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Regulatory Circuits Controlling Vascular Cell Calcification

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Abstract

Vascular calcification is a common feature of chronic kidney disease, cardiovascular disease, and aging. Such abnormal calcium deposition occurs in medial and/or intimal layers of blood vessels as well as in cardiac valves. Once considered a passive and inconsequential finding, the presence of calcium deposits in the vasculature is widely accepted as a predictor of increased morbidity and mortality. Recognition of the importance of vascular calcification in health is driving research into mechanisms that govern its development, progression, and regression. Diverse, but highly interconnected factors, have been implicated, including disturbances in lipid metabolism, oxidative stress, inflammatory cytokines, and mineral and hormonal balances, which can lead to formation of osteoblast-like cells in the artery wall. A tight balance of procalcific and anticalcific regulators dictates the extent of disease. In this review, we focus on the main regulatory circuits modulating vascular cell calcification.

INTRODUCTION

Vascular calcification is now recognized as an active process regulated at the systemic, cellular and molecular levels (Figure 1). Clinically termed “calcific vasculopathy,” it is considered a strong predictor of cardiovascular events, independently of traditional risk factors [1–3]. Patients with end stage renal disease have the most extensive calcific vasculopathy, which may account for cardiovascular disease being their most common cause of death [4, 5]. Calcium deposits impact vascular function by impairing vessel compliance [6, 7]. However, their effects on plaque stability remain under investigation. Advances over the last two decades, based on animal and cell culture studies, have accelerated our understanding of the mechanisms of vascular cell calcification. In this review, we focus on the main regulatory circuits including mineral and hormonal imbalances as well as inflammatory agents.

Pathobiology

Vascular calcification shares risk factors with atherosclerosis, including oxidative stress, lipid modification, and inflammation. All three components are known to co-localize with ectopic calcification in vivo [8, 9]. In subjects with atherosclerotic disease, calcium deposits are found mainly in the intimal layer of the vessel wall (Figure 2a) and in valve leaflets. Lipids and lipoproteins accumulate in the subendothelial space of arterial walls and undergo non-enzymatic oxidation by reactive oxygen species [10–13] produced by metabolic activity of surrounding cells [14]. The resulting lipid oxidation products induce the production of inflammatory cytokines, which boost cellular metabolic activity, amplifying the oxidative

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response [15]. Pro-osteogenic signaling and mineralization in aortic valve leaflets also co-localize with sites of high oxidant stress and diminished antioxidant reserve in hyperlipidemic mice [16]. Similarly, in hypercholesterolemic animals, osteogenic differentiation and mineralization in valve leaflets co-localize with oxidant stress and lipids, and are mitigated by antioxidant treatment [17] or reducing plasma lipid levels [18]. Notably, it also has been proposed that mineralization is a cause rather than a consequence of atherosclerosis, wherein calcium phosphate crystals elicit a pro-inflammatory response from monocyte-macrophages [19].

In subjects with renal disease, calcium deposits are found mainly in the medial layer of the vessel wall (Figure. 2b). Previously referred to as “Monckeberg’s calcinosis” and now known as uremic vasculopathy, it is associated with imbalances in mineral metabolism, specifically hypercalcemia and hyperphosphatemia, which correlate strongly with all-cause and cardiovascular mortality in patients on dialysis [20, 21]. Under normal physiological conditions, calcium/phosphate metabolism is tightly regulated by vitamin D, calcitonin, fibroblast growth factor-23 (FGF-23), and parathyroid hormone (PTH). In addition to mineral and hormonal imbalances, patients with renal disease also have high levels of oxidant stress and inflammatory cytokines [22–24], which are closely associated with vascular and valvular calcification [25]. When uremia is induced in hyperlipidemic mice, they develop arterial calcification at both atheromatous and atheroma-free sites [26]. Recent studies have shown that antioxidants inhibit uremia-induced calcification in mice [27].

