Regulation of Vascular Calcification
Roles of Phosphate and Osteopontin

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Abstract—Vascular calcification is prevalent in aging as well as a number of pathological conditions, and it is now recognized as a strong predictor of cardiovascular events in the general population as well as diabetic and end-stage renal disease patients. Vascular calcification is a highly regulated process involving inductive and inhibitory mechanisms. This article focuses on two molecules, phosphate and osteopontin, that have been implicated in the induction or inhibition of vascular calcification, respectively. Elevated phosphate is of interest because hyperphosphatemia is recognized as a major nonconventional risk factor for cardiovascular disease mortality in end-stage renal disease patients. Studies to date suggest that elevated phosphate stimulates smooth muscle cell phenotypic transition and mineralization via the activity of a sodium-dependent phosphate cotransporter. Osteopontin, however, appears to block vascular calcification most likely by preventing calcium phosphate crystal growth and inducing cellular mineral resorption. (Circ. Res. 2005;96:717-722.)

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Calcification of the cardiovascular system is associated with a number of diseases, including end-stage renal disease (ESRD) and cardiovascular disease. Calcium phosphate deposition is the hallmark of vascular calcification and can occur in the blood vessels, myocardium, and cardiac valves. Calcium phosphate deposits are found in distinct layers of the blood vessel and are associated with specific pathologies. Intimal calcification is observed in atherosclerotic lesions,1,2 whereas medial calcification is common to the arteriosclerosis observed with age, diabetes, and ESRD.3,4 Intimal and medial calcification may occur independently of each other. In ESRD patients, intimal and medial calcification have been observed in affected vessels,5,6 although the etiological and clinical significance of this finding is not yet clear.

Vascular calcification can lead to life-threatening organ dysfunction depending on its extent and the organ affected. For example, calcification of cardiac valve leaflets is recognized as a major mode of failure of native and bioprosthesis valves.7,8 Furthermore, vascular calcification is responsible for calcific uremic arteriolopathy, a necrotizing skin condition associated with extremely high mortality rates.9 Finally, idiopathic infantile arterial calcification, a genetic disease characterized by arterial calcification, fibrosis, and stenosis, leads to premature death in afflicted neonates.10

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In contrast, age and vascular disease-related vascular calcifications were previously considered benign. However, recent clinical studies have challenged this dogma. Calcification has been positively correlated with coronary atherosclerotic plaque burden, increased risk of myocardial infarction, and plaque instability. Furthermore, in the Rotterdam Coronary Calcification Study, a large population-based study, graded associations between coronary calcification score and stroke were identified. Similarly, medial arterial calcification is strongly correlated with coronary artery disease and future cardiovascular events in type I diabetic subjects, and is a strong prognostic marker of cardiovascular disease mortality in ESRD patients. These findings may be explained by growing evidence that vascular medial calcification in large arteries leads to increased stiffness and therefore decreased compliance of these vessels. These mechanical changes are associated with increased arterial pulse wave velocity and pulse pressure, and lead to impaired arterial distensibility, increased afterload favoring left ventricular hypertrophy, and compromised coronary perfusion. Thus, intimal and medial calcifications may contribute to the morbidity and mortality associated with cardiovascular disease.

It is becoming increasingly clear that vascular calcification is an actively regulated process that may be initiated by a number of different, nonmutually exclusive mechanisms. These mechanisms have been extensively reviewed elsewhere and include: (1) loss of mineral inhibiting factors; (2) induction of bone formation; (3) cell death; and (4) circulating nucleational complexes (ie, aggregates of calcium phosphate and proteins released from remodeling bone that may initiate ectopic mineralization). Abnormalities in mineral metabolism that enhance the calcium × phosphate product (Ca × P) may further exacerbate vascular calcification initiated by any of these mechanisms. This article focuses on recent evidence implicating elevated phosphate as a major inductive factor for vascular calcification and osteopontin as an inducible inhibitor of vascular calcification, and our current understanding of their mechanisms of action.

