Vascular Calcification: In Vitro Evidence for the Role of Inorganic Phosphate

CECILIA M. GIACHELLI
Bioengineering Department, University of Washington, Seattle, Washington

Abstract. Uremic patients are prone to widespread ectopic extraskeletal calcification resulting from an imbalance of systemic inorganic phosphate (Pi). There can be serious consequences of this process, particularly when it results in the calcification of the vasculature. A recent study examined the response of cultured human aortic smooth muscle cells to varying levels of extracellular Pi. Cells that were exposed to Pi levels similar to those seen in uremic patients (>1.4 mmol/L) showed dose-dependent increases in cell culture calcium deposition. The results of this study also defined the role of elevated phosphate in transforming the vascular phenotype of these cells to an osteogenic phenotype, such that a predisposition for calcification was created. Pi-induced changes included increased expression of the osteogenic markers osteocalcin and core-binding factor-1 genes, the latter of which is considered a “master gene” critical for osteoblast differentiation. These changes occur early after exposure to high phosphate levels and seem to be mediated by a sodium-dependent phosphate co-transporter, Pit-1 (Glvr-1). Calcification of vascular cells also seems to occur in the absence of a mineral imbalance but in the presence of platelet-derived growth factor, a potent atherogenic factor. Taken together, these data suggest that calcification of vascular cells can occur early in a phosphate-rich environment similar to that seen in patients with renal failure and in a platelet-derived growth factor–rich atherosclerotic region under normal phosphorus conditions. From a clinical viewpoint, it seems that early control or prevention of hyperphosphatemia may reduce coronary calcification and its associated morbidity and mortality for patients on dialysis.

Cardiovascular disease is prominent in ESRD (chronic kidney disease stage 5). Nearly half of the deaths in dialysis patients in 1999 were attributed to cardiovascular causes (1). It has also been documented that hyperphosphatemia is prevalent in patients with chronic renal failure (2) and that hyperphosphatemia is linked to increased risk of cardiovascular mortality in these patients (3–5). The cellular and molecular correlates of this linkage are now being elucidated and indicate that hyperphosphatemia can lead to vascular calcification or deposition of calcium phosphate mineral, generally hydroxyapatite, in cardiovascular tissues such as arteries, cardiac muscle, and heart valves, including prosthetic valves.

Elevated serum phosphate levels in uremic patients have been highly correlated with vascular calcification (5). High levels of calcium and phosphate can induce vascular calcification (6). Uremic patients are prone to ectopic calcification (3), defined as inappropriate mineralization in soft tissues (7). Ectopic calcification can be metastatic or dystrophic. Uremic patients are predisposed to metastatic calcification (3), defined as a systemic mineral imbalance associated with widespread ectopic calcification (7). This predisposition occurs when the calcium-phosphorus product is elevated (3). Ectopic calcification presents a particular clinical problem when it occurs in the vasculature of uremic patients, and it contributes to both the morbidity and mortality associated with ESRD (3,4).

Vascular calcification has been related to an increased risk of cardiovascular morbidity and complications such as atherosclerotic plaque burden (4,8,9), myocardial infarction (10,11), coronary artery disease (12,13), postangioplasty dissection (14), and increased ischemic episodes in peripheral vascular disease (15). It has also been found to be a powerful independent marker of coronary heart disease events in patients with diabetes (12). Recent studies also indicate that coronary calcification may be predictive of or associated with sudden cardiac death (16,17). Indeed, both the Framingham risk index and coronary calcification score as measured by electron beam computed tomography have been shown to have prognostic value for cardiovascular events (17). Finally, strong associations among arterial calcification, stiffness, pulse pressure, and mortality in dialysis patients have been noted and likely further contribute to the high rates of cardiac and peripheral ischemic disease and left ventricular hypertrophy in this population (18–20).

In light of these risks, it is important to limit vascular calcification in the dialysis population. Understanding the role of phosphate and improving our ability to manage hyperphosphatemia is an essential part of this effort. This article reviews data from a cellular and mechanistic viewpoint to explain the events that regulate the entry of inorganic phosphate (Pi) into vascular cells, the subsequent genetic and biochemical response within these cells, and the physiologic or pathologic outcome of such responses. These data clearly define a role for
phosphate in the mineralization of aortic smooth muscle cell (SMC) and provide biochemical evidence for the role of hyperphosphatemia in transforming vascular cells into osteoblast-like cells, thus increasing the risk of calcification and cardiovascular disease.

