

# Vascular Calcification in Chronic Kidney Disease

By Neal X. Chen and Sharon M. Moe

Dialysis patients have increased cardiovascular morbidity, mortality, and vascular calcification, and the latter appears to impact the former. Recent evidence indicates that vascular calcification is an active, cell-mediated process. Osteoblast differentiation factor *Cbfa1* and several bone-associated proteins (osteopontin, bone sialoprotein, alkaline phosphatase, type I collagen) are present in histologic sections of arteries obtained from patients with end-stage renal disease (chronic kidney disease stage V [CKD-V]). This supports the theory that vascular smooth muscle cells can dedifferentiate or transform to osteoblast-like cells, possibly by up-regulation of *Cbfa1*. In *in vitro* experiments, addition of pooled serum from dialysis patients (versus normal healthy controls) accelerated mineralization and increased expression of *Cbfa1*, osteopontin, and alkaline phosphatase in cultured vascular smooth muscle cells. Clinically, the pathogenesis of vascular calcification is not completely understood, although increased levels of phosphorus and/or other potential uremic toxins may play an important role by transforming vascular smooth muscle cells into osteoblast-like cells. Presumably, once this process begins, increased serum calcium X phosphorus product, or calcium load from binders, accelerates this process. In addition, it is likely that circulating inhibitors of calcification are also important. Further understanding of the pathophysiology of vascular calcification is needed to intervene appropriately.

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**V**ASCULAR CALCIFICATION in general, and coronary calcification in particular, is present in almost all subjects over age 65, and is more prevalent and severe in patients with chronic kidney disease stage V (CKD-V).<sup>1-3</sup> The assessment of coronary arteries by new noninvasive imaging techniques such as electron beam computed tomography scan and intravascular ultrasound has heightened the awareness that over 70% of atherosclerotic plaques observed in the aging population are calcified.<sup>4</sup> In the general population, epidemiologic studies have shown that vascular calcification is correlated positively with atherosclerotic plaque burden and is associated with an increased risk for myocardial infarction,<sup>5,6</sup> peripheral ischemic vascular disease,<sup>7</sup> and morbidity after angioplasty.<sup>8</sup> Furthermore, in a recent study of 79 sudden cardiac death cases, both the Framingham risk index and coronary calcification were shown to be predictive of future cardiovascular events.<sup>9</sup> Another form of vascular calcification, medial calcification, also is predictive of general cardiovascular morbidity and mortality. Lehto et al,<sup>10</sup> in a study of 1,059 patients with type II diabetes, showed that medial artery calcification was a strong independent predictor of total cardiovascular and coronary heart disease mortality, and also a significant predictor of future stroke and amputation.

Although arterial calcification has been recognized for more than 150 years,<sup>11</sup> the basic pathogenesis of this disorder still is not understood completely. Until recently, vascular calcification had been regarded as a passive process associated with

atherosclerosis or normal aging, resulting from a nonspecific response to tissue injury or necrosis. Now there is strong evidence that vascular calcification is an active and regulated process that shares features with normal embryonic bone formation and bone repair.<sup>1,3,12-19</sup>

## PATHOLOGY OF VASCULAR CALCIFICATION

Pathologically, there are 2 patterns of vascular calcification (Fig 1). Typical atherogenesis is found in large vessel disease and coronary arteries and is associated with lipid-laden macrophages and intimal hyperplasia. Calcification is evident later in the disease course in grade IV and V lesions by Stary's<sup>20</sup> classification. Intimal calcification has a diffuse, punctate morphology and appears as aggregates of calcium crystals. The process then extends from the intima into the media.<sup>20</sup> The result is calcification of existing atheromatous plaques, leading to occlusive coronary artery disease, angina, and infarction. In contrast, medial calcification (also called Mönckeberg's calcification or me-

