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Role of Calcium-Phosphate Product and Bone-Associated Proteins on Vascular Calcification in Renal Failure

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Cardiovascular events are the most frequent cause of death in patients with chronic renal failure (1–2). Heterotopic calcification of blood vessel walls occurs frequently with advanced age, atherosclerosis, and diabetes mellitus (3). In chronic renal failure, autopsy (4–5) and clinical investigations (6) have documented a higher prevalence of coronary plaques in dialyzed patients compared with the nonuremic population. The risk factors that contribute to the higher prevalence of atherosclerotic lesions in chronic renal failure include dyslipidemia, hyperhomocysteinemia, and hypertension (3). In addition, hyperphosphatemia and increased calcium-phosphate product are important contributors to vascular calcifications in patients with uremia (7).

A new, noninvasive imaging technology, electron beam computed tomography, has demonstrated that both hyperphosphatemia and increased calcium-phosphate product cause a progressive increase in calcium deposition in the coronary arteries and mitral and aortic valves in patients with advanced renal failure (8). Hyperphosphatemia associates with ectopic calcifications, increased risk of calciphylaxis, and calcinosis (9–10). Figure 1 illustrates direct and indirect mechanisms for hyperphosphatemia to increase calcium-phosphate product. High levels of phosphate, due to phosphorus retention, worsen the secondary hyperparathyroidism commonly present in renal failure through direct and indirect mechanisms. High phosphate enhances parathyroid cell proliferation and parathyroid hormone (PTH) synthesis and secretion directly and indirectly through both a reduction in serum calcitriol and ionized calcium levels and a reduction of skeletal resistance to PTH. The resultant high PTH causes osteitis fibrosa and bone loss and therefore further increases in calcium-phosphate product (11–12). In addition to its effects on bone, high PTH, by elevating cytosolic calcium, could cause microcalcifications, as has been demonstrated in lung (13) and skin (14). Also, PTH impairment of lipid metabolism and immune cell function (15) could directly contribute to enhance cardiovascular calcification. The relative contribution of hyperphosphatemia, high calcium-phosphate product, and secondary hyperparathyroidism to enhance vascular calcification, as well as the mechanisms involved, are incompletely understood.

In recent years, vascular calcification was shown to involve not a passive deposition of calcium-phosphate crystals on atherosclerotic vessels but an active process in which vascular cells elicit osteoblastic functions (16). Furthermore, there is a paradoxical coincidence of vascular mineralization with bone loss in human and animal models, which suggests that the same factors that induce high-turnover bone disease in renal failure could also mediate vascular calcification. This review presents the emerging understanding of the mechanisms by which hyperphosphatemia and elevated Ca × P product affect vascular calcification. In addition, we present the biologic interactions and functional parallels between bone and vascular tissues in bone-associated proteins and PTH-related peptide (PTH-rP).

Phosphate Regulation of Vascular Calcification

Hyperphosphatemia associates with prosthetic cardiac valve calcification and osteocalcin deposition in experimental animals with normal renal function (17). Recent studies have demonstrated phosphate regulation of vascular calcification and provided some insights into the mechanisms for phosphate induction of metastatic calcifications. *In vivo* studies by Kuro-o *et al.* (18), in the KLOTHO-gene mutant mice with a phenotype that resembles human aging, demonstrated that, in the presence of normal serum creatinine, albumin, cholesterol, and triglyceride levels and with only a mild increase in serum calcium levels (from 9.5 to 10.6 mg/dl), a two-fold increase in serum phosphate levels resulted in increased calcium-phosphate product as well as the development of vascular calcifications and osteoporosis that were clearly unrelated to malnutrition, abnormal lipid metabolism, or chronic renal failure. Because hyperphosphatemia was the main determinant of the increased Ca × P product, Jono *et al.* (19) assessed the contribution of hyperphosphatemia *per se* on vascular calcification. High phosphorus levels in the incubation media (2 mmol/L P) enhanced calcification in human aortic smooth-muscle cells. Phosphate-containing mineral deposition, assessed by von Kossa staining and light microscopy, was predominant in the extracellular regions of the cultures, with the greatest accumulation at sites of cell multilayering (19). Furthermore, these studies demonstrated that high phosphate directly increases human aortic smooth-muscle cells calcification and the expression of the osteoblast-specific genes *Osf2/Cbfa-1* and *osteocalcin* (19) (Figure 2). Both effects of high P are

