Hyperphosphatemia and Vascular Calcification in End-Stage Renal Disease

Yoshiki Nishizawa, MD, Shuichi Jono, MD,* Eiji Ishimura, MD,* and Atsushi Shioi, MD†*

Vascular calcification is a common finding in atherosclerosis and a serious problem in uremic patients. Because of the correlation of hyperphosphatemia and vascular calcification, the ability of extracellular inorganic phosphate levels to regulate human aortic smooth muscle cell (HSMC) culture mineralization in vitro was examined. HSMC cultured in media containing normal physiologic levels of inorganic phosphate (1.4 mM) did not mineralize. In contrast, HSMC cultured in media containing phosphate levels comparable with those seen in hyperphosphatemic individuals (>1.4 mM) showed dose-dependent increases in mineral deposition. Mechanistic studies showed that elevated phosphate treatment of HSMC also enhanced the expression of the osteoblastic differentiation markers osteocalcin and osf2/Cbfa-1. The effects of elevated phosphate on HSMC were mediated by a sodium-dependent phosphate cotransporter (NPC) as indicated by the ability of the specific NPC inhibitor phosphonoformic acid to dose-dependently inhibit phosphate-induced calcium deposition as well as osteocalcin and Cbfa-1 gene expression. The NPC in HSMC was identified as Pit-1, a member of the novel type III NPCs. These data suggest that elevated phosphate may directly stimulate HSMC to undergo phenotypic changes that predispose to calcification and offers a novel explanation of the phenomenon of vascular calcification under hyperphosphatemic conditions. Furthermore, we examined the factors affecting peripheral vascular calcification in 332 nondiabetic hemodialysis patients. There were 45 nondiabetic patients with vascular calcification. In multivariate logistic regression, the significant factors affecting vascular calcification were advanced age, longer duration of hemodialysis, increased phosphate concentrations, male gender, and lower predialysis diastolic pressure. Our findings suggest that an elevated phosphate level may directly stimulate HSMC to undergo phenotypic changes that predispose to calcification and offer a novel explanation of the phenomenon of vascular calcification under hyperphosphatemic conditions.

VASCULAR CALCIFICATION REFERS to the deposition of calcium phosphate minerals, most often hydroxyapatite, in cardiovascular tissues, including arteries, heart valves, and cardiac muscle. Vascular calcification is often encountered in the development of atherosclerotic intimal lesions and is a common consequence of aging.[1] In diabetic patients and individuals with renal failure, vascular calcification contributes to both the morbidity and the mortality associated with these diseases.[2] For example, vascular calcification is positively correlated with increased risk of myocardial infarction and increased risk of dissection after angioplasty.[3] Moreover, calcification is a major cause of failure for both native and tissue prosthetic heart valves, affecting 1% to 2% of the aging population.[4]

Until recently, vascular calcification was considered to be a passive, degenerative, and end-stage process of vascular disease. However, the observation of matrix vesicles, bone morphogenetic proteins, and noncollagenous bone matrix proteins such as osteopontin, osteonectin, osteocalcin, and matrix Gla protein (MGP) in calcified vascular tissues has challenged this paradigm.[5] Likewise, vascular media-derived cell cultures have the capacity to express alkaline phosphatase, osteocalcin, and osteopontin, and to calcify their extracellular matrix under appropriate condi-

**Department of Metabolism, Endocrinology and Molecular Medicine, Osaka City University Graduate School of Medicine, Osaka, Japan.*

†*Department of Cardiovascular Medicine, Osaka City University Graduate School of Medicine, Osaka, Japan.*

Address reprint requests to Yoshiki Nishizawa, MD, Department of Metabolism, Endocrinology and Molecular Medicine, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. E-mail: jono@med.osaka-cu.ac.jp

1051-2276/05/1501-0034$30.00/0

doi:10.1053/j.jrn.2004.09.027

tions.[6–8] Perhaps the most compelling evidence for active regulation of vascular calcification, however, has come from recent genetic studies in mice. The MGP-null mouse shows extensive calcification of the arterial tree, indicating that MGP, which is constitutively expressed in arterial smooth muscle cells, is normally an important inhibitor of vascular calcification.[9] In addition, several other mutant mouse strains, including the KLOTHO mouse deficient in β-glucosidase,[10] the carbonic anhydrase II mutant,[11] desmin-null mouse,[12] and the osteoprotegerin-null mouse,[13] all show enhanced susceptibility to vascular calcification. Finally, structures identical to bone and bone marrow are occasionally found in advanced atherosclerotic lesions, calcified cardiac valves, and Monckeberg sclerosis.[14] These findings suggest that vascular calcification is in fact an actively regulated process in which vascular cells may acquire osteoblast-like functions.