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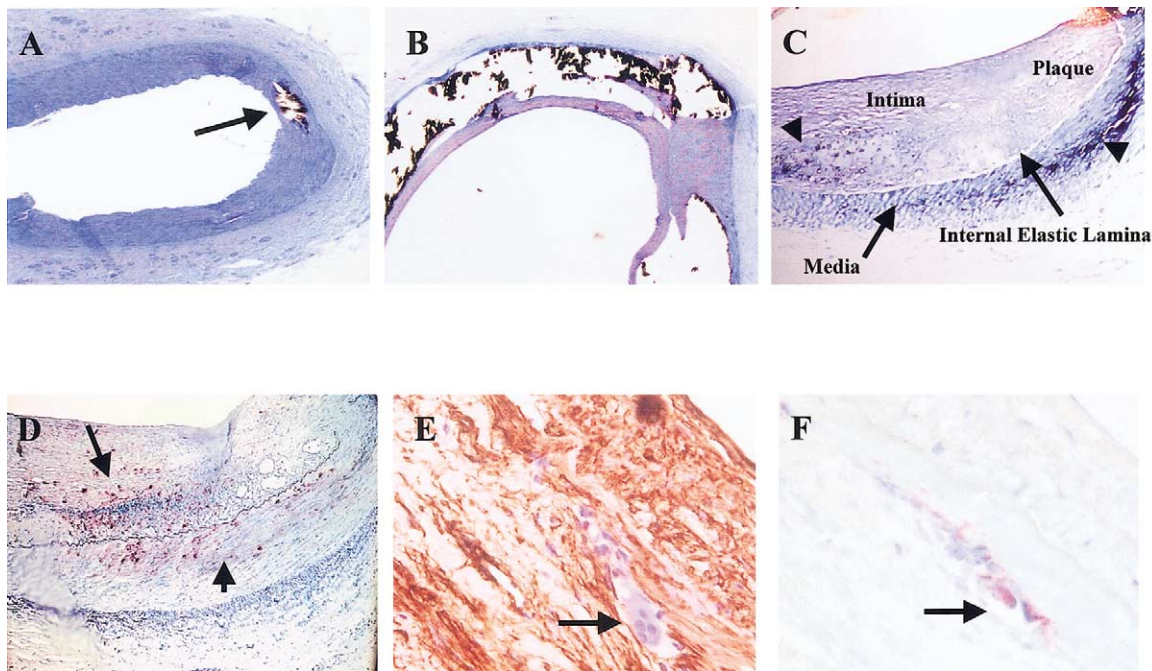
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**Fig 1. Medial compared with intimal calcification. (A-C) MacNeal stain (olivine blue and silver stain) of inferior epigastric artery from CKD-V patients. (A and B) Medial calcification (black color, arrow) begins in the medial layer and does not protrude into the lumen or affect the intimal layer. (C) In contrast, with typical atherosclerotic disease there is intimal proliferation and plaque formation that extends into the lumen, and therefore is detected as a filling defect on angiography. The calcification, detected in black (arrowheads), is seen adjacent to an intimal plaque and in the medial layer, showing that both processes can occur in the same artery. (D) The same artery as in C, showing expression of Cbfa1 by in situ hybridization in areas of calcification in both the media (arrowhead) and intima (arrow). (E) The medial layer of an inferior epigastric artery immunostained for type I collagen (brown). The presence of a multinucleated giant cell (arrow) is observed in the medial layer. (F) Same multinucleated cell from E showing positive staining for tartrate-resistant acid phosphatase, indicating an osteoclast-like cell (A-D reprinted from Moe et al<sup>19,67</sup> with permission).**

dial calcinosis) occurs independently of intimal calcification/atherosclerosis. Medial calcification occurs initially in the media of the vessel, usually at the internal elastic lamina and is not associated with lipid-laden macrophages or intimal hyperplasia.<sup>15,21</sup> As it progresses, a dense circumferential sheet of calcium crystals forms in the center of the media, bound on both sides by vascular smooth muscle cells, and may contain bone trabeculae and osteocytes.<sup>15</sup> Mönckeberg's calcification is described most commonly in distal vessels of patients with diabetes and renal failure.<sup>22</sup> It is now clear that medial calcification is very common in multiple arteries in patients with CKD-V.

The gold standard diagnostic tool for atherosclerotic calcification is angiography because the plaque protrudes into the lumen, producing a filling defect. In contrast, medial calcification is not readily detectable by angiography because no fill-

ing defect is produced (Fig 1). However, both forms of calcification can be detected by imaging techniques such as electron beam computed tomography scan,<sup>23</sup> spiral computed tomography,<sup>24</sup> and ultrasound.<sup>17,25</sup> The result of medial calcification is hard arteries, with increased pulse pressure, decreased compliance, and a resultant inability to compensate appropriately with increased blood flow during times of stress. Our recent studies evaluating the inferior epigastric artery in patients with CKD-V undergoing a renal transplant indicated that a Mönckeberg's pattern is also very prevalent in other nondistal, small arteries.<sup>18,19</sup> In addition, calcific uremic arteriolopathy (CUA or calciphylaxis) represents a form of medial vascular calcification.<sup>18</sup> Histologically, CUA is characterized by medial calcification of cutaneous and subcutaneous arteries and arterioles, with resultant tissue ischemia leading to skin ulceration. Gastro-