**Role of Phosphate**

Hyperphosphatemia is commonly observed in renal disease, especially in ESRD patients. Elevated serum phosphorus (in the form of phosphate) is a major risk factor for vascular calcification and cardiovascular mortality in these patients. Although elevations in the Ca × P may thermodynamically drive calcification, growing evidence indicates that direct effects of elevated phosphate on vessel wall cells may be more important in regulating the propensity of the vessel to calcify. We and others have found that heterogeneous, uncloned populations of vascular smooth muscle cells (VSMCs) do not spontaneously mineralize in culture, but can be induced to mineralize by elevating phosphate levels in the culture medium to those typically observed in hyperphosphatemic individuals (>2 mmol/L). Under these conditions, the extracellular matrix surrounding the VSMCs undergoes calcification with features similar to that observed in bone and in pathological vascular calcification in vivo, including the presence of calcifying collagen fibers, matrix vesicles, and bioapatite. In VSMC cultures, calcification does not appear to require apoptosis or to be associated with apoptotic cells (Giachelli and Li unpublished observations), although induction of apoptosis may accelerate calcification.

Concomitant with induction of VSMC mineralization, treatment with elevated phosphate induces cultured VSMCs to undergo a profound phenotypic transition. Under normal phosphate conditions, VSMCs express smooth muscle lineage markers representative of the contractile phenotype, including smooth muscle (SM) α-actin and SM22α. After treatment with elevated phosphate, there is a dramatic loss of these smooth muscle cell (SMC) lineage markers, and simultaneous gain of osteochondrogenic markers such as osteopontin, Cbfa1/Runx2, alkaline phosphatase, and osteocalcin. Importantly, almost identical changes in smooth muscle gene expression are observed in biopsy specimens from ESRD patients with calciphylaxis and with calcified inferior epigastric arteries. Finally, in an experimental mouse model of vascular calcification, the matrix Gla protein null (MGP−/−) mice, spontaneous vascular calcification was found in mice older than 2 weeks of age. Before mineralization, the vessels appear normal and medial SMCs express abundant SM lineage genes. However, as mineralization of the elastic lamellae ensues, medial cells lose SM α-actin and SM22α expression and gain expression of osteopontin, alkaline phosphatase, and Cbfa1/Runx2 (Speer and Giachelli, unpublished observation). Furthermore, analysis of older MGP−/− mice with advanced vascular calcification showed clusters of cells with chondrocytic features including type II collagen expression.

Based on these findings, we have hypothesized that VSMCs have the capacity to undergo modulation from a contractile to an osteochondrogenic phenotypic state that is controlled by local environmental cues such as elevated phosphate levels (Figure). According to gene expression patterns, this phenotypic state appears to be distinct from the previously characterized synthetic/dedifferentiated state seen in arteries injured by chemicals, disease, or trauma. The osteochondrogenic state may be exquisitely designed to repair and/or adapt to a mineralizing microenvironment, with...
enhanced expression of a number of mineral regulating molecules including the mineralization inhibitor, osteopontin, discussed later. Recent studies suggest that other molecules that promote or inhibit vascular calcification may also act, in part, by regulating VSMC phenotypic change, including elevated calcium29 and BMP-7.46

Mechanisms controlling this phenotypic transition in response to elevated phosphate in VSMCs are currently undergoing active investigation. We have determined that mineralization and VSMC phenotypic modulation in response to elevated phosphate are dependent on the activity of sodium-dependent phosphate cotransporters in the cells. Sodium-dependent phosphate cotransporters use the sodium gradient to actively transport phosphate into the cell. Three types of sodium dependent phosphate cotransporters have been identified based on structure, tissue expression, and regulation, and each family contains several members identified from various species. The type I and type II sodium-dependent phosphate cotransporters are predominantly expressed in intestine and kidney, and function to control phosphate reabsorption by these tissues.37,38 The type III sodium-dependent phosphate cotransporters are represented by Pit-1 (also named Glvr-1 and SLC20A1) and Pit-2 (also named Ram-1 and SLC20A2).39 These proteins are more ubiquitously expressed in tissues including kidney, heart, lung, brain, liver, and bone.40 Although the physiological functions of type III sodium-dependent phosphate cotransporters have not yet been identified, they may serve a more generalized function to allow phosphate movement into cells in support of oxidative phosphorylation. Although type III sodium-dependent phosphate cotransporters appear to be constitutively expressed in many tissues, phosphate deficiency39 and certain cytokines like insulin-like growth factor, transforming growth factor-β, and platelet-derived growth factor41–43 induce expression, whereas PTH reduces expression.44 We have found that Pit-1 and Pit-2 are the only sodium-dependent phosphate cotransporters expressed in human VSMCs29 (Li and Giachelli, unpublished data). Inhibition of sodium-dependent phosphate transport by phosphonoformic acid, a generic sodium-dependent phosphate cotransporter inhibitor, blocked elevated phosphate-induced SMC mineralization.26,29,45 Furthermore, elevated phosphate-induced Chfα-1 and osteocalcin expression were also inhibited by phosphonoformic acid.26,45 Most recently, we have found that suppression of endogenous Pit-1 expression by small interfering RNAs inhibits SMC mineralization in response to elevated phosphate, and overexpression of either Pit-1 or Pit-2 is able to rescue the phosphate-induced mineralization in Pit-1–deficient cells.46 In addition, Suzuki et al showed that vasopressin induces SMC mineralization through the enhancement of the phosphate transport activity of Pit-1.47 These findings suggest that Pit-1 and phosphate transport play a crucial role in SMC mineralization and phenotypic modulation by elevated phosphate.

