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Vascular Calcification: The Killer of Patients with Chronic Kidney Disease

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ABSTRACT

Cardiovascular complications are the leading cause of death in patients with chronic kidney disease (CKD). Vascular calcification is a common complication in CKD, and investigators have demonstrated that the extent and histoanatomic type of vascular calcification are predictors of subsequent vascular mortality. Although research efforts in the past decade have greatly improved our knowledge of the multiple factors and mechanisms involved in vascular calcification in patients with kidney disease, many questions remain unanswered. No longer can we accept the concept that vascular calcification in CKD is a passive process resulting from an elevated calcium-phosphate product. Rather, as a result of the metabolic insults of diabetes, dyslipidemia, oxidative stress, uremia, and hyperphosphatemia, "osteoblast-like" cells form in the vessel wall. These mineralizing cells as well as the recruitment of undifferentiated progenitors to the osteochondrocyte lineage play a critical role in the calcification process. Important transcription factors such as Msx 2, osterix, and RUNX2 are crucial in the programming of osteogenesis. Thus, the simultaneous increase in arterial osteochondrocytic programs and reduction in active cellular defense mechanisms creates the "perfect storm" of vascular calcification seen in ESRD. Innovative clinical studies addressing the combined use of inhibitors that work on vascular calcification through distinct molecular mechanisms, such as fetuin-A, osteopontin, and bone morphogenic protein 7, among others, will be necessary to reduce significantly the accrual of vascular calcifications and cardiovascular mortality in kidney disease. In addition, the roles of oxidative stress and inflammation on the fate of smooth muscle vascular cells and their function deserve further translational investigation.

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Cardiovascular complications are the leading cause of death in patients with chronic kidney disease (CKD).[1] Vascular calcification is a common complication in CKD, and London *et al.*[2] demonstrated that the extent and histoanatomic type of vascular calcification are predictors of subsequent vascular mortality. The contribution of traditional risk factors such as hypertension, aging, smoking, diabetes, and abnormal lipid metabolism does not fully explain the high frequency of cardiovascular disease, indicating that some other distinct pathogenesis may be involved.[2–6]

Two major types of vascular calcification are distinguished by their location and association with atherosclerotic plaque formation. One type, atherosclerotic calcification, is located in the intimal layer and is associated with atherosclerosis. Atherosclerotic calcification involves cellular necrosis, inflammation, and lipid deposition.[7–9] As lesions progress, osteogenesis, including osteoblast induction and lamellar bone formation, becomes increasingly evident. The other type is Monckeberg sclerosis, in which amorphous mineral forms circumferentially along or within one or more elastic lamellae of the medial layer. Also known as medial artery calcification, this type is more prevalent in patients with diabetes and CKD.[7,8] Most *in vitro* studies examining vascular calcification have been performed in vascular smooth muscle cells (VSMCs), which are the major component of the medial arterial layer. For many years, vascular calcification was thought to be a passive process resulting from elevated serum phosphate (P^{2-}) levels and an increase in the calcium phosphate product ($Ca^{2+} \times P^{2-}$), resulting in oversaturated plasma.[10–12] Recent studies, however, revealed a link between vascular calcification and osteogenesis. Many key regulators of bone formation and bone structural proteins are expressed in both calcified medial arterial layers and atherosclerotic plaques,[13–24] suggesting that vascular calcification is an active process. In addition, there is growing evidence that physiologic inhibitors of vascular calcification also exist.[25–28]

Hyperphosphatemia and an elevated ($Ca^{2+} \times P^{2-}$) associate with cardiovascular

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mortality in patients with CKD.[29–32] In dialysis patients, vascular calcification is associated with hypercalcemia, hyperphosphatemia, an elevated ($Ca^{2+} \times P^{2-}$), and ingested oral calcium (Ca^{2+}).[4,33,34] In addition, coronary artery calcification occurs much earlier in pediatric patients undergoing dialysis than in the general population, and its progression positively correlates with serum P^{2-} levels, the ($Ca^{2+} \times P^{2-}$), and daily Ca^{2+} intake.[35,36] Moreover, clinical studies[37] demonstrated a decreased mortality in dialysis patients ingesting the non–Ca^{2+}-containing phosphate binder sevelamer compared with patients ingesting Ca^{2+}-based phosphate binders. Other studies showed that dialysis patients treated with sevelamer had little or no progression of vascular calcification when compared with those treated with Ca^{2+}-containing phosphate binders, even when control of serum P^{2-} levels in both groups was equivalent.[38,39] Thus, a disturbance in Ca^{2+} and P^{2-} metabolism plays a crucial role in the progression of vascular calcification in patients with CKD.

Active vitamin D compounds are commonly used for the treatment of secondary hyperparathyroidism. Low levels of serum calcitriol are associated with an increased risk for vascular calcification[40,41] and contribute to cardiovascular-related mortality in patients with CKD.[2–6] Furthermore, active vitamin D compounds provide a survival benefit for patients with CKD that is independent of serum Ca^{2+}, P^{2-}, and parathyroid hormone (PTH) levels.[42–46] Because vitamin D receptor (VDR) activation is beneficial in patients with CKD not only for the suppression of serum PTH levels but also for improved survival, continuous efforts have been made to develop new vitamin D analogs with lower calcemic and phosphatemic activities.[47]

In this article, we discuss the proposed mechanisms by which vascular calcification progresses in patients with CKD. These pathophysiologic mechanisms are broadly divided into three groups: The induction of osteoblastic transdifferentiation of VSMCs, the osteogenic lineage allocation and differentiation of multipotent vascular progenitors such as pericytes and calcifying vascular cells, and the loss of inhibitors of vascular calcification (Figure 1). We also examine the role of vitamin D in vascular calcification.