intestinal perforation caused by medial calcification of arteries also has been reported. Thus, various clinical outcomes can result from calcification of multiple vessels, representing a continuum of pathologies from vascular calcification depending on which artery is involved. However, it is important to emphasize that there are 2 distinct forms of arterial calcification: intimal/atheromatous and medial calcification. Both types are very common in CKD-V, both can co-exist in the same vessel, and both have undesirable hemodynamic consequences (Fig 2).

#### PATHOPHYSIOLOGY OF VASCULAR CALCIFICATION

Pathologic analysis of arteries shows that vascular calcification is associated with the production of bone proteins by vascular smooth muscle cells, such as osteopontin, bone sialoprotein, bone morphogenetic protein 2, alkaline phosphatase, matrix gla protein, osteocalcin, and type I collagen.<sup>12,13,15,26</sup> Knock-out mice models provide further evidence of the importance of these proteins and the presence of a regulated process. Animals deficient in osteoprotegerin and matrix-gla protein have impaired bone mineralization and osteopenia.<sup>27,28</sup> In addition, these animals also have vascular calcification. Many other knock-out mice have been found to have vascular calcification,<sup>29</sup> supporting the theory that some proteins appear to provide protection from vascular calcification. Furthermore, epidemiologic data<sup>30,31</sup> shows a correlation between osteoporosis and atherosclerosis, suggesting that when bone does not mineralize properly, the vessels do and vice versa. These data show that the calcification of vascular tissue is an active process, similar to that which occurs in bone and that the processes are interrelated.

Normal bone mineralization refers to the ordered deposition of hydroxyapatite on a type I collagen matrix.<sup>32</sup> Histologically, mineral initially appears in matrix vesicles. These vesicles are membrane-bound bodies that exocytose from the plasma membrane of osteoblasts and migrate to the loose extracellular matrix space of bone. Many noncollagenous matrix proteins involved in apatite nucleation have been identified, including osteopontin, osteonectin, and bone sialoprotein. These collagen and noncollagenous proteins appear concurrent with matrix vesicle formation and are felt to regulate the mineralization process in

bone through a balance of proteins that promote, and others that inhibit, mineralization. These same processes appear to regulate vascular calcification. Matrix vesicles, which initiate the calcification in normal mineralizing endochondral bone, have been found in calcifying arteries,<sup>18,33</sup> and the mineral identified in calcified arteries is hydroxyapatite, similar to that found in bone.<sup>34</sup> The increased expression of type I collagen, which is known to be critical in the formation of the initial calcified structure in bone, is up-regulated in atherosclerotic plaque<sup>35</sup> and in medial calcification.<sup>19</sup> In vitro, type I collagen promotes calcification of vascular smooth muscle cells.<sup>36</sup> Bone sialoprotein is an acidic extracellular matrix glycoprotein that can bind to collagen and can nucleate hydroxyapatite in bone.<sup>37</sup> Normal arteries express minimal or no bone sialoprotein whereas expression of bone sialoprotein is up-regulated in the media from patients with distal Mönckeberg's sclerosis<sup>15</sup> and in medial calcification of arteries in patients with CKD-V.<sup>19</sup>

Osteopontin can bind calcium and mediate cell adhesion in bone, and the colocalization of osteopontin with biomineralization in hard tissue, and its ability to bind and potentially orient calcium, suggests that osteopontin might function to promote calcification in vivo.<sup>38</sup> However, in vitro, in vascular smooth muscle cells, osteopontin inhibits mineral deposition and blocks hydroxyapatite crystal growth,<sup>39</sup> but only when phosphorylated.<sup>40</sup> This suggests that posttranslational modification of osteopontin may provide further control of mineralization. A recent in vivo study in which matrix-gla and osteopontin knock-out mice were cross-bred showed that the matrix-gla<sup>-/-</sup> and osteopontin<sup>-/-</sup> mice had increased and earlier calcification of arteries than the matrix-gla<sup>-/-</sup> alone. These data suggests that osteopontin is an inducible inhibitor of vascular calcification.<sup>41</sup> Thus, mineralization occurs in both bone and arteries when the balance of calcification-promoting collagen and noncollagenous proteins, compared with calcification-inhibiting noncollagenous proteins, favors promotion over inhibition. Further studies undoubtedly will provide continued clarification of this complex regulation.

