Mutant FGF23 Prevents the Progression of Chronic Kidney Disease but Aggravates Renal Osteodystrophy in Uremic Rats

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(Received July 10, 2008)

Summary Phosphorus is one of the important factors that accelerate the progression of chronic kidney disease. Phosphorus restriction or phosphate binders have been reported to have the ability to prevent the progression of chronic kidney disease. FGF23 is a circulating factor that regulates renal phosphorus reabsorption and 1α-hydroxylase activity. We focused on the phosphaturic activity of FGF23 and investigated whether a pharmacological dose of FGF23 is beneficial to the progression of renal insufficiency in uremic rats. To this end, we administered one of the mutant FGF23 expression plasmids into irreversible Thy1 rats. Chronic renal failure rats were established by intravenous injection of anti-rat CD90 (Thy1.1) monoclonal antibody to unilaterally nephrectomized Wistar rats. The rats were then intravenously injected every 2 wk with a naked DNA solution containing 10 μg of MOCK vector or a mutant FGF23 expression plasmid for 13 wk. Renal function was assessed biochemically and histopathologically. Mutant FGF23 significantly decreased serum creatinine and serum urea nitrogen. The marked glomerular sclerosis observed in uremic rats receiving the MOCK vector was ameliorated in rats treated with mutant FGF23. However, mutant FGF23 not only significantly decreased serum 1,25(OH)2D and calcium but also aggravated high-turnover renal osteodystrophy from extremely high levels of PTH. These results might be a result of the mechanisms of FGF23 such as phosphaturic activity and lowering the level of 1,25(OH)2D. In conclusion, mutant FGF23 prevented the progression of chronic renal failure by regulating serum phosphorus but aggravated renal osteodystrophy from the lowered levels of 1,25(OH)2D.

Key Words fibroblast growth factor 23, 1,25(OH)2D, phosphorus, renal osteodystrophy

Disorders of bone and mineral metabolism are found in patients with chronic kidney disease (CKD) and are associated with pathogenesis of secondary hyperparathyroidism. Recent investigations have demonstrated that these abnormalities are also associated with higher mortality due to cardiovascular disease in patients with end stage renal disease (1, 2). In patients with CKD who are not yet on dialysis, phosphorus restriction may help to protect the residual renal function (3). Previous studies using animal models showed the importance of phosphorus regulation. In the chronic renal failure models, a low phosphate diet led to the prevention of the progression of renal failure independent of protein and calorie intake (4). Furthermore, phosphate binders are reported to prevent the progression of renal insufficiency (5–7).

Fibroblast growth factor 23 (FGF23) is a novel, secreted protein that consists of 251 amino acids and shares the sequence homology of the fibroblast growth factor family (8). FGF23 is predominantly expressed in osteocytes but is also expressed in thymus, lymph nodes, and brain (9, 10). Transgenic mice that overexpress FGF23 exhibit decreased urinary phosphate reabsorption, hypophosphatemia, low serum 1,25(OH)2D levels, and rickets (11, 12), whereas FGF23 null mice exhibit hyperphosphatemia and elevated serum 1,25(OH)2D levels, and abnormal skeletogenesis (13, 14). Thus FGF23 is a hormone-like factor that regulates phosphorus homeostasis and vitamin D metabolism.

We focused on the phosphaturic activity of FGF23 and investigated whether pharmacological doses of FGF23 have beneficial effect against the progression of renal insufficiency in uremic rats. To clarify the effects of FGF23, we administered a mutant FGF23 expression plasmid into irreversible Thy1 rats (15), which are characterized by mesangial cell proliferation and matrix expansion accompanied by persistent proteinuria, hypertension, and a moderate decline in renal function (16). Our results indicate that mutant FGF23 has a bidirectional effect in uremic rats: prevention of the progression of renal insufficiency from phosphaturic activity and, from the lowered levels of 1,25(OH)2D, aggravation of renal osteodystrophy (ROD) including ostitis fibrosa and the activation of osteoblasts and...
osteoclasts. The results suggest that phosphaturic factors or agents might be novel therapeutic approaches to CKD.

