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Cardiovascular Pathology 13 (2004) 63–70

CARDIOVASCULAR PATHOLOGY

Regulation of cardiovascular calcification

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Received 18 September 2003; received in revised form 27 October 2003; accepted 3 November 2003

Abstract

Vascular calcification is highly correlated with cardiovascular disease (CVD) and is a significant predictor of cardiovascular events, especially in high risk patients such as the end stage renal disease (ESRD) population. Vascular calcification can lead to serious problems including valve stenosis, decreased vascular compliance, calciphylaxis, and even sudden death. However, the contribution of vascular calcification to progression of atherosclerosis is unknown and needs more study. Biochemical, histological, and genetic studies indicate that vascular calcification is actively regulated and involves both positive and negative modulators. Several nonmutually exclusive theories to account for vascular calcification based on current studies are discussed.

Keywords: Modulator; Mutant mouse; Regulation; Vascular calcification

1. Clinical relevance of cardiovascular calcification

Cardiovascular calcification refers to pathological calcium phosphate deposition in the blood vessels, myocardium, and cardiac valves. Clinical consequences of cardiovascular calcification depend on its extent and the organ affected. In the heart, calcification and subsequent stiffening, thickening, and tearing of the valve leaflets have long been known as a major mode of failure of both native as well as bioprosthetic cardiac valves [1,2]. In small arterioles, vascular medial calcification is responsible for calcific uremic arteriolopathy (also called calciphylaxis), an almost-always fatal skin necrotic condition seen in a small but significant percentage of dialysis patients [3]. Vascular medial calcification leading to a stenosing, fibroproliferative arterial process is also the major finding and cause of death in the rare genetic disorder, idiopathic infantile arterial calcification [4].

In contrast to the above observations, calcification of blood vessels commonly seen with aging, uremia, diabetes, and atherosclerosis has been considered, for the past century, a benign finding. This perception is quickly changing as technological advances in noninvasive measurement of vascular calcification, particularly electron beam computed tomography (EBCT), have allowed rapid and sensitive measurements to be correlated to a growing list of clinical events and cardiovascular risk. In coronary arteries, calcification is positively correlated with atherosclerotic plaque burden [5,6], increased risk of myocardial infarction [7–9], and increased risk of dissection following angioplasty [10]. Whether calcification is related to plaque stability, however, is less clear, although recent studies indicate that coronary calcification may be associated with and/or predictive of sudden cardiac death. Using autopsy specimens, intimal calcification was found to be a reliable marker of plaque instability, defined as plaques that have undergone rupture [11]. In addition, in a study of 79 adults with sudden cardiac death, both the Framingham risk index and coronary calcification (as measured by EBCT) were demonstrated to be predictive of future cardiovascular events [12]. Whether these findings relate to increase in plaque instability is controversial; indeed, a recent study using finite element analysis suggested that intimal calcification did not appreciably change the stress profiles of fibrous caps compared to lipid pools [13], though solid shear stresses were not considered thus limiting interpretation of the study.

While some of these findings may relate to the correlation of vascular calcification with extent of underlying arterial disease, it is also possible that vascular calcification itself may contribute to initiation or progression of cardiovascular disease (CVD). This possibility seems particularly plausible in the case of vascular calcification associated with chronic kidney disease (CKD). Over the last 2 years, a

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1054-8807/04/$ – see front matter
doi:10.1016/S1054-8807(03)00130-3

number of studies have highlighted the epidemic of vascular calcification and CVD mortality in CKD. Nearly half the deaths in dialysis patients are due to CVD. In fact, the risk of CVD mortality in adult CKD patients is 20–30 times higher than the risk in the general population [14]. High ischemic heart disease mortality rates are partly attributable to increased aortic medial calcification that leads to increased arterial wall stiffness, increased pulse pressure, and decreased perfusion of coronary arteries during diastole [15–17]. Thus, stiffening of compliance arteries through calcification probably underlies increased coronary ischemic syndromes, including myocardial infarction and left ventricular hypertrophy, in CKD patients. This constellation of cardiovascular problems may also help explain morbidity and mortality associated with diabetes and aging in general where increased vascular calcification is prevalent [18,19].

