Vascular Calcification and Uremia: What Do We Know?

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Key Words

Vascular calcification • Chronic kidney disease • Pyrophosphate • Osteoprotegerin • Fetuin A • Matrix γ -carboxyglutamic acid protein • Bone morphogenetic protein 7

Abstract

In the last decade, the nephrology community has focused its attention on the main cause of morbidity and mortality in chronic renal failure patients: cardiovascular disease. In addition, recent studies pointed out that vascular calcification (VC) is a major cause of cardiovascular disease in the dialysis population. Interestingly, the pathogenesis of VC and soft tissue calcification in chronic kidney disease (CKD) has been extensively investigated. Nowadays we know that VC is associated not only with passive calcium phosphate deposition, but also with an active, cell-mediated process. To better understand the pathogenesis of VC in CKD, numerous regulatory proteins have been studied, because of their ability to inhibit mineral deposition in the vessels. We here examine the state of the art of those substances recognized as regulatory key factors in preventing VC in uremic conditions, such as fetuin A (α_2 -Heremans-Schmid glycoprotein), matrix γ -carboxyglutamic acid protein, pyrophosphate, osteoprotegerin and bone morphogenetic protein. We conclude that at present it is too early to introduce these novel markers into clinical practice.

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Introduction

Chronic kidney disease (CKD) patients develop atherosclerotic vascular disease earlier and faster than the general population [1]. In CKD-induced cardiovascular disease, ‘classic’ and ‘nontraditional’ risk factors of vascular calcification (VC) have to be considered [2]. In fact, severe VC is a common event in CKD [3, 4]. These patients develop extensive medial calcification, which causes increased arterial stiffness and severer cardiovascular disease that induces a higher morbidity and mortality [5, 6].

A mounting number of VC risk factors are involved in the dialysis population: age, gender, dialysis vintage, inflammation, mineral metabolism abnormalities and diabetes [7, 8]. In addition to these well-known features of CKD patients, in the last decade new pathogenetic ideas have been emerging in the nephrology community to better understand extraskeletal calcification. In fact, although the calcification process has not been completely elucidated, it is now clear that it does not merely consist in passive deposition of calcium phosphate crystals; on the contrary, it is a well-organized process, involving cell activity and specific protein synthesis [9].

Accordingly, several bone-related proteins are now certified for their capacity of inducing or inhibiting the process of extraskeletal calcification, and their potential role as ‘protective’ or ‘noxious’ proteins associated

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with VC in CKD needs to be clarified. Analyzing those substances recognized as regulatory key factors in preventing VC in uremic conditions, such as fetuin A (α_2 -Heremans-Schmid glycoprotein, AHSG), matrix γ -carboxyglutamic acid protein (MGP), pyrophosphate, osteoprotegerin (OPG) and bone morphogenetic protein (BMP) 7, as 'the 5 principal actors' inhibiting VC in uremic conditions, a number of questions arise:

(1) What do we really know about each of them, and how can we put all of them together?

(2) Which protein plays the most important role in this disease?

(3) Should nephrologists include the measurement of the serum levels of these proteins amongst the routine tests for the workup of their patients?

Phosphate as a Major Promoter of VC

Recently, very intriguing in vitro studies by Giachelli [10] and co-workers in Seattle demonstrated that high phosphorus levels in culture media (2 mmol/l) stimulate VC. The pathogenesis of phosphate-induced VC has been investigated in depth [11], and in vitro studies have indicated that high phosphate increases VC via direct induction of the osteoblast-specific gene *Cbfa1*, which regulates the expression of several BMPs [12]. In CKD the expression of these proteins was also induced by uremic serum independently of phosphate concentrations, in an in vitro model of bovine vascular smooth muscle cells (VSMCs) [13]. Importantly, Moe et al. [14] showed increased *Cbfa1* and osteopontin expression in the media and intima of calcified epigastric arteries from uremic patients. Clearly, these data suggest that VC is an active process. The cellular formation of a bone-like structure in calcified vessel walls indicates that the uremic environment and elevations in serum phosphate levels may be regulating factors. Considering the concentration product of serum calcium and phosphate, physicochemical crystallization would occur continuously if it were not inhibited by specific macromolecules.

At present, 5 substances have been extensively investigated because of their inhibitory property on VC. In fact, in experimental animal models, either the absence or the reduction of AHSG, MGP, pyrophosphate, OPG and BMP-7 have been associated with vascular and extracellular calcium deposition (table 1). Price et al. [15] postulated the formation of a stable high-molecular-weight complex of a calcium phosphate mineral phase and the 2 inhibitory proteins AHSG and MGP in normal serum.

