Review

Fibroblast Growth Factor (FGF)-23 and Hypophosphatemic Rickets/Osteomalacia

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Introduction

RICKETS and osteomalacia are related diseases characterized by impaired mineralization of bone matrices. The main causes of these disorders are hypophosphatemia and deficient actions of vitamin D metabolites. Chronic hypophosphatemia causing rickets/osteomalacia derives either from insufficient intestinal absorption of phosphate or from renal phosphate leak (Table 1). Although detailed mechanisms for maintenance of serum calcium are well known at present, knowledge concerning regulation of phosphate metabolism is clearly inadequate. Actually, serum phosphate level is believed to be determined mainly by maximal tubular reabsorption of phosphate (TmP/GFR). Several humoral factors such as parathyroid hormone (PTH), glucocorticoid and insulin-like growth factor-I, modulate TmP/GFR. However, the mechanism of abnormally reduced TmP/GFR in several hypophosphatemic diseases is unknown. Recent genetic, biochemical and physiological studies revealed that a novel humoral factor is involved in the pathogenesis of hypophosphatemic rickets/osteomalacia. In this brief review, we summarize the current concepts concerning the pathophysiology of hypophosphatemic rickets/osteomalacia and discuss unanswered questions.

Hypophosphatemic rickets/osteomalacia

There are several kinds of diseases that cause hypophosphatemic rickets/osteomalacia (Table 1).

Of these, tumor-induced rickets/osteomalacia (TIO), autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) and X-linked hypophosphatemic rickets/osteomalacia (XLH) show very similar biochemical features. These diseases are characterized by hypophosphatemia due to reduced TmP/GFR. In addition, hypophosphatemia usually stimulates renal 25-hydroxyvitamin D 1α-hydroxylase and increases serum 1,25-dihydroxyvitamin D [1,25(OH)2D] level. In contrast, patients with TIO, ADHR or XLH show inappropriately normal or low serum 1,25(OH)2D level. Therefore, regulatory mechanisms for both tubular reabsorption of phosphate and vitamin D metabolism are deranged in these hypophosphatemic diseases. The similarity of biochemical features suggests the existence of a common pathophysiological mechanism for the development of hypophosphatemia and abnormal vitamin D metabolism in these three diseases.

Pathogenesis of TIO

TIO is one of paraneoplastic syndromes. Patients with TIO exhibit severe muscle weakness and sometimes become bedridden. There is a marked decrease of bone mineral density and fractures are frequent. Hence, TIO is a devastating disease with significant morbidity. In contrast, when the responsible tumor is completely removed, these symptoms will dramatically improve and there will be a complete cure. Therefore, correct diagnosis and management of TIO are clinically very important. The most frequent cause of TIO is benign mesenchymal tumor with vascular component like hemangio-pericytoma. In addition, some malignant tumors including prostate cancer, osteosarcoma, chondro-
blastoma, fibrosarcoma and oat cell carcinoma are reported to bring about TIO. Furthermore, non-tumorous lesions like epidermal nevi and fibrous dysplasia also are described as causes for TIO [1]. More than one hundred cases of TIO have been described in the literature [1]. However, because diagnosis of TIO is sometimes very difficult, it is highly likely that some patients with TIO are misdiagnosed or undiagnosed. Therefore, the precise frequency of this disease should be higher. Patients with TIO may be misdiagnosed to have neuromuscular diseases if serum phosphate level is not correctly evaluated. Even when hypophosphatemia is found, the diagnosis of TIO at present depends on discovery and removal of the responsible tumor. However, the responsible tumor for TIO is sometimes too small or in difficult locations to be identified by palpation or X-ray examination. We have experienced a patient with TIO whose tumor was first identified by magnetic resonance imaging skeletal survey [2]. This diagnostic approach had been useful in other two patients with TIO [3]. Because some responsible tumors for TIO are in bones, magnetic resonance imaging skeletal survey should be of value in at least some patients with TIO. Furthermore, even if patients with hypophosphatemic rickets/osteomalacia have tumors, we cannot be sure that the tumors are causing TIO. Clearly, a specific biochemical diagnostic method for TIO is needed.

