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Increased circulatory level of biologically active full-length FGF-23 in patients with hypophosphatemic rickets/osteomalacia

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Hypophosphatemic rickets/osteomalacia with inappropriately low serum 1,25-dihidroxyvitamin D level is commonly observed in X-linked hypophosphatemic rickets/osteomalacia, autosomal dominant hypophosphatemic rickets/osteomalacia and tumor-induced osteomalacia. Although the involvement of a newly identified factor, FGF-23, in the pathogenesis of ADHR and TIO has been suggested, clinical evidence indicating the role of FGF-23 has been lacking. We have previously shown that FGF-23 is cleaved between Arg^{179} and Ser^{180}, and this processing abolished biological activity of FGF-23 to induce hypophosphatemia. Therefore, sandwich ELISA for biologically active intact human FGF-23 was developed using two kinds of monoclonal antibodies that requires the simultaneous presence of both the N-terminal and C-terminal portion of FGF-23. The serum levels of FGF-23 in healthy adults were measurable and ranged from 8.2 to 54.3 ng/L. In contrast, those in a patient with TIO were over 200 ng/L. After the resection of the responsible tumor, the elevated FGF-23 level returned to normal level within 1 h. The increase of serum concentrations of 1,25-dihidroxyvitamin D and phosphate, and the decrease of serum 24,25-dihydroxyvitamin D followed the change of FGF-23. In addition, the elevated serum FGF-23 levels were demonstrated in most patients with XLH. It is likely that increased serum level of FGF-23 contributes to the development of hypophosphatemia not only in TIO but also in XLH.

Introduction

Rickets and osteomalacia are characterized by impaired mineralization of bone matrices. There are three diseases that share common clinical features including hypophosphatemia due to renal phosphate wasting and inappropriately low serum 1,25-dihydroxyvitamin D level for hypophosphatemia. Those diseases are X-linked hypophosphatemic rickets/osteomalacia (XLH), autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) and tumor-induced rickets/osteomalacia (TIO) (1). Recent studies indicated that ADHR derives from missense mutations in FGF-23 gene (2) and FGF-23 is overexpressed in tumors responsible for TIO (3). In addition, we have shown that administration of recombinant FGF-23 induces phosphaturia and hypophosphatemia in mice (4). Proteolytic consensus site was identified in FGF-23 protein and the cleavage of FGF-23 between Arg^{179} and Ser^{180} abolished its effect to cause hypophosphatemia (5). In addition, implantation of CHO cells stably expressing human FGF-23 mimicked almost all features of TIO in mice (4). These results indicate that excess actions of full-length uncleaved FGF-23 cause hypophosphatemic rickets/osteomalacia. However, it is not known whether circulatory level of FGF-23 is actually elevated in patients with ADHR or TIO. In addition, although the responsible gene for XLH was identified as a gene with homologies to endopeptidases and named phosphate-regulating gene with homologies to endopeptidases on the X-chromosome (PHEX) (6), the role of FGF-23 in the development of hypophosphatemia in XLH is completely unknown. To address these issues, we developed sandwich enzyme-linked immunosorbent assay (ELISA) for human FGF-23 using two monoclonal antibodies to FGF-23. The results indicate that biologically active uncleaved FGF-23 is present in normal circulation. In addition, circulatory level of FGF-23 is increased in a patient with TIO and returned to normal level soon after surgery. Furthermore, serum level of FGF-23 was high in patients with XLH during treatment. These results indicate that hypophosphatemic rickets/osteomalacia may be caused by excess activity of full-length FGF-23.

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Materials and Methods

Antigens

Recombinant human FGF-23 with or without histidine-tag sequence at the C-terminus was purified from conditioned media of cultures of CHO cells stably expressing each recombinant protein as described previously (4).

Monoclonal antibodies

Two rounds of hybridoma production were conducted to obtain monoclonal antibodies against human FGF-23. In the first experiment, six Balb/c mice were immunized with 20 μg each of purified recombinant FGF-23 protein with histidine-tag sequence in RIBI adjuvant (Corixa, WA) four times at intervals of a week. In the second experiment, seven Balb/c mice were immunized by the four times intraperitoneal injections with 20 μg each of purified recombinant human FGF-23 protein in RIBI adjuvant at intervals of a week. The spleen cells from immunized mice were fused with mouse myeloma cell line, sp2/0 (American Type Culture Collection:

CRL1581) in a ratio of 5:1 using polyethylene glycol. The culture supernatants were screened by ELISA using microplates coated with recombinant human FGF-23 protein. FN1 and FC1 antibodies derived from experiment 1 and 2, respectively.

