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REVIEW ARTICLE

The FGF23 and Klotho system beyond mineral metabolism

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Abstract FGF23 is a bone-derived hormone that acts primarily on the kidney to induce phosphaturia and suppress synthesis of 1,25-dihydroxyvitamin D_3. The unique feature of FGF23 is that it requires Klotho as an obligate co-receptor. The FGF23–Klotho system has emerged as an endocrine axis indispensable for maintaining phosphate homeostasis. Mineral and bone disorders associated with chronic kidney disease (CKD-MBD) can be viewed as a series of events triggered by a compensatory response of the FGF23–Klotho system to excess phosphate intake relative to the residual nephron number. Furthermore, the fact that disruption of the FGF23–Klotho system causes phosphate retention and a syndrome resembling aging in mammals has led to the notion that phosphate accelerates aging. The aging-like pathology caused by phosphate, or phosphatopathy, may be unique to the higher organisms having the *Klotho* gene and provides new insights into the molecular mechanism of aging in humans.

Keywords FGF23 · αKlotho · Phosphatopathy

Discovery of Klotho

The *Klotho* gene was discovered in 1997 as a gene whose expression was defective in a mouse strain exhibiting complex phenotypes resembling human aging [1]. The founder of this strain was a transgenic mouse carrying a transgene designed to express rabbit sodium-proton exchanger-1 under the control of human elongation factor-1α promoter [2]. The transgene was transmitted to the offspring with Mendelian inheritance; however, its expression was undetectable. Thus, this strain was of no use for the initial purpose of the experiment but, intriguingly, developed multiple aging-like symptoms when homozygous for the transgene.

The reason for silencing the transgene turned out to be extensive methylation [3]. Methylation of the transgene is often observed in transgenic mice and regarded as a universal defense mechanism inhibiting exogenous gene expression. In this strain, the methylation extended from the transgene to an adjacent unknown gene, which was later named *klotho* after a Greek goddess who spins the thread of life. The methylation reached the CpG island in the promoter region and the 1st exon of the *klotho* gene, resulting in a severe hypomorphic allele. Consequently, mice homozygous for the transgene, or *kl/kl* mice, were defective in *klotho* gene expression.

The phenotypes observed in *kl/kl* mice included a shortened life span, growth retardation, atrophy of multiple organs (gonads, thymus, skin, and fat), vascular calcification, cardiac hypertrophy, emphysematous lung, osteopenia, sarcopenia, hearing disturbance, and cognition impairment. These phenotypes appeared shortly after weaning and progressed rapidly as if aging were accelerated. The *klotho* gene encoded a single-pass transmembrane protein and was expressed predominantly in the kidney. Despite its tissue-specific expression, Klotho deficiency affects various tissues that did not express Klotho, suggesting involvement of a humoral factor(s) in Klotho protein function.

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Identification of Klotho as FGF23 receptor

In 2000, fibroblast growth factor-23 (FGF23) was identified as a bone-derived hormone responsible for autosomal dominant hypophosphatemic rickets (ADHR) [4]. ADHR is characterized by disturbed bone mineralization associated with low serum levels of phosphate and active vitamin D (1,25-dihydroxyvitamin D_3). Patients with ADHR carry missense mutations in the *FGF23* gene (R176Q, R179Q, R179W), which confer resistance to inactivation by proteolytic cleavage on FGF23 protein [5]. As a result, ADHR patients have elevated serum FGF23 levels. FGF23 acts on the kidney to function as a phosphaturic hormone and as a counter-regulatory hormone for 1,25-dihydroxyvitamin D_3. Thus, pathophysiology of ADHR can be explained by hyper-FGF23-emia.

In 2006, the physiological receptor for FGF23 was identified as FGFR–Klotho complexes [6]. This discovery was prompted by the fact that FGF23 knockout mice and *kl/kl* mice exhibited identical phenotypes. FGF23 knockout mice showed high serum levels of phosphate and 1,25-dihydroxyvitamin D_3 as expected [7]. An unexpected finding was that they developed aging-like phenotypes including growth retardation, organ atrophy (thymus and gonads), and a shortened life span. On the other hand, *kl/kl* mice were known to show hyperphosphatemia and hypervitaminosis D besides the aging-like phenotypes [8]. These observations led to the hypothesis that Klotho might function as a receptor for FGF23. In fact, Klotho forms constitutive complexes with particular FGFR isoforms (FGFR1c, FGFR3c, and FGFR4), to which FGF23 can bind with high affinity [6]. Namely, Klotho functions as an obligate co-receptor for FGF23.