The complete cure of TIO by removal of the responsible tumor indicates that the tumor secretes some humoral factor that causes phosphaturia and derangement of vitamin D metabolism. This hypothetical humoral factor causing TIO has been called phosphatonin [4]. As regards the phosphaturic activity of phosphatonin, there are several reports that showed inhibitory activity in conditioned media of tumor cells responsible for TIO on phosphate transport of kidney cells [5-8]. Opposum kidney (OK) cells or human proximal tubular cells were used in those assays. However, the phosphaturic factor causing TIO has not been cloned using these in vitro assays. Physiological proximal tubular phosphate transport is mediated by sodium-phosphate co-transporter type IIa [9]. On the other hand, there are at least four types of sodium-phosphate co-transporters [10]. In a preliminary study, we examined the expression of sodium-phosphate co-transporters in primary cultured renal proximal tubules and commercially available OK cells. We could easily find the expression of type III co-transporter while that of type IIa was barely detectable. Similar lack of expression of sodium-phosphate co-transporter type IIa in primary cultured proximal tubular cells and renal cell lines has also been described [11]. Therefore, because cultured kidney cells do not necessary express type IIa co-transporter, it is difficult to establish an in vitro assay that accurately evaluates physiological proximal tubular phosphate reabsorption. This is probably one of the reasons why the phosphaturic factor causing TIO has not been cloned so far.

In an attempt to identify the phosphaturic factor, we employed a different approach [12]. We made a cDNA library from a tumor responsible for TIO. We then screened the cDNA library with two kinds of probes. One probe was made from the cDNA of the tumor causing TIO and the other one was from the

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<td>Inadequate phosphate absorption</td>
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<td>Fanconi syndrome</td>
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*Excluding derangements of actions of vitamin D metabolites
cDNA of adjacent normal bone. By screening of about 320,000 clones, we picked up 456 clones that hybridized with the probe from the tumor and that did not hybridize with the probe from the normal bone. After sequencing these clones, cDNAs that are dominantly expressed in the tumor causing TIO were aligned according to the frequency of appearance. The most frequently cloned cDNA was dentin matrix protein-1 (DMP-1) followed by heat shock protein-90 and osteopontin. Two novel cDNAs were observed at that time. That turned out to be identical with fibroblast growth factor (FGF)-23 [13, 14] and matrix extracellular phosphoglycoprotein (MEPE) [15]. We then examined the biological activity of clones whose functions were not known. Because an appropriate and reproducible in vitro assay system for phosphate transport could not be established in our laboratory, we used a mouse model system. When Chinese hamster ovary (CHO) cells that stably expressing DMP-1 or MEPE were implanted into nude mice and allowed to form tumors, serum phosphate did not decrease. However, when CHO cells expressing FGF-23 were used, serum phosphate was clearly lower than that of nude mice without tumor cells or mice with wild-type CHO cells. This reduction of serum phosphate was associated with increased urinary excretion of phosphate and high serum level of alkaline phosphatase (Table 2). In addition, serum 1,25(OH)2D level was clearly lower and renal expression of 25-hydroxyvitamin D 1α-hydroxylase was reduced in mice with CHO cells expressing FGF-23. Furthermore, bone mineral density and ash content of bone were apparently decreased in mice with exposure to FGF-23. Histological analysis of bone indicated that there was a widening of growth plate and an increase of osteoids in mice with CHO cells expressing FGF-23 (Table 2). Thus, continuous production of FGF-23 by implanted cells reproduced biochemical and histological features of TIO. It also is recently reported that several tumors causing TIO overexpress FGF-23 [16]. These results indicate that TIO is caused by overexpression of FGF-23 (Fig. 1).

![Image](image.jpg)

**Fig. 1.** Hypothetical model of pathogenesis for XLH and TIO. In normal condition, phosphatonin (PTN) is degraded by PHEX protein and phosphate reabsorption in proximal tubules is maintained. Overproduction of the putative phosphaturic factor called phosphatonin causes hypophosphatemia in tumor-induced rickets/osteomalacia (TIO). It has been shown that FGF–23 is the causative factor of TIO. In X-linked hypophosphatemic rickets/osteomalacia (XLH), mutated PHEX cannot cleave phosphatonin and evoke phosphaturia and hypophosphatemia by inhibitory activity of phosphatonin on renal phosphate reabsorption. It is not clear at the moment whether this humoral factor in XLH is FGF–23 or not. Pi: inorganic phosphate

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<th>Table 2. Characteristics of patients with TIO and mice with CHO cells stably expressing FGF–23.</th>
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<td>Hypophosphatemia</td>
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<td>Phosphaturia</td>
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<td>Low serum 1,25(OH)2D</td>
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<td>High alkaline phosphatase</td>
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<td>Low bone mineral density</td>
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Pathogenesis of ADHR

FGF-23 was actually cloned by two groups in the year 2000. FGF-23 was first cloned in mouse by homology to FGF-15 [14]. It was shown in mouse that FGF-23 is expressed in brain, especially in ventrolateral thalamic nucleus, thymus, small intestine and heart. FGF-23 was also cloned as the responsible gene for ADHR, another hypophosphatemic disease [13]. ADHR is a rare hypophosphatemic rickets with clinical features similar to XLH. Although detailed analysis of ADHR family suggests some differences in clinical presentation between XLH and ADHR [17], it is difficult to discriminate these two diseases if sufficient family history cannot be obtained. An international collaborative group identified FGF-23 as the responsible gene for ADHR by positional cloning [13]. Human FGF-23 cDNA encodes a protein with 251 amino acids. FGF-23 has a signal peptide at its N-terminus and shows a homology to FGF family members especially in the middle portion of the protein. Expression of human FGF-23 is observed in heart, liver, thyroid/parathyroid, small intestine, testis, skeletal muscle and fetal chondrocytes [13]. We also have observed expression of FGF-23 in heart, liver, lymph node and thymus [12]. However, expression of FGF-23 is not observed in kidney, indicating that abnormal phosphate and vitamin D metabolism in ADHR also is caused by a humoral mechanism.