Importantly, vascular calcification shares many features with bone formation including histopathological features, mineral composition, and initiation mechanisms. In 1863, Virchow described calcium deposits in vessel walls as “ossification not mere calcification” [28]. The recapitulation of osteogenesis is observed in histological analysis of arteries from human subjects, about 15% of which contain ectopic osteochondrogenic tissues, including osteoid, osteocytes, osteoblasts, osteoclasts and chondrocytes [29, 30]. In mice, vascular calcification primarily takes the form of cartilaginous metaplasia (Figure. 2c), following a recessive inheritance pattern with variable penetrance, and occurring most often at sites of fluid mechanical stress due to disturbed flow such as along the aortic valves and the innominate arteries [31, 32].

With respect to mineral composition, energy-dispersive electron analysis and nuclear magnetic resonance (NMR) imaging have been used to define the structure, stoichiometry, and elemental composition of the mineral formed in arteries, identifying it primarily as hydroxyapatite, the same mineral as in bone [33, 34]. Such findings accentuate the highly overlapping mechanisms that govern pathologic vascular calcification and osteogenesis. There are differences, however, in the lipid composition of intimal versus medial calcification – intimal calcified lesions contain cholesterol and cholesterol esters in addition to fatty acids that are found in medial calcified lesions and bone [35].

Vascular calcification appears to involve similar mechanisms as skeletal bone formation. For example, Bonucci [36] and Anderson [37] showed that skeletal mineralization is initiated with the release of matrix vesicles from osteoblasts and chondrocytes, 50–500 nm in size, which serve as nucleation sites for hydroxyapatite mineral. Anderson and colleagues subsequently demonstrated nearly identical matrix vesicles in human calcific atherosclerotic lesions [38]. Cholesterol is an integral component of matrix vesicles, and its upregulation is essential for mineralization in vascular cells [39, 40]. Under normal conditions, the matrix vesicles released by VSMCs are enriched in the mineralization inhibitors, matrix GLA protein (MGP) and fetuin, but these are lost under conditions of hypercalcemia [41].

Vascular calcification models

Both cell culture and mouse models have been used for mechanistic studies of vascular calcification. Several mouse models for atherosclerotic and uremic-induced calcification have been established. Hyperlipidemic mouse models, including those deficient in low-density lipoprotein receptor or in apolipoprotein E, are widely used for studying atherosclerotic calcification, whereas renal ablation and an adenine-supplemented diet are used to induce uremic medial calcification [26, 42].

Tissue culture models of both vascular and valvular calcification include medial vascular smooth muscle cells (VSMCs), aortic myofibroblasts, valvular interstitial cells, and pericytes from human, bovine, and rodent tissues [43–48]. These cell types appear to be related to one another, and they are essentially the non-endothelial cells of the vasculature. In light of this similarity, in this review, we may refer to them loosely as “vascular cells.” One of the first in vitro calcification models was established by Schor and colleagues using bovine pericytes [49]. We subsequently isolated a clonal subpopulation of bovine aortic medial cells that spontaneously undergo calcification in culture, termed “calcifying vascular cells” [34]. These cells express potent osteogenic factors, including bone morphogenetic protein-2 (BMP-2) and core binding factor alpha 1 (Cbfa1; also known as runt-related transcription factor 2, Runx2). In addition, they express osteoblastic differentiation markers, including alkaline phosphatase (also known as tissue nonspecific alkaline phosphatase), MGP, osteopontin, type I collagen, and osteocalcin [34, 44]. The osteochondrogenic potential of VSMCs has also been shown in mice expressing beta-galactosidase-labeled VSMCs [50].