Inorganic Phosphate Levels as an Important Regulator of Vascular Calcification

The molecular mechanisms regulating vascular calcification remain obscure. A clue to this process, however, is suggested by several observations linking serum phosphate levels with a tendency toward vascular calcification. First, a high serum phosphate level is highly correlated with the extent of vascular calcification and vascular disease.[15,16] One of the most common causes of hyperphosphatemia is chronic renal failure and subsequent kidney dialysis, in which serum inorganic phosphate levels can typically exceed 2 mM.[16,17] Vascular calcification observed in these patients is routinely referred to as metastatic calcification because it occurs in the presence of a systemic mineral imbalance. Second, in both experimental animals and in children, prosthetic valve calcification is correlated with elevated phosphate levels.[18,19] Third, as mentioned above, the KLOTHO mutant mouse develops extensive vascular medial calcification and has twice the serum phosphate levels found in wild-type mice.[10] Finally, local disturbances of calcium and phosphate metabolism in atherosclerotic plaques have been suggested to contribute to the development of vascular calcification.[20] Thus, it is hypothesized that an important regulator of vascular calcification is the level of inorganic phosphate.

Inorganic Phosphate Levels Regulate Vascular Smooth Muscle Cell Calcification

We hypothesized that vascular smooth muscle cells (HSMC) might respond to elevated extracellular Pi levels by increasing promineralizing molecules, thereby leading to vascular calcification. The role of inorganic phosphate in vascular HSMC mineralization was investigated using an in vitro model system that was shown to mimic many of the features seen in human metastatic vascular calcification in vivo. We found that HSMC cultured in media containing normal serum phosphate levels do not mineralize. In contrast, HSMC treated with media containing phosphate levels comparable with those seen in hyperphosphatemic patients accumulated significant levels of apatitic mineral in the their matrices. Inorganic phosphate increased HSMC calcification in a time- and dose-dependent manner, and mineralization induced by inorganic phosphate was similar to that observed in other culture systems[8] and calcified vascular tissues in vivo.[21,22] In patients with chronic renal failure treated by hemodialysis, hyperphosphatemia is commonly associated with widespread vascular calcification.[23] In fact, recent studies indicate a striking association of serum phosphorus levels with mortality risk in chronic hemodialysis patients,[16,24,25] probably a result of the increased calcinosis, calciphylaxis, and secondary hyperparathyroidism typically observed in these patients. When combined with the data from our studies, these observations support the concept that inorganic phosphate levels may directly regulate vascular calcification.

Evidence for a Sodium-Dependent Phosphate Cotransporter System in HSMC

How might HSMC sense elevated phosphate levels? To determine the mechanism by which HSMC sense elevated Pi levels, we examined phosphate uptake in the HSMC using radiolabeled Pi. HSMC took up Pi in a sodium-dependent and Pi gradient-dependent manner. These properties are consistent with the presence of a

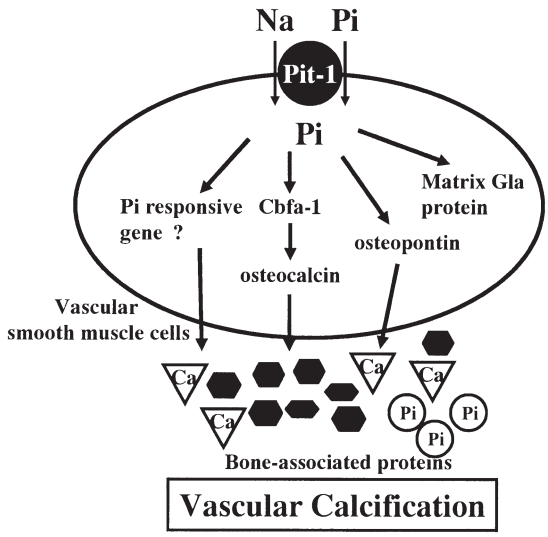

Figure 1. Phosphate regulation of smooth muscle cell mineralization. Increased Pi transport leads to increased intracellular Pi in smooth muscle cells. By an as-yet-unknown mechanism, elevated intracellular Pi levels activate specific signaling pathways that increase osteogenic gene expression, including Cbfa-1 and osteocalcin, and stimulate secretion of potential mineral nucleating molecules such as calcium-binding proteins. The net effect is enhanced susceptibility to vascular calcification.

sodium-dependent phosphate cotransporter (NPC). Three types of NPCs have been identified to date and are grouped according to homology. Type I and type II NPCs are predominantly restricted to the kidney and intestine, whereas type III NPCs are ubiquitous. This family includes NPCs that act as receptors for gibbon ape leukemia virus (Glvr-1, Pit-1) and amphotropic murine retrovirus (Ram-1, Pit-2). Of these known NPCs, only Pit-1 was identified in our HSMC as well as in human fetal aorta.

