Mechanistic Insights into Vascular Calcification in CKD

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ABSTRACT
Cardiovascular disease begins early in the course of renal decline and is a life-limiting problem in patients with CKD. The increased burden of cardiovascular disease is due, at least in part, to calcification of the vessel wall. The uremic milieu provides a perfect storm of risk factors for accelerated calcification, but elevated calcium and phosphate levels remain key to the initiation and progression of vascular smooth muscle cell calcification in CKD. Vascular calcification is a highly regulated process that involves a complex interplay between promoters and inhibitors of calcification and has many similarities to bone ossification. Here, we discuss current understanding of the process of vascular calcification, focusing specifically on the discrete and synergistic effects of calcium and phosphate in mediating vascular smooth muscle cell apoptosis, osteochondrocytic differentiation, vesicle release, calcification inhibition, expression, senescence, and death. Using our model of intact human vessels, factors initiating vascular calcification in vivo and the role of calcium and phosphate in driving accelerated calcification ex vivo are described. This work allows us to link clinical and basic research into a working theoretical model to explain the pathway of development of vascular calcification in CKD.


There is a well defined association between declining renal function and increasing cardiovascular morbidity and mortality both in the general population1 and in CKD patients.2,3 The prevalence and progression of vascular calcification increases dramatically once a patient is on dialysis,4 and the vascular phenotype of even young dialysis patients can be compared with that of octogenarians.5 CKD patients have the perfect storm of risk factors for the development of cardiovascular disease (CVD), and important modifiable risk factors include dysregulated mineral metabolism with high circulating levels of calcium and phosphate.6,7

However, exposure to high calcium and phosphate does not simply lead to a passive dumping of calcium and phosphate in dead or dying tissues. Recent evidence clearly establishes that vascular calcification is a highly regulated cell-mediated process that has many similarities to bone formation. Central to the calcification process is the vascular smooth muscle cell (VSMC).

In this review, we describe a model of intact human vessels from CKD and dialysis patients that allows us to study the earliest changes involved in the initiation of calcification in vivo.4 Ex vivo culture of these vessels in uremic conditions8 corroborates findings from in vitro and animal studies, showing that calcification in intact vessels is a vesicle-mediated process coincident with the phenotypic transformation of VSMCs to bone-like cells. Importantly, clinico-pathological correlations of ex vivo studies with clinical and epidemiologic findings that implicate the role of dialysis-related factors in driving calcification are described. Emerging concepts including the effects of vitamin D on the vessel wall, VSMC aging, and a potential role of the endothelium in mediating VSMC calcification are also briefly discussed.

SITES OF CALCIFICATION AND THEIR CLINICAL CONSEQUENCES

Two distinct, but sometimes overlapping, sites of calcification have been described in CKD patients: arterial intimal and medial calcification. Intimal calcification is seen with advancing age, hypertension, dyslipidemia, and smoking and takes the form of atherosclerotic vascular disease.9 It is a patchy and discontinuous process that involves inflammatory macrophages and VSMCs in lipid-rich regions of the atherosclerotic plaque. In contrast, medial calcification, also known as Mönckeberg’s sclerosis, is typically seen in diabetes and CKD. It involves sheet-like calcification in the tunica media with a concentric thickening of the vessel wall10 without involvement...
of the intima, and was originally assumed to be a degenerative age-related problem.\textsuperscript{11}

In adolescents and young adults with CKD, intimal calcification is rarely seen, but in adult CKD patients, because a number of traditional Framingham risk factors for atherosclerosis are also present, varying combinations of intimal and medial calcification may coexist. It is difficult to clearly differentiate intimal from medial calcification using current imaging modalities; however, the clinical consequences differ. Medial calcification leads to increased vascular stiffness,\textsuperscript{12} systolic hypertension, and left ventricular hypertrophy, whereas intimal calcification is associated with ischemic heart disease. One study suggests that hemodialysis patients with predominant intimal calcification have a higher relative risk of mortality than those with predominant medial calcification.\textsuperscript{13}

Although the differences between intimal and medial calcification imply different etiologies, the cell type that is central to all vessel wall calcification is the VSMC.