2. Location and morphology of vascular calcification

Several studies have characterized the mineral component of vascular calcification and found that the major calcium phosphate phase is bioapatite, similar to that observed in bone [20,21]. The location as well as morphology of calcified deposits in blood vessels is diverse. In atherosclerotic lesions, calcification is mainly found in the intima in a dispersed punctate form in the early stage of the disease and, as the process proceeds, the aggregates of calcium phosphate crystals deposit to produce larger patchy stippled crystals associated with the necrotic regions of atheromas. Furthermore, a significant number of calcified vascular and valvular lesions also show outright ossification, complete with bone marrow, cartilage, and mature lamellar bone [22,23]. The second histological site of vascular calcification is the media, known as Monckeberg's medial sclerosis. Such calcification occurs independently of intima calcification and is associated with elastin/collagen-rich extracellular matrix. Typical morphology of such calcification in the early stage of the disease is linear deposits along the elastic lamina throughout most of the medial width, and in advanced lesions, the media was filled with circumferential rings of mineral. In some cases, osteocytes and bone trabeculae with bone marrow were observed at the later stages of the disease [24]. Medial calcification is prominently found in the aged, diabetic, and uremic patient [25,26] and believed at least partially to be the cause of high CVD mortality in CKD patients as described above. In uremic patients with atherosclerosis, no doubt a mixture of these two types of calcification occurs.

3. Molecular mechanisms of vascular calcification

For the past century, vascular calcification was considered a degenerative process leading to uncontrolled precipitation of calcium phosphate associated with tissue necrosis and/or metabolic calcium and phosphate imbalance. However, this paradigm has been largely revised based on several key observations indicating that ectopic mineralization is actually a highly regulated process and may share mechanisms with bone formation. Morphological features of calcified blood vessels share several similarities to bone, including the presence of bioapatites, matrix vesicles, and presence of cells that when cultured under appropriate conditions, can form a mineralized matrix [27–29]. In fact, outright bonelike tissues have been identified in human lesions of Monckeberg's sclerosis, atherosclerosis, and aortic stenosis [22,30,31]; cartilaginous metaplasia is routinely seen in atherosclerotic and calcified vessels in murine models [32,33] and has been reported in calcified heart valves [22]. In an effort to explain these observations, four different yet nonmutually exclusive theories regarding the cause of vascular calcification have been put forward: (1) loss of inhibition,(2) induction of bone formation (3) circulating nucleational complexes, and (4) cell death. Each of these theories is illustrated in Fig. 1 and discussed, respectively, below.

3.1. Theory 1: loss of inhibition

It is becoming increasingly clear that most body fluids and organs normally contain inhibitors of calcium phosphate deposition, explaining why they do not spontaneously mineralize even though body fluids are supersaturated with respect to calcium and phosphate. A growing number of such molecules have been identified using mouse mutational analyses. Table 1 lists a number of specific mouse mutants, which present with enhanced cardiovascular calcification as part of their phenotype, indicating that these proteins are also normally important in suppressing vascular calcification.