There are a number of possible origins for cells that produce mineral in the vasculature in vivo. As noted above, resident vascular wall cells may be responsible [50–52]. It is also possible that circulating progenitor cells contribute directly by migrating into the artery wall then differentiating [53]. By nanoparticle imaging, circulating monocytes colocalize with aortic calcification in vivo and may contribute indirectly by inducing osteochondrogenic differentiation of resident VSMC [54]. Bone marrow transplant techniques have been used to resolve these questions, however the findings are difficult to interpret since the transplanted cells are typically injected directly into the circulation [55] or into the myocardium [56].

Regulatory factors

Extracellular matrix calcification is governed by both procalcific and anticalcific factors. Pathologic conditions conducive to ectopic calcification may be due to downregulation of anticalcific factors and/or upregulation of procalcific factors.

I. Procalcific

a. Inflammatory factors: Vascular and valvular cells are regulated at the cellular and molecular levels by inflammation. Inflammatory cytokines, oxidized lipids and oxidant stress promote VSMC expression of osteogenic transcription factors Cbfa1/Runx2, BMP-2, Msx2, osterix, Sox9, osteopontin, osteocalcin, and alkaline phosphatase, while reducing VSMC expression of SM22alpha and SMalphactin [30, 57–60]. Such a phenotypic change in VSMCs may explain the bone-like architecture observed in intimal calcification. In the murine model of uremia, phenotypic changes in VSMC occur prior to calcium deposition, accompanied by elastin degradation, possibly due to upregulation of proteases, including matrix metalloproteinases [61].

Oxidant stress and oxidized lipids are known to induce osteochondrogenic differentiation of vascular cells in vitro [62–65]. Induction of vascular cell calcification by oxidative stress

involves modulation of Cbfa1/Runx2 via PI3 kinase/Akt signaling [66]. We and others have found that macrophages induce osteogenic differentiation of VSMC via a soluble inflammatory cytokine, tumor necrosis factor- α (TNF- α) [67, 68]. In vitro studies show that TNF- α induces the pro-calcific factor, alkaline phosphatase, and mineralization in VSMC [67, 69] and aortic valve myofibroblasts [70]. A recent study show that TNF- α , through the NF- κ B signaling pathway, promotes phosphate-induced calcification by reducing efflux of a calcification inhibitor, pyrophosphate, and its transport protein, ankylosis protein homolog [71]. An elegant demonstration of the in vivo contribution of inflammatory cytokines was provided by Towler's laboratory using mice with vascular-specific overexpression of TNF- α [72]. They further demonstrated that TNF- α induces the pro-osteogenic Msx2/wnt pathway in vascular cells via TNF receptor-1-mediated redox signaling [51]. Experiments using neutralizing factors confirm the important role of oxidant stress and inflammation in vascular calcification. For example, antioxidants block vascular cell calcification in vitro [64]; anti-TNF- α antibody, infliximab, inhibits vascular calcification in vivo [72]; and the lipid-solvent, ethanol, blocks calcification of bioprosthetic aortic valves ex vivo [73].

b. Calcium and phosphate minerals: We and others have recently found that calcium phosphate nanocrystals can initiate the osteogenic program in VSMC, including upregulation of BMP-2 and osteopontin, under conditions characterized by elevated extracellular phosphate, calcium or both [74, 75]. Although the mechanism of nanocrystal effects on gene expression is not clear, one possibility is that they enter the nucleus and interact with histones, as suggested by Nabiev and colleagues [76]. Alternatively, calcium phosphate nanocrystals smaller than 300 nm may be endocytosed by VSMC, causing cytoplasmic calcium bursts leading to apoptosis [77]. Debris from cell death and apoptotic bodies may also serve as foci for apatite nucleation. Increased extracellular calcium levels also regulate expression of calcification inhibitors, such as osteoprotegerin and MGP [41, 78]. In an ex vivo model, vessel rings from renal patients show increased susceptibility to calcium- and phosphate-induced mineralization, as well as greater apoptosis and release of vesicles in comparison with vessel rings from healthy subjects [79]. It also has been proposed that "calcium phosphate crystal nuclei" released during bone turnover enter the circulation and lodge in the elastic lamellae of arteries and other soft tissues [80]. Also known as nucleational complexes, these could explain the epidemiological association between osteoporosis and vascular calcification.

