PAPER: URAEMIC VASCULAR CALCIFICATION

SM Moe, Associate Professor of Medicine and Associate Dean for Research, Indiana University School of Medicine, Indianapolis, United States of America

INTRODUCTION

Cardiovascular disease is the leading cause of death in patients undergoing dialysis,[1] but this cannot be fully explained by the traditional risk factors of diabetes, hypertension, family history, hyperlipidemia and smoking.[2] Dialysis-specific cardiovascular risk factors, such as elevations in oxidative stress, homocysteine, and advanced glycation end-products[3,4] have been suggested. In addition, hyperphosphataemia, hypercalcaemia, and elevations in the serum calcium x phosphorus (Ca x P) product have all been associated with increased mortality in patients undergoing dialysis,[5] with cardiovascular disease the primary cause of death associated with hyperphosphataemia.[6]

PATHOLOGY OF VASCULAR CALCIFICATION

In the general population, vascular disease of a variety of different types may be associated with calcification:

1. Atherosclerotic disease is characterised by fibrofatty plaque formation, and based on autopsy data and animal models, calcification was thought to occur late in the course of the disease[7,8] These plaques can protrude into the arterial lumen, leading to a filling defect on angiography. However, recent advances in imaging and intravascular ultrasound have demonstrated that atherosclerosis can also be a circumferential lesion, with calcification occurring earlier in the course of the disease.[9]
2. Arteriosclerosis affects the medial layer of elastic arteries, thickening and stiffening them. This manifests clinically as elevated pulse pressure and increased pulse wave velocity, and is commonly associated with systolic hypertension in the elderly. Systolic hypertension is known to be associated with increased mortality,[10] and for a given level of systolic hypertension, the risk of cardiovascular mortality is further increased by elevations in the pulse pressure.[11] Arteriosclerosis may be associated with calcification, and the degree of calcification correlates with the pulse pressure in the general population.[12]
3. Mönckeberg's calcification, or medial calcinosis affects smaller elastic arteries.[13] This disease is more common in patients with diabetes, renal failure and advanced ageing. Contrary to previous teaching, recent studies have found that this form of medial calcification in distal vessels is associated with increased all-cause and cardiovascular mortality in diabetic patients.[14,15]

IMAGING OF VASCULAR CALCIFICATION

Plain radiographs can be used to assess the prevalence of vascular calcification, although they lack the sensitivity to allow quantitation of calcium longitudinally. While there is some distinct appearance of medial compared to intimal calcification on plain radiographs,[16] the reproducibility across multiple research sites has not been evaluated. Ultrasound can assess the magnitude of vascular calcification, but also only allows semi-quantitative assessments.

More recently ultra-fast computed tomography (CT) scans with electrocardiogram (ECG) gating have allowed imaging of coronary arteries only in diastole, avoiding motion artifact. Electron beam CT scanning (EBCT) has been extensively used for the assessment of vascular calcification,[17] but is not widely available. Two alternative techniques for gating to ECG tracings during spiral CT have been described. In prospective gating, images are only acquired during diastole. In retrospective gating, images are taken throughout the cardiac cycle, and subsequently diastolic images are selected by matching to ECG tracings.[18,19] These techniques have allowed reproducible quantitation of coronary artery and aorta calcification, but unfortunately do not allow differentiation of medial from intimal calcification.

VASCULAR CALCIFICATION IN DIALYSIS PATIENTS

The high prevalence of vascular calcification in patients with chronic kidney disease (CKD) is not a new observation. Ibels *et al.* in 1979[20] demonstrated that both renal and internal iliac arteries of patients undergoing a renal transplant had increased atherogenic/intimal disease and increased calcification (detected by chemical methods) compared to transplant donors. In addition, the medial layer was thicker and more calcified in the uraemic patients compared to the donors.[20] A more recent study evaluated coronary arteries obtained at autopsy in dialysis patients compared to age matched, non-dialysis patients who had died from a cardiac event.[21] This study found a similar magnitude of atherosclerosis (plaque burden and intimal thickness) in the dialysis patients compared to controls, but the plaque was more heavily calcified in the dialysis patients. In addition, morphometry of the arteries demonstrated increased medial thickening,[21] although calcification in the medial layer did not appear to be increased in the proximal 2–3 cm of each artery. The distal vessel was not specifically evaluated. Thus, there is histological

evidence for increased arterial calcification in coronary, renal and iliac arteries from patients on dialysis compared to non-dialysis patients.