Phosphatopathy

The phosphaturic activity of FGF23 is based on its ability to suppress phosphate reabsorption by down-regulating Na-dependent phosphate co-transporter type IIa/c (Npt2a and Npt2c) expressed on the apical membrane at proximal tubules [9]. The counter-regulatory activity of FGF23 against 1,25-dihydroxyvitamin D_3 is based on its ability to suppress expression of 1α-hydroxylase encoded by the *Cyp27b1* gene and to induce expression of 24-hydroxylase encoded by the *Cyp24* gene [9], which are essential for synthesis and degradation of 1,25-dihydroxyvitamin D_3, respectively. Therefore, the pathophysiology of mice lacking FGF23 or Klotho is (1) phosphate retention due to impaired urinary phosphate excretion and (2) vitamin D intoxication due to increased synthesis and decreased degradation of 1,25-dihydroxyvitamin D_3.

Which is responsible for the accelerated aging, phosphate retention or vitamin D intoxication? Some laboratories rescued the aging-like phenotypes in mice lacking FGF23 or Klotho by placing them on a vitamin D-deficient diet [8] or by ablating the vitamin D receptor gene [10] or the *Cyp27b1* gene [11]. These observations were thought to support the hypothesis that the aging-like phenotypes might be attributed to vitamin D intoxication. However, these interventions lowered serum phosphate levels and failed to exclude the possibility that phosphate retention was actually responsible for the rescue. Other laboratories succeeded in slowing down the accelerated aging in these mutant mice by placing them on a low phosphate diet [12] or by ablating the *Npt2a* gene [13]. These interventions solved phosphate retention, but further increased the serum 1,25-dihydroxyvitamin D_3 levels in response to the danger of phosphate deficiency. Nonetheless, the aging-like phenotypes were rescued, indicating that phosphate retention, but not vitamin D intoxication, was primarily responsible for the accelerated aging.

These observations have established the notion that phosphate accelerates aging. However, it may evoke semantic discussion to designate mice lacking FGF23 or Klotho as a state of accelerated aging, because their phenotypes are not always observed in normal mice during a natural course of aging. Thus, a new term "phosphatopathy" has been proposed and defined as the aging-like pathology that can be ameliorated by restriction of phosphate intake [14].

Phosphatopathy is universally observed in patients with chronic kidney disease (CKD). CKD patients suffer from increased mortality, vascular calcification, cardiac hypertrophy, osteopenia, and sarcopenia associated with decreased Klotho expression [15]. When CKD progresses to end-stage renal disease (ESRD), hyperphosphatemia ensues [16]. Importantly, phosphate-lowering therapy improves the clinical outcomes of ESRD patients [17]. The fact that CKD patients and Klotho-deficient mice share many common features has led to the notion that CKD can be viewed as a clinical model of "accelerated aging" [18].

Mechanism of phosphatopathy

High extracellular phosphate is toxic to the cell. Apoptosis is induced when vascular endothelial cells are exposed to high-phosphate medium [19]. Vascular smooth muscle cells cultured in high-phosphate medium undergo osteoblastic transformation characterized by down-regulation of smooth muscle markers such as α-smooth muscle actin and SM22α, and reciprocal up-regulation of osteoblast markers such as RUNX2 and osteopontin, culminat-

ing in matrix mineralization or calcification [20]. It has been believed that these deleterious effects are triggered by excess phosphate entry into the cell through an Na-dependent phosphate co-transporter type III (a.k.a. PiT-1) because of the following observations: First, phosphonoformic acid (PFA), which was believed to inhibit PiT-1, suppressed smooth muscle cell calcification [21]. Second, knock-down of PiT-1 expression by siRNA in smooth muscle cells suppressed calcification [22]. However, Villa-Bellosta provided unequivocal evidence showing that PFA inhibited Npt2a/c but not PiT-1 [23]. In addition, using a mutated PiT-1 that lacks phosphate transport activity, Giachelli and her colleagues showed that forced-expression of the mutated PiT-1 in PiT-1-deficient smooth muscle cells restored the ability of high-phosphate medium to induce calcification [24]. These observations argue against the notion that phosphate entry into the cell is required for calcification.