High levels of serum calcium and phosphate are strongly associated with cardiovascular mortality in renal patients [20, 81]. Treatment of VSMC with inorganic phosphate promotes matrix mineralization (Figure 3). It has been shown that inorganic phosphate is internalized by VSMC via the type III sodium-dependent phosphate cotransporter, PiT-1 [82]. The increase in phosphate uptake induces Cbfa1/Runx2 with ensuing calcification in VSMC [82, 83]. Phosphate-induced calcification of VSMC is mediated by mitochondrial reactive oxygen species, which activate the NF- κ B pathway [84]. In addition, inorganic phosphate and protein-kinase A agonists synergistically promote calcification of VSMC by suppressing levels of extracellular pyrophosphate, an inhibitor of calcification [85].

c. Hormones: Hyperparathyroidism is another major risk factor for medial calcification in renal disease [86]. Nephrectomized rodents that received PTH infusions had increased aortic expression of Cbfa1/Runx2 and calcium deposition regardless of phosphate intake [87, 88]. Similarly, the severity of medial calcium deposition in the aorta correlates with the levels of serum PTH in uremic rats with ovariectomy [89]. Interestingly, it is bone loss from the cortical, rather than trabecular, compartments that is significantly correlated with aortic calcification [89, 90]. We previously showed that chronic stimulation of the protein kinase A pathway, the main signaling pathway for PTH, has paradoxical effects on matrix

mineralization of vascular and bone cells [44, 91]. Inhibition of serum PTH levels either by parathyroidectomy [92] or a calcimimetic agent [93] ameliorates aortic calcification in uremic mice.

Both in vitro and in vivo studies have linked levels of vitamin D metabolites with vascular calcification [94–96]. In renal patients, vascular disease severity corresponds inversely with levels of the storage form of vitamin D, 25-hydroxycholecalciferol [97]. In contrast, knockout mouse models of FGF-23 and Klotho, which exhibit increased arterial calcification, have elevated vitamin D levels due to increased expression of renal 25-hydroxyvitamin D-1-alpha-hydroxylase, which converts the storage to the active form [98, 99]. In mice, deficiency in both FGF23 and renal 1-alpha-hydroxylase appears to blunt arterial calcification [98]. Others have shown that a high cholesterol diet augments vitamin D-induced vascular calcification via oxidative stress [100]. The apparent paradoxical findings between vascular calcification and serum vitamin D levels, especially in patients with renal disease, may stem from the multiple endocrine regulatory feedback loops that control levels of PTH and precursors of vitamin D in a complex manner.

II. Anti-calcific—Calcium deposition in extraskeletal tissues is inhibited by both circulating hormonal factors as well as local factors. Knockout mouse models have implicated MGP, osteopontin, osteoprotegerin, ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), fetuin, klotho/FGF23, and Notch as inhibitors of vascular calcification.

a. MGP: MGP is an extracellular matrix protein that accumulates near the borders of calcified plaques [101]. It is progressively expressed during osteoblastic differentiation of VSMC [44] and vascular pericytes [102]. Paradoxically, MGP-deficient mice have extensive arterial calcification, aortic dissection, and death at 6–8 weeks [103]. Conversely, MGP transgenic mice have reduced calcification as well as reduced atherosclerosis [104]. The latter, unexpected finding supports the findings of Nadra and colleagues that calcium mineral crystals may have a causal role in atherogenesis [19]. Restoration of MGP expression driven by SM22alpha promoter in MGP deficient mice reverses the arterial phenotype [105]. For functional activity, MGP requires vitamin K-dependent gamma-carboxylation [106]. Inhibiting gamma-carboxylation of MGP by warfarin reduces its ability to inhibit matrix mineralization, possibly through inhibiting binding to potent osteogenic factor, BMP [106, 107]. This may account for the induction of vascular calcification by the vitamin K inhibitor, warfarin, in rodents [108, 109]. Other evidence suggests that MGP is able to inhibit calcification independently of its effect on BMP-2 [110, 111].