DOES OUR CHOICE OF PHOSPHATE-LOWERING THERAPY MAKE THE PROBLEM WORSE?

In recent years, the mainstay of phosphate-binding therapy to control blood phosphate levels in renal failure has been oral calcium compounds. Could increased calcium intake be contributing to the calcification seen in dialysis patients? Braun *et al.*[22] demonstrated that coronary artery calcification by EBCT increased with advancing age in patients on dialysis and that the calcification scores were two to fivefold greater in dialysis patients than age matched individuals with normal renal function and angiographically proven coronary artery disease. Goodman *et al.*[23] demonstrated that advanced calcification can also occur rapidly in the coronary arteries of children and young adults, and found a relationship between calcification score and increasing doses of calcium-containing phosphate binders, as well as increased Ca x P product. Subsequently, a prospective study[24] randomised haemodialysis patients to calcium-containing phosphate binders or to sevelamer, a non-calcium phosphate binder, and found that those on sevelamer were protected from the rise in the coronary artery and aorta calcification that was seen by EBCT at 26 and 52 weeks in those on calcium-containing binders. Both groups had similar, good control of serum phosphorus. There was more hypercalcaemia and over-suppression of parathyroid hormone in the calcium binder arm, and a lowering of the low-density lipoprotein (LDL) cholesterol in the sevelamer arm. Whether the mechanism of the difference was excess calcium intake, the impaired bone remodeling as a result of low parathyroid hormone level, or the LDL cholesterol lowering is not known. Most likely, it is a combination of these factors.

Several other authors have determined risk factors associated with coronary artery calcification or the degree of calcification (reviewed in Hujairi NM *et al.*[25]). Only age and duration of dialysis have been consistently identified. Mineral metabolism factors such as elevated phosphorus, elevated Ca x P, or calcium load from phosphate binders, have not been uniformly identified as risk factors (Table 1).

IS VASCULAR CALCIFICATION AS BAD FOR DIALYSIS PATIENTS AS FOR THE GENERAL POPULATION?

While coronary calcification is associated with poor outcomes in the general population,[26] this relationship has not been demonstrated in the dialysis population, where the process may be different, and may not have the same causes and significance. However, in a recent study in a small cohort of haemodialysis patients coronary artery calcification scores were higher in patients who died or were hospitalised in the 15 months following the baseline scan, than in those who were alive or not hospitalised.[27] These results need to be confirmed in a larger study, but it seems intuitive that such excessive coronary artery calcification cannot be good.

In contrast to coronary artery calcification, data in dialysis patients has demonstrated that patients with intimal calcification of the femoral artery shown by plain radiograph, was associated with increased all-cause and cardiovascular mortality compared to those with medial calcification, which in turn were statistically greater than those with no calcification.[16] These data have been recently duplicated by Adragao *et al.* using hand and

TABLE 1
Risk factors for vascular calcification in CKD.

Risk factor	Intimal/atherosclerotic calcification	Medial/Mönckeberg's calcification
Dyslipidaemia	Yes	No
Advanced age	Yes	Yes
Elevated blood pressure	Yes	Reciprocal (medial lesions worsen blood pressure)
Male	Yes	No
Smoking	Yes	No
Inflammation	Yes (local)	Yes (systemic mediators)
Diabetes/glucose intolerance	Yes	Yes
Reduced GFR	No	Yes
Hypercalcaemia	No	Yes
Positive balance	No	Yes
Hyperphosphataemia	Yes	Yes
PTH abnormalities	No	No
Vitamin D administration	No	Yes
Duration of treatment with dialysis	No	Yes

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pelvic radiographs.[28] In addition, calcification of the larger arteries is associated with increased pulse wave velocity and pulse pressure[29, 30] suggesting that calcification can lead to arterial stiffness. Increased pulse wave velocity[30] and pulse pressure[31] are associated with increased mortality in dialysis patients. Alterations in mineral metabolism appear to be more uniformly associated with increased calcification in peripheral arteries, in addition to advancing age and increased duration of dialysis.[16, 32] Table I identifies risk factors associated with both intimal calcification and medial calcification in dialysis patients based on data reviewed during a consensus conference held by the National Kidney Foundation in 2003.[33] As is clear from Table I the pathogenesis of all forms of vascular calcification is multi-factorial.

PATHOGENESIS OF VASCULAR CALCIFICATION: A RECIPROCAL RELATIONSHIP WITH BONE?