Sandwich ELISA for the detection of human FGF-23 protein

FGF-23 levels in samples were determined by the sandwich ELISA as previously reported (7) with some modifications as follows. Each well of the 96-well ELISA plate was coated by FN1 antibody by incubating with 50 μl of FN1 antibody (10 μg/ml) in 50 mM $NaHCO_3$ over 12 hours at 4°C. The wells were then blocked by incubating with a commercially available reagent (Superblock; Pierce, IL) for 30 min at room temperature. After removal of the blocking reagent, the wells were washed with Tris-buffered saline (TBS) containing 0.1% Tween 20 (T-TBS). Then purified recombinant human FGF-23 protein or samples were added to the wells and incubated for 1 hour. After washing with T-PBS four times, 10 μg/ml of biotinylated FC1 antibody in 10% blocking reagent was added and incubated for 1 hour. The wells were then washed with T-TBS four times, streptavidin-horse radish peroxidase (HRP) in 10% blocking reagent was added and incubated for 30 min at room temperature. The wells were washed four times and incubated with 50 μl of substrate solution (Tetramethylbenzidine; Dako, Glostrup, Denmark) for 30 min. The reaction was stopped by adding 50 μl of sulfonic acid. The absorbance at 450 nm of each well was measured with microplate reader (MTP-300; Corona, Hitachinaka, Japan).

Subject

After informed consent was obtained, we measured FGF-23 levels in 104 healthy controls, one patient with TIO and 6 patients with XLH. Healthy controls included 30 males (age 24 to 59) and 74 females (age 21 to 63) and sera were used for FGF-23 measurement. A thirty-five-year old female patient was admitted to our hospital complaining bone pain and muscle weakness. She showed typical features of hypophosphatemic osteomalacia (Table 1). After surgical removal of the mixed-type mesenchymal tumor in the lower extremity, her symptom completely disappeared. Serum and plasma samples were obtained before and after the surgery of this patient. We also measured serum FGF-23 levels in 6 patients with XLH. Nucleotide sequences of PHEX gene of these patients were determined as described (8) and summarized in Table 2. Cases 1 and 2, and 4 and 5 are familial cases.

Results

Construction of Sandwich ELISA for FGF-23

Since previous studies indicated that the cleavage between Arg^{179} and Ser^{180} of FGF-23 abolished its effect to cause hypophosphatemia (5), we selected two monoclonal antibodies so that specifically detect uncleaved FGF-23 in the presence of inactive fragments. Out of 13 monoclonal antibodies obtained, two antibodies (FN1 and FC1) were suitable for the construction of sandwich ELISA. FN1 and FC1 antibodies recognized N-terminal and C-terminal fragments, respectively. A typical standard curve using purified recombinant FGF-23 is shown in Figure 1A. FGF-23 concentrations of 104 healthy controls ranged from 8.2 to 54.3 ng/L (mean +/- SE; 28.9 +/- 1.1). Age or gender-dependent difference in serum FGF-23 level was not observed among the subjects analyzed (Fig. 1B).

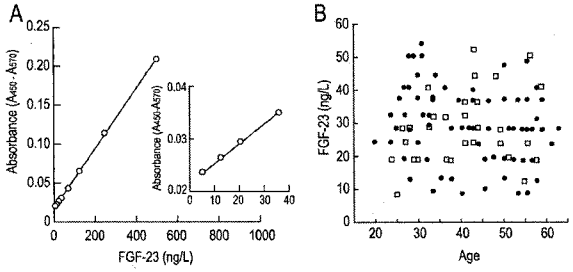

Figure 1. (A) Dose-response curve of recombinant human FGF-23 in human serum. The results are expressed as the means of duplicate measurements. (B) Relationship between serum FGF-23 levels and ages in healthy subjects (74 females; closed circle, 30 males; open square).

Changes of serum FGF-23 level in a patient with TIO

To investigate the involvement of FGF-23 in TIO, we applied the sandwich ELISA to sera from a patient with TIO. Clinical findings of the patient were summarized in Table 1. The patient was under treatment with neutral phosphate and 1α-hydroxyvitamin D_3 before surgery. After the removal of a mixed-type mesenchymal tumor in the right popliteal fossa, serum phosphate and $1,25(OH)_2D$ levels rapidly increased without the administration of phosphate and 1α-hydroxyvitamin D_3.

Table 1. Clinical findings of a patient with TIO.

	Before operation	After operation	Reference range
Serum values			
Calcium (mmol/L)	2.30	2.25	2.13 - 2.55
Phosphorus (mmol/L)	0.45	1.39	0.81 - 1.45
ALP (U/L)	960	1387	75 - 234
intact PTH (ng/L)	34	36	10 - 65
$1,25(OH)_2D$ (pmol/L)	22.9	172	39 - 156
TmP/GFR (mmol/L)	0.16	1.45	0.74 - 1.39

Serum values and TmP/GFR at the time of admission and on day 14 after the surgery are listed.

