FGF-23, vitamin D and calcification: the unholy triad

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Introduction

Recent studies have changed the commonly held view that phosphate is mostly needed for normal skeletal growth and development. Extensive research in the last decade has identified numerous other essential dynamic functions for phosphate, ranging from signaling to energy metabolism. Abnormal phosphate homeostasis potentially affects functional activities of almost any organ system. Despite its wide biological importance and significance, the regulation of phosphate homeostasis is not yet clearly understood. Recent studies have identified a number of molecules with phosphaturic activities, including fibroblast growth factor-23 (FGF-23) [1], frizzled-related protein 4 [2] and matrix extracellular phosphoglycoprotein [1,3]. Among these recently identified molecules, so far FGF-23 has been implicated in various human diseases, including autosomal dominant hypophosphatemic rickets (ADHR) [4], oncogenic osteomalacia (OOM) [5], X-linked hypophosphatemia (XLH) [6], chronic renal diseases [7] and familial tumoral calcinosis (FTC) [8].

Phosphate homeostasis

The maintenance of phosphate homeostasis is of crucial biological importance, as it is required for generation and transformation of cellular energy and eventual cell survival and proliferation. It is also essential for determining the activity of proteins, and mineralization of skeletal tissues. Calcium and phosphate levels are well controlled in the normal healthy state. Until recently, phosphate homeostasis had been thought to be regulated by molecules that are involved in maintaining calcium homeostasis, such as vitamin D and parathyroid hormone (PTH), by exerting either direct or indirect effects on serum phosphate. Clinical and experimental studies have shown that dietary restriction of phosphate reduced serum PTH concentration, and delayed or improved secondary hyperparathyroidism, independent of serum levels of calcium or calcitriol [9,10]. Similarly, an abnormally low level of serum phosphate, in spite of a normal serum level of calcium, in rare genetic disorders like ADHR, XLH or OOM is not always consistent with the commonly held notion of a passive inverse regulation of serum phosphate by serum calcium. Recently, FGF-23 has been identified as one of the key molecules involved in the regulation of phosphate homeostasis [11,12] without directly affecting calcium homeostasis.

FGF-23

FGF-23 is a recently identified member of the FGF family, and thought to be actively involved in phosphate homeostasis and skletogenesis [13]. Although sequence comparisons have found high homology of FGF-23 to FGF-19 and FGF-21 [13], FGF-23 is the only member of the FGF family that contains a proconvertase processing site. FGF-23 is a 30 kDa secreted protein that is processed by a pro-convertase type enzyme into two smaller fragments of ~18 kDa (amino fragment) and 12 kDa (carboxy fragment). The biological significance of these FGF-23 fragments and its specific receptors are not yet clearly defined, although several studies have shown that FGF-23 could exert its biological activities through binding with the known receptors for other members of the FGF family [14,15]. Activating mutations in the proprotein convertase cleavage site of the FGF-23 gene were identified as the molecular basis for ADHR [4]; these mutations have been shown to prevent proteolytic

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cleavage of the FGF-23 protein, the net effect being an enhanced biologic activity of FGF-23 [16], with resultant hypophosphataemia in affected patients. Conversely, inactivating mutations in the FGF-23 gene were shown to be associated with FTC [8], an autosomal recessive disorder characterized by hyperphosphatemia and ectopic calcifications due to reduced biological activity of FGF-23. Recent animal studies with genetic modifications of FGF-23 have shown similar phenotypes as those of human diseases; for instance transgenic mice overexpressing FGF-23 showed hypophosphataemia, reduced serum 1,25(OH)₂D₃ levels and rickets [17,18]. Opposite effects were documented in Fgf-23 null mice, with high serum phosphate levels, possibly due to increased renal reabsorption of phosphate, and increased vitamin D activity leading to soft tissue calcifications [19,20].

**Soft tissue calcification**

Abnormal tissue calcification is a major cause of mortality in a wide range of diseases. It may be associated with almost 50% of cardiovascular deaths in dialysis patients. Recent studies have emphasized the need for vigorous control of hyperphosphatemia to improve survival of patients with end stage renal diseases (ESRD), but long-term medication with unphysiologic doses of active vitamin D metabolites to treat patients with secondary hyperparathyroidism is not always effective, and may raise the risk of cardiovascular death due to abnormal calcification via an increase in the calcium–phosphate product. Although it is essential to effectively treat ESRD patients with secondary hyperparathyroidism, it is also crucial to minimize soft tissue calcification.