Uremic conditions associated with hyperphosphatemia and hypercalcemia may tend to make the complex unstable and decrease both AHSG and MGP serum levels. Moreover, mice deficient in AHSG develop ectopic calcification when fed a high-calcium and high-phosphorus diet, indicating that AHSG is a systemically acting inhibitor of calcification. In contrast, MGP acts as VC inhibitor locally.

Importantly, current research involves efforts to define the complex interactions between cellular and molecular mediators of VC and, in particular, the role of endogenous calcification inhibitors. Therefore, we decided to focus our review on the recent insights into these 5 substances, in order to evaluate their clinical relevance.

Fetuin A

AHSG, also known as human fetuin A, is an important inhibitor of extraskeletal calcification. Similarly to other acute-phase reaction proteins, AHSG is synthesized by the liver, and its concentration falls during the cellular immunity phase of inflammation [16]. In vitro, AHSG inhibits the de novo formation and precipitation of calcium phosphate, but it does not exert any effects on hydroxyapatite once it is formed [17]. AHSG antagonizes the antiproliferative action of transforming growth factor β_1 , and blocks osteogenesis and deposition of calcium-containing matrix in dexamethasone-treated rat bone marrow cells [18]. AHSG knockout mice develop extensive ectopic calcifications in the myocardium, kidney, lung, tongue and skin, even if calcification of larger vessels is conspicuously absent [19].

Hemodialysis patients with lower serum AHSG levels have increased mortality due to cardiovascular events [20]. This observation by Ketteler et al. [20] suggests that AHSG is involved in preventing the accelerated extracellular calcification observed in CKD. A recent study in a population of 115 hemodialysis patients supports this hypothesis, as VC was associated not only with increasing age and a history of cardiovascular events, but also with abnormal values of inflammatory markers, such as reduction in AHSG and albumin, and an increase in C-reactive protein and fibrinogen [21]. Moreover, a recent prospective multicenter cohort study in both hemodialysis and peritoneal dialysis patients showed the association of an increase in the serum AHSG levels of 0.1 g/l and the reduction in all-cause mortality of 13% [22].

Indeed, in a recent study, Stenvinkel et al. [23] demonstrated that CKD patients with elevated inflammatory

Table 1. Inhibitory proteins of VC with a potential role in uremic conditions

	Class	Chemistry	Production	Distribution	Action	Null type	Notes
AHSG [15–23]	Cystatin superfamily	62-kDa glycoprotein	Liver	Ubiquitous	Direct Ca inhibition; indirect: TGF- β antagonism	Diffuse systemic calcification (<i>DBA/2</i> strains)	Acts under special conditions
MGP [24–33]	GLA protein	Noncollagen bone protein, 84 aa; 14 kDa; 5 γ -GLA residues	VSMCs, chondrocytes	Calcified tissues (cartilage, vessels)	Inhibition of calcification	Elastic and muscular arterial calcifications, aortic rupture; osteopenia, fractures	Requires vitamin-K-dependent γ -carboxylation
PP [34–44]	Inorganic plasma solute	Chain of P linked through oxygen	VSMCs, chondrocytes, osteoblasts	Ubiquitous	Inhibits any type of mineralization		Hydrolyzed by alkaline phosphatase
OPG [45–61]	TNF R superfamily	Glycoprotein, 401 aa; 60 kDa M; 120 kDa D	Heart, arteries, veins, bone marrow, lung, kidney, intestine	Several tissues (bone, vessels); circulating cytokine R	Inhibits osteoclastogenesis, protective for vascular system (endothelial cell survival factor; dendritic cell stimulation)	High-turnover osteoporosis, fractures; VC (aorta and renal arteries, elastic types)	Not required for embryonic bone
BMP-7 [62–70]	TGF- β superfamily	Protein, 431 aa; 7-cystatin-residue ring	Embryo: skeleton, kidney, eye, CV; adult: kidney	Collecting tubules, glomerulus, adventitia	Cell proliferation/differentiation/apoptosis; osteoblast and VSMC differentiation; embryo: renal morphogene; adult: renal tubule differentiation	Small dysgenic kidneys (uremia), eye abnormalities, polydactyly	Anti-TGF- β effect; anti-inflammatory effect (IL-1, -6 and -8 inhibition)

Chemical, physiological and pathophysiological characteristics of AHSG, MGP, pyrophosphate, OPG and BMP-7. PP = Pyrophosphate; Ca = calcium; TGF- β = transforming growth factor β ; GLA = glutamic acid; aa = amino acids; P = phosphate; TNF = tumor necrosis factor; M = monomeric; D = dimeric; R = receptor; CV = cardiovascular; IL = interleukin.

markers and with the AHSG 256Ser allele had lower serum AHSG levels and higher all-cause and cardiovascular mortality rates.