**METHODS**

*Chronic renal failure model.* All experiments were performed using male Wistar rats. 7 wk old, purchased from Japan SLC, Inc. (Shizuoka, Japan). They were housed in cages and allowed unlimited access to a normal rodent diet (CE-2, Ca 1.2%, P 1.0%, CLEA Japan, Inc., Shizuoka, Japan) and tap water. Irreversible Thy1 nephrectomy and sham operation were performed under ether anesthesia. Uninephrectomized rats were injected intravenously with 1 mg/mL/kg anti-rat CD90 (Thy1.1) monoclonal antibody, clone MRC OX-7 (Cedarlane Laboratories Ltd., Ontario, Canada), and sham-operated rats were injected with 1 mL/kg of vehicle (phosphate buffered saline). The care of the animals and the present protocols complied with the “General Consideration for Animal Experiments” and were approved by Chugai Pharmaceutical’s Ethics Committee for Treatment of Laboratory Animals.

**Plasmid vectors and naked DNA injection method.** Construction of plasmid vectors expressing mutant FGF23 was described previously (17). Briefly, the plasmids were digested with EcoRI and the fragments then inserted into a unique EcoRI site between the CAG promoter and a 3′-flanking sequence of a rabbit β-globin gene in the pCAGGS3 expression plasmid, which was kindly provided by Dr. Miyazaki (18). A naked DNA injection solution containing plasmid vector was prepared using the TransIT® In Vivo Gene Delivery system (Mirus, Madison, WI, USA) immediately before injection. Rats were intravenously injected every 2 wk with 12 mL of naked DNA injection solution containing 10 μg of expression plasmid, pCGFM2 (mutant FGF23) or empty pCAGGS3 plasmid (MOCK) (17).

**Experimental design.** We examined the efficacy of mutant FGF23 to prevent the progression of renal failure by using the naked DNA injection method described above. To measure biochemical parameters, serum was collected from the jugular vein under ether anesthesia 2 wk after the operations were performed. The uremic rats were divided into four groups, allocated with respect to serum creatinine concentration, and then intravenously injected with an expression plasmid every 2 wk. Serum was collected from the jugular vein under ether anesthesia at 2, 6, 10, and 13 wk after the first DNA injection.

**Biochemical parameters.** Serum and urine samples were analyzed in a Hitachi 7170E automatic analyzer (Hitachi Co. Ltd., Tokyo, Japan) for phosphorus, calcium, creatinine, and urea nitrogen. Serum PTH levels were measured using Rat Intact PTH ELISA Kit (Immutopics, San Clemente, CA, USA). Serum 1,25(OH)2D was determined by 1,25(OH)2D RIA kit TFB (TFB, Tokyo, Japan).

**Kidney and femur histopathology.** Kidney and femur were fixed with 20% neutral buffered formalin, embedded in paraffin, cut into 4-μm sections, and stained with periodic acid-Schiff (PAS) and hematoxylin-eosin stains. Kossa staining was also performed to examine ectopic calcification of the kidney. The samples were examined histopathologically. Kidney lesion including glomerulosclerosis, degeneration of tubules, interstitial fibrosis and inflammatory cell infiltration was graded into five stages of severity: not remarkable (−), very slight (±), slight (+), moderate (++), severe (+++) (16). Femur lesion including osteitis, osteoblast activation and osteoclast activation was also graded into the same five stages. Blind analysis was done on all sections by the same observer.

**Statistics.** All values are expressed as the mean±SE. Statistical analysis was performed using an unpaired t-test and Dunnett-Hsu multiple comparison test. p<0.05 was considered statistically significant.

**RESULTS**

**Effect of mutant FGF23 on the levels of serum parameters in uremic rats**

Changes in serum phosphorus are shown in Fig. 1a. The level of serum phosphorus increased progressively

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**Fig. 1.** Mutant FGF23 significantly decreased serum phosphorus (a) and calcium (b). Values are expressed as means±SE. Open circle (○), sham rats injected with MOCK vector (n=7); closed circle (●), uremic rats injected with MOCK vector (n=9); closed square (■), uremic rats injected with mutant FGF23 vector (n=7). #p<0.05, *p<0.05 vs MOCK-treated group.
for 13 wk in uremic rats treated with the MOCK vector. Mutant FGF23 significantly decreased serum phosphorus at 2, 6, and 10 wk. Mutant FGF23 also significantly decreased serum calcium from the 2nd to 13th week (Fig. 1b).