Growing evidence suggests that type III sodium-dependent phosphate cotransporters are likely to be important mediators of cell-mediated matrix mineralization in general. In osteoblasts, Pit-1 mRNA levels increase and correlate with differentiation and mineralization.48 Furthermore, inhibition of sodium-dependent phosphate cotransporters by phosphonoformic acid also blocked phosphate-induced mineralization and osteopontin (OPN) expression in this cell type.49,50 Finally, phosphate uptake as well as mineralization of chondrocyte-derived matrix vesicles have been shown to depend on the activity of phosphate transporters, including type III sodium-dependent phosphate cotransporters.51,52 Matrix vesicles are thought to be key nucleating structures during endochondral ossification, and matrix vesicles have been described in calcified vascular lesions53 and cultured SMCs.28 Thus, these studies suggest that phosphate must be transported through mineral-forming cells, perhaps via matrix vesicles, to participate in matrix mineralization, and additionally that phosphate may have a unique signaling role(s) in these cells.

Although this review has focused on potential roles of VSMCs, it is important to stress that other cell types may also contribute to the osteochondrogenic processes observed in vascular calcification. In a series of elegant experiments, Demer et al identified and cloned a population of bovine arterial medial cells, termed calcifying vascular cells, that spontaneously form nodules that mineralize in vitro under long-term culture.54,55 These cells lack characteristic VSMC markers and display pericyte-like properties early in culture, and develop osteoblastic features, including expression of alkaline phosphatase, osteocalcin, and mineralization, with time in culture. Nodulation and mineralization of calcifying vascular cells are modulated by a large number of proatherogenic factors, and have more recently been shown to undergo additional developmental fates, including leiomyogenesis, depending on the culture conditions.55 Thus, these cells behave like pericytes that have long been postulated as a reservoir of multipotent stem cells in adults and can be induced to differentiate into multiple lineages, including osteoblasts.56 Likewise, Towler et al have described vascular myofibroblasts expressing Msx2 associated with mineralization in diabetic low-density lipoprotein receptor-null mice.57–59 These data support the presence of non-SM–derived, pluripotent stem cell-like populations within the artery wall capable of osteogenic differentiation that might be involved in vascular calcification under pathological conditions. Thus, lineage studies are critically needed to determine conditions and disease states under which pluripotent stem cells, phenotypic modulation of SMCs, or both contribute to vascular calcification.

Role of Osteopontin

It has long been known that blood and body fluids are at or near saturation with respect to calcium and phosphate levels, suggesting that mechanisms must exist to prevent ectopic calcification. Human and mouse genetic findings have now determined that most tissues, including blood vessels, normally express inhibitors of mineralization, and lack of these molecules ("loss of inhibition") leads to calcification. In humans, a dramatic example of the importance of this mechanism is genetic deficiency of the small molecule, pyrophosphate, that leads to idiopathic infantile arterial calcification.10,60 In this condition, calcification of the arteries leads to a severe fibroproliferative vascular disease that
culminates in heart failure soon after birth. Thus, pyrophosphate has emerged as a major regulator of vascular calcification during human development. A model mimicking the human disease, the tip-toe walking mouse, has a naturally occurring mutation in nucleotide pyrophosphate/phosphodiesterase I, which leads to pyrophosphate deficiency. In addition to articular cartilage calcification, ankylosis, and increased cementum, these animals display vascular calcification.61-66 A growing number of other putative calcification inhibitory molecules have been identified using mouse mutational analyses, including MGP, β-glucosidase, carbonic anhydrase II, fetuin, osteoprotegerin, desmin, and Smad 6.34,66-73 Mutant mice deficient in these molecules present with enhanced cardiovascular calcification as part of their phenotype and demonstrate that specific proteins and small molecules are normally important in suppressing ectopic calcification, including vascular calcification.