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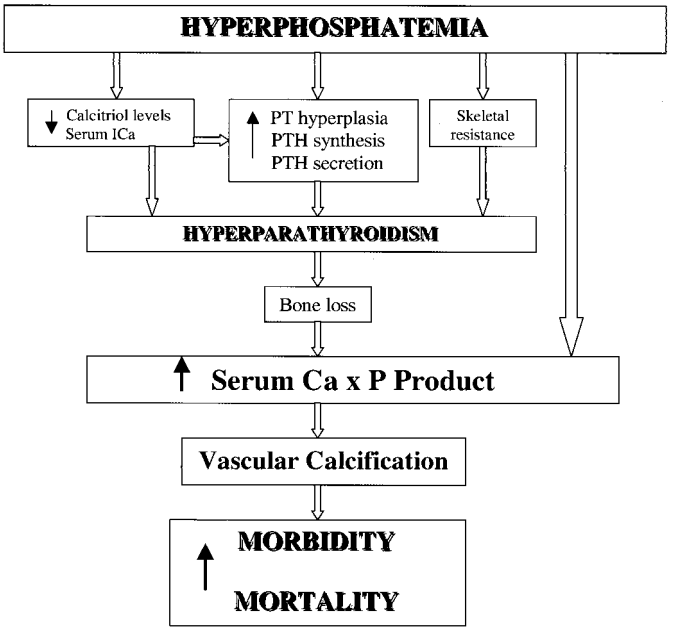

Figure 1. Potential side effects of hyperphosphatemia in renal failure.

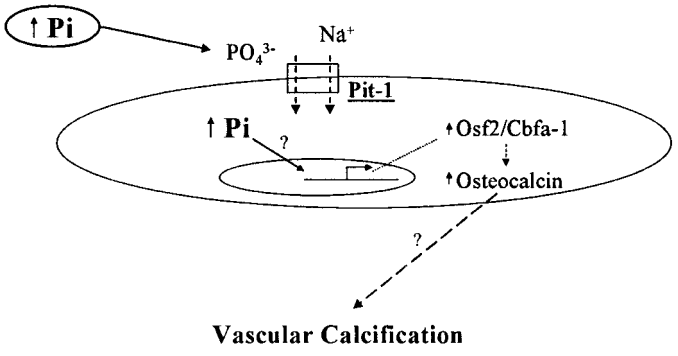

Figure 2. Phosphate regulation of vascular smooth muscle-cell calcification. In human smooth-muscle cells in culture, high-phosphate medium (2.0 mmol/L) increases calcification by enhancing intracellular phosphorus concentration and inducing osteoblast-specific genes *Osf2/Cbfa-1* and *osteocalcin,* which directly associate with ectopic calcification. Both effects are mediated by the sodium-dependent phosphate cotransporter (NPC) Pit-1 (member of type III family of NPC). Adapted with permission from Jono *et al.* (19).

mediated by the sodium-phosphate cotransporter Pit-1. *Osf2/Cbfa-1* is the only transcriptional activator of osteoblasts differentiation in postnatal life (20). Moreover, *Cbfa-1* regulates the expression of osteocalcin (21), one of the more important osteoblast-specific genes *in vitro* and *in vivo.* These reports suggest that, in addition to elevating Ca × P product, high P *per se* could induce vascular calcification. New studies are mandatory to address the contribution of increased phosphate levels in the regulation of Pit-1 function and *Osf2/Cbfa-1* and *osteocalcin* expression and therefore in bone formation and arterial calcification in advanced renal failure.

Phosphate Regulation of Vascular Cell Proliferation

Abnormal proliferation of vascular smooth-muscle cells (VSMC) is another contributor to the development of atherosclerotic lesions. Cell cycle activity is regulated by proteins of the growth suppressor family of cyclin-dependent kinase inhibitors, including $p21^{Cip1/WAF1}$. p21 has been implicated in arresting VSMC growth through a thrombospondin-1–associated mechanism (22). Increases in p21 expression limit intimal cell proliferation in response to arterial injury (23) and correlate with growth arrest in several other cell types (24). Increases in p21 induce G1 arrest and blocked entry into S phase by the inactivation of cyclin–cyclin-dependent kinase complexes. Enhanced p21 also inhibits DNA replication by preventing the formation of a trimer of proliferating cell nuclear antigen, an essential cofactor for the activity of DNA polymerase delta.