As shown in Fig. 2a, the level of serum PTH was elevated in the uremic rats treated with the MOCK vector compared with the normal control and considered to be caused by progression of chronic renal failure. Interestingly, the level of serum PTH in the mutant FGF23-treated group was markedly higher than that of the MOCK vector-treated group at 2 and 13 wk. On the other hand, the level of 1.25(OH)\(_2\)D in the mutant FGF23-treated group significantly decreased compared to the MOCK vector-treated group at 2 wk (Fig. 2b).

**Effect of mutant FGF23 on the renal function**

The levels of serum creatinine and urea nitrogen were measured to assess the renal function of uremic rats (Fig. 3). Serum creatinine and urea nitrogen levels in the uremic rats treated with MOCK vector significantly increased throughout the experimental period compared to the normal control. Mutant FGF23 signifi-
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Rapid intravenous bolus injection (12 mL/head in the present study) is required to express the mutant FGF23 gene, and so we examined the effect of bolus injection on the progression of renal failure. There were no differences between the uremic rats treated with MOCK vector and the disease control group (no injection) in the levels of either serum creatinine or urea nitrogen. Other biochemical parameters and pathological examinations also showed no differences between these two groups (data not shown).

Kidneys were examined histopathologically at 13 wk (Table 1). Marked glomerulosclerosis was observed in uremic rats treated with MOCK vector (Fig. 4b). These changes were ameliorated in rats treated with mutant FGF23 (Fig. 4c). Furthermore, tubulointerstitial damage, including tubular degeneration, interstitial inflammatory cell infiltration, and interstitial fibrosis, was observed in uremic rats treated with MOCK vector, and this damage was also ameliorated in the mutant FGF23-treated rats.

**Effect of mutant FGF23 on renal osteodystrophy in femur**

We also performed histopathological analysis of the femur (Table 2). Moderate levels of high-turnover renal osteodystrophy (ROD), including ostitis fibrosa and activation of osteoblasts and osteoclasts, were observed in uremic rats treated with MOCK vector (Fig. 5b). Surprisingly, this high-turnover ROD was accelerated in the mutant FGF23-treated group (Fig. 5c).

Table 2. Incidence of histopathological change in femur in irreversible Thy1 rat.

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* p<0.05 vs MOCK control.

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Fig. 4. Histopathology of kidney at 13 wk (periodic acid-Schiff stain). (a) Sham-operated rats treated with MOCK vector, (b) uremic rats treated with MOCK vector, and (c) uremic rats treated with mutant FGF23 vector. Note that mutant FGF23 ameliorated glomerular and tubular damage. Objective lens ×20.
DISCUSSION

In patients with CKD, nutritional therapy, especially a low protein diet, may help to protect residual renal function. Recently, we reported that the beneficial effect of a low protein diet is associated with phosphorus content from a study using irreversible Thy1 rats fed six different isocaloric diets, including three levels of protein and two levels of phosphorus (19). The results indicated that the molecules with phosphaturic activity are potential novel therapeutic agents.

FGF23 possesses bidirectional potency from phosphaturic activity and from lowering the level of 1,25(OH)2D. In the present study, mutant FGF23 was found to prevent the progression of renal insufficiency but to aggravate ROD in uremic rats, biological effects considered to be the result of the bidirectional potency of FGF23. In FGF23-treated uremic rats, the serum parameters of renal function such as creatinine and urea nitrogen and pathological changes in the kidney such as glomerulosclerosis and tubular degeneration were significantly improved (Fig. 3, Table 1). These data indicate that mutant FGF23 prevented the progression of renal failure biochemically and histopathologically. On the other hand, in mutant FGF23-injected rats, severe typical high-turnover ROD, characterized by osteitis fibrosa and the activation of osteoblasts and osteoclasts with elevated rates of bone formation, was observed.