MOLECULAR MECHANISMS INVOLVED IN VASCULAR CALCIFICATION

Ectopic Osteogenesis

Many bone-associated proteins, including osteocalcin (OC),[48] osteopontin (OPN),[14,16,23,24] matrix γ-carboxyglutamic acid protein (MGP),[19] and osteoprotegerin (OPG)[49] are expressed in atherosclerotic plaques and associate with atherosclerotic calcification. These factors also relate to medial layer calcification (Monckeberg sclerosis), which was confirmed by the deletion of the target gene in mice and by *in vitro* studies using VSMCs. Although the basic processes, especially the initial steps, are different, some processes in atherosclerotic calcification and medial layer calcification may overlap when the initial step in vascular calcification occurs in aortic tissues.

Cbfa1/Runx2, a specific transcription factor for osteoblastic differentiation, has an important role in vascular calcification. This protein is essential for the differentiation of osteoblasts from their mesenchymal precursors. Cbfa1/Runx2-null mice completely lack functional osteoblasts and display profound mineralization and skeletal defects.[50] In humans, mutations in the Cbfa1/Runx2 locus cause cleidocranial dysplasia, an autosomal dominant disease characterized by the absence of clavicles, open fontalles, supernumerary teeth, and short stature.[51] Multiple signal transduction pathways that relate to posttranslational modification such as phosphorylation or protein–protein interaction are involved in the transcriptional activity of Cbfa1/Runx2. The mitogen-activated protein kinase (MAPK) pathway, which is activated by signals from the extracellular matrix (fibroblast growth factor 2 [FGF-2], bone morphogenic proteins (BMPs), and PTH plays a crucial role in the induction of Cbfa1/Runx2 activity, which results in the induction of osteoblastic differentiation,[52] as does hydrogen peroxide (*vide infra*). As a key factor in bone formation, the activation of Cbfa1/Runx2 by the aforementioned factors, is thought to play an important role in vascular calcification. In patients with CKD, it is well established that hyperphosphatemia is associated with the development of vascular calcification.[14–33] It was previously thought that high serum phosphate levels caused vascular calcification by simply exceeding ($Ca^{2+} \times P^{2-}$) solubility, resulting in precipitation. Recently, though, studies in VSMCs showed that high extracellular phosphate levels induce VSMCs to transform into osteoblast-like cells, suggesting that the processes of vascular calcification are active. Elevated extracellular P^{2-} levels accelerate mineralization of VSMCs; are associated with the induction of Cbfa1/Runx2; and increase bone-associated proteins such as OC, OPN, and alkaline phosphatase (ALP).[26,53–55]

At this point, however, it is important to highlight that, although osteogenic mechanisms participate in most if not all major forms of vascular calcification, not all vascular calcification leads to vascular ossification, particularly the deposition of lamellar or woven bone. For example, only 13% of calcified heart valves show evidence of bone formation, even though BMP expression is evident.[56] The processes directing true ossification may relate to angiogenic signals provided by hypoxia induced factor α and vascular endothelial growth factor, important mediators of bone formation and osteogenic–angiogenic coupling.[57]

Elastin Degradation

Because the initial step in medial calcification is in part associated with the degradation of elastin, representing elastic fibers with linear mineral deposits along elastic lamellae,[58] the degradation of elastin is thought to contribute to the osteogenic process in aortic tissue. Elastin is the most abundant protein in the walls of the arteries, which are subjected to pulsative pressure generated by cardiac contraction.[59] Elastin constitutes 90% of elastic fibers and 10% of microfibrillar glycoproteins, such as fibrillins and microfibrillar-associated