Three mutations of FGF-23 gene were reported in three families with ADHR. However, it was not clear how mutations of FGF-23 gene affect phosphate and vitamin D metabolism. When FGF-23 with 6xHis tag sequence at its C-terminus was produced by CHO cells and conditioned medium was evaluated by Western blot analysis using anti-His antibody, two immunoreactive proteins were detected [12]. The larger protein with molecular mass of about 30 kDa had 25Tyr at its N-terminus indicating that this protein is a mature form of FGF-23 lacking signal sequence. The smaller protein with molecular mass of about 10 kDa had 180Ser at its N-terminus. The preceding amino acid sequence of 180Ser is 176Arg-177His-178Thr-179Arg and agrees with the proteolytic consensus sequence of RXR (Fig. 2). Mutations found in ADHR patients occur either in 176Arg or 179Arg. Therefore, it is possible that mutations in FGF-23 gene prevent proteolytic processing of the FGF-23 protein and somehow enhance the activity of FGF-23 to cause phosphaturia. Actually, when mutant FGF-23 protein in which both 176Arg and 179Arg were replaced by Gln was expressed and the conditioned medium was analyzed by Western blotting, the C-terminal fragment of FGF-23 with 180Ser at its N-terminus could not be observed [18]. Therefore, while overproduction of FGF-23 by tumor cells causes TIO, mutations of FGF-23 gene caused by a humoral mechanism.

![Fig. 2. Structure of FGF-23 protein. When recombinant FGF-23 with 6xHis tag sequence at its C-terminus was expressed in CHO cells and conditioned medium was analyzed by Western blotting using anti-His antibody, two protein products were observed (shaded area). The larger product was mature FGF-23 lacking the signal sequence. The smaller one was C-terminal fragment of FGF-23 starting at 180Ser. The preceding amino acid sequence of 180Ser is 176Arg-177His-178Thr-179Arg and agrees with proteolytic consensus site. Mutations in FGF-23 gene found in ADHR patients occur either in 176Arg or 179Arg. Amino acids are shown with a single letter.](image-url)
seem to result in ADHR by impairing protein processing and probably enhancing phosphaturic activity of FGF-23 protein.

**Pathogenesis of XLH**

XLH is the most frequent cause of vitamin D-resistant rickets and shows similar biochemical features to TIO and ADHR. Patients with XLH usually exhibit bone deformity and/or growth retardation during infancy. The international collaborative group identified the responsible gene for XLH by positional cloning in 1995 [19], and named it PHEX (phosphate regulating gene with homologies to endopeptidases on the X chromosome). Since the identification of PHEX gene, many abnormalities of PHEX gene were reported in patients with XLH and compiled in a database (http://data.mch.mcgill.ca/phexdb/). These include missense and nonsense mutations, insertions and deletions, and splicing abnormalities of mRNA. The presence of nonsense mutations of exon 1 or 2 in patients with XLH that should drastically alter the structure of the PHEX protein indicates that XLH is caused by inactivation of PHEX activity. PHEX gene product is considered to have one membrane-spanning region [20]. Because of its homology to other endopeptidases such as neutral endopeptidase and endothelin-converting enzyme 1, PHEX protein was supposed to have proteolytic activity. Actually, PHEX protein was shown to degrade peptides from PTH or PTH-related protein [21, 22]. However, it is largely unknown how abnormal PHEX proteins impair the phosphate metabolism. The expression of PHEX is reported in bone, teeth, brain, muscle, testis, ovary and parathyroid gland, but not in kidney [22-26]. This means that abnormal PHEX protein itself cannot directly invoke derangements of phosphate and vitamin D metabolism in kidney. Therefore, it has been hypothesized that there is a humoral phosphaturic factor that is inactivated by PHEX protein in healthy individuals. Abnormal PHEX proteins seen in XLH patients seem to be unable to degrade the factor and evoke hypophosphatemia by excess phosphaturic action of this humoral factor. Although it is not clear at the moment whether this factor in XLH is the same as that causing TIO, the phosphaturic factor in XLH was assumed to be phosphatonin in some reports [27] (Fig. 1). This hypothetical humoral mechanism for the development of hypophosphatemia in XLH is supported by several investigations. The Hyp mouse is one of murine homologues of XLH and is shown to have a deletion of the 3' region of the Phex gene. [23]. When a normal mouse and a Hyp mouse were connected by parabiosis experiment, serum phosphate of the normal mouse decreased [28]. In addition, even when kidneys from normal mice were transplanted into Hyp mice, hypophosphatemia of Hyp mice did not improve [29]. In contrast, phosphaturia was not observed when kidneys from Hyp mice were transplanted into normal mice. Finally, when normal kidney was transplanted into a XLH patient with chronic renal failure, hypophosphatemia occurred [30]. These results indicate that there is no intrinsic defect of phosphate reabsorption in kidney of Hyp mice or XLH patients. Thus, identification of this humoral factor causing phosphaturia in XLH is indispensible for understanding pathogenesis of XLH. However, the identity of the phosphaturic activity causing XLH has yet to be clarified thus far.