b. Osteopontin: Osteopontin, first identified as a protein bridge linking osteoclasts to mineral in bone tissue, is enriched in phosphorylated residues and is a prominent component of mineralized extracellular matrix [112]. Its expression is upregulated in calcified arteries although its function appears to limit the extent of calcium deposition [113]. Full-length osteopontin promotes cell attachment and migration [114, 115]. In osteopontin-deficient hyperlipidemic mice, osteochondrogenic transition of VSMC and fibrosis is enhanced [6]. In humans, fragmented, rather than full-length, osteopontin is enriched in calcific aortic valves, where it has proinflammatory properties, activating Wnt signaling and inducing aortic calcification [6].

c. Osteoprotegerin: Osteoprotegerin is a soluble decoy receptor of RANKL, an inducer of osteoclastic differentiation. The critical role of osteoprotegerin in regulating vascular calcification is best appreciated from the unexpected phenotype of osteoprotegerin deficient mice: extensive medial calcification [116]. However, it is not clear whether the medial calcification is a direct effect of OPG deficiency in vascular cells or an indirect result of the

osteoporotic phenotype (i.e., hypercalcemia) resulting from osteoclastic overactivity. In an interesting conundrum, serum osteoprotegerin levels are elevated in patients with cardiovascular diseases, further complicating the interpretation of its function in the vasculature [117–121]. This may result from a negative feedback circuit with insufficient gain, given that OPG treatment reduces atherosclerotic calcification in mice and warfarin/vitamin D-induced calcification in rats [109, 122], and given that OPG blocks calcification by upregulating IGF1 receptors and enhancing VSMC survival [123].

d. Fetuin: Fetuin (aka, alpha 2-Heremans-Schmid) is a circulating glycoprotein produced mainly by the liver in adults [124]. Evidence for its anti-calcific role stems from the phenotype of the fetuin-deficient mice, which includes ectopic calcification in soft tissues [125, 126]. It appears that fetuin inhibits mineralization by more than one mechanism. First, it has been shown that fetuin restricts apatite crystal growth by constraining the mineralization within the collagen fibril [127]. Secondly, fetuin, which gets internalized by VSMC and incorporated into matrix vesicles, impairs the ability of matrix vesicles to nucleate calcium-phosphate crystals. Serum levels of fetuin-1 are reduced in patients with ESRD, and the levels correlate with coronary and valvular calcification [128–130]. The reduced levels of serum fetuin may be a result of clearance of the protein after it forms a complex with mineral, since the level of fetuin-mineral complex has been positively correlated with increased artery calcification [131].

e. Klotho and FGF-23: Klotho is transmembranous protein involved in suppressing ageing phenotypes, including arteriosclerosis, osteoporosis, and a short lifespan [132–134]. Klotho enhances binding of FGF-23 to its receptor FGFR [135] and regulates reabsorption of phosphate in the kidney [99]. Evidence suggests that Klotho also reduces oxidative stress by inhibiting insulin/IGF-1 signaling [136]. Mice deficient in either Klotho or FGF23 have hyperphosphatemia and develop vascular calcification [99, 137]. In patients and mice with renal disease, Klotho levels are reduced, and treating VSMC with Klotho inhibits calcification induced by inorganic phosphate [138]. Consistent with those findings, knocking down Klotho in human aortic VSMC results in upregulation of Cbfa1/Runx2, suppression of SMC markers and acceleration of calcium deposition [139].