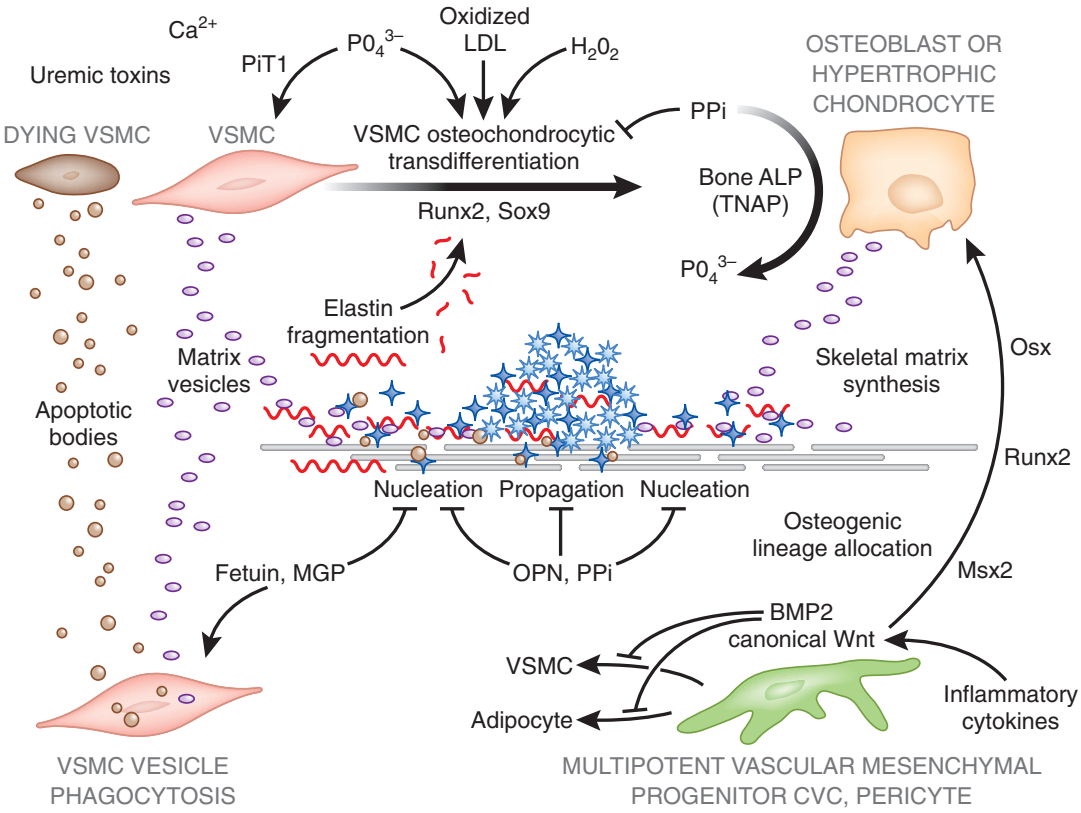

Figure 1. Cell fate, function, and phenotype in vascular calcification. In response to uremic toxins or elevated calcium and phosphate levels, VSMCs elaborate lipidaceous vesicle from apoptotic cells or produce matrix vesicle. The latter, approximately one third the diameter of apoptotic bodies, are much more efficient in nucleating mineral deposition. These vesicles nucleate calcium deposition in the form of a poorly crystalline hydroxyapatite, associated with the elastin-rich extracellular matrix of arteries. The process of elastinolysis not only creates sites for vesicle, mediated nucleation, but also released EDPs that promote osteochondrogenic "transdifferentiation" of VSMCs. This latter process is stimulated by oxidized LDL and ROS, *viz.*, hydrogen peroxide. With osteochondrogenic differentiation, gene expression profiles change dramatically, with induction of bone ALP, production of a highly collagenous extracellular matrix, and elaboration of matrix vesicle. Bone ALP locally degrades inorganic pyrophosphate, an important inhibitor of mineralization and transdifferentiation. In addition, multipotent vascular mesenchymal progenitors called calcifying vascular cells (CVC) or pericytes can yield cells of the osteoblast and chondrocyte lineage. This occurs through paracrine BMP and canonical Wnt signals that "shunt" these proliferating progenitors away from other fates, such as the mature VSMC, and toward osteogenic lineages. Inflammatory cytokines such and TNF play critical roles. Of note, as shown by Shanahan's group,[19,20] the phagocytic clearance of matrix vesicles by VSMCs is critical in limiting the number of sites that nucleate mineral deposition. In severely advanced atherosclerotic lesions, cholesterol crystals have also been shown to nucleate calcium phosphate deposition as well. See text for details.

glycoproteins, which form microfibrills of elastic fibers. Matrix metalloproteinases (MMPs) have an important role in the degradation of elastin.[60–62] MMP-2 and MMP-9 bind and degrade insoluble elastin to generate soluble elastin peptides.[63] These elastin peptides bind the elastin laminin receptor (ELR), which is located on the surface of VSMCs.

The degradation of elastin also induces the overexpression of TGF-β. TGF-β1 not only plays an important role in osteoblast differentiation[64] but also accelerates the calcification of VSMCs. The transduction pathway of both the ELR and the TGF-β receptor involves the activation of MAPK, which induces Cbfa1/Runx2 activation.[52] Thus, the activation the ELR or the TGF-β receptor in VSMCs may result in the induction of Cbfa1/Runx2 through MAPK phosphorylation and sequentially initiate the transformation of VSMCs into osteoblast-like cells. Because the signal transduction pathway for both the ELR and the TGF-β receptor are implicated in the osteogenic process in VSMCs, the degradation of elastin plays a crucial role, especially in the initial step of medial calcification.[65,66]

INDUCER OF VASCULAR CALCIFICATION

Ca^{2+} and P^{2-} Status

Compared with the general population, patients with CKD have a disproportionately high occurrence of vascular calcification. One hypothesis to account for this is the altered Ca^{2+} and P^{2-} metabolism seen in these patients. This is the most important contributor to the progression of vascular calcification in the uremic condition. Extracellular P^{2-} promotes the mineralization of VSMCs in both dosage- and time-dependent man-