The changes of serum FGF-23 level in this patient were determined by this ELISA. As expected, serum concentrations of FGF-23 were obviously increased before surgery. The FGF-23 in serum drastically decreased after the resection of the tumor. Time course of the changes of FGF-23 and other biochemical parameters after removal of the tumor was shown in Figure 2. The serum concentration of FGF-23 had already decreased even at 30 min after the surgery and returned to the normal range at 60 min (Fig 2C). The increment of serum phosphate appeared at 6 h. In the vitamin D metabolites, the

concentration of serum $1,25(OH)_2D$ first increased after 3 h (Fig. 2D). At this time, serum 24,25-dihydroxyvitamin D [$24,25(OH)_2D$] did not change. Serum $24,25(OH)_2D$ decreased by 51% at 9 hours after the surgery.

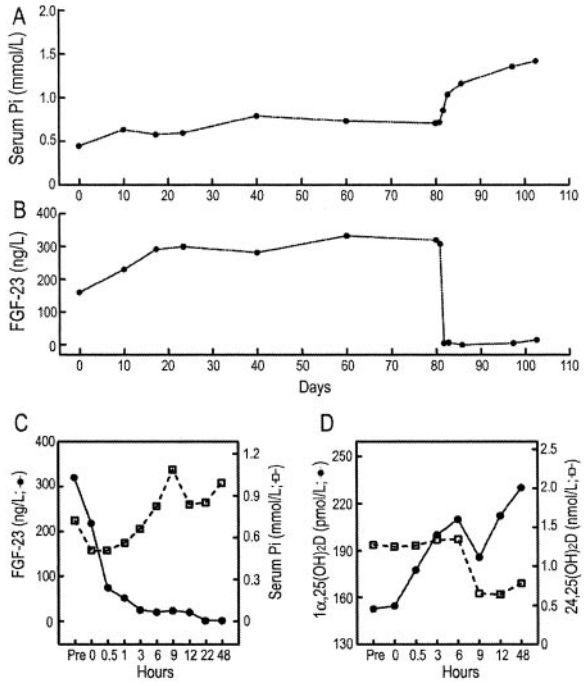

Figure 2. Time courses of changes in serum levels of phosphate (A) and FGF-23 (B) in the patient with TIO during the hospitalization. Operation was performed on day 81. Changes in serum levels of FGF-23, phosphate and vitamin D metabolites just after the surgery was shown in C and D (C: phosphate; open square, FGF-23; closed circle, D: $1,25(OH)_2D$; closed circle, $24,25(OH)_2D$; open square). $1,25(OH)_2D$ and $24,25(OH)_2D$ were isolated from sera by HPLC and quantified by RIA and competitive protein-binding assay, respectively.

Detection of circulatory FGF-23 protein by Western blotting

To confirm the elevated level of uncleaved FGF-23 in the circulation of the patient with TIO, we employed immunoprecipitation and Western blotting for FGF-23 in plasma. When plasma from a healthy control and the patient with TIO before the surgery was immunoprecipitated using FN1 or FC1 antibody and blotted using FC1 antibody, a protein with molecular weight of about 32.5 kDa commonly appeared (Fig. 3). The mobility of this protein corresponded to that of recombinant full-length FGF-23 (4). In addition, Western blotting confirmed that plasma of the patient with TIO contained much more amount of uncleaved FGF-23 than that of the healthy control. Furthermore, it was shown that these plasma contained C-terminal fragments of FGF-23 just as recombinant FGF-23 was expressed in mammalian cells in vitro and that the combination of FN1 and FC1 antibodies detected only uncleaved FGF-23 in samples. These results confirm that this ELISA actually detects full-length FGF-23 and that the cleavage of FGF-23 occurs in vivo as well as in vitro.

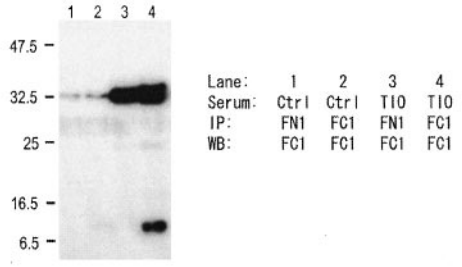

Figure 3. Detection of circulatory FGF-23. FGF-23 in plasma from a healthy control (lane 1 and lane 2) or the patient with TIO (lane 3 and lane 4) (0.4 ml each) was immunoprecipitated with FN1 or FC1 antibody, and were analyzed by Western blotting with FC1 antibody.