One of the first of these genes to be discovered was matrix Gla protein (MGP). MGP is a 10-kDa protein containing 5 γ-carboxyglutamic acid (GLA) residues and is normally expressed at high levels in cartilage and smooth muscle. Mice with a null mutation in MGP ($MGP^{-/-}$) were found to die within the first 2 months of age due to arterial rupture and heart failure as a result of extensive calcification of the large elastic and muscular arteries. In addition, these mice showed inappropriate cartilage calcification and osteopenia [33]. MGP is a calcium-binding protein by virtue of its GLA residues. Thus, a potential mechanism of MGP action in inhibiting calcium phosphate deposition is calcium chelation [45]. Alternatively, circulating complexes of MGP and calcium phosphate have been identified, suggesting that MGP is involved in calcium phosphate clearance [46]. In addition, MGP binds to elastin [47] and may normally mask mineral nucleation sites. In contrast to its mineral-binding properties, MGP was also found to inhibit bone morphogenetic protein-2 activity via matrix association and thus inhibit osteogenic differentiation [48]. Regardless of the mechanism, it is clear that MGP is a major inhibitor of both

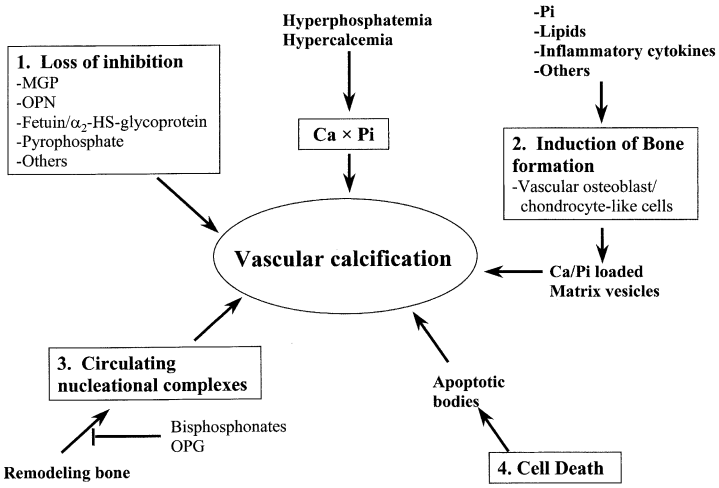

Fig. 1. Schematic illustration summarizing four current theories regarding molecular mechanisms of vascular calcification. (1) Loss of inhibition—loss of constitutively expressed inhibitors of calcium phosphate deposition leads to default mineralization. (2) Induction of bone formation—phenotypic transition of SMCs or other vascular precursor cells to osteoblast/chondrocyte-like cells promotes bone formation recapitulating bone developmental mechanisms. (3) Circulating nucleational complexes—ectopic mineralization due to deposition of calcium phosphate crystal-containing circulating complexes generated by active bone resorption. (4) Cell death—apoptotic bodies and/or necrotic cell debris act as nucleation sites in damaged tissues.

arterial and cartilage calcification and a major regulator of bone and vascular homeostasis.

Fetuin, also known as α_2-HS-glycoprotein, is a ubiquitous protein found at high concentrations in blood. Fetuin is also a potent inhibitor of calcium phosphate precipitation and accounts for about 50% of the mineral inhibitory activity of the blood. In the blood, fetuin circulates in a complex with MGP and calcium phosphate precipitates and has thus also been suggested as participating in a mineral clearance mechanism [46]. Mice lacking fetuin develop soft tissue and intravascular calcification, especially when challenged with Vitamin D [36]. Of particular interest, a recent cross-sectional study in 312 stable hemodialysis patients showed that fetuin concentrations were significantly lower in patients on dialysis and correlated with increased risk of cardiovascular death [49]. Thus, fetuin deficiency may contribute to excess vascular calcification observed in end stage renal disease (ESRD) patients.

Another interesting protein is osteopontin (OPN). We [50–52] and others [53–57] have reported that OPN is abundant at sites of calcification in human atherosclerotic plaques and in calcified aortic valves but is not found in normal arteries. OPN is an acidic phosphoprotein normally found in mineralized tissues such as bones and teeth and thought to be involved in regulation of mineralization by acting as an inhibitor of apatite crystal growth, as well as promoting osteoclast function through the $\alpha_v\beta_3$ integrin (reviewed in Ref. [58]). While OPN knock-out ($OPN^{-/-}$) mice do not have an overt bone or vascular phenotype, they are completely protected from ovariectomy-induced