Regardless of the precise mechanism of mineralization, osteoblasts appear critical in the process in bone by secreting collagen and noncollagenous proteins, and providing the matrix vesicle for initiation of mineralization. Osteoblasts differentiate

from a pluripotent mesenchymal stem cell to a mature osteoblast with different morphology, and gene and matrix protein production in each phase of differentiation, bone formation, and mineralization. These same stem cells can differentiate to vascular smooth muscle cells (VSMCs). Primary VSMCs from explants of medial tissue of both normal and diseased arteries transform into phenotypically distinct cells capable of calcification *in vitro*.<sup>12</sup> These modified VSMCs facilitate mineralization *in vitro* by forming nodules similar to those produced by osteoblasts, producing bone-associated proteins and forming matrix vesicles. A variety of stimuli have been shown to induce or modulate phenotypic transformation of VSMCs to osteoblast-like cells with subsequent mineralization *in vitro*, including phosphorus, oxidized low-density lipoprotein, calcitriol, parathyroid hormone, and parathyroid hormone-related peptide.<sup>42-45</sup> Jono *et al*<sup>46</sup> have shown that phosphorus-induced calcification was dependent on the sodium-phosphate cotransporter. Furthermore, exogenous phosphate added to human VSMCs cultures up-regulated *Cbfa1* expression,<sup>46</sup> a transcription factor critical for osteoblast differentiation and the expression of the bone matrix proteins osteopontin, osteocalcin, and type I collagen.<sup>47</sup> *Cbfa1* knock-out mice fail to form mineralized bone, proving that *Cbfa1* is the switch that turns a pluripotent stem cell into an osteoblast.<sup>48</sup> Thus, the *in vitro* data in VSMCs support that phosphorus can lead to calcification, and that phosphorus can induce *Cbfa1* and the expression of bone matrix proteins. Hyperphosphatemia, a laboratory abnormality common in CKD-V patients, therefore could represent an important stimulus by which uremia predisposes to excessive vascular calcification.

#### VASCULAR CALCIFICATION IN PATIENTS WITH END-STAGE RENAL DISEASE

Dialysis patients are known to have many cardiovascular risk factors such as diabetes, hypertension, and increased levels of homocysteine and oxidized lipids. Cardiovascular disease is the leading cause of death in patients with CKD-V,<sup>49</sup> but the traditional Framingham risk factors do not completely account for this increasing mortality.<sup>50</sup> In addition to these traditional vascular risk factors, there is increasing evidence that increased serum phosphorus, serum calcium X phosphorus product, and/or calcium load in the form of calcium-containing phosphate binders are associated with vas-

cular disease, including coronary artery calcification by electron beam computed tomography,<sup>51,52</sup> calcific uremic arteriopathy (calciphylaxis),<sup>18</sup> carotid and aortic calcification,<sup>17,53</sup> hemodynamic abnormalities,<sup>54</sup> and valvular disease.<sup>55</sup> In addition, increased phosphorus and calcium X phosphorus products are associated with increased mortality,<sup>56</sup> principally owing to cardiovascular death.<sup>57</sup> These studies suggest a relationship of these laboratory values and positive calcium and phosphorus balance with vascular disease. Chertow *et al*<sup>58</sup> recently showed that the noncalcium phosphate binder sevelamer can arrest coronary artery and aorta vascular calcification in CKD-V patients, whereas calcium-based phosphate binders increased calcification in both coronary arteries and the aorta (25% versus 6% for coronary arteries; 28% versus 5% for the aorta). A baseline assessment of these patients determined that approximately 80% had significant coronary artery calcification, and that the severity of calcification correlated with a history of cardiac disease. Furthermore, Blacher *et al*<sup>59</sup> found that the risk for cardiovascular and all-cause mortality was associated with the severity of arterial calcification by ultrasound. We also have found that the coronary calcification score assessed by spiral computed tomography scan in hemodialysis patients was greatest in those who died or were hospitalized during a follow-up of 15 months versus those who did not, and that the process slowed or stopped after renal transplantation.<sup>60</sup> These results confirm the importance of mineral metabolism in cardiovascular disease and vascular calcification. However, vascular calcification was also a common finding before the use of calcium-containing phosphate binders, when aluminum binders predominated,<sup>61</sup> and duration of dialysis predominated as a risk factor in cross-sectional studies,<sup>17,18,23,51,53,54,62</sup> thus other uremic factors are likely to also contribute.