In addition to the indisputable pathologic role of hyperphosphatemia, recent studies have detected an important role of osteogenic molecules in the formation of abnormal vascular calcification [21]. For instance, upregulation of core binding factor 1 (osteoblast transcription factor), alkaline phosphatase, osteopontin and osteocalcin in vascular smooth muscle cells exposed to high phosphate led to the formation of abnormal hydroxyapatite, implicating osteogenic differentiation [22]. Similarly, bone morphogenic protein-2–homeobox protein MSX-2 signaling has also been shown to promote such osteogenic differentiation [23]. Furthermore, recently identified calcification inhibitors, including matrix gla protein (MGP), osteoprotegerin (OPG), fetuin-A and pyrophosphate are implicated in ectopic calcification as well. For example, MGP null mice were shown to develop severe calcification of aorta and cartilage [24]. Similarly, OPG null mice were found to have extensive calcification in aorta and renal arteries [25], while spontaneous soft tissue calcification was detected in fetuin-A deficient mice [26]. In vivo ablation of ank gene (a transmembrane protein that regulates tissue pyrophosphate level) resulted in periarticular calcification [27]. FGF-23, a mostly bone-derived circulating factor, was found to produce extensive soft tissue calcification when deleted in mice (Fgf-23⁻/⁻) [19,20]. It thus appears increasingly likely that some of the factors that are essential for osteogenesis are also actively involved in the formation of abnormal soft tissue calcification [28].

**FGF-23 and soft tissue calcification**

The interrelation between FGF-23, vitamin D and soft tissue calcification is not yet clear. Although the dramatic impact of abnormal tissue calcification on the prognosis of haemodialysis patients has become progressively clear, the potential contribution of vitamin D metabolites to the genesis and progression of this complication has not been studied extensively, maybe due to our inability to monitor coronary artery calcifications on a regular basis. However, the recent development of electron beam computed tomography has provided a means to quantify coronary artery calcifications in vivo, and this tool, among others, has helped us to recognize that an elevated calcium–phosphate product and the dietary calcium load thought to be of safe range are actually not as safe as anticipated, warranting further molecular studies to understand the complex process of cardiovascular calcification. The recently generated Fgf-23 null mouse has provided a unique tool to study the in vivo effects of vitamin D in development of abnormal soft tissue calcification in a hyperphosphatemic microenvironment, a situation close to the condition of dialysis patients receiving vitamin D treatment.

**Fgf-23 null animals**

We and others have recently generated Fgf-23 null mice [19,20]. Homozygous mutant mice were significantly smaller in size than wild-type littermates. A significant increase in serum phosphate was detected in Fgf-23 null mice, with no significant change in serum PTH. Fgf-23 null mice developed extensive vascular and visceral calcifications by 6 weeks of age, involving cardiac vessels, valves, kidney, lung and skeletal muscle (Figure 1). This was associated with increased renal expression of 1α-hydroxylase [1α(OH)ase] and high serum 1,25-dihydroxyvitamin D₃ levels. The lifespan of Fgf-23 null mice was significantly reduced (maximum 13 weeks). Since homozygous ablation of Fgf-23 resulted in increased synthesis of active vitamin D metabolites, we presumed that hyperphosphatemia, excessive skeletal mineralization and soft tissue calcifications in Fgf-23 null mice were partly mediated through increased activity of vitamin D.

**Fgf-23/1α(OH)ase null animals**

To test the hypothesis that altered phosphate homeostasis, skeletal mineralization and soft tissue calcification in Fgf-23 null mice were partly regulated
through enhanced vitamin D activity, we decided to generate a new double knockout mouse line by genetically ablating the $1\alpha$(OH)ase gene from Fgf-23 null mice. We have crossbred our Fgf-23 mutant mice with $1\alpha$(OH)ase null mice [29]. This new mouse line, deficient in both Fgf-23 and $1\alpha$(OH)ase [Fgf-23$^{-/-}$/1$\alpha$(OH)ase$^{-/-}$], did not only reverse hyperphosphatemia and lower serum calcium levels, but more importantly eliminated soft tissue calcification that was evident in Fgf-23 null mice (Figure 1), suggesting that the lack of Fgf-23, through a negatively regulated circuit of 1,25-dihydroxyvitamin D3 synthesis was responsible for abnormal tissue calcification.

These in vivo experiments re-emphasized two important issues: first, in presence of hyperphosphatemia, widespread tissue calcification is intensified by active vitamin D metabolites; and secondly, such pathological calcification could be prevented by reducing their synthesis. Whether the use of synthetic vitamin D analogues such as paricalcitol is associated with less soft tissue calcification in uremic patients than the active compound 1,25-dihydroxyvitamin D3 remains to be seen [30].

**Conclusion**

In contrast to the animal models in which soft tissue calcification develops via vitamin D toxicity with an increase in serum calcium, the Fgf-23 null mouse provides an interesting tool for the study of vitamin D effects in a hyperphosphatemic microenvironment, a situation close to that of dialysis patients receiving vitamin D treatment. Experimental studies in vivo using compound Fgf-23/1$\alpha$(OH)ase null mice allow us to demonstrate a direct link between enhanced vitamin D activity and soft tissue calcification. The results of such genetic manipulation studies provide a molecular basis for soft tissue calcifications that are widely documented in ESRD patients treated with unphysiologic doses of vitamin D metabolites. They underscore the importance of optimal dosing regimens and controlled treatment schedules of vitamin D metabolites in dialysis patients to avoid extraskeletal calcification.

**Conflict of interest statement.** None declared.

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