Interestingly, in uremic rats, whereas phosphate restriction upregulates parathyroid p21 expression, thus suppressing uremia-induced parathyroid growth, high dietary phosphorus prevents the increase in parathyroid p21 necessary to compensate the mitogenic stimuli induced by the onset of renal failure (25). A similar regulation of p21 expression by phosphorus concentrations in vascular cells could modulate proliferation rates, thus providing an additional mechanism for phosphorus to contribute to the cardiovascular pathology of renal failure.

Bone-Associated Proteins in Vascular Calcification

Numerous studies have supported a new definition of arterial calcification as an organized process with similarities to bone formation and a striking association between bone loss and ectopic calcifications. The current understanding of the association between bone morphogenetic proteins and vascular calcification in atherosclerotic lesions in humans and animal models is presented below and summarized in Figure 3. Although increased expression of osteocalcin, osteonectin, bone morphogenic protein type 2a (BMP-2a) and alkaline phosphatase associates with enhanced vascular calcification, higher content of osteopontin, matrix Gla protein (MGP), and osteoprotegerin inhibits mineral deposition.

Osteopontin has been associated with both bone mineralization and ectopic calcification. Two recent studies have investigated the expression of osteopontin in calcified arteries (26–27). Wada *et al.* (28) proposed that osteopontin acts as an inhibitor of calcification of VSMC cultures. Jono *et al.* (29) demonstrated that the phosphorylation of osteopontin was a required step for inhibition of vascular cell calcification in VSMC. Thus, osteopontin is not only a potent bone mineralization regulator but is also an important inhibitor of ectopic calcifications.

Gla proteins also participate in the pathophysiology of osteoporosis and in the prevention of vascular calcification (30). MGP is an extracellular matrix protein with high affinity for hydroxyapatite. Mice that lack MGP develop arterial calcification as well as inappropriate bone formation with pathologic fractures during the first 2 mo of life (31). These findings illustrate that, similar to osteopontin, MGP is required to promote normal bone formation and also to inhibit vascular calcification.