**MODEL SYSTEMS TO STUDY VASCULAR CALCIFICATION**

Animal knock-out models\textsuperscript{14–16} human VSMC explant cultures,\textsuperscript{10,17–22} and, most recently, organ culture of vessel rings\textsuperscript{4,23} have all been used to study vascular calcification, and each model offers unique advantages. Animal knock-out models provide insights into the effects of single gene defects and the crucial role of calcification inhibitors such as Fetuin-A,\textsuperscript{14} matrix Gla protein (MGP),\textsuperscript{16} and osteoprotegerin (OPG)\textsuperscript{15} in regulating ectopic calcification. Major mechanistic insights into the process of vascular calcification have also come from \textit{in vitro} studies, in particular, studies utilizing human VSMCs in culture that spontaneously convert to an osteochondrocytic phenotype,\textsuperscript{24}–\textsuperscript{26} forming multicellular nodules that spontaneously calcify, due to apoptosis and vesicle release by the resident nodular VSMCs.\textsuperscript{19} These membrane vesicles and debris form the initial nidus for mineral nucleation and subsequent calcification.\textsuperscript{19} However, VSMCs cultured from explants rapidly lose their contractile properties \textit{in vitro} and lack the matrix and tissue architecture of a normal vessel wall, which can limit this model.

In contrast, vessel rings cut from medium-sized muscular arteries and cultured \textit{ex vivo} have an intact matrix structure including elastic lamellae, and their VSMCs can maintain a normal contractile phenotype for prolonged periods in culture.\textsuperscript{4,24} Utilizing human vessels allows the study of a slowly progressive disease like CKD as well as examining the effects of dialysis on the vessels. Vessels from children provide an ideal model to study uremic influences on the vessel wall because they are free of pre-existing diseases like diabetes, dyslipidemias, and uncontrolled hypertension. In addition, vessels derived from CKD patients can be experimentally manipulated \textit{ex vivo} using this model system.

Both VSMC explants and the human vessel model have been used to provide insights into the roles of calcium and phosphate in mediating VSMC calcification and results from these studies are discussed in detail below.

**VASCULAR CALCIFICATION IS SIMILAR TO PHYSIOLOGIC BONE OSSIFICATION**

In bones and teeth where calcification is required, resident cells develop specific mechanisms to enable mineral nucleation and crystal growth in the extracellular matrix. Ectopic vascular calcification follows a very similar process to physiologic bone formation. VSMCs are of mesenchymal origin and under stress can differentiate to different mesenchymal-derived cell types such as osteoblasts, chondrocytes, and adipocytes, leading to calcification, altered matrix production, and lipid accumulation.\textsuperscript{24} At sites of calcification, VSMCs undergo an osteochondrocytic phenotypic change and upregulate expression of mineralization-regulating proteins that are normally confined to bone and cartilage.\textsuperscript{10} These proteins include a number of transcription factors, such as Runx2 (previously known as Cbfa-1), Osterix, Msx2, and Sox9, that induce differentiation of VSMCs to an osteochondrocytic phenotype.\textsuperscript{22,25} To create a microenvironment that is permissive for calcification, specialized membrane-bound bodies called matrix vesicles, serve as nucleation sites for hydroxyapatite.\textsuperscript{20,26} Under normal conditions, VSMC-derived vesicles do not calcify because they are loaded with mineralization inhibitors such as MGP and Fetuin-A.\textsuperscript{21} However, on exposure to high extracellular calcium, or with intracellular calcium release, and when calcification inhibitor levels are low, VSMCs produce mineralization-competent vesicles that contain preformed hydroxyapatite.\textsuperscript{20,27} To enable crystal growth, vesicles contain alkaline phosphatase (ALK), which creates a phosphate source by degrading pyrophosphate, a potent inhibitor of hydroxyapatite crystal growth. Matrix vesicles also contain annexins, sodium-dependent phosphate transporters, and concentrate phospholipid components on the vesicle membrane such as phosphatidylserine.\textsuperscript{28} Recent magnetic resonance spectroscopy studies show that both the hydroxyapatite microcrystalline structure as well as the glycosaminoglycan scaffold of calcified vessels are identical to those in bone.\textsuperscript{29} This phenotypic plasticity of VSMCs plays an important role in vascular repair, but the endpoint of dedifferentiation may also be VSMC death.