ners by increasing the influx of P^{2-} into VSMCs, which leads to the induction of osteoblastic-differentiation factors such as Cbfa1/Runx2 and OC.[54] The blockade of Pit-1, a type III sodium-dependent phosphate co-transporter, impairs this mineralization, suggesting that elevated extracellular P^{2-} concentrations induce the mineralization of VSMCs through the activation of Pit-1. Furthermore, Pit-1 upregulates in calcified aortic tissue from uremic rats with hyperphosphatemia,[67] and inhibition of Pit-1 activity by small interfering RNA prevents the induction of Cbfa1/Runx2 and osteoclast expression in VSMCs even under high extracellular P^{2-} concentrations.[68] Accordingly, elevated extracellular P^{2-} concentration modulates the transformation of VSMCs into osteoblast-like cells by mediating Pit-1 activity. In addition to extracellular P^{2-}, Ca^{2+} accelerates the mineralization of VSMCs.[69,70] Ca^{2+}-induced mineralization is dependent on the function of Pit-1.[70] Long-term treatment of human VSMCs with elevated calcium induces the expression of Pit-1, suggesting that both P^{2-} and Ca^{2+} activate Pit-1 and increase the influx of P^{2-} into VSMCs additively and synergistically. Recently, a functional Ca^{2+}-sensing receptor was shown to express in VSMCs. Compared with normal individuals, the expression of this Ca^{2+}-sensing receptor is downregulated in VSMCs from patients with CKD.[71] Thus, the extracellular Ca^{2+} and Ca^{2+} receptor axis may have a role in the progression of vascular calcification.

Membrane-bound matrix vesicles and apoptotic bodies also have important roles in the initiation of vascular calcification. Vesicles and apoptotic bodies are released from living and dying VSMCs, respectively, and can be the nidus for mineral nucleation.[72–74] These elements are released by increases in extracellular Ca^{2+} and P^{2-} and can initiate mineralization of VSMCs. A study by Reynolds *et al.*[75] highlight an important role for the VSMCs in the metabolism of membrane-bound matrix vesicles. VSMCs avidly phagocytose membrane-bound matrix vesicles in a fetuin-dependent manner. Thus, apoptosis of VSMCs in ESRD, that is promoted by hyperphosphatemia and uremic toxins, not only liberates apoptotic bodies that can nucleate mineralization but also gives rise to the demise of cells that clear the membrane-bound matrix vesicles and apoptotic bodies.[75] Because of the mechanistic "inflection point" that occurs with VSMC apoptosis, even in the absence of osteogenic transdifferentiation, VSMC physiology and function are critical in the pathogenesis of uremic calcification.[75]

Uremic Toxins

Compared with serum from nonuremic individuals, uremic serum increases the mineralization of VSMCs and upregulates the expression of Cbfa1/Runx2 and its target protein OPN, regardless of the serum P^{2-} concentration.[76,77] Furthermore, blocking Cbfa1/Runx2 activity decreases uremic serum-induced ALP and OC expression in VSMCs. Uremic toxins upregulate Cbfa1/Runx2, which is mediated by cAMP/protein kinase A (PKA).[76,77] Uremic serum also increases the secretion of a crucial mediator of osteoblastic differentiation, BMP-2, from VSMCs, resulting in the mineralization of VSMCs.[76,77] This suggests the activation of Cbfa1/Runx2 by uremic toxins mediates cell signaling through cAMP/PKA and BMP-2, additively or synergistically. In uremic serum, not only P^{2-} but also BMP-2 may have a role as a mediator in the transformation of VSMCs into osteoblast-like cells.[78]

Oxidative Stress and Inflammation

The uremic state is characterized by increased oxidative stress. Oxidative stress is the net balance between oxidant production and antioxidative activity.[79–82] Pro-oxidants include reactive nitrogen species and reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide. Hydrogen peroxide and xanthine/xanthine oxidase dosage-dependently increase intracellular oxidative stress (as determined by 2,7 dichlorofluorescein fluorescence) and enhance ALP activity in VSMCs.[83] These effects of ROS are attenuated by antioxidants, suggesting the involvement of oxidative stress in the osteoblastic differentiation of vascular cells. This increased oxidative burden results in the formation of oxidized LDLs, which have been shown to stimulate dedifferentiation of VSMCs into a bone phenotype.[84] Conversely, oxidative stress has an inhibitory effect on the differentiation of osteoblasts and bone marrow stromal cells. This reciprocal response of oxidative stress in vascular and bone cells may explain the clinical aspect of osteoporosis, which demonstrates both calcification (vessel) and decalcification (bone).

Importantly, the ROS hydrogen peroxide was recently shown to promote osteogenic transdifferentiation of VSMCs, including upregulation and activation of Runx2/Cbfa1 in concert with matrix mineral deposition.[85] The Akt signaling cascade is particularly important for osteogenic H_2O_2 signaling in VSMCs and is not associated with apoptosis-induced mineralization mechanisms. Moreover, recent studies highlighted the accumulation of ROS at sites of vascular calcium accrual in humans.[86] The biologic diversity of ROS generation noted already indicates that, although initially heterogeneous, convergent pathways mediate active mineralization through osteochondrogenic transcriptional programming.

Because the oxidative stress that induces osteoblastic differentiation of VSMCs is often the result of the inflammatory process, inflammatory cytokines themselves have been implicated in vascular calcification. It has also been suggested that TNF-α has a crucial role in vascular calcification.[87–90] The osteoblastic differentiation of VSMCs, as assayed by ALP activity and mineral deposition, is induced by TNF-α in a dosage-dependent manner. This induction by TNF-α is mediated through the cAMP pathway, and cAMP stimulates the osteoblastic differentiation of VSMCs. Furthermore, TNF-α enhances the DNA binding of Cbfa1/Runx2, activated protein 1, and cAMP responsive element binding protein, which are important transcription factors in osteoblastic differentiation.[81–90]