#### PATHOPHYSIOLOGY OF VASCULAR CALCIFICATION IN CKD-V

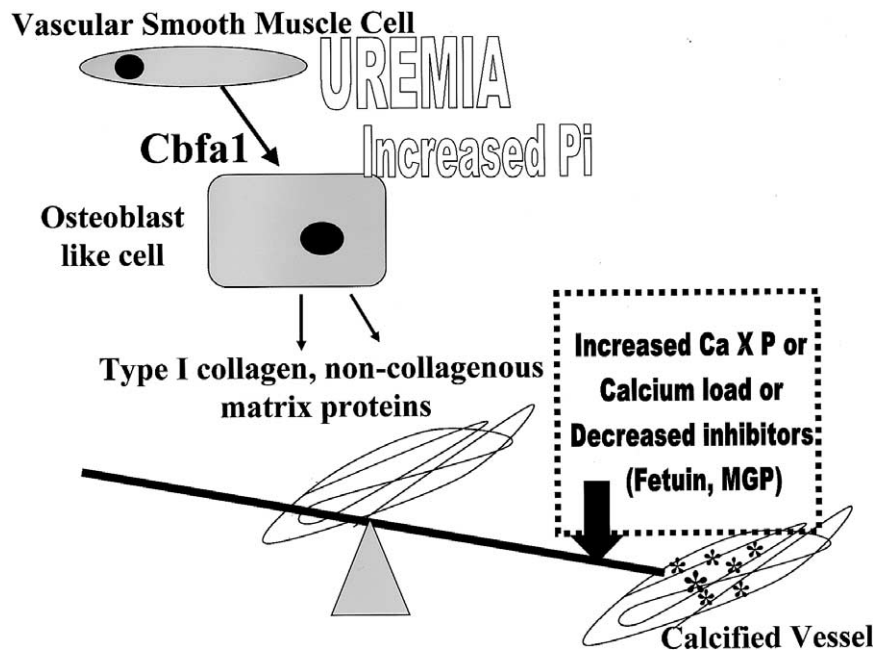
Extraskelatal calcification affecting dialysis patients previously was thought to be secondary to passive precipitation of supersaturated mineral content as assessed by calcium X phosphate product. We have performed histologic analysis of vascular calcification from specimens obtained from dialysis patients. In CUA, or calciphylaxis, medial calcification in calciphylaxis was accompanied by expression of osteopontin<sup>18</sup> and bone sialoprotein

and osteonectin (unpublished observation). Electron microscopy of CUA specimens revealed the presence of matrix vesicles identical to those observed in human bone.<sup>18</sup> Hyperphosphatemia and increased calcium X phosphate product were associated with development of CUA,<sup>18</sup> emphasizing the role of phosphorus in uremic vascular calcification. We then evaluated prospectively inferior epigastric arteries obtained from CKD-V patients at the time of renal transplantation. The degree of vascular calcification by both spiral computed tomography and histologic stains for calcification was proportional to the expression of the bone matrix proteins osteopontin, bone sialoprotein, alkaline phosphatase, and type I collagen. Furthermore, 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.<sup>19</sup> Furthermore, we have shown the presence of a multinucleated, tartrate-resistant, acid-phosphatase, osteoclast-like cell in an area of medial calcification, supporting that not only bone formation but perhaps bone resorption can occur in calcified arteries (Figs. 1E and 1F). These results confirm a cell-mediated, osteogenic process in vascular calcification in CKD-V patients, similar to findings in vessels of nondialysis patients with both calcified atherosclerotic coronary arteries<sup>12,13,26,63,64</sup> and medial calcinosis in small distal vessels.<sup>15</sup>