Effect of Excess Pi in Vascular Cell Culture Media

Recent evidence dispels the classically held view that vascular calcification is a passive, degenerative, end-stage process of vascular disease. Evidence now points to vascular calcification as an actively regulated process akin to bone mineralization (7). Both pro- and anticalcifying mechanisms have been found to play an active role in mineral deposition in vascular cells. Exploring the hypothesis that human aortic SMC (HSMC) in culture would respond to elevated Pi levels by increasing pro-mineralization factors, we have examined the response of cultured HSMC to increasing concentrations of Pi in the medium and found that cells that are exposed to physiologic levels of Pi (1.4 mmol/L) grow normally and do not undergo mineralization (21). In contrast, cells that are grown in the presence of higher Pi concentrations (up to 2 mmol/L) similar to those seen in individuals with hyperphosphatemia, show an increased deposition of calcium into vascular cells. This deposition occurs in a time- and dose-dependent manner (Figure 1). No spontaneous deposition of calcium occurred in the calcification media or in media supplemented up to 10 mmol/L Pi, indicating that vascular cells (or cell-derived matrix) were essential for mineralization. After 10 d of culture at Pi >1.4 mmol/L, calcified cells developed granular deposits that were identified as phosphate-containing material by positive von Kossa staining. The granules were primarily associated with extracellular matrix, with the greatest accumulation occurring in areas of cell multilayering. Transmission electron microscopy and electron diffraction of specific sites verified an apatitic mineral phase, matrix vesicles, and calcified collagen fibers. The calcification seemed to be a general effect of vascular smooth cells, because cells from various sources—primary and immortalized human fetal and adult tissues and aortic and coronary atherosclerotic plaque—all exhibit similar behavior (6,21). These data provided evidence to support the tenet that calcification of vascular SMC could occur with increasing frequency in an environment of increasing phosphate concentration. This accumulation of calcium can increase with time and can result in calcium deposition primarily in the extracellular matrix of vascular cells regardless of age of tissue and vascular cell origin.

Mechanistic Evidence for Hyperphosphatemia-Induced Calcification

Results from several in vitro studies have indicated that Pi stimulates SMC to undergo phenotypic changes that predispose them to calcification (21–23). The transcription factor core-binding factor-1 (Cbfa-1) has been found to regulate osteocalcin, osteopontin, and type I collagen gene expression. This factor is an absolute requirement for osteoblast differentiation (24). The expression of the osteogenic markers osteocalcin and Cbfa-1 is strongly induced in the presence of elevated phosphate (21). In cell culture studies, induction of these osteogenic markers occurred as early as 24 h after treatment with Pi at a concentration of 2 mmol/L. Similarly, in a study of bovine aortic SMC, mineralization of SMC in culture was associated with the dramatic loss of smooth muscle–specific gene expression (smooth muscle lineage markers SM22 and α smooth muscle actin) in the presence of an organic phosphate donor, β glycerophosphate (6). A third study (23) has shown that medial cells from calcified arteries of matrix gla-protein (a calcification inhibitor) null mice express high levels of osteopontin and Cbfa-1, as well as decreased levels of α smooth muscle actin, when compared with SMC from noncalcified wild-type blood vessels. It is interesting that evidence for similar expression patterns in calcified human arteries have recently been reported. Osteopontin levels were increased (25,26) and α smooth muscle actin levels were decreased (25) in calcified medial layers of cutaneous blood vessels in patients with calcific uremic arteriolopathy. Furthermore, staining for osteopontin and other bone matrix molecules was strongly correlated with medial calcification in epigastric arteries of dialysis patients (27). These data support the concept that SMC undergo phenotypic conversion to osteogenic cell type in the presence of hyperphosphatemia in both animals and humans. Recently, in situ hybridization and immunostaining have shown that Cbfa-1 and osteopontin are selectively expressed in the media and intima of calcified but not uncalcified inferior epigastric arteries from uremic patients (28). In addition, pooled uremic sera and nonpooled control human sera were found to induce the expression of Cbfa-1 in bovine vascular

![Graph](https://via.placeholder.com/150)


*P<0.01 vs 1.4mM.*
SMC in a time-dependent, non-phosphorus-mediated mechanism. As in the bone, Cbfa-1 seems to be a key regulatory factor in vascular calcification, being upregulated by uremic toxins in dialysis patients.

It seems that the effects of hyperphosphatemia are mediated by a sodium-dependent phosphate co-transporter (NPC) that facilitates entry of Pi into vascular cells (21). Three types of NPC have been defined in humans. Types I and II are most common in the kidney and intestine. Type III is expressed throughout the body (29). PCR and Northern blot analysis have identified the NPC in HSMC as Pit-1 (Glr-1), which is a type III NPC. No transcripts were found for any other NPC in HSMC in culture (21).

For determining whether the phenotypic change seen in SMC culture calcification was regulated by the NPC system, the NPC-specific inhibitor phosphonoformic acid (PFA) was added to the culture medium. Gene expression was noted in the presence and in the absence of PFA. PFA almost completely inhibited Pi uptake in HSMC. The phosphate-induced expression of osteogenes (as indicated by osteocalcin and Cbfa-1 markers) was also inhibited by PFA in a dose-dependent manner. A second NPC inhibitor, arsenate, was also found to inhibit the expression of osteogenes, validating the hypothesis that Pi entry into cells and the subsequent activation of osteogenic genes is dependent on NPC-regulated cell entry (6,21).