In addition to the osteogenic transdifferentiation of VSMCs, Demer and colleagues[91,92] highlighted the contributions

of pluripotent vascular mesenchymal progenitors to the vascular calcium accrual. Calcifying cells of Demer are mural multipotent mesenchymal progenitors related to the microvascular pericyte. In addition to VSMC, osteogenic, chondrogenic, and adipogenic lineages are derived from pericytes. Tintut *et al.*[91] estimated that between 10 and 30% of mural VSMCs are in fact calcifying vascular cells. Thus, in addition to VSMC osteochondrogenic "transdifferentiation," proliferative expansion and osteogenic lineage allocation of pericytic VSMCs can contribute to the osteochondrogenic cell populations of the vessel wall. Recently, the procalcific Wnt signaling cascades activated in diabetic vascular disease was identified as being entrained by the low-grade inflammation of diabetes[92] and contributing to osteochondrogenic differentiation of vascular pericytes.[93] Indeed, administration of infliximab, a specific inhibitor of TNF-α signaling, significantly reduced high-fat diet–induced aortic calcium accrual, and the upregulation of the BMP2-Msx2-Wnt signaling contributes to the osteogenic programming of multipotent vascular mesenchymal progenitors.[92] The precise ontogeny and sources of these progenitors, potentially arising from circulating osteogenic progenitors,[94] has yet to be fully established.

Other Inducers

Because leptin is mainly cleared by the kidney, its serum concentrations is increased in patients with CKD.[95] Leptin can induce osteoblastic differentiation and the mineralization of VSMCs.[96] Glucocorticoids and glucose also induce the osteoblastic differentiation of VSMCs.[97,98]

INHIBITORS OF ECTOPIC OSTEOCHONDROGENIC MINERALIZATION

That MGP, OPN. and OPG gene-null mice show massive vascular calcification indicates that these genes work as inhibitors of vascular calcification. Thus, in addition to ectopic osteogenesis by which vascular calcification progresses in patients with CKD, a lack of inhibitors of calcification is another important mechanism behind vascular calcification.

Matrix γ-Carboxyglutamic Acid Protein

MGP was originally isolated from bone. MGP-null mice have medial layer vascular calcification of the aorta and its branches and develop aortic rupture as a result. MGP requires vitamin K–dependent γ-carboxylation to be fully functional.[99] Non–γ-carboxylated MGP but not γ-carboxylated MGP is associated with vascular calcification.[100–102] MGP binds BMP-2 to mediate the osteoblastic differentiation of VSMCs and inhibits the activity of BMP-2 in the differentiation of mesenchymal cells.[103] Furthermore, MGP expression is downregulated and osteoblastic markers such as collagen type II and OC are upregulated in calcified vessels. MGP also binds Ca^{2+} crystals and inhibits crystal growth.[102] Taking these facts into consideration, MGP has a role in maintaining the normal phenotype of VSMCs and in preventing their osteoblastic differentiation; however, as Shanahan's group discovered,[75] MGP along with fetuin plays a critical role in the regulation of membrane-bound matrix vesicle biology. Given the multiple roles for MGP in controlling VSMC physiology, the consequences of coumadin treatment on the risk for calcific uremic arteriolopathy may be related to perturbation of MGP functions.

Osteopontin

OPN is an acidic phosphoprotein that is expressed in mineralized tissues and inhibits the mineralization of tissues by blocking hydroxyapatite formation and by activating osteoclast function.[104] Although OPN is not expressed in normal vessels, abundant OPN is found in calcified arteries, indicating that OPN is a regulator of vascular calcification. When OPN-null mice, which have no significant vascular calcification, are bred to MGP-null mice in whose medial layer vascular calcification spontaneously develops, vascular calcification in the offspring is enhanced, suggesting that OPN has an inhibitory effect on vascular calcification *in vivo*.[21] OPN inhibits the mineralization of VSMCs by binding to the mineralized crystal surface.[105–106] This is independent of extracellular P^{2-} concentration and ALP activity. Moreover, phosphorylation of OPN is necessary for its inhibitory effect on the mineralization of VSMCs.[106] The function of OPN is thought to represent an adaptive response to counteract the progression of vascular calcification.

Of note, however, it is now clear that OPN has multifunctional roles in vascular physiology.[104] In addition to its actions that promote calcium egress and inhibit mineralization, OPN has emerged as a proinflammatory cytokine that enhances vascular remodeling and angiogenesis, in part through the activation of MMPs cleaved by thrombin. Indeed, broad-spectrum MMP inhibitors reduce vascular calcium accrual in preclinical models.[107] Thus, the proangiogenic action of cleaved OPN on vascular matrix calcium deposition facilitates vascular matrix mineralization, unlike the inhibition observed with full-length phosphorylated OPN.[104,105]

Osteoprotegerin

OPG inhibits osteoclast differentiation and is a crucial modulator of bone resorption through its action as a decoy receptor for the receptor activator of NF-κB ligand (RANKL).[108,109] OPG-null mice develop severe medial layer calcification, along with mural T cell infiltration. Mice deficient in both OPG and apolipoprotein E (apoE) have progressive calcification of atherosclerotic lesions compared with that of mice deficient in apoE alone (apoE-null), suggesting OPG acts as an inhibitor of vascular calcification *in vivo*.[110] OPG was shown to inhibit ALP activity in aortic tissue and prevent the progression of medial layer vascular calcification.[111] Similar results were observed in the diabetic Ldlr−/− mouse model, showing again OPG administration diminishes vascular calcium accumulation, potentially through immunomodulatory actions upon diet-induced low-grade mural inflammation.[112] Although little is known about the direct effect of OPG on VSMCs, it is important to understand further its role in osteogenesis and its involvement in

the inflammatory response, which is important for the osteoblastic differentiation of VSMCs.