**Future directions**

It has been shown that FGF-23 is involved in the development of at least two hypophosphatemic diseases, TIO and ADHR. However, there still remain many unanswered questions. Clinical similarity between TIO, ADHR and XLH suggests that there is a common pathophysiological mechanism in these diseases. Therefore, it is necessary to evaluate whether PHEX protein can degrade FGF-23. Actually, a recent report showed that in vitro translated tagged FGF-23 protein can be degraded by in vitro translated PHEX protein [31]. In contrast, mutant FGF-23 protein found in ADHR patients was not degraded by PHEX in this assay [31]. These results agree with the concept that FGF-23 is a substrate for PHEX protein. However, RXXR motif is not a site that is reported to be cleaved by PHEX [21, 22]. Because PHEX and FGF-23 are expressed in different tissues, FGF-23 should be secreted into general circulation if the PHEX protein processes FGF-23. On the other hand, RXXR motif is known to be recognized by proteases like furin [32]. It is likely that FGF-23 protein can be cleaved at the RXXR motif by ubiq-
uitously expressed enzymes other than PHEX protein because the processing was observed when FGF-23 is expressed in CHO, OK, COS and 293 cells [12, 16]. Therefore, if inability of mutant PHEX to correctly process FGF-23 protein is the cause of XLH, the specific interaction between PHEX and FGF-23 proteins needs to be shown. In addition, it should be clarified why other proteases that recognize RXXR motif cannot substitute for PHEX.

We have shown in vivo biological activity of FGF-23. However, only a little is known about the actual target cells of FGF-23. A recent in vitro study showed that the conditioned medium of COS cells expressing either wild-type FGF-23 or mutant FGF-23 found in ADHR patients inhibited phosphate transport of OK cells [31]. In addition, this inhibition of phosphate transport was blocked by adding heparin [31]. This means that the activity of FGF-23 is inhibited by heparin. However, we could not demonstrate the inhibitory activity of recombinant FGF-23 on phosphate uptake by OK cells in our laboratory [12]. Because there are subclones of OK cells, this discrepancy may be explained by the difference in cells used. Furthermore, it is possible that the conditioned medium of COS cells contains other factors that modulate activity of FGF-23. In contrast, heparin augments biological activity of other FGF family members such as FGF-2 [33]. Clearly we need more in vitro systems to evaluate the activities of FGF-23. The physiological function of FGF-23 also is another problem. FGF-23 certainly plays important roles in the development of TIO and ADHR when overexpressed or mutated. However, it is possible that the physiological role of FGF-23 has nothing to do with phosphate and vitamin D metabolism. Knockout mice for FGF-23 would shed light on this matter.

Clinically, the establishment of an assay system for FGF-23 in blood is very important, as it should enable biochemical diagnosis of TIO and ADHR. There is no specific biochemical diagnostic method for TIO at present. Therefore, if the responsible tumor cannot be found, patients with acquired hypophosphatemic rickets/osteomalacia are sometimes considered to have "idiopathic" hypophosphatemic rickets/osteomalacia [34]. When these patients are shown to have high blood level of FGF-23, we should vigorously search for the responsible tumor. The assay system for FGF-23 will eventually contribute to the establishment of a clearer classification of hypophosphatemic diseases. It also should be examined whether FGF-23 is involved in other diseases with abnormal phosphate and vitamin D metabolism.

Conclusion

Two diseases have been shown to be caused by overexpression or mutations of FGF-23 gene in these past two years. However, there still remain many important questions, to which we will certainly find answers in the near future. FGF-23, its analog or antagonist may be useful for treatment of some diseases with abnormal phosphate and vitamin D metabolism. Such advances will radically change the diagnostic approach and clinical management of patients with abnormal phosphate metabolism.

References

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