**CELLULAR MECHANISMS OF CALCIUM- AND PHOSPHATE-INDUCED CALCIFICATION**

The uremic milieu is a hostile environment that contains a large number of risk factors and reduced levels of physiologic inhibitors that allow the initiation and progression of vascular calcification. Clinical and epidemiologic studies consistently show that high circulating calcium and phosphate levels associate with calcification.\textsuperscript{2,6,7} Whereas calcium and phosphate can have distinct effects on VSMCs, their synergistic effects, in particular through hydroxyapatite nanocrystal formation,\textsuperscript{30,31} have also recently been shown to drive accelerated
calcification. The key processes include osteochondrocytic differentiation, apoptosis, and vesicle release and perturbation of calcification inhibitor levels.

**Calcium and Phosphate Uptake by VSMCs**

High serum phosphate levels are almost ubiquitous in advanced CKD. In addition, CKD patients are thought to have increased total body calcium levels, and chronic exposure to high circulating calcium and phosphate levels associates with the greatly increased calcification risk in this cohort. Importantly, studies in vitro and in animal models show the initiation of calcification requires an increased uptake of calcium and phosphate by the VSMCs. Phosphate enters through the sodium-dependent phosphate cotransporters, PiT-1 and PiT-2, in a time and concentration-dependent manner. Calcium uptake by VSMCs is required for their contractility, and is therefore tightly regulated by the calcium-sensing receptor (CaSR), as well as by voltage-gated calcium channels that mediate extracellular calcium uptake. The CaSR is capable of sensing changes in extracellular calcium concentrations in the millimolar range; its key role in the physiologic state is to regulate vascular myogenic tone. When VSMCs are exposed to high extracellular calcium in vitro, expression of the CaSR is downregulated. Ablation of CaSR function increases VSMC calcification, whereas calcimimetics, drugs that increase the sensitivity of the CaSR to calcium, reduce calcification. Calcium channels can be targeted by calcium channel blockers, drugs that are routinely used in the treatment of hypertension; in animals made hypercalcemic with vitamin D treatment, calcium channel blockers ameliorate calcification, and in a clinical trial in hypertensive patients, calcium channel blockers slow down the progression of calcification.

**Role in Osteochondrocytic Differentiation of VSMCs**

Phosphate also regulates the osteochondrocytic conversion of VSMCs, which involves the upregulation of osteochondrocytic genes with simultaneous down-regulation of smooth muscle cell genes. The key transcription factor that is essential for this process is Runx2. Osteoblastic VSMCs produce ALK, that in turn inactivates the mineralization inhibitors pyrophosphate and osteopontin and releases free phosphate. Several studies have shown that exposure of uremic animals to high phosphate leads to arterial medial calcification; calcification can be reversed, with a corresponding downregulation of Runx2, with a phosphate binder.

In part, bone morphogenic protein (BMP) signaling activates the transcriptional activity of Runx2. In response to vascular injury, the dedifferentiated VSMC increases osteogenic signaling through BMP-2, and BMP-2 is expressed in the atherosclerotic intima. When VSMCs are cultured in serum from uremic patients, it results in increased Runx2 expression that is inhibited by the BMP-2 inhibitor, noggin. Synthetic hydroxyapatite nanocrystals and isolated high-phosphate-induced nanocrystals have also been shown to upregulate the gene expression of BMP-2 and osteopontin in VSMCs, thereby driving the vicious cycle of accelerated calcification.