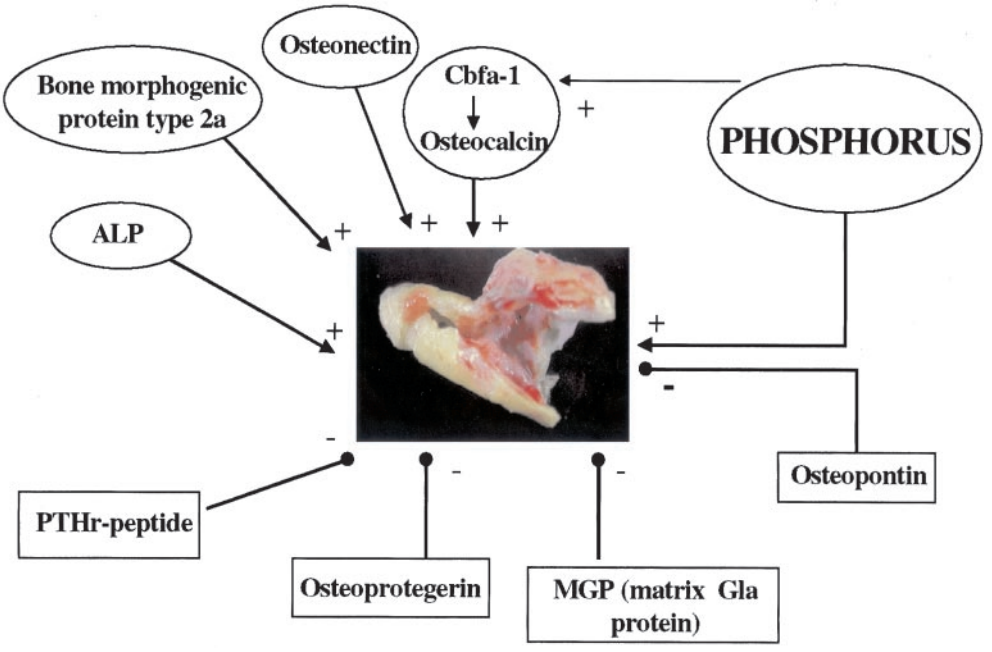

Figure 3. Vascular calcification and bone-associated proteins. Bone-associated proteins elicit opposing effects on vascular calcification. Osteocalcin, osteonectin, bone morphogenic protein-2a, and alkaline phosphatase (ALP) are inducers of calcification. In contrast, osteopontin, matrix Gla protein (MGP), osteoprotegerin, and parathyroid hormone–related peptide (PTHr-peptide) inhibit calcification. In addition to a direct effect on calcium deposition, high phosphorus could enhance active calcification through induction of *Cbfa-1* and *osteocalcin* expression. Osteopontin and osteocalcin are the two most critical bone-associated proteins involved in vascular calcification, and their regulation in chronic renal failure should be further examined. Adapted from Tierney *et al.* (55).

Osteoprotegerin (OPG), a protein that inhibits osteoclastogenesis, is an additional player in vascular calcification. In OPG-deficient mice, Bucay *et al.* (32) observed a decreased bone density and increased vascular calcification of the aorta and renal arteries. Studies by Min *et al.* (33) indicate that OPG plays an important role in both pathologic and physiologic calcification processes. Similar to osteopontin and MGP, OPG appears to inhibit ectopic calcification.

In contrast to the protective effects of osteopontin, MGP, and OPG, increases in other bone matrix proteins, osteocalcin, osteonectin, BMP-2a, and alkaline phosphatase, directly correlate with vascular calcification. Osteocalcin was found in calcified atherosclerotic plaques and calcified areas within glutaraldeyde-preserved porcine valves (34). In addition, increased bone formation has been demonstrated in osteocalcin-deficient mice (35). Studies to clarify the role of osteocalcin in vascular calcification have demonstrated that high phosphate increased human aortic smooth-muscle cells calcification by enhancing osteocalcin and *Osf2/Cbfa-1* expression (19). These findings suggest a direct association between osteocalcin and ectopic calcifications.

Osteonectin, also known as SPARC or BM40, is a protein that is localized mainly in mineralized tissues such as bone, cartilage, and teeth (36). Bini *et al.* (37) identified osteonectin, osteocalcin, and osteopontin in advanced atherosclerotic plaques with dystrophic calcification in human thrombotic carotid arteries. Osteonectin was found in endothelial cells, VSMC, and macrophages, and its expression associates with calcium-phosphate deposition (37). Although osteonectin seems to associate directly with ectopic calcification, the mechanisms for osteonectin-mediated vascular calcification remain unclear. Another protein, BMP-2a, which has a potent osteoblast-differentiating function (38), was found in human atherosclerotic plaques. A direct association appears to exist between BMP-2a and vascular calcification (39). Finally, alkaline phosphatase (ALP) activity may play a role in calcification of artery cell walls in humans. Shioi *et al.* (40) demonstrated that increased levels of ALP accelerate calcification in bovine vascular smooth-muscle cells. Moreover, levamisole, an ALP inhibitor, blocked bovine vascular smooth-muscle cell calcification in a dose-dependent manner (40). The presence of ALP in human atherosclerotic lesions (41) suggests an active role in the promotion of vascular calcification. More studies are necessary to identify the precise role of bone-associated proteins in the pathogenesis of vascular calcification and the contribution of altered expression and/or function of these proteins in chronic renal failure to the higher incidence of vascular calcification in patients with uremia.

Role of PTH-rP in Vascular Calcification

One more protein appears to play an important role in modulating calcification of VSMC: the PTH-rP. In an *in vitro* calcification model, Jono *et al.* (42) showed the inhibitory action of PTH-rP in bovine vascular smooth-muscle cells. In

addition, the inhibition of calcification by either etidronate or levamisole involves enhancement of PTH-rP secretion (42).

This *in vitro* relationship between PTH-rP expression and vascular calcification has been observed also in an *in vivo* model. Nakayama *et al.* (43) showed that, in coronary atherosclerotic smooth-muscle cells, PTH-rP expression correlated with the severity of atherosclerosis. However, PTH-rP expression was higher in uncalcified coronary atherosclerotic lesions than in calcified VSMC (43).

