metabolic changes leading to altered FGF23 level. Second, it is not clear how the processing of FGF23 protein is controlled and why the processing between Arg¹⁷⁹ and Ser¹⁸⁰ is necessary. Furthermore, intracellular signaling molecules beyond *Klotho*-FGFR1c complex induced by FGF23 are largely unknown. Future studies clarifying these issues will hopefully help to establish novel therapeutic measures for deranged phosphate and vitamin D metabolism.

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