Table 1
Vascular calcification phenotypes in mouse mutant strains

Gene	Mouse mutant	Phenotype	References
Carbonic anhydrase II (Car2)	$Car2^{-/-}$	Osteopetrosis, calcification of small arteries and arterioles, especially in the kidney	[34]
Fetuin/α_2-HS-glycoprotein	$Fetuin^{-/-}$	Vascular and soft tissue calcification	[35,36]
Fibrillin-1	MgΔ/mgΔ, mgR/mgR	Aortic aneurysm, medial arterial calcification	[37]
β-Glucosidase	$Klotho^{-/-}$	Vascular calcification, aging	[38]
MGP	$MGP^{-/-}$	Vascular, valve and cartilage calcification	[33]
OPN	$OPN^{-/-}$	Enhanced calcification of subcutaneously implanted valve; increased medial calcification in $MGP^{-/-}$ background.	[39,40]
	$OPN^{-/-}$ $MGP^{-/-}$		
OPG	$OPG^{-/-}$	Osteoporosis, vascular calcification	[41]
PC-1, NPP1, Npps	ttw/ttw	Articular cartilage calcification, arterial calcification	[42,43]
Smad6/Madh6	Madh6-mutant	Endocardial cushion defects, aortic ossification	[44]

osteoporosis [59] and PTH-induced bone resorption [60], confirming the important function of OPN in mediating bone resorption. Interestingly, in a subcutaneous implantation model, a 5- to 10-fold greater calcification was achieved in glutaraldehyde-fixed porcine aortic valve leaflets explanted from $OPN^{-/-}$ mice versus OPN wild types ($OPN^{+/+}$). Subsequent histological analysis of the calcified leaflets from $OPN^{+/+}$ animals showed colocalization of OPN and calcium deposits, suggesting the inhibitory role of OPN. In addition, heterozygous mice showed early calcification of implants at 14 days, with subsequent regression at 30 days. The regression was further found to correlate with the accumulation of OPN and carbonic anhydrase II (CAII) expressing monocyte-derived cells, including macrophages and foreign body giant cells, and subsequent acidification of the implants [39]. Finally, the role of OPN in calcification of native blood vessels has been determined in an $MGP^{-/-}$ $OPN^{-/-}$ double knockout model. $MGP^{-/-}$ $OPN^{-/-}$ mice showed an accelerated and enhanced vascular calcification compared to $MGP^{-/-}$ $OPN^{+/+}$ mice; arterial calcification of $MGP^{-/-}$ $OPN^{-/-}$ mice was twice as much as $MGP^{-/-}$ $OPN^{+/+}$ at 2 weeks and over 3 times as much at 4 weeks [40]. These studies showed that OPN acts as an inducible inhibitor in an adaptive response of vascular injury, not only through inhibiting crystal growth but also promoting active regression.

Lastly, mice carrying a Car2 null allele ($Car2^{-/-}$) lack CAII protein and show age-dependent medial calcification in arterioles and small arteries including renal hilar and arcuate arteries [34]. CAII appears in abundance in various epithelial and nonepithelial cells including monocyte-derived macrophages, foreign body giant cells, and osteoclasts. The enzyme catalyzes the $H_2O+CO_2 \leftrightarrow H^+ + HCO_3^-$ reaction and thus provides protons and bicarbonate ions to the local microenvironment. In osteoclasts, active CAII allows massive acid secretion into a sealed microenvironment known as the resorption lacunae and together with specialized proteinases that degrade the organic matrix, the bone mineral is dissolved and resorbed by the cells [61]. Whether this activity applies to vascular mineral is unclear, although a syndrome of osteopetrosis and cerebral calcification was observed in a rare inherited disease as a result of CAII deficiency [62].