Fetuin-A

Fetuin-A (α2-Heremans-Schmid glycoprotein) is a Ca^{2+}-binding protein found in serum and produced predominantly by the liver.[113] Fetuin-A–null mice develop massive pulmonary, vascular, and other tissue calcification accompanied by renal dysfunction, but the calcification intriguingly spares the aorta.[114] Whereas MGP, OPN, and OPG are local factors involved in vascular calcification and function at the site of calcification, fetuin-A is a circulating inhibitor of vascular calcification. VSMCs can take up serum fetuin-A and pool it in intracellular membrane-bound matrix vesicles. As previously stated, these vesicles are released from VSMCs and become the nidus for mineral nucleation. These released vesicles have abundant fetuin-A and abrogate the ability of regular membrane-bound matrix vesicles to form hydroxyapatite crystal.[75] The uptake of fetuin-A by VSMCs is also induced by extracellular Ca^{2+} but not by extracellular P^{2-}. This fetuin-A uptake increases the amount of Ca^{2+} entering VSMCs and is mediated by annexin Ca^{2+} channel activity, facilitating its inhibitory role in VSMC mineralization.[115]

Pyrophosphate

Pyrophosphate (PPi) is also the major inhibitor of vascular calcification and acts by inhibiting hydroxyapatite crystal formation.[116] PPi is generated from the hydrolysis of nucleotide triphosphates by the nucleotide pyrophosphatase phosphodiesterase family (NPP). An important member of the NPP family is NPP-1. The lack of PPi generation as a result of the inactivation of NPP-1 causes extended medial layer calcification, which reflects idiopathic infantile arterial calcification.[117] NPP-1–null mice also develop mineralization of VSMCs with the induction of cartilage-specific genes.[118] Mice defective in Ank, a transporter of PPi, also develop medial layer calcification. In contrast to VSMCs, it has been shown that blocking PPi generation is necessary to induce aortic ring calcification even with high concentrations of Ca and P.[119]

Moreover, the mechanisms of PPi-dependent control of vascular calcium accrual encompass the inhibition of VSMC osteochondrogenic transdifferentiation,[118,120,121] the same processes that are promoted by P^{2-}.[26] The signaling mechanisms responsible for this important biologic activity of PPi are as yet unknown but may provide insights useful for the development of novel pharmacotherapeutic agents based on bisphosphonate structures.[120]

Others

N-3 fatty acids and IGF-I also inhibit osteoblastic differentiation and mineralization of VSMCs. Mice deficient in Smad6, Klotho, FGF-23, or carbonic anhydrase II develop extensive vascular calcification.[121–126] The Klotho–FGF-23 axis requires renal 1-α hydroxylase activity, indicating the contributions of endogenous calcitriol.[125] To clarify clinical relevance, the precise role of these vascular calcification inhibitors in CKD need more exploration.

ROLE OF VITAMIN D IN VASCULAR CALCIFICATION

Vascular calcification is a risk factor for cardiovascular mortality. In patients with CKD, adjusted cardiovascular mortality is 10 to 20 times higher than in the general population.[1] Medial layer calcification is very common in patients with CKD. The specific vascular calcification in patients with CKD is calciphylaxis, which also occurs in medial layer. This medial layer calcification is associated with high mortality in patients with CKD.[127] Calcitriol and its analogs are widely used to manage secondary hyperparathyroidism. There is some controversy as to whether active vitamin D compounds directly accelerate vascular calcification.

VSMCs possess 25-hydroxyvitamin D3–1α hydroxylase[128] and the VDR,[129–131] indicating that vitamin D has a role in VSMC function and physiology. Calcitriol upregulates VDR expression in VSMCs, and VDR activation is widely known to modulate cellular proliferation and differentiation. Calcitriol suppresses VSMC proliferation, mediating the inhibition of EGF, which is implicated in cell proliferation.[132] One vitamin D analog, paricalcitol, modulates genes implicated in cell differentiation and proliferation.[133] In contrast, other studies have reported the stimulation of VSMC proliferation by calcitriol.[133,134] Calcitriol also has been shown to induce the expression of OPN, a local inhibitor of vascular calcification in VSMCs.[135] Calcitriol increases VSMC calcification by increasing the RANKL/OPG ratio.[136] Calcitriol at a concentration range of 10^{-7} to 10^{-9} M also increases VSMC calcification in a dosage-dependent manner and is accompanied by an induction of ALP activity and a suppression of PTH-related peptide (PTHrp) secretion of VSMCs.[137] The inhibitory effect of PTHrp on VSMC calcification is mediated by both cAMP/PKA and PKC signaling.[137] Importantly, the concentrations of calcitriol used in these *in vitro* studies are supraphysiologic.[138] Thus, it is unclear whether these *in vitro* results are relevant *in vivo*.