HSMC Culture Mineralization Is Dependent on NPC Function

To determine whether NPC function was important in HSMC culture calcification, we used the NPC inhibitor phosphonoformic acid (PFA). In the presence of PFA, HSMC Pi uptake was almost completely abolished. Inhibition of transport was dose-dependent, and half-maximal inhibition occurred between 0.1 and 0.5 mmol/L PFA. Consistent with a role in culture mineralization, PFA completely inhibited HSMC calcification. PFA effects were dose-dependent and half-maximal between 0.1 and 0.5 mmol/L PFA.

How might elevated phosphate and NPC activity induce HSMC-mediated mineralization? We speculate that under conditions of high extracellular phosphate or enhanced cellular NPC levels, intracellular levels of inorganic phosphate are increased via the action of Pit-1. This may lead to mechanisms initiating promineralizing metabolic processes within the cell. One of these mechanisms may be increased elaboration of an extracellular matrix that is prone to mineralize. In support of this idea, we found that elevated phosphate levels stimulated expression of both Cbfa-1 and its downstream transcriptional target, osteocalcin, in HSMC. Osteocalcin is a major noncollagenous protein found in bone matrix, and is believed to regulate mineralization.[5] Cbfa-1 is an osteoblast-specific transcription factor required for osteoblast differentiation, bone matrix gene

Table 1. Factors Affecting Vascular Calcification in Nondiabetic Hemodialysis Patients (n = 332)

	Odds Ratio	95% Confidence Interval	*P* Value
Age (y)	1.082	1.039-1.127	.0001
Duration of dialysis (y)	1.128	1.065-1.195	.0001
Phosphate (mg/dL)	1.784	1.264-2.520	.0010
Male (versus female)	3.380	1.289-8.860	.0019
Diastolic blood pressure (mm Hg)	0.972	0.953-0.9993	.0075
Systolic blood pressure (mm Hg)	1.020	0.9997-1.043	.0906
Smoking (versus nonsmoking)	1.417	0.577-3.480	.3741
Calcium (mg/dL)	0.846	0.459-1.558	.5918
Kt/V	0.778	0.087-6.990	.8224
Intact parathyroid hormone (pg/mL)	1.000	0.998-1.002	.9277
		$R^2 = 0.231$*	$P < .0001$

*R^2, multiple coefficient of variation.

expression, and consequently bone mineralization.[26] Cbfa-1 has been previously shown to directly regulate the expression of the major components of bone matrix, including collagen type I, osteocalcin, and osteopontin.[27] Thus it is likely that phosphate-signaled increases in Cbfa-1 gene expression in HSMC leads to enhanced transcription and secretion of an osteoid-like extracellular matrix that contributes to enhanced calcification under hyperphosphatemic conditions (Fig 1).

Increased Phosphate Level Is Associated With Vascular Calcification in Dialysis Patients

Vascular calcification, which significantly increases cardiovascular and other causes of mortality,[28] is highly prevalent in dialysis patients. Factors affecting vascular calcification in dialysis patients include advanced age, derangement of calcium-phosphate metabolism,[29] and diabetes.[30] We examined 332 nondiabetic patients (192 male and 140 female, 59 ± 13 years). Hand roentgenography was performed, and visible vascular calcification of the hand arteries was evaluated. There were 45 nondiabetic patients with vascular calcification (13.6%). Multivariate logistic regression analyses were performed to explore the combined impact of factors affecting vascular calcification. The significant factors affecting vascular calcification in nondiabetic patients were advanced age, longer duration of hemodialysis, increased phosphate concentrations, male gender, and lower predialysis diastolic pressure (Table 1). In the present study, the level of phosphate was significantly higher in nondiabetic patients with vascular calcification than in nondiabetic patients without vascular calcification ($P = .0003$). In the former group, although the Ca×P product was higher $P = .0058$), the statistical significance of the level of phosphate was greater. This finding emphasizes that hyperphosphatemia is an important risk factor in diabetic patients with end-stage renal disease. Our data suggest that elevated phosphate level may directly stimulate HSMC to undergo phenotypic changes that predispose to calcification and offer a novel explanation of the phenomenon of vascular calcification under hyperphosphatemic conditions.