Involvement of FGF-23 in X-linked hypophosphatemic rickets/osteomalacia

The similarities of clinical features of TIO and XLH suggest that there is a common pathogenetic mechanism for these diseases. Although the responsible gene for XLH was identified as PHEX, it has been unclear how insufficient PHEX gene product causes hypophosphatemia and rickets/osteomalacia. To investigate whether FGF-23 is also involved in the pathogenesis of XLH, serum concentrations of FGF-23 in XLH patients were determined. Blood samples from 6 patients with XLH, whose mutations in PHEX gene were confirmed by DNA sequencing, were subjected to the ELISA. As shown in Table 2, most patients exhibit elevated FGF-23 levels compared to those of healthy adult subjects and average level of FGF-23 in patients with XLH was significantly higher than that of healthy subjects ($p < 0.0001$ by Student's t-test).

Table 2. FGF-23 in patients with XLH.

	Age	Sex	FGF-23 (ng/L)	Mutation in PHEX gene
Case 1	3 months 1 year	M	111.3 380.3	2071-1g→a (intron 20)
Case 2	38 years	F	39.0	2071-1g→a (intron 20)
Case 3	5 years 13 years	F	55.7 68.6	849+1g→a (intron 7)
Case 4	15 years	F	115.0	2071-2a→g (intron 20)
Case 5	18 years	F	107.6	2071-2a→g (intron 20)
Case 6	67 years	F	96.5	Q189X (exon 5)

Case 1 is a son of Case 2. Case 4 and Case 5 are sisters.

Discussion

In the present study, we developed a sandwich ELISA for FGF-23 using a pair of monoclonal antibodies with high

affinity to FGF-23. Since major processing site that abolish the biological activity of FGF-23 has been identified, we produced ELISA to measure biologically active uncleaved FGF-23. The sensitivity of this assay was sufficient to detect serum levels of FGF-23 in healthy adults. Western blotting indicated that biologically active uncleaved FGF-23 exists in normal plasma as well as cleaved fragments suggesting that FGF-23 is a physiological humoral factor. Age- or gender-dependent differences in the FGF-23 level were not observed in our subjects. However, it is possible that children with higher circulatory phosphate level may have different value of FGF-23. In a patient with TIO, serum FGF-23 level was clearly elevated as expected. Normalization of serum FGF-23 level after the surgery indicated that the tumor secreted FGF-23 into circulation. Reduction of FGF-23 levels after the surgery preceded the changes of serum phosphate and vitamin D metabolites. These changes is probably induced by the removal of FGF-23, because decreases of serum phosphate and $1,25(OH)_2D$ were induced by the administration of recombinant FGF-23 (4). These findings in addition to the previous studies clearly demonstrated that tumor-derived uncleaved FGF-23 is a causative factor of TIO.

Hypophosphatemic rickets/osteomalacia with inappropriately low $1,25(OH)_2D$ is commonly observed in TIO, ADHR, and XLH. Recent studies indicate that ADHR is associated with the misssense mutations in FGF23 gene that result in the destruction of protease recognition motif, RXXR. Since the cleavage at this site inactivate FGF-23 and the mutant FGF-23 proteins with the reported mutations are resistant to the cleavage, it is implied that the missense mutations result in the increased serum level of biologically active uncleaved proteins if expression level of FGF-23 does not change by the mutations. Although we do not have patients with ADHR, this issue will be clarified using this ELISA. On the other hand, the responsible gene for XLH is PHEX. Although there are several reports that showed proteolytic activity of PEHX, the physiological substrate for PHEX has still been unknown. It has been suggested that an unidentified phosphaturic factor is present in the circulation of patients with XLH (9). Because of similarities in clinical features of XLH and TIO, 'phosphatonin' has been used to describe this unidentified circulating factor in TIO and XLH (10). However, it has been unclear whether humoral phosphaturic factors in TIO and XLH are identical or not. Our results clearly indicate that circulatory level of biologically active FGF-23 is increased in most patients with XLH. These results strongly suggest that excess action of full-length FGF-23 is a common fundamental mechanism for development of hypophosphatemic rickets/osteomalacia in XLH, TIO and ADHR. However, there was a relatively wide variation of FGF-23 levels in patients with XLH. Further study is necessary to clarify the mechanism of high circulatory level of FGF-23 in patients with XLH.

In conclusion, we have established sandwich ELISA that specifically measure biologically active uncleaved FGF-23. Uncleaved FGF-23 is present in normal circulation and elevated level of uncleaved FGF-23 is observed in patients with TIO and XLH. Excess action of FGF-23 may be a common mechanism for the development of hypophosphatemic rickets/osteomalacia in TIO, XLH and ADHR.

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