Regular tissue culture medium contains $\sim$1 mM phosphate and $\sim$2 mM calcium. These concentrations are similar to the serum levels in humans, and close to the solubility limit. Therefore, addition of phosphate to the medium can induce formation of calcium-phosphate (CaPi) precipitates. CaPi precipitates have the ability to induce cellular damage and innate immune responses [25–27]. In the serum-containing medium, CaPi precipitates are absorbed by serum protein fetuin-A and prevented from growing into large CaPi crystals. These CaPi-laden fetuin-A molecules aggregate spontaneously to form nanoparticles, which are dispersed in the medium as colloids. These colloidal particles are designated as calciprotein particles (CPPs) [28]. Although less active than inorganic CaPi precipitates, CPPs can also induce cellular damage and innate immune responses [27]. Then, which is responsible for the phosphate toxicity, soluble phosphate or insoluble CaPi?

Several lines of evidence show that insoluble CaPi is the true culprit for the deleterious effects of phosphate. First, the osteoblastic transformation of smooth muscle cells was induced by insoluble CaPi without increasing the phosphate concentration [25, 26]. Second, smooth muscle cell calcification induced by high-phosphate medium did not occur when insoluble CaPi was removed from the medium by centrifugation or when formation of insoluble CaPi was inhibited by PFA [23]. PFA does not inhibit phosphate transfer by PiT-1 but inhibits CaPi precipitation. Since PiT-1 was originally identified as a retrovirus receptor [29], it is intriguing to speculate that PiT-1 may function as a receptor mediating the effects of insoluble CaPi on smooth muscle cells. Because insoluble CaPi in the blood exists not as inorganic CaPi precipitates but as CPPs, this review uses CPPs to represent insoluble CaPi hereafter.

Recent clinical studies demonstrated that serum levels of CPPs were increased with decline of renal function and associated with clinical indices of arterial stiffness, vascular calcification, and chronic inflammation independently of renal function [30, 31]. Considering the ability of CPPs to induce smooth muscle cell calcification and innate immune responses in vitro, the association of serum CPP levels with vascular calcification and chronic inflammation may not merely be correlation but causation. Taken together, CPPs may be regarded as a "pathogen" and a novel therapeutic target of phosphatopathy.

FGF23-independent action of Klotho

Klotho protein is a substrate of α-, β-, and γ-secretases and subject to ectodomain shedding on the cell surface, resulting in secretion of the entire extracellular domain into the extracellular space [32, 33]. In fact, secreted Klotho is detected in the blood, urine, and cerebrospinal fluid [34]. Thus, Klotho protein exists in two forms: membrane Klotho and secreted Klotho. The membrane Klotho functions as an obligate co-receptor for FGF23, whereas the secreted Klotho functions as a humoral factor independently of FGF23.

The secreted Klotho protein regulates the activity of various ion channels and transporters. It activates a calcium channel TRPV5 (transient receptor potential cation channel subfamily V member 5) [35, 36] and a potassium channel ROMK1 (renal outer medullary potassium channel 1) [37], whereas it inactivates a calcium channel TRPC6 (transient receptor potential cation channel, subfamily C, member 6) [38] and Npt2a/c independently of FGF23 [39]. These promiscuous activities may be explained by the fact that the extracellular domain of Klotho has homology to family I glycosidases and potentially functions as an enzyme and/or a lectin that hydrolyzes and/or binds to a particular glycosidic linkage(s). Specifically, secreted Klotho has sialidase activity that hydrolyzes the α-glycosidic linkage between sialic acid and galactose (SAα2 → 6Gal), in the N-linked glycans of TRPV5 [36]. TRPV5 is expressed on the apical membrane of distal tubules and functions as the major entry gate of transcellular calcium reabsorption. Removal of sialic acid residues by sialidase activity of secreted Klotho exposes galactose residues as reducing terminals, to which galectin-1 can bind. Galectin-1 is a galactose-binding lectin abundantly present in the extracellular matrix. As galectin-1 forms dimers, secreted Klotho triggers the formation of TRPV5–galectin-1 complexes on the cell surface, thereby preventing internalization and tethering TRPV5 on the cell surface. Because the activity of TRPV5 is primarily regulated by the number of TRPV5 inserted in the plasma membrane, secreted Klotho increases cellular calcium uptake through TRPV5. The same mechanism operates in ROMK1 activation by secreted Klotho [37].