Epidemiological studies in post-menopausal women and the ageing general population have demonstrated that patients with osteoporosis have increased atherosclerosis and, more recently, increased coronary artery calcification.[34–8] The ability of bone to mineralise appears to peak between 25–35 years old. Thereafter, bone mineral content decreases gradually, with a 5-year acceleration at the time of menopause in women.[39] Interestingly, coronary artery calcification progresses from between the ages of 25–35 years until death.[40] Drake *et al.* recently found genetic evidence linking atherosclerosis with osteoporosis in mice with diet induced atherosclerotic disease,[41] and several knock-out mice models demonstrate a linkage between bone demineralisation and vascular calcification (reviewed in Wallin R *et al.*[42]). Furthermore, treatments aimed at improving bone density have been found helpful in preventing vascular calcification in animal models, including bisphosphonates.[43–6] This same inverse relationship has been found in dialysis patients, as Braun *et al.* also found a significant correlation between increased coronary artery calcification by EBCT and decreased bone mineral density by CT in a cross-sectional analysis.[22] Thus, it is plausible that in the ageing general population, and in patients with CKD, there is preferential mineralisation in vascular tissue instead of bone. Alternatively, if bone is abnormal, mineralisation occurs in vascular tissue. The latter may be an important factor in patients with renal disease as bone remodelling is clearly impaired and associated with calcification.[47]

VASCULAR CALCIFICATION IS A COMPLEX AND REGULATED PROCESS

Previous dogma was that vascular calcification represented cell necrosis, or simply an artifact of serum supersaturation for calcium and phosphate (which is obviously increased in renal failure), and thus a passive deposition. However, recent evidence suggests that this is a complex, regulated process. Animal knock-out models have demonstrated that selective deletion of many genes, including matrix gla protein (MGP),[48] osteoprotegerin[49] and others (reviewed in Wallin R *et al.*[42]), leads to vascular calcification. Pathologic analysis of specimens from both coronary arteries and peripheral arteries of non renal failure patients demonstrates the presence of bone proteins in areas of calcification.[50–4] Furthermore, it was demonstrated that vascular smooth muscle cells (VSMC) isolated from human or bovine arteries were capable of mineralising *in vitro*[50, 55] in a similar manner to osteoblasts.[56] These data demonstrate that both intimal and medial calcification are regulated processes that parallel osteogenesis in several respects. Furthermore, using this *in vitro* VSMC model, several of the non-traditional risk factors associated with cardiovascular disease and/or vascular calcification in CKD patients have been found to increase mineralisation including elevated phosphorus,[57] decreased parathyroid hormone and parathyroid hormone-related peptide,[58] calcitriol,[59] uraemic serum,[60] advanced glycation end-products,[61] alterations of lipoproteins,[62–4] and homocysteine.[65]

DIALYSIS PATIENTS EXPRESS BONE PROTEINS IN BLOOD VESSELS

To examine the pathophysiology of vascular calcification observed in dialysis patients, we examined arteries histologically.[66, 67] We found expression of bone proteins in calcified arteries from patients with calcific uraemic arteriolopathy (calciphylaxis), and in the inferior epigastric arteries from dialysis patients undergoing renal transplantation.[67]

The presence of positive immunostaining for these bone proteins was found more frequently than was overt calcification, which suggests that the deposition of these proteins precedes calcification.[67] Thus, *ex vivo* findings suggest that the initial changes that occur in the vessels of dialysis patients are the deposition of these bone matrix proteins, followed by calcification.

It is hypothesised that uraemia induces calcification. In order to further understand the mechanism by which this 'bone like' process occurs in blood vessels, we incubated bovine vascular smooth muscle cells (BVSMC) in the presence of normal human pooled serum compared to pooled human serum from haemodialysis patients on dialysis for at least two years (to eliminate residual renal function).[60] Using these pooled sera *in vitro,* we demonstrated that uraemic serum led to increased and accelerated calcification in BVSMC *in vitro.* Furthermore, the uraemic serum upregulated the expression of osteopontin in BVSMC compared to normal serum, a process that was partially dependent on both alkaline-phosphatase and sodium-phosphate co-transport.[60] Of importance, the final media concentration of phosphorus was similar in the BVSMC cultures with 10% normal and those with 10% uraemic serum (~0·5 mM),[60] well below levels known to induce calcification in the work by Jono

et al.[57] This confirms observations in non-dialysis patients that VSMC can behave like osteoblasts. Given that both cell types originate from the same mesenchymal stem cell, this change may well represent the expression of normally repressed genes.