e. ENPP1: Several lines of evidence suggest that pyrophosphate, a potent inhibitor of vascular calcification, is generated by the membrane bound enzyme, ENPP1 [140]. ENPP1 is expressed at the shoulder of atherosclerotic lesions, and its expression level is decreased in calcified plaques [141]. In patients with renal disease, plasma pyrophosphate levels are inversely correlated with the presence of calcific vasculopathy but are not affected by dialysis [142]. Importantly, deficiency of ENPP1 leads to widely disseminated arterial medial calcification, in genetically engineered mice as well as in humans, a condition now known as generalized arterial calcification of infancy [143]. Cecil and Terkeltaub further showed that the increased aortic calcification in Enpp1 deficient mice is due to reduced expression of the inhibitory factor, soluble receptor for advanced glycation endproducts [144]. Significant arterial and joint calcifications are also seen in patients with frameshift mutations in ecto-5'-nucleotidase gene (NT5E), encoding a key enzyme, CD73 that converts AMP to adenosine in the ENPP1 metabolic pathway [145]. Direct administration of pyrophosphate inhibits uremic and vitamin D-induced aortic calcification without affecting bone turnover in rats [146].

f. Notch: The Notch family of transmembrane receptors plays an important role in development by regulating cell differentiation. Mutations in Notch1 have been found in families with aortic valve calcification [147, 148], and the endothelial-specific deletion of a Notch ligand, Jag1, in mice causes valvular calcification [149]. One individual with Alagille

syndrome, an autosomal-dominant disorder associated with Jag1 gene, was reported as having aortic calcification [150]. These in vivo findings suggest an inhibitory effect, however, in vitro studies show stimulatory effects of Notch signaling in osteoblastic differentiation of VSMC, osteoblasts and multipotent mesenchymal cells [151–155]. This difference between in vivo and in vitro findings is likely due to the developmental stages of the affected cells.

SUMMARY

Work of many investigators over the past two decades has brought attention to the importance of calcific vasculopathy in the morbidity and mortality of cardiovascular disease, especially in those with renal disease. Advanced imaging techniques have permitted rapid detection of mineralization in patients, but no specific treatment strategies are available. The phenomenon appears to involve development of osteoblast-like cells in the artery wall, driven by several interdependent mechanistic pathways with similarities to those of embryonic osteogenesis. Further elucidating and controlling these regulatory circuits – inflammatory cytokines, hormonal imbalances, dyslipidemia, mineral imbalances, and oxidant stress – are promising directions for understanding and mitigating the growing prevalence of calcific vasculopathy in our aging population.

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LIST OF ABBREVIATIONS

BMP-2	Bone morphogenetic protein 2
Cbfa1	Core binding factor alpha 1
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1
FGF-23	Fibroblast growth factor 23
MGP	Matrix GLA protein
NMR	Nuclear magnetic resonance
PTH	Parathyroid hormone
Runx2	Runt-related transcription factor 2
TNF-α	Tumor necrosis factor-alpha
VSMCs	Vascular smooth muscle cells

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Figure 1.

Schematic diagram of regulation of vascular cell calcification by systemic and local factors, including activators such as oxylipids (produced under increased oxidant stress), cytokines (produced by lipid-laden macrophages), hormones, calcium/phosphate (Ca/Pi) nanocrystals and inhibitors.

Figure 2a

Figure 2b

Figure 2c

Figure 2. Types of vascular calcification: (a) intimal atherosclerotic calcification (arrow) of human femoral artery by von Kossa staining; (b) medial calcification (arrows) of murine artery by hematoxylin and eosin staining, magnification 100X; and (c) calcified cartilaginous metaplasia (arrows) of murine aortic root by hematoxylin and eosin (top) and von Kossa (bottom) staining, magnification 100X.

Figure 3.

In vitro matrix mineralization (arrow) of murine VSMC in response to inorganic phosphate treatment indicated by calcein (green, calcium mineral), Alexa Fluor 568 conjugated to phalloidin (red, actin network), and DAPI (blue, nuclei), magnification 100X.