Price and Lim [24] have extensively investigated the mechanisms by which proteins inhibit VC. They showed the existence of a high-molecular-weight complex of a calcium phosphate mineral phase and the 2 inhibitory proteins AHSG and MGP in the serum of rats treated with bone-active bisphosphonate etidronate [25]. Furthermore, in a recent in vivo study, Price et al. [25] found that vitamin-D-induced VC is associated with a 70% reduction in serum AHSG, probably due to the clearance of the fetuin-mineral complex from serum. Importantly, this high-molecular-weight complex has been detected only in experimental animal models that received high doses of etidronate, but never in humans affected by VC.

The potential role of AHSG in VC pathogenesis in CKD patients is still poorly understood. As AHSG binds calcium and hydroxyapatite, fetuin knockout mice have soft tissue calcifications, and serum AHSG levels are reduced in both uremic and inflammatory conditions, loss of serum and local AHSG may directly promote VC in uremic patients.

Matrix γ -Carboxyglutamic Acid Protein

Extracellular MGP is a member of the vitamin-K-dependent protein family, which has a high affinity for calcium and phosphate. Its role in the prevention of VC was suggested by the findings in MGP-deficient mice, which develop diffuse arterial calcification, osteoporosis and pathological fractures during their first 2 months of life [26]. In addition in MGP-deficient mice that develop VC, Murshed et al. [27] demonstrated that this protein acts locally and not systemically.

The role of MGP in the pathogenesis of osteoporosis has been established [28], but its effects in CKD patients are still unclear.

Its inhibitory mechanism on mineralization appears to be mediated by BMP-2 [29]. Wajih et al. [30] have recently shown that MGP is found both in the cytosolic and in the microsomal fraction obtained by differential centrifugation of human VSMC homogenate together with AHSG, and they have demonstrated serum AHSG uptake by these cells. AHSG uptake and secretion by these cells could represent an additional protective mechanism against arterial calcification.

The localization of MGP and other bone matrix proteins, such as osteopontin, has been investigated by Canfield et al. [31], in calcified vessels of different sizes affected by different types of lesion (atherosclerosis, chronic vascular rejection in renal allografts and calcific uremic arteriolopathy, i.e. calciphylaxis) as well as in normal vessels. MGP was not detected in normal blood vessels; its expression was enhanced at loci of arterial calcification independently of their nature, suggesting that calcification in vessels of different sizes occurs by a similar pathogenetic mechanism involving MGP [31].

An association between polymorphisms of the MGP gene and myocardial infarction has been described in low-risk individuals [32]. Furthermore, their distribution has proved to differ significantly in CKD/hemodialysis patients as compared to healthy controls, and particular alleles are associated with an increase in cardiovascular events in hemodialysis patients [33]. Potentially, the identification of polymorphisms of the MGP gene and their association with cardiovascular morbidity is a critical step towards the understanding of the pathogenetic mechanisms of VC in CKD and dialysis patients.

Recently, Jono et al. [34] have reported an association between serum MGP levels and coronary artery calcification, detected by electron beam computed tomography, in 115 subjects with suspected coronary artery disease and normal renal function. Patients with coronary artery calcification had lower serum MGP levels compared to those with no calcium in the coronary tree, providing further support to the hypothesis that MGP plays a role in the prevention of VC [34].

At present the diagnostic value of circulating MGP levels in uremic and nonuremic subjects is limited by the fact that the assays that are currently available cannot differentiate between carboxylated (active) and undercarboxylated (inactive) forms of MGP, as the prevalent synthesis of the latter form is associated with intimal and medial calcification [35].

Pyrophosphate

At variance with other compounds, inorganic pyrophosphate (iPP) is not a protein and its inhibitory effect on cartilage calcification and oxalate crystallization has been known for many years [36, 37]. In addition, it has been demonstrated that iPP inhibits aortic calcification induced experimentally by vitamin D administration in rats [38]. Indeed, even low plasma concentrations, in the

order of 2–4 μM , are sufficient to prevent crystallization in calcium- and phosphate-saturated solutions [39].