Another important molecule is OPN. OPN is an acidic phosphoprotein normally found in mineralized tissues such as bones and teeth, and it is involved in regulation of mineralization by acting as an inhibitor of apatite crystal growth, as well as promoting osteoclast function through the α,β integrin.74 Although OPN is not found in normal arteries, we75-77 and others78-82 have reported that OPN is abundant at sites of calcification in human atherosclerotic plaques and in calcified aortic valves. In addition, OPN levels are greatly elevated in the spontaneously mineralizing arteries of MGP−/− mice.27 These findings suggest that OPN may be an important regulator of arterial mineral deposition under conditions of injury and disease.

To examine the role of OPN in vascular calcification, we bred OPN-null mice (OPN−/−) that have no overt vascular phenotype to MGP−/− mice in which vascular calcification spontaneously develops. Mice deficient in both MGP and OPN (MGP−/−OPN−/−) showed accelerated and enhanced vascular calcification compared with mice deficient in MGP alone (MGP−/−OPN+/+)71 These studies indicate that OPN is an inducible inhibitor of vascular calcification in vivo and may play an important role in the adaptive response of the body to injury and disease. In light of our previous in vitro findings, part of the inhibition of arterial calcification in MGP−/− mice may be accounted for by the potent apatite inhibitory activity of phosphorylated OPN.30,83 Furthermore, our most recent studies point to a novel role for OPN in promoting ectopic mineral resorption as well.

In a subcutaneous implantation model, a 5- to 10-fold greater calcification was observed in glutaraldehyde-fixed porcine aortic valve leaflets explanted from OPN−/− mice versus OPN wild-types (OPN+/+), verifying again the inhibitory effect of OPN in calcification in vivo. More importantly, heterozygous mice showed early calcification of implants at 14 days, with subsequent regression at 30 days. The regression was found to correlate with the accumulation of OPN and carbonic anhydrase II expressing monocyte-derived cells, including macrophages and foreign body giant cells, and with subsequent acidification of the implants.79 Rescue of the calcification phenotype in the OPN−/− subcutaneous implantation model could be achieved by the administration of exogenous OPN. Significant inhibition of calcification in glutaraldehyde-fixed bovine pericardial implants was achieved compared with controls by delivering soluble phosphorylated rat recombinant OPN via injection at the implant site (72% inhibition) or adsorption to the implant surface (91% inhibition). Reduced phosphorylation and inactivation of the arginine-glycine-aspartate motif in adsorbed OPN resulted in significant loss of inhibition, indicating that the optimal anti-calcific effect required sufficient phosphorylation (10 to 14 phosphate groups/molecule of OPN) and a functional RGD motif. More importantly, quantitative immuno- staining showed a strong positive correlation between carbonic anhydrase II expression localized to glutaraldehyde-fixed bovine pericardial implants and adsorbed OPN having sufficient phosphorylation and a functional RGD motif.84 These studies suggest that OPN acts as an inducible inhibitor of calcification not only by inhibiting crystal growth but also by promoting active regression.

**Conclusions**

Vascular calcification is highly correlated with cardiovascular disease mortality, especially in ESRD and diabetic patients. In addition to the devastating effects of inappropriate biomineralization seen in cardiac valvulopathies, calciphylaxis, and idiopathic infantile arterial calcification, vascular calcification is now recognized as a marker of atherosclerotic plaque burden as well as a major contributor to loss of arterial compliance and increased pulse pressure seen with age, diabetes, and renal insufficiency. The presence of inducers, such as phosphate, and inhibitors, such as pyrophosphate and osteopontin, are likely to control whether calcification occurs under pathological conditions. Furthermore, arterial wall cells appear to play a particularly important role in mediating vascular calcification. Understanding the origins of the cells participating in osteochondral tissue formation, and mechanisms controlling their differentiation may aid in the development of novel therapeutic strategies to prevent and potentially reverse vascular calcification, which is an urgent need in the ESRD population.

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**References**


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