In animal models of CKD, it has been reported that calcitriol treatment results in the development of vascular calcification.[139,140] The dosages of calcitriol used in all of these studies, however, are so high that they induce a significant increase in the ($Ca^{2+} \times P^{2-}$), which could easily by itself lead to vascular calcification. Thus, it still remains unclear whether therapeutic doses of calcitriol, which suppress PTH without hypercalcemia and hyperphosphatemia, can induce vascular calcification; however, vitamin D analogs, such as maxacalcitol, paricalcitol, and doxercalciferol, have differential effects on vascular calcification in uremic animal models.[138,140] Whereas a high dosage of calcitriol (125 ng/kg, intravenously, three times a week for 2 wk) increased the degree of aortic calcification, maxacalcitol had no effect even at dosages that produced comparable serum Ca^{2+}, P^{2-}, and ($Ca^{2+} \times P^{2-}$) levels.[140]

Paricalcitol and doxercalciferol, two analogs in the vitamin D_2 family, have different effects on vascular calcification in uremic rats.[141,142] We first tested a low

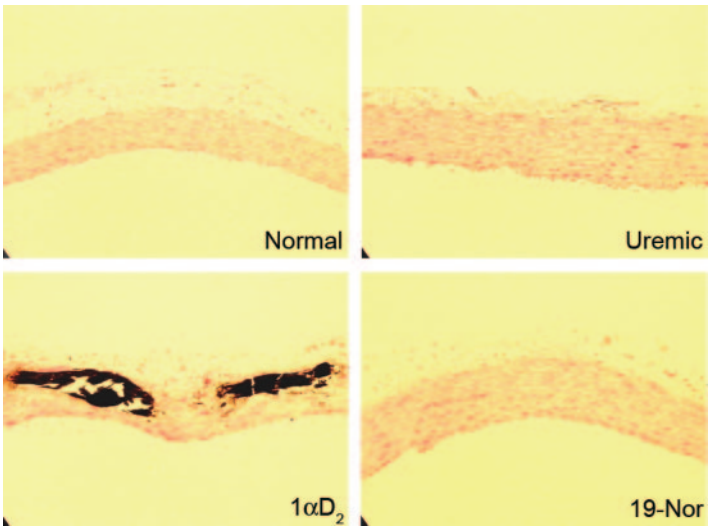

Figure 2. Representative microphotograph of Von Kossa staining of aorta from uremic rats treated with vehicle (UC), 0.04 μg/kg calcitriol (1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]), 0.16 μg/kg doxercalciferol [1α(OHD_2)], or paricalcitol [19-nor 1,25$(OH)_2D_2$]. Each drug was given intraperitoneally three times a week for 1 mo. Magnification, ×400.[141]

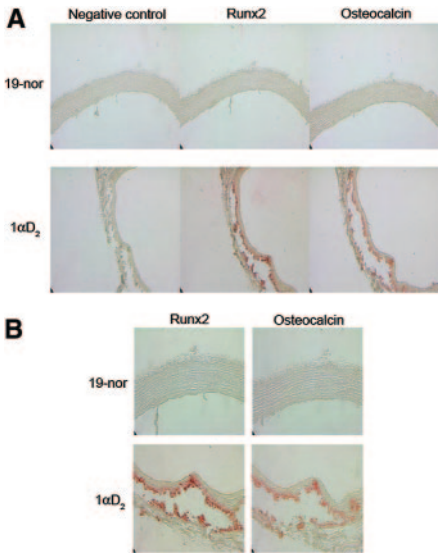

Figure 3. Representative microphotograph of immunohistochemistry for RUNX2 and OC in aorta from uremic rats treated with 0.10 μg/kg 1α(OH) D_2 or 0.16 μg/kg 19-nor-1,25$(OH)_2D_2$. Each drug was given intraperitoneally three times a week for 1 mo. Magnifications: ×100 in A; ×200 in B.[141]

dosage of calcitriol (0.04 μg/kg intraperitoneally three times per week for 1 mo) in the five-sixths nephrectomy rat model and found it induced massive aortic calcification accompanied by a marked increase in serum Ca^{2+}, P^{2-}, and ($Ca^{2+} \times P^{2-}$).[141] Thus, it is difficult to determine whether calcitriol directly induces vascular calcification or the effect is due to hypercalcemia, hyperphosphatemia, and the increased ($Ca^{2+} \times P^{2-}$) seen in these rats. We then compared the effect of equal dosages of paricalcitol and doxercalciferol (0.16 μg/kg intraperitoneally three times per week for 1 mo) on vascular calcification in this animal model. Compared with paricalcitol, doxercalciferol markedly increased ($Ca^{2+} \times P^{2-}$). This would explain why substantial vascular calcification is observed in rats treated with doxercalciferol but not paricalcitol (Figure 2). When dosages of these two analogs were adjusted so that ($Ca^{2+} \times P^{2-}$) was the same, doxercalciferol (0.10 μg/kg intraperitoneally three times per week for 1 mo) still increased aortic Ca^{2+} content, whereas a higher dosage of paricalcitol (0.24 μg/kg intraperitoneally three times per week for 1 mo) did not. Furthermore, Wu-Wong *et al.*[142] similarly demonstrated that a high dosage of these analogs (0.67 μg/kg intraperitoneally three times per week for 12 d) induced comparable hypercalcemia and hyperphosphatemia, although aortic Ca^{2+} content was much higher in doxercalciferol-treated uremic rats than in those receiving paricalcitol. Thus, differential effects on vascular calcification exist between these two analogs, which are independent of ($Ca^{2+} \times P^{2-}$). We demonstrated that doxercalciferol strongly induced the expression of Cbfa1/Runx2 and OC, whereas paricalcitol did not (Figure 3). Because in osteoblastic cells Cbfa1/Runx2 interacts with the VDR to upregulate the OC gene,[143] VDR activation by doxercalciferol may more strongly stabilize OC transcription in calcifying aortic tissue as a result of a longer half-life of doxercalciferol. There also may be differences in the acceleration of osteoblastic differentiation of VSMCs between the two analogs. Alternatively, paricalcitol has been shown, by itself, not to induce vascular calcification. This has been shown to be associated with the suppression of the increase in pulse pressure that develops in uremic rats.[136]