Produced by VSMCs, chondrocytes and osteoblasts, iPP is distributed ubiquitously. Its formula $[\text{P}_2\text{O}_7^{4-}]$ and reactive chemical nature suggest that it is a compound used to bind or deliver oxygen and phosphate at tissue level for rapid employment. Several intracellular enzymatic reactions are responsible for its production [40]; in particular, 3 compounds are significantly involved: ectonucleotide pyrophosphate phosphodiesterase 1 which is considered the rate-limiting enzyme for intracellular iPP synthesis; a transmembrane protein encoded by the progressive ankylosis locus which is responsible for iPP shuttling into the extracellular space, and tissue nonspecific alkaline phosphatase which is responsible for the degradation of excess extracellular iPP [41]. Interestingly, a deficit of the progressive ankylosis transmembrane protein in transgenic mice results in periarticular hydroxyapatite deposition and arthritis [42], and a loss of function mutation of ectonucleotide pyrophosphate phosphodiesterase 1 has recently been described in idiopathic infantile arterial calcification [43]. Characteristically, children with this disease develop severe calcification of the internal elastic laminae of small, medium and large-size arteries, together with myointimal proliferation. The inhibitory role of iPP in the appearance of VC has been shown also in an animal model of medial calcification in the rat, in which local consumption of pyrophosphate by alkaline phosphatase resulted in calcification of the elastic lamina [44].

Plasma iPP is normally cleared by the kidney [45]. However, serum levels in hemodialysis patients have been shown to be reduced and diminish even further after each dialysis session, which clears about one third of the existing amount [46].

As a whole, the calcification-inhibitory action of iPP in vivo is well documented. However, its simple chemical composition and heterogeneous metabolism, as well as the local nature of its action, hinder the development of a preparation for clinical use considerably.

Osteoprotegerin

In the late nineties a cytokine, named osteoclast-inhibiting factor, was characterized by three different research groups for its significant and specific capacity of inhibiting osteoclastogenesis [47–49]. After the protective role of this protein in osteoporosis had been confirmed by the development of the disease in knockout

animals [50], the protein was given the new name of OPG. Besides osteoporosis resulting from excessive osteoclast activity, OPG-null animals develop diffuse calcifications of elastic vessels, such as the aorta and renal arteries [50]. However, even if significant amounts of OPG are produced by the osteoblast-like cells committed to calcify, resulting in increased serum OPG levels, also a positive association may be possible.

OPG is produced not only by osteoblasts, but also by many different tissues and cell types, including the lung, kidney, intestine and endothelial cells. Its biological effects are still not completely understood, but it is certainly involved in the inhibition of apoptosis [51]. Involvement of OPG in VC has been reaffirmed by the discovery of its mRNA in calcified, but not in normal human arteries [52].

Circulating levels of this cytokine have been associated with the presence and severity of coronary calcification [53] and with progressive atherosclerosis [54] in non-uremic subjects. In renal patients, increased levels have been associated with abnormal aortic calcifications in patients on dialysis [55] and with coronary artery calcifications in both dialysis and transplantation patients [56]. Similarly positive is the association of serum OPG levels with all-cause and cardiovascular mortality both in patients on dialysis [57] and in transplantation patients [58]. Serum levels of OPG are influenced by several factors, in particular, especially in uremia, by creatinine clearance [58] and possibly bone turnover [59–62]. A progressive increment in OPG with age has been reported in normal [55, 59, 61] and uremic [57, 59, 63] subjects and indicates OPG as a theoretical common marker for 2 typical hallmarks of aging: osteoporosis and calcification. Additional investigations are required before serum OPG can be used in clinical practice.

Bone Morphogenetic Protein 7

Recently, exciting new data from the group of Keith Hruska in Saint Louis [64–66] have suggested that a new player, BMP-7, is involved in the process of VC in chronic renal failure, as well as in the pathogenesis of renal osteodystrophy.

BMPs are members of the transforming growth factor β superfamily of cytokines and consist of a group of at least 15 morphogenes involved in intracellular messaging.

BMP-7 is a crucial element for the development of kidneys, eyes and bones [67]. In the skeleton, BMP-7 de-

ficiency produces a patterning defect causing extra digits and ribs to be formed and leads to a deficiency of precursor cell commitment to the osteoblastic differentiation and mineralization program. In the adult, BMP-7 maintains a role in osteoblast function, suggesting a hormonal role in bone metabolism. Interestingly, BMP-7 expression decreases early in the course of renal failure [68].

This state of BMP-7 deficiency has important consequences in the pathogenesis and treatment of chronic renal insufficiency [69], but is also very interesting for the pathogenesis and treatment of VCs. Indeed, BMP-7 maintains VSMC differentiation and prevents their transformation into cells with an osteoblastic phenotype [64, 70]. Thus, the state of BMP-7 deficiency, characteristic of chronic renal failure, could favor VC, especially within the context of atherosclerotic lesions.