We previously showed that mutant FGF23 inhibited Na/Pi cotransport activity, the expression of NaPi2a and NaPi2c mRNA and protein in renal brush border membrane vesicles (17, 20). In the present study, the hypophosphatemia observed in the mutant FGF23-treated uremic rats might be mainly due to the inhibition of the renal Na/Pi cotransporter.

Previous reports show that FGF23 binds to some of the FGF receptors, such as FGFR1c, -3c and -4c in vitro, but the physiological role of these receptors is still controversial. Furthermore the signaling pathways of FGF23 remain unclear (21). If the FGF23 signaling pathway could be separated into a phosphaturic pathway and a 1α-hydroxylase pathway, the former would be the ideal target for therapeutic agents.

The mechanism by which hypophosphatemia may prevent the progression of CKD remains unknown. Previous experimental data suggested that nephrocalcinosis, calcification of the kidney, is involved in the mechanism of phosphorus toxicity (3, 22). The phosphorus toxicity appears to be related to the induction of calcium phosphorus precipitation, resulting in tubulo-interstitial disease. Further study is necessary to clarify the mechanism of the beneficial effects of hypophosphatemia.
The extremely elevated level of serum PTH found in the early stage was thought to be the cause of severe ROD (Fig. 5c, Table 2). Recently, Ben-Dov et al. (23) reported that FGF23 suppress both PTH secretion and PTH gene expression through the MAPK pathway in normal rats. In the present study, the reason the level of serum PTH increased in spite of the administration of mutant FGF23 is that mutant FGF23 suppressed 1,25(OH)2D and serum calcium by lowering the level of 1α-hydroxylase. As for the suppression of 1α-hydroxylase, it was reported that mice bearing FGF23-expressing CHO cells showed suppressed expression of 25-hydroxyvitamin D 1α-hydroxylase mRNA in the kidney and a low concentration of 1,25-dihydroxyvitamin D (24). Furthermore, because 1,25(OH)2D is activated by 1α-hydroxylase in renal proximal tubular cells, patients with renal insufficiency show low levels of 1,25(OH)2D. Therefore we speculated that mutant FGF23 decreases serum calcium more severely in uremic rats with renal tubular damage by suppressing 1α-hydroxylase. Our results suggest that the hypocalcemia following a decrease in 1,25(OH)2D levels in the early stage might induce severe ROD in mutant FGF23-treated uremic rats.

In the present study, we used the naked DNA injection method. This technique avoids the risk of phenotypic abnormalities during development that frequently occurs in transgenic animals. Furthermore, this method is useful to examine the biological roles of long-term delivery secretory proteins in rats (25). Because the half-life of FGF23 in the circulation has been estimated to be approximately 21.5 min (26), it might be difficult to assess the efficacy of FGF23 using recombinant protein over a longer period. We used mutant FGF23 and not wild-type FGF23 because FGF23 mutants such as R176Q, R179Q and R179W, but not wild-type FGF23, induced hypophosphatemia in intact mice using the naked DNA injection method in our previous study (17). Proteolytic processing of FGF23 may also play an important role in regulating its biologic activity. Because FGF23 is cleaved between Arg179 and Ser180, a process that abolishes biologic activity, mutant FGF23s become resistant to the intracellular proteolytic cleavage from the proteolytic processing. The point mutations at this site result in autosomal dominant hypophosphatemic rickets, showing the importance of the site. The mutant FGF23 we use in this experiment has been found in patients with ADHR.

In conclusion, the present study demonstrated that mutant FGF23 has bidirectional effects in renal failure rats: prevention of the progression of renal insufficiency from phosphaturic activity and aggravating ROD by lowering the level of 1,25(OH)2D.

Acknowledgments

We are very grateful to Dr. Jun-ichi Miyazaki (Osaka University) for providing us the pCAGGS3 expression vector. We also thank Dr. Naoshi Horiba (Chugai Pharmaceutical Co., Ltd.) for providing technical advice during the course of these studies. We also thank Dr. Toshio Mori (Chugai Research Institute for Medical Science, Inc.) for technical assistance with the histopathology.

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Mutant FGF23 prevents renal failure but not ROD.