Clearly, these findings showed the *in vitro* and *in vivo* association between PTH-rP expression and arterial calcification. However, the mechanisms involved, as well as the regulation of the expression of PTH-rP and its receptor, the PTH receptor, during vascular calcification in renal failure, remain to be clarified.

Treatment of Hyperphosphatemia in Renal Failure

Because vascular calcifications are predictive of higher morbidity and mortality, the control of serum phosphorus and calcium-phosphate product is important in the prevention of arterial calcifications in chronic renal failure (7). The current treatment for the hyperphosphatemia of chronic renal failure consists of dietary phosphate restriction, dialysis treatment, and administration of phosphate binders (aluminum salts, calcium carbonate, or acetate).

The main limitation for effective phosphate restriction in patients undergoing dialysis is that it requires dietary protein restriction. Basically, the achievement of a hypophosphoric diet is against a protein intake of 1.0 to 1.2 g/kg per d, which is recommended to avoid malnutrition. An average daily and weekly P balance is depicted in Figure 4. In a patient undergoing dialysis who ingests the adequate amount of protein, the average daily phosphate intake is 1 to 1.2 g. With an average 60% P absorption, the net balance is 5 g of phosphate per week.

During hemodialysis treatment, phosphate clearance is efficient only during the first 2 h. Serum phosphorus levels do not change during the second half of dialysis (44). Because hemodialysis removes ~900 mg of phosphate three times weekly, >90% of patients with uremia require phosphate binders to avoid hyperphosphatemia (45). In fact, to achieve a "normal" phosphorus level that is below 5 mg/dl, 13 to 18 g of calcium carbonate are required daily (46).

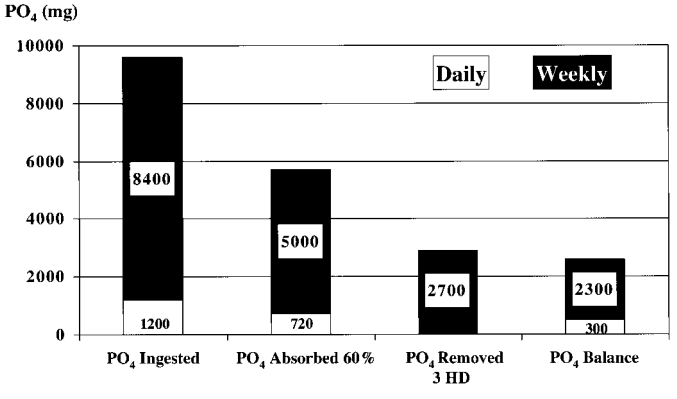

Figure 4. Phosphate balance in patients undergoing hemodialysis (HD). From a weekly intake of phosphate of ~8.4 grams (1.2 g/d), 60% is absorbed, rendering a net absorption of 5.0 g/wk (0.7 g/d). Because P removed by standard HD (3 times weekly, 4 h for each session) is ~2.7 g, a positive P balance is ~2.3 grams weekly (300 mg/d).

Recent studies have described the limitations of calcium-based phosphate binders and the calcium overload in patients with advanced renal failure (8,47). Patients undergoing dialysis ingest 500 to 800 mg of calcium daily. Additional sources contribute to the high calcium load: calcium in the dialysate, calcium supplements, and calcium-based phosphate binders. Hsu *et al.* (48) demonstrated that patients undergoing dialysis are in a positive calcium balance and that the excess calcium is present in the gastrointestinal tract and soft tissues. In addition, in patients with uremia, there is an association between elemental calcium intake from calcium-based phosphate binders and dietary phosphorus as protein (46).

The problem of calcium load in renal failure was emphasized 35 yr ago by Clarkson *et al.* (49). In patients with advanced renal failure, the ingestion of 20 g of calcium carbonate daily for 18 to 30 d caused an important increase in calcium absorption. Furthermore, increased calcium absorption was also present in normal controls (49). Clarkson in 1966 concluded: "Unless all of the calcium which is absorbed is laid in bone the calcium content of the soft tissues may rise and renal function deterioration increase" (49).