To understand further the mechanism by which uremia and/or hyperphosphatemia induces vascular calcification, bovine VSMCs were incubated in the presence of pooled normal human serum versus pooled serum from hemodialysis patients on dialysis for at least 2 years (to eliminate residual renal function).<sup>65</sup> The results show that uremic serum levels increased and accelerated calcification *in vitro* compared with healthy (nonuremic) serum. Furthermore, despite low final concentrations of phosphorus in the media, uremic serum up-regulated the expression of the bone transcription factor *Cbfa1* and its downstream protein product osteopontin in bovine VSMCs.<sup>65,66</sup> The addition of inorganic phosphorus failed to augment the *Cbfa1* and osteopontin expression induced by uremic serum, suggesting that uremic factor(s) other than hyperphosphatemia participate in the development of vascular calcification. This was supported further by the finding that the effects of uremic serum were inhibited only partially by blocking the so-

dium/phosphate cotransporter, whereas the same inhibitor completely prevented the effect of phosphorus in normal human serum.<sup>65</sup> It therefore appears that vascular calcification may be accelerated by uremic serum, possibly by up-regulating *Cbfa1*, leading to accelerated transformation of VSMCs into osteoblast-like cells. This process is mediated only partially by hyperphosphatemia and additional factors present in uremic serum may accelerate the differentiation. We also recently have shown that the osteoblast transcription factor *Cbfa1* was expressed (by *in situ* hybridization, immunostaining, and reverse-transcription polymerase chain reaction of tissue) in both the media and the intima in calcified vessels from renal transplant patients, but there was only minimal expression in noncalcified vessels.<sup>67</sup> This supports our hypothesis that *Cbfa1* is a key regulatory factor in the vascular calcification observed in dialysis patients. Our findings of *Cbfa1* and the downstream proteins osteopontin and type I collagen in areas of both intimal and medial calcification in CKD-V patients suggests that regardless of the disease initiating process, the progression to calcification may indeed be caused by expression of *Cbfa1* and dedifferentiation of VSMCs into osteoblast-like cells. Recently, Tyson et al<sup>68</sup> also showed expression of *Cbfa1* alkaline phosphatase, bone sialoprotein, and osteocalcin were expressed by reverse-transcription polymerase chain reaction in calcified, but not noncalcified, arteries with atherosclerotic disease from non-CKD-V patients. The downstream protein products of *Cbfa1* include alkaline phosphatase, osteopontin, osteocalcin, and type I collagen. As described earlier, these proteins are not only markers of osteoblast differentiation, but also are regulators of calcification. It is of interest that *Cbfa1* regulates proteins that both promote and inhibit calcification, implying a natural mechanism for controlling the extent of calcification.

Lastly, in addition to locally produced inhibitors, there is evidence for the presence of circulating inhibitors of vascular calcification. Fetuin-A (AHSG or  $\alpha_2$ -HS glycoprotein) levels are decreased in CKD-V patients with CUA, and the knock-out mice have extraskelatal calcification in the presence of hypercalcemia.<sup>69,70</sup> The administration of osteoprotegerin and bisphosphonates also can prevent the vascular calcification observed in rats given high doses of vitamin D and warfarin.<sup>71-73</sup> Warfarin interferes with  $\gamma$ -carboxylation of matrix-gla protein,<sup>74</sup> another protein that ap-



**Fig 2.** Hypothetic mechanism of vascular calcification in dialysis patients. We hypothesize a three step mechanism in the induction of uremic vascular calcification: First, vascular smooth muscle cells dedifferentiate to become osteoblast-like. Second, these osteoblast-like cells lay down a matrix of collagen and non-collagen proteins that serve as a nidus for subsequent mineralization. Third, this nidus becomes mineralized when the pro-mineralizing forces (increased  $\text{Ca} \times \text{P}$ , or increased calcium load) outweigh the antimineralizing forces of inhibitors (fetuin and matrix gla protein).

pears to inhibit vascular calcification, because the matrix-gla knock-out mice develop extensive medial vascular calcification.<sup>27</sup> Clearly, there are multiple mechanisms to regulate extraskelatal calcification and we are only beginning to understand this complex process.

#### CONCLUSION

Vascular calcification is common in patients with CKD-V as well as the general aging population, and is an active process. An initial step in the process of calcification of arteries may be dedifferentiation of VSMCs to osteoblast-like cells via up-regulation of *Cbfa1*. In vitro studies suggest that increased phosphorus as well as other as yet unidentified uremic toxins can induce differentiation. These osteoblast-like cells are capable of producing bone matrix proteins, which subsequently may regulate mineralization. Once mineralization is initiated, increased calcium X phosphorus product or calcium load may accelerate the process. Similar to findings in bone, the mineralization proceeds when the balance of promoting proteins outweighs inhibitory proteins (Fig 2). Further under-

standing the mechanism by which vascular calcification occurs should offer the potential hope of developing therapeutic strategies to arrest this deadly process.

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