3.2. Theory 2: Induction of bone formation

As mentioned above, ectopic bone, in addition to diffuse matrix mineralization, is often found in calcified arteries [22]. In addition, several different groups have confirmed that isolated arterial medial cells can be induced to mineralize in vitro under the appropriate conditions [29,63,64]. Furthermore, SMC undergo a striking phenotypic transition to cells resembling osteo/chondrogenic precursors both in vivo and in vitro under mineralizing conditions [27,65]. Thus, it has been proposed that vascular calcification may represent a recapitulation of embryonic bone formation. Whether pluripotent stem cells, transdifferentiation of SMC, or both mechanisms are involved is not yet known, but data supporting these possibilities have emerged.

Bostrom et al. [27,63] identified and isolated a clonal population of arterial medial cells, termed calcifying vascular cells (CVC), that spontaneously formed nodules that mineralized in vitro under long-term culture. These cells showed osteoblastic features, including expression of alkaline phosphatase and osteocalcin. The CVC have more recently been shown to undergo additional developmental fates including adipogenesis depending on the culture conditions [66]. Thus, these data support the presence of a pluripotent stem cell-like population within the artery wall capable of osteogenic differentiation that might be involved in vascular calcification under pathological conditions.

In contrast to CVC, heterogenous uncloned populations of SMC do not spontaneously mineralize in culture but can be induced to mineralize by elevating phosphate levels in the medium (either in the form of the organic phosphate donor, beta glycerophosphate, or inorganic phosphorus [64,67]). Of particular relevance to vascular calcification in the CKD patients, phosphate levels in the hyperphosphatemic range (>2 mM) induced calcification of bovine and human aortic smooth muscle cells via a mechanism sensitive to inhibitors of sodium-dependent phosphate cotransport (NPC) [67]. Furthermore, elevated phosphate levels induced SMCs to undergo a phenotypic transition characterized by loss of smooth muscle cell markers (SM α-actin and SM22α) and gain of osteogenic markers (OPN, Cbfa-1, alkaline phosphatase, and osteocalcin) [65]. The phenotypic transition was also inhibited by NPC inhibitors, suggesting that increased phosphate uptake may lead to transdifferentiation of smooth muscle cells to a prominer-alizing cell type similar to osteoblasts or chondrocytes [67]. Almost identical changes in cell lineage marker expression were observed in the calcified blood vessels of $MGP^{-/-}$ mice [65] and SMCs isolated from the calcified arteries of $MGP^{-/-}$ mice (Speer and Giachelli, unpublished data) and also in biopsy specimens from patients with calciphylaxis [68]. More interestingly, analysis of calcified arteries of the older $MGP^{-/-}$ mice showed the existence of cells with chondrocytic features, including hypertrophic chondrocyte-like cells surrounded by a typical metachromatic cartilage matrix [33].

Evidence in support of the transdifferentiation of vascular SMCs was also raised by Schulick et al. [69] in a different animal model. Using an in vivo gene delivery model, an adenoviral vector expressing active transforming growth factor-β1 (TGF-β1), rat arterial SMCs were found to have lost their lineage markers; about 10–25% of all intimal and medial cells were chondrocyte-like, and cartilaginous metaplasia appeared as a result of local accumulation of active TGF-β1. Cartilage gives rise to bone via a process known as endochondral bone formation and is the major developmental mechanism for long bone formation. Indeed, TGF-β1 was found to present in calcified aortic valve and to colocalize

with calcification and bone formation in atherosclerotic lesions [70,71].

Finally, both stimulators and inhibitors of CVC and SMC mineralization in vitro have been identified and are listed in Table 2. Further work in characterizing these molecules is needed and may aid in the development of novel therapeutic agents to prevent or regress vascular calcification, especially in high-risk populations.