In addition, to control the progression of secondary hyperparathyroidism, oral or intravenous administration of $1,25(OH)_2D_3$ is commonly used. Because $1,25(OH)_2D_3$ increases intestinal calcium absorption and calcium and phosphate mobilization from bone, vitamin D therapy further increases the risk of development of vascular calcification in patients who have an already enhanced calcium-phosphate product, especially in those who receive calcium salts as phosphate binders.

Despite this modern therapy, more than half of these patients do not achieve good control of serum phosphorus, calcium-phosphate product, and PTH levels. New phosphate binders, poly-allylamine hydrochloride (sevelamer HCl, Renagel; Genzyme, Cambridge, MA) and trivalent iron–containing compounds, have been developed. These compounds do not contain aluminum or calcium and therefore lack the side effects associated with classical phosphate binders. In the case of sevelamer HCl, in addition to effectively reducing serum levels of phosphorus (31%) and PTH (22%) with no hypercalcemia (Figure 5), it ameliorates lipid abnormalities (50). A reduction of ~30% in low-density lipoprotein cholesterol and an 18% increase in high-density lipoprotein cholesterol concentration accompanied sevelamer treatment (50). These findings suggest the potential of this new phosphate binder to reduce atherosclerotic lesions and vascular calcification.

In the case of trivalent iron–containing compounds, in normal and uremic rats (51), effective inhibition of intestinal phosphate absorption was demonstrated for both iron(III) oxide-hydroxide dextran (52) and Fe(III) citrate: Fe(III) chloride (51). Despite no reduction in serum phosphorus, both compounds markedly reduced urinary phosphate excretion. In 13

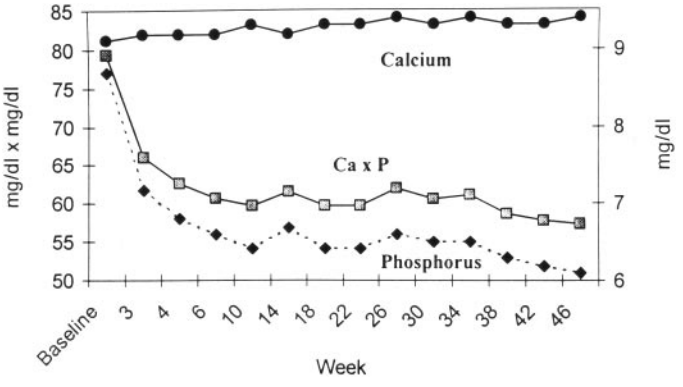

Figure 5. Control of serum phosphorus in patients with advanced renal failure. Effects of sevelamer HCl on serum calcium, phosphorus, and calcium-phosphate product in a group of 192 patients undergoing HD. (Modified and reprinted with permission, Ref. [50]).

predialysis patients, with a plasma phosphate level of 2.2 mM, polynuclear iron hydroxide decreased serum phosphate levels by 20% in 4 wk with only mild laxative side actions (53). Studies in 32 patients undergoing dialysis with plasma phosphate levels of 2.64 mM showed Fe(III) polymaltose complex to cause an average 0.38-mM reduction in P by 8 wk, a decrease such that *P* did not reach statistical significance and there was no effect on serum PTH (54). Further information on the safety of these Fe(III) compounds is mandatory.

In patients with chronic renal failure, increased serum Ca × P product and hyperphosphatemia are important contributors to the higher incidence of arterial calcifications and cardiovascular events. Hyperphosphatemia, by accelerating the progression of secondary hyperparathyroidism, increases serum PTH and bone loss. High PTH itself induces increases in intracellular calcium and abnormal lipid metabolism that promote soft tissue calcifications. Phosphorus-induced and PTH-induced bone loss elevates Ca × P product and, most likely, the expression of factors that mediate the strong association between bone loss and arterial calcification, such as bone-associated proteins. Direct effects of phosphorus on vascular pathology include the regulation of vascular cell proliferation as well as the induction of the expression of the osteoblast-specific bone-forming proteins, Cbfa-1 and osteocalcin. Clearly, the control of serum phosphorus levels in patients with uremia may reduce vascular calcification not only by decreasing calcium-phosphate product but also by reducing serum PTH, thus ameliorating the active and yet incompletely understood processes common for vascular calcification and bone loss.

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