**Role in Apoptosis and Vesicle Release**

Calcium plays a key role in inducing apoptosis and in the formation and release of hydroxyapatite laden matrix vesicles that, in turn, form mineral nucleation sites to promote further calcification. Although calcium has been implicated as a major nucleator of crystalline hydroxyapatite, calcium and phosphate are synergistic in promoting VSMC calcification. When VSMCs in vitro are incubated in high calcium conditions, apoptotic cell death releases more calcium, which in turn may drive further apoptosis. Apoptotic bodies form a nidus for calcification and the propensity of these to calcify is markedly increased after calcium and phosphate treatment. Just as matrix vesicle calcification in bone is promoted by high calcium levels, high cytosolic calcium levels can change the intrinsic properties of VSMC-derived matrix vesicles and induce them to calcify. High calcium levels lead to annexin 6-phosphatidylserine nucleation complexes and enhanced matrix metalloproteinase-2 activity, which leads to elastin degradation and calcification. More importantly, exposure to high calcium levels will eventually deplete the endogenous calcification inhibitor, MGP, from matrix vesicles; when Fetuin-A levels are also reduced as in dialysis patients, vesicles contain preformed crystalline hydroxyapatite or become mineralization competent, thereby promoting further calcification. Interestingly, these processes are not driven to any great extent by phosphate alone, even at high concentrations.

More recently, some studies suggest that calcium-phosphate nanocrystals drive the vicious cycle of progressive calcification. One study reported that calcium-phosphate nanocrystals undergo lysosomal degradation by VSMCs, leading to very high intracellular calcium levels and subsequent cell death. The potency of calcium crystals was dependent on their size; small crystals of <1 μm diameter were more likely to undergo lysosomal phagocytosis. This study supports the clinical finding of accelerated calcification in dialysis patients. Once a nidus of calcification forms in the extracellular matrix of VSMCs, its uptake and phagocytosis will promote further calcification. Second, the osteochondrocytic differentiation of VSMCs was also induced by calcium-phosphate nanocrystals, rather than soluble phosphate alone. Calcium-phosphate nanocrystals upregulated BMP-2 expression and osteopontin levels increased but this was not associated with a concomitant increase in Runx2.

**ACCELERATED CALCIFICATION IN VESSELS FROM CHILDREN ON DIALYSIS: CLINICOPATHOLOGICAL CORRELATIONS**

Our group developed a model of intact human vessels removed at the time of routine surgery and described the changes
in medium-sized muscular arteries of CKD and dialysis patients both in vivo\(^1\) and after ex vivo manipulations.\(^8\) Vessels were taken at the time of transplantation (inferior epigastric artery) or peritoneal dialysis catheter insertion (omental arteries) and compared with mesenteric vessels removed from healthy controls during planned intra-abdominal surgery. The patient cohort was well characterized with clinical and biochemical data as well as vascular scans, allowing for translational studies with detailed clinicopathological correlations. All sampled vessels were studied at baseline and after ex vivo organ bath cultures, to mimic the uremic milieu, and assayed for quantification of the calcium load and ALK activity as well as detailed histology, immunohistochemistry, and electron microscopy. This work corroborates previous clinical data as well as in vitro studies and animal experiments, and provides quantitative evidence that calcium accumulation in the vessel wall begins predialysis\(^{48-50}\) and that factors specific to the dialytic milieu trigger accelerated calcification.\(^2,32,51\)

**In Vivo Changes in Predialysis and Dialysis Vessels**

Predialysis CKD vessels show calcium accumulation in the vessel wall that correlates with patient serum calcium \(\times\) phosphate levels over the preceding year (Figure 1).\(^4\) This was not associated with histologic signs such as osteochondrocytic differentiation, vesicle release, change in smooth muscle cell number, or perturbation in calcification inhibitors. Clinical studies suggest that predialysis CKD patients have preserved levels of the mineralization inhibitors Fetuin-A, OPG, MGP, and pyrophosphate,\(^52-54\) thereby protecting them from exposure to high calcium \(\times\) phosphate levels. In contrast, the calcium load in dialysis vessels was almost twice as high as in predialysis vessels, and strongly correlated with the time on dialysis. These findings are in keeping with clinical studies that report low levels of calcification in predialysis CKD patients, with calcification significantly increasing on dialysis.\(^50\) Our data confirm that mere exposure to a high calcium \(\times\) phosphate milieu is not sufficient to drive accelerated calcification, and that exposure to the dialysis milieu leads to key changes in the vessel wall that potentiate calcification.