In patients with CKD, bone mineral disorders are correlated with higher mortality, which is mainly the result of the development of cardiovascular diseases.[12,29–32] Thus, there is a very close relationship between mineral-bone disorders in CKD and cardiovascular diseases including vascular calcification. Vitamin D compounds have an important role in this relationship, because they are widely used for the treatment of secondary hyperparathyroidism and have calcemic and phosphatemic actions. Apparently, these compounds can induce vascular calcification through their calcemic and phosphatemic actions. Oversuppression of PTH by vitamin D compounds leads to low-turnover bone disease, typically adynamic bone disease, which is associated with vascular calcification.[144] Even though vitamin D analogs were designed to suppress PTH with less calcemic and phosphatemic actions, they sometimes induce hypercalcemia and hyperphosphatemia. Both hypercalcemia and hyperphosphatemia associate with vascular calcification in patients with CKD[2,33,34] as well as *in vitro* and *in vivo* studies; however, there is no clear evidence that vitamin D compounds directly induce vascular calcification in patients with CKD.

Low serum calcitriol levels are associated with an increased risk for vascular calcification in the general population.[41] A significant decrease in serum calcitriol levels is observed at the early stages of

CKD.[145,146] As stated, VSMCs express hydroxyvitamin D3–1α hydroxylase[128] and the VDR.[130,131] This evidence suggests that altered local actions of vitamin D on vascular cells contributes to the development of vascular disease, including vascular calcification, in patients with CKD. Furthermore, recent clinical observations demonstrate that vitamin D analogs provide a survival benefit for patients with CKD independent of serum Ca^{2+}, P^{2-}, and PTH levels.[42–46] This beneficial effect results, in part, from the corrective actions of vitamin D analogs, which activate the VDR in vascular cells locally as well as systemically. Further investigation is necessary to clarify the precise mechanisms by which these vitamin D analogs have beneficial effects on the cardiovascular complications seen in patients with CKD and how this affects the mortality of patients with CKD.

CONCLUSIONS

Although research efforts in the past decade have greatly improved our knowledge of the multiple factors and mechanisms involved in vascular calcification in patients with CKD, many questions remain unanswered. No longer can we accept the concept that vascular calcification in CKD is a passive process resulting from an elevated ($Ca^{2+} \times P^{2-}$). Rather, as a result of the metabolic insults of diabetes, dyslipidemia, oxidative stress, uremia, and hyperphosphatemia, "osteoblast-like" cells form in the vessel wall. These mineralizing cells as well as the recruitment of undifferentiated progenitors of the osteochondrocyte lineage play a critical role in the calcification process. Important transcription factors such as osterix,[147] Msx 2,[92,148] and RUNX2[52,67,76] are crucial in the osteogenic programming. Importantly, as shown by Shanahan and colleagues,[19,20,74] changes in VSMC matrix vesicle metabolism induced by hyperphosphatemia and uremia profoundly impair the arterial surveillance normally provided by mural smooth muscle, such as the phagocytic removal of procalcified matrix vesicles and apoptotic bodies. In addition, multifunctional roles have been shown for serum fetuin-A in inhibiting human vascular smooth muscle cell calcification (Figure 1).[75,113,115] Thus, the simultaneous increase in arterial osteochondrocytic programs and reduction in active cellular defense mechanisms creates the "perfect storm" of vascular calcification seen in ESRD.

The hemodynamic consequences of vascular calcification are the loss of arterial elasticity, increase in pulse wave velocity, development of left ventricular hypertrophy, decrease in coronary artery perfusion, and myocardial ischemia and failure.[2,34] These alterations are the main causes of mortality in the vast majority of patients with CKD. It is not unusual to see coronary calcification scores grater than 1000 units, an "exorbitant" value in very young patients maintained on hemodialysis. Thus, the mortality rates of patients who survive CKD and receive hemodialysis are striking; for example, a 30-yr-old patient with ESRD has the life expectancy of that of an 80-yr-old person with normal renal function. Currently, physicians are maximizing the efforts to control ($Ca^{2+} \times P^{2-}$). Although of critical importance, this approach has been insufficient to have a clinical impact on the progression of vascular calcification once initiated.

Innovative clinical studies addressing the combined use of inhibitors that work through distinct molecular mechanisms on vascular calcification such as fetuin-A, OPN, and BMP-7, among others, will be necessary to reduce significantly vascular calcification accrual and cardiovascular mortality in CKD. In addition, the roles of oxidative stress and inflammation on smooth vascular cell fate and function deserve further translational investigation.

Patient-oriented research is necessary to determine the extent to which arterial compliance, Windkessel function, and tissue perfusion can be meaningfully reversed by egress of vascular mineral deposits. An interdisciplinary working group in mineral metabolism and vascular disease would help both focus and advance the field. As physician-scientists with expertise in mineral metabolism, addressing these research challenges presents a unique opportunity to contribute to improving the cardiovascular health of patients with CKD.

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