The transcription factor Core binding factor alpha 1 (Cbfa1) is thought to be the 'switch' that turns a pluripotent stem cell into an osteoblast during normal fetal development. Cbfa1 knock-out mice fail to form mineralised bone, proving that Cbfa1 is critical for the initial differentiation of osteoblasts.[68, 69] In addition, arteries from the MGP knock-out mice lose smooth muscle markers and gain expression of Cbfa1 as they progressively mineralise their arteries.[70] Work by Giachelli *et al.* has clearly demonstrated that phosphorus induces the expression of Cbfa1 in cultured VSMC.[57,71] It was pertinent to ask if it was the phosphorus in the uraemic serum or other factors that were important in uraemia serum induced vascular calcification. We found that in cultured BVSMC incubated with normal human serum there was an upregulation of Cbfa1 by RT-PCR in the presence of phosphorus in the form of beta-glycerophosphate. However, in BVSMC incubated with pooled uraemic serum, Cbfa1 expression was already upregulated to the same level, and additional phosphorus failed to augment expression further.[72] Thus, uraemic toxins other than serum phosphorus can induce Cbfa1, and calcification, *in vitro*. *Ex vivo* evidence has also been found of the expression of Cbfa1 in VSMC adjacent to both medial and intimal calcification in inferior epigastric arteries obtained at the time of kidney transplant.[72] Further supporting this observation, expression of Cbfa1 has also been observed in calcification of atherosclerotic plaques from patients without CKD.[73] Taken together, these results support that Cbfa1 may be a key regulatory factor in the pathogenesis of vascular calcification in dialysis patients. Thus, uraemic toxins, and/or elevations in serum phosphorus (and likely multiple other factors) induce this osteoblast phenotype via upregulation of Cbfa1. These cells can then lay down a matrix of collagen and non-collagenous proteins in the arterial wall, providing the scaffolding on which calcification can then occur.

INHIBITORS OF VASCULAR CALCIFICATION IN DIALYSIS PATIENTS

Vascular calcification is very prevalent, but not uniform, in dialysis patients. Depending on the series, an average of 17% of dialysis patients have no vascular calcification, and remain free of it on follow-up.[25] While youth gives some protection, other factors must be involved, and natural inhibitors of vascular calcification have come under scrutiny.

Matrix gla protein is a locally produced calcification inhibitor. We measured serum levels of MGP in dialysis patients, and found no significant correlation between serum MGP levels and coronary artery or aorta calcification score by spiral CT in a cohort of dialysis patients,[74] despite findings of correlation in non-CKD patients.[75] However, we then examined sections from the inferior epigastric artery of dialysis patients and demonstrated that MGP expression correlated with the presence of calcification by Von Kossa staining.[74] The increased MGP expressed locally during vascular calcification may limit the extent of calcification since MGP can bind to bone morphogenic protein-2 (BMP-2), a pro-mineralisation factor.[76]

Fetuin-A (AHSG or a2-HS glycoprotein) is a circulating inhibitor of calcification which inhibits the *de novo* formation and precipitation of calcium phosphate.[77] It is mainly produced in the liver in adults[78] and is abundant in plasma. In bone marrow stromal cells, fetuin-A binds to BMP-2 and transforming growth factor β, inhibiting mineralisation, and suppressing the expression of bone matrix proteins.[79, 80] Fetuin-A knock-out mice have extra-skeletal calcification in the presence of hypercalcaemia or when cross-bred on a mouse strain with a pre-disposition to calcification.[81] In humans, expression of fetuin-A is inversely correlated with the acute phase response. A study by Ketteler *et al.* has demonstrated that fetuin concentration in serum of dialysis patients was inversely related to C-reactive protein. Furthermore, low fetuin-A levels were associated with increased cardiovascular mortality.[82] Serum from dialysis patients with the syndrome of cutaneous vascular occlusion with calcification known as calcific uraemic arteriolopathy, or calciphylaxis, had impaired *ex vivo* capacity to inhibit hydroxyapatite precipitation, which could be normalised by the addition of purified fetuin-A.[81] However, serum fetuin-A levels are not uniformly low in dialysis patients,[82] thus, fetuin-A deficiency may only be a factor in some patients, or perhaps there is a relative deficiency of fetuin-A given the high serum concentration of calcium and phosphorus. The precise role of fetuin-A and other inhibitors in the vascular calcification in CKD patients remains to be determined, but clearly, there are multiple mechanisms to regulate extra-skeletal calcification: this complex process is only beginning to be understood.