To further investigate the mechanisms that drive accelerated calcification in dialysis vessels, we performed detailed histology and immunohistochemistry to examine the mechanisms that have previously been shown to be important in in vitro calcification models. Dialysis vessels show VSMC loss that was confirmed to be due to apoptotic cell death. Apoptosis was not seen in predialysis vessels with a similar calcium load, implying that dialysis-related factors cause VSMC apoptosis. In addition, hydroxyapatite laden vesicles are evident on electron microscopy and the vesicle components Fetuin-A and MGP are deposited in calcified areas. Clinical studies report that dialysis patients have reduced Fetuin-A levels,\(^55\) and MGP is in its undercarboxylated state,\(^56\) which has a greatly reduced capacity to inhibit calcification.\(^57\) VSMC death will not only reduce the production of calcification inhibitors like MGP,\(^21,58\) but can also increase local concentrations of calcium,\(^47\) thereby promoting the vicious cycle of VSMC death, vesicle release, and accelerated calcification. Previous studies using VSMCs in culture suggest that apoptotic bodies form a nidus for hydroxyapatite deposition,\(^19,20\) but this is the first study to show that calcification in vivo is a vesicle-mediated process.

In dialysis vessels, calcification is accompanied by increased ALK activity and associates with concomitant upregulation of the master bone regulator, Runx2, confirming in vivo osteoblastic conversion of VSMCs. Raised ALK levels are described in dialysis patients\(^59\) and in those with calcific uremic arteriopathy.\(^60\) In addition, a previous study showed mechanical injury to the vessel associates with raised ALK and calcification.\(^21\)

On histology, von Kossa staining shows hydroxyapatite crystal deposition only within the tunica media in all of the calcified vessels, implying that, at least in the earliest stages and in young CKD patients, calcification is arteriosclerotic rather than atherosclerotic. Furthermore, there was no neointima formation.

Figure 1. Mechanisms of calcification in VSMCs in culture and in intact vessel rings in predialysis and dialysis vessels. Superscript numbers indicate references to relevant articles.
and no evidence of inflammation characteristic of intimal calcification, wherein macrophage infiltration and inflammation are the earliest events preceding osteogenesis in atherosclerotic plaque, and TNF-α-dependent Msx2–Wnt signaling cascades trigger calcification.

Calcium- and Phosphate-Mediated Calcification in Predialysis and Dialysis Vessels Ex Vivo

To identify the factors that lead to VSMC death in dialysis, we cultured vessel rings from healthy controls as well as predialysis and dialysis patients in high calcium or high calcium and phosphate media up to 21 days in order to mimic the uremic milieu (Figure 2). The control vessels were completely resistant to calcification even after prolonged culture in highly calcifying conditions, suggesting that normal VSMCs possess intact inhibitory pathways that prevent calcification. In contrast, predialysis and dialysis vessels show a time-dependent accumulation of calcium that is significantly greater in dialysis vessels under identical ex vivo conditions. It is thought that previous exposure in vivo to the CKD milieu may have damaged the VSMCs or compromised their inhibitory mechanisms, thereby priming the vessels for calcification. In addition, vessels with histologically overt (von Kossa positive) calcification in calcium-phosphate media, suggesting that once a nidus for calcification is formed it acts to accelerate further calcification.

Recent studies suggest a role for calcium in mediating apoptosis and vesicle release. To determine the potencies of calcium and phosphate at inducing calcification, we incubated vessel rings in media with different calcium and phosphate contents but with an equivalent calcium-phosphate product of 5.4 mM. In the presence of high calcium and phosphate (2.7 mM calcium and 2 mM phosphate), both predialysis and dialysis vessels showed significantly greater calcification than in media with high phosphate (1.8 mM calcium and 3 mM phosphate), highlighting the potency of calcium at inducing calcification. Furthermore, the high calcium media caused greater VSMC apoptotic cell death. In high calcium media, dialysis vessels showed a 30% reduction in VSMC numbers due to apoptotic cell death, and calcification could be inhibited by the pan-caspase inhibitor ZVAD. This wave of rapid apoptosis is seen concomitantly with the formation of the first crystalline nidus of calcification observable by electron microscopy and supports in vitro studies showing that lysosomal degradation of calcium-phosphate nanocrystals promotes apoptosis.