In addition, secreted Klotho inhibits the activity of several growth factors including IGF1 (insulin-like growth factor-1) [40], Wnt [41], and TGFβ1 (transforming growth factor-β1) [42]. TGFβ1 is identified as the most potent inducer of epithelial-to-mesenchymal transition (EMT), the initial step towards fibrosis and cancer metastasis. Binding of TGFβ1 to TGFβ receptor type II (TGFβR2) recruits and phosphorylates TGFβ receptor type I (TGFβR1), which activates the SMAD signaling pathway to exert the TGFβ1 action. Secreted Klotho directly binds to TGFβR2 and inhibits the binding of TGFβ1 to TGFβR2, thereby attenuating the TGFβ1 activity [42]. The Wnt family of ligands is also known as potent inducers of EMT. Secreted Klotho also attenuates the Wnt activity by directly binding to Wnt3 and Wnt4 [41]. Thus, secreted Klotho functions as a humoral EMT inhibitor. In fact, when injected into mice, secreted Klotho protein ameliorated renal fibrosis induced by unilateral ureteral obstruction and cancer metastasis induced by transplantation of Lewis lung carcinoma cells [42]. It remains to be determined whether secreted Klotho functions as a lectin that binds to glycans of TGFβR2 and Wnt. It also remains to be determined whether secreted Klotho binds to IGF1 and/or IGF1 receptor. However, the ability of secreted Klotho to inhibit IGF1 activity contributes to the inhibition of TRPC6, because IGF1 is the major humoral regulator that promotes translocation of TRPC6 to the plasma membrane [38]. These FGF23-independent actions of secreted Klotho may contribute to extended life span [40] and improved cognitive function [43] in transgenic mice that overexpress Klotho.

CKD and the FGF–Klotho endocrine system

Regardless of the underlying disease, CKD can be viewed as the process of progressive reduction of the functional nephron number. Despite substantial loss of nephron, hyperphosphatemia is observed only in ESRD patients (CKD stage 4 or 5), indicating that phosphate homeostasis is maintained until stage 4–5 during CKD progression. This is because decrease in the nephron number is compensated by increase in the phosphate excretion per nephron, which is attained by increase in FGF23. In fact, FGF23 start increasing as early as stage 2 [44]. The increase in FGF23 leads to the decrease in 1,25-dihydroxyvitamin D_3, which then increases the parathyroid hormone. In fact, the changes in these hormones during CKD progression occur in this order [44]. Therefore, the trigger of CKD–mineral and bone disorder (CKD–MBD) can be regarded as the increase in FGF23 in an effort to compensate for the excess phosphate intake relative to the residual nephron number. This notion implies that phosphate restriction should be considered for normophosphatemic CKD patients with hyper-FGF23-emia to prevent CKD-MBD [28, 45]. This notion awaits justification in clinical trials.

Closing remarks

It has been widely known that the elements of the sea water and those of the human body are similar. This is consistent with the notion that the life is derived from the sea. In fact, 9 out of the 10 most abundant elements are identical between the sea water and the human body. The only difference is phosphorus. Phosphorus is rich in the human body, but rare in the sea water, suggesting that the organism starts accumulating phosphorus at some time point during evolution. It was the Devonian period (~400 million years ago) when the bony fish (Osteichthyes) was evolved [16]. The organisms before the bony fish had the cartilage or the shell made of calcium carbonate. However, these skeletons were not as hard as the bone made of calcium phosphate or hydroxyapatite. Acquisition of the hard bone made of hydroxyapatite may be a prerequisite for the evolution of the terrestrial organisms necessary to support their body without the help of buoyancy. Intriguingly, the ortholog of the Klotho is absent in the organisms without the bone made of CaPi, indicating that the *Klotho* gene has evolved for phosphorus. In this sense, the notion that phosphate accelerates aging may be applicable only for the organisms that have the *Klotho* gene. Accordingly, phosphatopathy may be regarded as a form of aging unique to vertebrates. Further research on phosphatopathy is expected to identify new therapeutic targets for human aging and age-related diseases.

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Compliance with ethical standards

Conflict of interest This supplement is supported by the grants from The Japanese Society for Kidney Bone Disease (JSKBD) and from the Research Meeting on Kidney and Metabolic Bone Disease.

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