Once the role of NPC in hyperphosphatemia-induced calcification was established, it was important to determine whether these proteins would also affect calcification in the absence of a mineral imbalance. Such calcification commonly occurs in atherosclerotic lesions. The atherogenic stimulus platelet-derived growth factor (PDGF) was studied in HSMC culture. It also induces the expression of Pit-1 mRNA and calcification of HSMC in a time- and dose-dependent manner. This PDGF-mediated calcification seems to occur even under normal phosphate conditions (6). These data provide a mechanistic basis for the increased calcification associated with atherosclerotic lesions, which typically contain high PDGF levels even when phosphate levels are within normal limits (30).

### Phosphate Regulation of SMC Mineralization: An Overview

On the basis of the data reviewed here, it is proposed that extracellular Pi is moved into intracellular compartments via NPC-mediated pathways (Figure 2). This intracellular movement is increased during hyperphosphatemia as seen in uremic patients (or during PDGF stimulation as seen in atherosclerotic lesions) and leads to the accumulation of intracellular phosphate. By pathways that have not yet been fully elucidated, the increased intracellular phosphate serves as a signal for osteogenic gene expression (Cbfa-1 and downstream targets osteopontin and osteocalcin) and as a suppressor of HSMC-specific gene expression, resulting in increased secretion of mineral-nucleating molecules (matrix vesicles, calcium-binding proteins, alkaline phosphatase, and collagen-rich extracellular matrix). These factors combine to transform the cell to be susceptible to vascular calcification (6).

The emerging picture of soft tissue calcification seems to be one that is actively regulated. The focus of this article has been on the active inducers of calcification. It should be added that definitive data on the presence of local and systemic inhibitors of calcification have been accumulating over the past several years and come largely from studies involving gene knockout mice. These data implicate the involvement of several gene products in ectopic calcification (Table 1) (31–37) and suggest that the matrix gla-protein gene, osteoprotegrin, and osteopontin may serve as natural inhibitors of cardiovascular calcification that may be either constitutively expressed or induced to prevent ectopic calcification. Indeed, vascular calcification may involve an active and dynamic balance of procalcifying and anticalcifying mechanisms.

### Conclusion

Uremic patients are prone to widespread ectopic and metastatic calcification as a result of mineral imbalances, particularly the imbalance of Pi. Serious consequences occur as a result of this. The in vitro data reviewed here provide biochemical evidence for the role of hyperphosphatemia in transforming cells from a vascular phenotype to an osteogenic phenotype, creating a predisposition for calcification. These changes occur early after exposure to high Pi levels and continue to accumulate over time and with increasing Pi concentrations.

It seems that the phenotypic transformation of HSMC in response to hyperphosphatemia is mediated by the NPC Pit-1, which predisposes SMC to undergo mineralization. Smooth muscle-specific gene expression may be downregulated, whereas osteoblast or chondrocyte-like gene expression may be upregulated through upregulation of Cbfa-1 and its down-

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**Figure 2.** Regulation of human smooth muscle cell mineralization by phosphate ion. Reprinted from Giachelli CM, Jono S, Shioi A, Nishizawa Y, Mori K, Morii H: Vascular calcification and inorganic phosphate. *Am J Kidney Dis* 38: S34–S37, 2001, with permission from the National Kidney Foundation.
stream genes, thus promoting mineralization. Pit-1 also seems to be able to affect calcification in the absence of a systemic mineral imbalance. In light of this in vitro evidence, early control or prevention of hyperphosphatemia may be key in reducing coronary calcification and the resulting morbidity and mortality as a result of cardiovascular disease for patients on dialysis.

References


Table 1. Genes associated with ectopic calcification

<table>
<thead>
<tr>
<th>Null Mutation</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>Matrix gla-protein (31)</td>
<td>Arterial, valve, and cartilage calcification</td>
</tr>
<tr>
<td>β-glucosidase (klotho) (32)</td>
<td>Vascular calcification, rapid aging</td>
</tr>
<tr>
<td>Desmin (33)</td>
<td>Neonatal cardiomyopathy with calcification</td>
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<tr>
<td>Carbonic anhydrase II (34)</td>
<td>Vascular calcification of small arteries</td>
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<tr>
<td>Fetuin (35)</td>
<td>Decreased serum hyaluronic acid inhibitory activity</td>
</tr>
<tr>
<td>Osteoprotegrin (36)</td>
<td>Osteoporosis, vascular calcification</td>
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<tr>
<td>Osteopontin (37)</td>
<td>Increased calcification of subcutaneously implanted bioprosthetic valve</td>
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