References

1. Blumenthal HT, Lansing AI, Wheeler PA: Calcification of the media of the human aorta and its relationship to intimal arteriosclerosis, aging and disease. Am J Pathol 20:665-687, 1944
2. Frink RJ, Achor RW, Brown AJ, et al: Significance of calcification of the coronary arteries. Am J Cardiol 26:241-247, 1970
3. Fitzgerald PJ, Ports TA, Yock PG: Contribution of localized calcium deposits to dissection after angioplasty. An observational study using intravascular ultrasound. Circulation 86:64-70, 1992
4. Loecker TH, Schwartz RS, Cotta CW, et al: Fluoroscopic coronary artery calcification and associated coronary disease in asymptomatic young men. J Am Coll Cardiol 19:1167-1172, 1992
5. Giachelli CM: Ectopic calcification: Gathering hard facts about soft tissue mineralization. Am J Pathol 154:671-675, 1999
6. Watson KE, Bostrom K, Rvindranath R, et al: TGF-b1 and 25-hydroxycholesterol stimulate osteoblast-like vascular cells to calcify. J Clin Invest 93:2106-2113, 1994
7. Shioi A, Nishizawa Y, Jono S, et al: Beta-glycerophosphate accelerates calcification in cultured bovine vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 15:2003-2009, 1995
8. Wada T, McKee MD, Steitz S, et al: Calcification of vascular smooth muscle cell cultures: Inhibition by osteopontin. Circ Res 84:166-178, 1999
9. Luo G, Ducy P, McKee MD, et al: Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature 386:78-81, 1997
10. Kuro OM, Matsumura Y, Aizawa H, et al: Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 390:45-51, 1997
11. Spicer SS, Lewis SE, Tashian RE, et al: Mice carrying a CAR-2 null allele lack carbonic anhydrase II immunohistochemically and show vascular calcification. Am J Pathol 134:947-954, 1989
12. Thornell LE, Carlsson L, Li L, et al: Null mutation in the desmin gene gives rise to a cardiomyopathy. J Mol Cell Cardiol 29:2107-2124, 1997
13. Bucay N, Sarosi I, Dunstan CR, et al: Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 12:1260-1268, 1998
14. Parhami F, Bostrom K, Watson K, et al: Role of molecular regulation in vascular calcification. J Atheroscler Thromb 3:90-94, 1996
15. Alfrey AC, Ibels LS: Role of phosphate and pyrophosphate in soft tissue calcification. Adv Exp Med Biol 103:187-193, 1978
16. Block GA, Port FK: Re-evaluation of risks associated with hyperphosphatemia and hyperparathyroidism in dialysis patients: Recommendations for a change in management. Am J Kidney Dis 35:1236-1237, 2000
17. Winchester JF, Rotellar C, Goggins M, et al: Calcium and phosphate balance in dialysis patients. Kidney Int Suppl 41:S174-178, 1993
18. Levy RJ, Qu X, Underwood T, et al: Calcification of valved aortic allografts in rats: Effects of age, crosslinking, and inhibitors. J Biomed Mater Res 29:217-226, 1995
19. Levy RJ, Schoen FJ, Levy JT, et al: Biologic determinants of dystrophic calcification and osteocalcin deposition in glutar-

aldehyde-preserved porcine aortic valve leaflets implanted subcutaneously in rats. Am J Pathol 113:143-155, 1983

20. Fleckenstein-Grun G, Fleckenstein A: Calcium—A neglected key factor in arteriosclerosis. The pathogenetic role of arterial calcium overload and its prevention by calcium antagonists. Ann Med 23:589-599, 1991

21. O'Brien ER, Garvin MR, Stewart DK, et al: Osteopontin is synthesized by macrophage, smooth muscle, and endothelial cells in primary and restenotic human coronary atherosclerotic plaques. Arterioscler Thromb 14:1648-1656, 1994

22. Fitzpatrick LA, Severson A, Edwards WD, et al: Diffuse calcification in human coronary arteries. Association of osteopontin with atherosclerosis. J Clin Invest 94:1597-1604, 1994

23. Sarkozi L, Szabo A: Effect of hemodialysis on the distribution of phosphates in blood. Clin Physiol Biochem 7:184-188, 1994

24. Block GA, Hulbert ST, Levin NW, et al: Association of serum phosphorus and calcium × phosphate product with mortality risk in chronic hemodialysis patients: A national study. Am J Kidney Dis 31:607-617, 1998

25. Goodman WG, Goldin J, Kuizon BD, et al: Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. N Engl J Med 342:1478-1483, 2000

26. Otto F, Thornell AP, Crompton T, et al: Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89: 765-771, 1997

27. Ducy P, Zhang R, Geoffroy V, et al: Osf2/Cbfa1: A transcriptional activator of osteoblast differentiation. Cell 89:747-754, 1997

28. Davies MR, Fruska KA: Pathophysiological mechanisms of vascular calcification in end-stage renal disease. Kidney Int 60:472-479, 2001

29. Cozzolino M, Dusso AS, Slatopolsky E: Role of calcium-phosphate product and bone-associated proteins on vascular calcification in renal failure. J Am Soc Nephrol 12:2511-2516, 2001

30. Raggi P, Boulay A, Chasan-Taber S, et al: Cardiac calcification in adult hemodialysis patients. A link between end-stage renal disease and cardiovascular disease? J Am Coll Cardiol 39:695-701, 2002