CONCLUSION

In conclusion, it is hypothesised that vascular calcification in dialysis may be a three-step process (Figure 1). First, VSMC are stimulated by uraemic toxins, including phosphorus, to transform into osteoblast-like cells. Cbfa1 may be critical for this differentiation, or it may be only a marker. These cells then lay down a bone matrix of type I collagen and non-collagenous proteins. The final step may be mineralisation of this matrix, in part through physiochemical processes, and in part guided by matrix proteins and osteoblast-like cells, and modulated by inhibitors. The abnormal bone remodelling that occurs in CKD accelerates this by

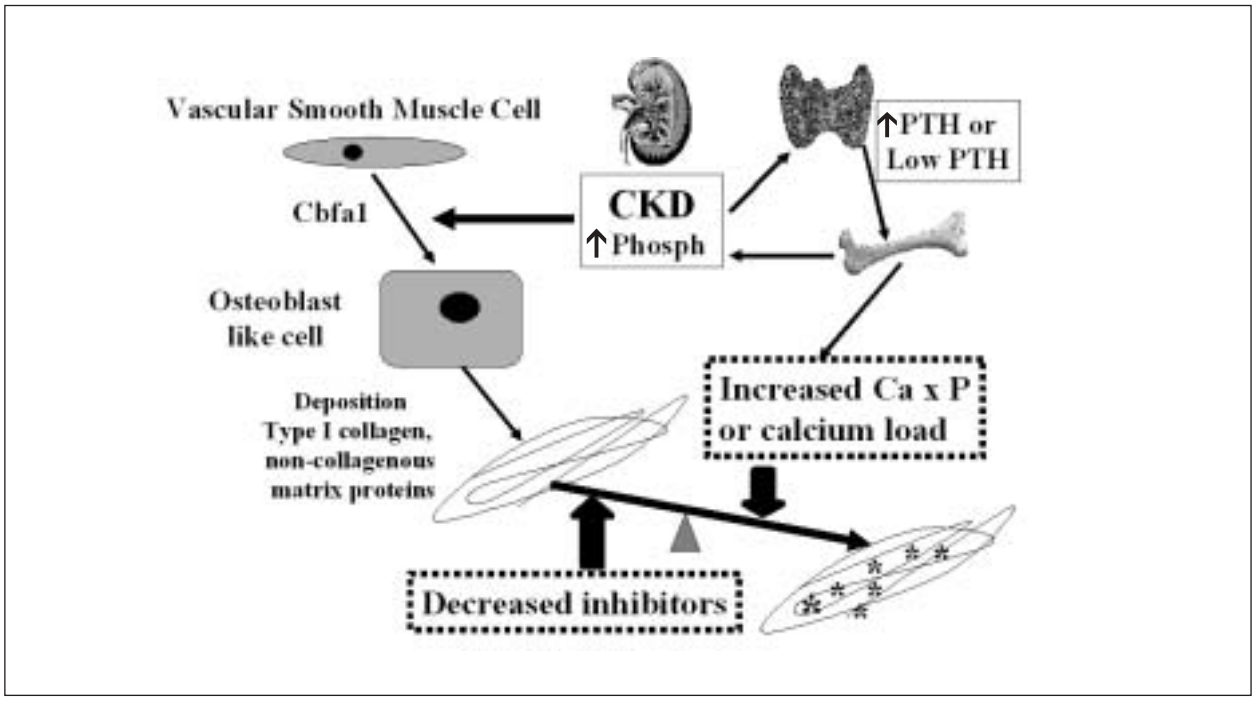

FIGURE 1

Hypothesis of mechanism of vascular calcification in CKD.

VSMC become osteoblast-like via upregulation of Cbfa1 in response to uraemic toxins and/or phosphorus. These cells can then lay down a matrix of collagen and non-collagenous protein. This matrix will become calcified when there is increased mineral availability or decreased inhibitors present. Abnormal bone in CKD contributes to the increased mineral availability due to hyperparathyroidism, with release of calcium and phosphorus from bone, or over-suppressed parathyroid hormone leading to an inability of bone to take up the buffer.

preventing bone from serving as the appropriate reservoir for excess mineral.

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