In renal osteodystrophy, BMP-7 affects osteoblast morphology and number, eliminates peritrabecular fibrosis, decreases bone resorption and increases bone formation in secondary hyperparathyroidism. Moreover, it restores normal rates of bone formation in adynamic bone disorder, as demonstrated in a renal ablation animal model [71]. In this study, adynamic bone disease was reversed with a physiological bone anabolic factor: the results of BMP-7 treatment differ from parathyroid hormone level elevation because bone resorption is not stimulated and increased bone formation rates are thereby truly anabolic. Therefore, BMP-7 is broadly efficacious in renal osteodystrophy. An important feature is that it increases the skeletal deposition of ingested phosphorus and calcium, improving ion homeostasis in CKD.

Given the known role of BMP-7 in osteoblast biology, it may seem paradoxical that it would reduce VC. However, the effects of individual BMP are highly dependent on the target cell type, the receptors expressed, and the state of differentiation and maturity [72]. Transdifferentiation of VSMCs into cells with an osteoblastic phenotype could be the critical first step in the pathogenesis of VC, and it is clear that BMP-7 has a positive influence on maintaining VSMC differentiation.

Suggesting that BMP-7 may be viewed as a fourth renal hormone, along with renin, erythropoietin and calcitriol, Davies et al. [65] demonstrated that BMP-7 is an effective treatment for VC in a murine model of atherosclerosis and chronic renal failure, a finding that may have important implications for the development of future therapies for this condition in humans.

Therapy

At present no specific treatment is available to upregulate the serum concentrations of AHSG, MGP, pyrophosphate, OPG and BMP-7. However, preliminary clinical studies demonstrated the possibility to prevent or slow the progression of VC in dialysis patients. Recently, Yuen et al. [73] have shown the natural history of coronary artery calcification progression in a cohort of patients converted to nocturnal hemodialysis (6–8 h/session, 5–6 sessions/week). After 1 year of nocturnal hemodialysis, these patients had decreased serum phosphate levels, low rates of coronary calcification progression and consequently low cardiovascular risk.

In the past, the standard treatment for the hyperphosphatemia in CKD consisted of dietary phosphate restriction, dialysis treatment and administration of phosphate binders (aluminum salts, calcium carbonate or acetate). Recent studies described the limitations of calcium salts as phosphate binders and the elevated calcium load in patients with advanced renal failure [74]. Moreover, with such a therapeutic approach more than 50% of patients did not achieve a good control of serum phosphate levels. Recently, new phosphate binders, that do not contain aluminum or calcium, and therefore lack the side effects associated with calcium-based phosphate binders, opened new perspectives in preventing VC in CKD. Recent studies have shown that new therapeutic tools may be useful to prevent VC in animals and humans [75].

Furthermore, recent studies analyzed new potential effects of vitamin D receptor activators and calcimimetics on preventing VC and cardiovascular disease in CKD. In experimental animal models, the limited hypercalcemic effect of a new vitamin D receptor activator, paricalcitol, results from a lower affinity for vitamin D receptor in intestine and bone. In addition, paricalcitol causes a selective vitamin D activation and a reduction in intesti-

nal calcium absorption. Recently, an in vitro study demonstrated that only calcitriol increased calcification of VSMCs cultured in calcification media, but this effect was not present when cells were incubated with paricalcitol [76]. Moreover, in an elegant in vivo study, Mizobuchi et al. [77] showed that both calcitriol and doxercalciferol significantly increased the serum calcium phosphate product and aortic calcium content in uremic rats. In contrast, paricalcitol had no effect on VC.

Finally, in uremic rats, the calcimimetic R-568 reduces elevated parathyroid hormone levels without inducing VC, prevents calcitriol-induced VC and decreases mortality [78].

Conclusion

In conclusion, we reviewed the clinical relevance of endogenous VC inhibitors and their role in uremic conditions, understanding that the knowledge of both cellular events and suspected molecular pathways that control VC are important to highlight some potential avenues for future research [79–81]. Although these substances are certainly associated with or involved in the pathogenesis of VC, none of them can be defined as ‘the marker’ of this important clinical condition. Available data indicate that a complex interplay among several ‘actors’ normally occurs at the tissue level to avoid pathological deposition of crystals. Accordingly it is predictable that some specific ‘mixture’ may favor crystal growth while others will not. The heterogeneity of assay results and the information provided by the protein levels at present do not justify this practice. However, in a near future, the contemporary assessment of several markers, such as the ones described above, will enable us to recognize patients at high risk of VC, who should be managed aggressively.

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