High calcium and phosphate media also promote vesicle release, but this is only seen in dialysis and not in normal vessels; electron microscopy confirms the presence of crystalline apatite in vesicles. Moreover, deposition of dysfunctional undercarboxylated MGP and extensive crystalline calcification of the extracellular matrix are present in areas of vesicle release in the dialysis vessels. Vesicle release is likely to be an adaptive response, because vesicles extrude calcium from the cell into the extracellular matrix, thereby protecting the cell from intracellular calcium overload. Healthy vessels have normal levels of the inhibitory proteins Fetuin-A and MGP that act to block mineral nucleation within vesicles, whereas inhibitors are lacking in dialysis vessels and vesicles become procalcific. Vesicle release may be an adaptive response of VSMCs to prolonged exposure to high calcium and phosphate levels. In support of this, we found that normal vessels did not show vesicle release, but on acute and prolonged exposure to high calcium and phosphate ex vivo, they developed intracellular calcium overload and mitochondrial calcification, a precursor of necrotic cell death.
Taken together, our model of intact human vessel vessels describes VSMCs in different vessel types that have fundamentally different responses to high calcium and phosphate as a result of phenotypic changes induced during long-term exposure to dysregulated mineral metabolism in vivo. Although these processes may provide survival benefits in predialysis, prolonged exposure to high and potentially increasing levels of calcium and phosphate, depletion of functional calcification inhibitors, and the presence of hydroxyapatite nanocrystals themselves can overwhelm adaptive responses in dialysis vessels, leading to a vicious cycle of cell death and calcification.

**EMERGING CONCEPTS IN VSMC CALCIFICATION**

Exciting new studies have focused on pathways that regulate phosphate homeostasis, and three key areas of work include the fibroblast growth factor 23 (FGF-23)–Klotho axis and premature vascular senescence, the procalcic as well as protective roles of vitamin D on the vasculature, and a potential role of the endothelium in vascular calcification. These concepts are only recently being explored in *in vitro* and animal studies, and further mechanistic insights may be possible using the vessel ring model.

**FGF-23–Klotho and Vascular Senescence**

Clinical and epidemiologic studies in the healthy elderly population show that phosphate uptake and enhancing VSMC osteochondrocytic differentiation.67 Phosphate has recently been shown to promote VSMC senescence by interfering with expression of a key longevity gene, *sirtuin 1*,68 therefore increased phosphate uptake in response to decreased Klotho may promote premature VSMC aging. Importantly, senescent VSMCs have been shown to increase expression of BMP2, a major driver of osteochondrocytic differentiation.69,70

**Effects of Vitamin D on the Vasculature**

Vitamin D receptor activators (VDRA) are routinely used for the treatment of secondary hyperparathyroidism in CKD, but there are conflicting data on their effects on the vasculature. VSMCs express the vitamin D receptor and have functional 1α-hydroxylase and 25-hydroxyase enzyme systems so that all vitamin D metabolites can be utilized by them in an autocrine/paracrine fashion.71 VDRAs promote calcification by upregulating Runx2, osterix, and osteocalcin72,73 and also increasing calcium transport into the VSMCs.74 However, other studies show that vitamin D has protective effects by increasing calcification inhibitory proteins such as MGP and osteopontin,75 reducing pro-inflammatory cytokines, such as IL-6, IL-1β, and TGF-β.76 Importantly, FGF-23 can downregulate the renal 1α-hydroxylase receptor, thereby reducing vitamin D production,77 suggesting a complex interplay between FGF-23–Klotho and vitamin D on the vasculature. The proposed mechanisms of VDRA action on the VSMC are shown in Figure 3.

Many of the above studies use supraphysiological doses of VDRAs,72,73 and it is suggested that therapeutic doses of calcitriol, which suppresses parathyroid hormone without systemic hypercalcemia and hyperphosphatemia, do not induce vascular calcification.78 In addition, differential effects on vascular calcification have been demonstrated between different VDRAs that are independent of calcium and phosphate effects; doxercalciferol, a D2 analog, was...
shown to induce the expression of Runx2 and osteocalcin,79 whereas paricalcitol did not induce these effects or cause osteoblastic differentiation.76,79 Moreover, clinical and in vitro studies suggest that there may in fact be a bimodal association of vitamin D levels with vascular measures such that both low and high levels of vitamin D associate with abnormal vascular effects.80,81 Further studies are required to determine the optimal VDRA and its optimal dose for vascular health.

Endothelial–Smooth Muscle Cell Cross-Talk

One of the earliest events in the development of CVD is damage to the endothelium leading to alterations in vasoactive mediators to favor vasoconstriction.82 Given the close proximity of endothelium and smooth muscle cells in blood vessels, it could be postulated that cross-talk between these two cell types may alter the progression of vascular calcification. Current evidence suggests that vasodilators such as nitric oxide inhibit calcification and osteoblast differentiation by attenuating TGF-β,83 whereas vasoconstrictors like endothelin84,85 may accelerate calcification.

A further cause of unsatisfactory vascular repair in CVD are disturbances in growth factors involved in the formation of vascular networks, predominately vascular endothelial growth factor-A (VEGF-A) and angiopoietins (Ang).86 Elevated Ang-2 levels are associated with raised cholesterol and OPG, as well as mediators of microinflammation in CKD patients and are strong predictors of long-term mortality, independent of arterial stiffness or vascular calcification.87 Clear evidence exists that VEGF-A and Ang are involved in bone formation88,89 and given the similarities between this process and vascular calcification, it could be hypothesized that vascular growth factors may also play a role in this process. Indeed, VEGF-A promotes the BMP-induced mineralization of cultured VSMC.90 VEGF-A and Ang could modulate calcification by affecting endothelia through binding to VEGFR1/2 or Tie-2, respectively, leading to changes in vasodilators or vasoconstrictors. Alternatively, there is evidence that VEGF and Ang receptors are expressed on VSMCs themselves91,92; therefore, vascular growth factors may directly alter the biology of these cells (Figure 4).

These data suggest that altering molecules involved in regulating angiogenesis and vascular tone may be a novel therapeutic strategy to modulate vascular calcification and that studies investigating endothelia-smooth muscle signaling in intact vessel rings, wherein there is close proximity of these cells, are warranted.

CLINICAL PERSPECTIVE

Clinical and cell biology studies provide converging evidence to suggest that calcification begins in predialysis CKD.4,48 Surrogate markers of calcification,2,93,94 as well as a quantitative assessment of the calcium load in arterial biopsy samples,4 strongly correlate with exposure to high circulating calcium and phosphate levels.95 Thus, measures to reduce serum phosphate must begin early in CKD. Some studies suggest that treatment with a noncalcium-containing phosphate binder can attenuate the progression of calcification,51 but this does not translate to improved survival96 and a recent cohort study suggests that early treatment with any type of phosphate binder can independently improve survival.97

Our studies suggest that calcium plays a major role in VSMC apoptosis and vesicle release, but also acts synergistically with phosphate to drive accelerated calcification. Serum calcium levels have been independently associated with increased mortality in hemodialysis patients,92,98 but in fact the greatest mortality risks occur when high calcium and phosphate coexist.7 Although free (ionized) serum calcium levels are tightly regulated, transient hypercalcemic episodes, particularly in dialysis patients, as seen during hemodialysis or with the use of vitamin D analogs or calcium-based phosphate binders, may potentially influence vascular calcification.99–101 Moreover, it is important to remember that serum calcium does not reflect the total body levels, and the majority of dialysis patients are thought to have high total body calcium stores102; careful calcium balance studies are required to better understand calcium homeostasis in CKD patients, including how much of the

Figure 4. Vascular growth factors and calcification. One of the earliest phases of CVD is disturbances in molecules that control blood vessel growth such as VEGF-A and Ang. These could modify vascular calcification either indirectly by altering endothelia biology and the expression of vascular tone mediators, which in turn affect the VSMC. Alternatively, VEGF and Ang may alter VSMC biology directly because their receptors are also found on these cells.
ingested calcium, administered through diet or as a phosphate binder, is absorbed and the fate of this absorbed calcium. Furthermore, in low bone turnover states, fluxes in serum calcium and phosphate cannot be buffered by adynamic bone, resulting in ectopic soft tissue and vascular calcification. It would therefore seem wise to limit the total calcium intake from phosphate binders in dialysis patients, particularly in those with persistent or recurrent hypercalcemia, arterial calcification, and adynamic bone disease as recommended by the Kidney Disease Improving Global Outcome guideline.

Calcification progresses rapidly on dialysis, and vessels from dialysis patients show apoptotic VSMC damage and vessel release, suggesting that in the dialysis milieu, damage-inducing agents in addition to continued exposure to high and possibly worsening calcium and phosphate lead to progressive VSMC damage and calcification. Clinical studies suggest that once calcification begins, it can progress rapidly on dialysis; in a cohort of young hemodialysis patients, those who had baseline coronary calcification doubled their calcification scores within 20 months. Similar findings are shown in incident dialysis patients wherein those with no coronary calcification at baseline do not develop significant calcification up to the 18-month follow-up. These data suggest that pre-existing arterial calcification is one of the key factors predicting further progression of vascular calcification in CKD patients, raising the possibility of some protective mechanisms or genetic factors in play that prevent some patients from developing vascular calcification despite exposure to the uremic milieu. However, it may also be that the initial nidus of calcification in the vessel drives accelerated calcification in the procalcific uremic milieu. Longitudinal follow-up studies correlating the baseline vessel calcium load with progressive changes in the carotid artery intima-media thickness and coronary artery calcification score on computed tomography scans may provide important information on factors driving accelerated calcification. It is clear that the current dialysis practice (hemodialysis and peritoneal dialysis) associates with unacceptably high cardiovascular morbidity and mortality rates. Intensified or long daily dialysis show significantly better survival outcomes as well as improved VSMC biology and should be offered to patients where possible. The effects of dialysis are at best only partially reversed after transplantation, implying that pre-emptive renal transplantation should be considered the gold standard of renal replacement therapy.

Notably, vessel biopsies from CKD and dialysis patients show that all pre-dialysis and 25% of dialysis vessels have normal carotid artery intima-media thickness despite calcium accumulation in the vessel wall. Moreover, vessel stiffness, as measured by pulse wave velocity, and coronary calcification on a computed tomography scan were seen in only two children with the highest vessel calcium loads. This suggests that our currently available clinical measures are not sensitive enough to detect early stages of calcification, and a normal/negative test should be interpreted with caution. Whereas some authors suggest that screening programs for the detection and surveillance of vascular calcification in general clinical practice may allow for more accurate risk stratification and monitoring of treatment, scarce clinical resources may be better utilized for preventative strategies to reduce the modifiable risk factors for calcification from early CKD stages.

FUTURE DIRECTIONS

As the management of patients with CKD continues to improve, they no longer die from renal failure but from CVD. Vascular calcification develops early in CKD, progresses inexorably on dialysis, and is only partially reversed after transplantation. Dialysis vessels appear to have lost protective mechanisms to limit calcification progression, and once a nidus of hydroxyapatite crystals forms, calcium begets calcium. Although there are multiple risk factors for calcification in CKD, calcium and phosphate drive key processes in the initiation and progression of calcification.

The vasculature remains a difficult tissue to target and currently there are no effective treatments available. Vitamin D analogs remain an exciting therapeutic option, with their beneficial effects on cardiac and vascular function. Newer therapeutic targets including calcification inhibitors may hold promise for the future. Meanwhile, the prevention of mineral dysregulation, starting from the earliest stages of CKD, remains key to the reduction of cardiovascular mortality in CKD patients.

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None.

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