3.3. Theory 3: circulating nucleational complexes

A growing number of studies have linked bone remodeling, specifically osteoclastic resorptive activity, with vascular calcification. First, mice deficient in osteoprotegerin (OPG), a soluble member of the TNFα family, are osteoporotic and show vascular calcification, suggesting that OPG and its regulators may be important in explaining the link between CVD and osteoporosis [41]. Indeed, recent studies have elucidated a key role for OPG as an osteoblast-derived inhibitor of osteoclast differentiation and function by virtue of its ability to bind receptor activator of NFκB ligand (RANKL), and thereby block function of receptor activator of NFκB (RANK) [89]. While much less is known about effects of OPG on vascular cells, Price et al. [94] have found that OPG and the bisphosphonates, alendronate and ibandronate inhibit arterial calcification in warfarin- and/or vitamin D-treated rats at doses comparable to those used to inhibit bone resorption [90,91]. In a subsequent study, they used a specific inhibitor of osteoclastic V-H^+-ATPase, SB 242784, and were able to block both vascular calcification and osteoclastic resorption in rats treated with toxic doses of vitamin D [92]. These findings have led to the hypothesis that vascular calcification is linked to osteoclastic resorption. Price et al. [92] suggest that soft tissue calcification is promoted by crystal nuclei generated at sites of bone resorption that travel in blood and lodge in soft tissue, thereby inducing tissue mineralization. Indeed, these authors have observed that under some circumstances, a complex consisting of a calcium phosphate mineral and the proteins fetuin and MGP are released from bone and can be detected in blood, and the release of the complex can be inhibited by osteoclastic inhibition [93]. This hypothesis is appealing given the link between postmenopausal osteoporosis and cardiovascular calcification [94] for which no mechanistic data are yet available. However, how such a circulating nucleating complex crosses the endothelial barrier is unknown. Furthermore, if bisphosphonates and other osteoclastic inhibitors prove to be effective against CVD in postmenopausal women, this would be useful as the latest studies indicate that hormone replacement therapy is negatively correlated to cardiovascular health [95].

3.4. Theory 4: cell death

Cell death has long been regarded as a major nucleational mechanism for vascular calcification, especially in the case of dystrophic calcification as seen in atherosclerotic lesions where large areas of necrosis are typically observed. It is known that dying cells become highly permeable to calcium and phosphate and may therefore concentrate these ions beyond their solubility product and facilitate homogeneous nucleation of crystals. In addition, phospholipid membranes may provide sites for heterogeneous nucleation and/or epitactic growth of calcium phosphate crystals [96]. Indeed, matrix vesicles, the known nucleation sites for calcium phosphate crystal formation in cartilage and bone, were observed in calcifying vascular lesions and appeared to be derived from dying SMCs [97]. The association of cell death with calcification of SMCs was also studied in vitro in cultured SMC nodules. Apoptosis was found to occur before the onset of nodular calcification of SMCs; and stimulation or inhibition of nodular apoptosis increased or decreased SMC calcification, respectively. More interestingly, apoptotic bodies isolated from SMC cultures were found to accumulate calcium. Like matrix vesicles, the calcium concentrated inside the apoptotic bodies was in crystallized forms [98]. These observations provide evidence that apoptotic bodies

Table 2
Modulators of vascular cell calcification in vitro

Stimulator	References	Stimulator	References	Inhibitor	References
Acetylated LDL[a]	[72]	25-OH cholesterol[a]	[63]	Adrenomedullin[b]	[73]
Advanced glycation endproducts[b]	[74]	Oxidative stress	[75]	Collagen IV[a]	[76]
collagen I[a]	[76]	Phosphate[b]	[64,67,77,78]	C-type natriuretic peptide[b]	[73]
Dexamethasone[b]	[79]	TGFβ[a]	[63]	Gas6/Axl[c]	[80]
17 β-estradiol[a]	[81]	TNFα[a]	[82]	High-density lipoprotein (HDL)[b]	[83]
Fibronectin[a]	[76]	TNFα + oncostatin[b]	[84]	Osteopontin[b]	[28,29]
IL-4 and IL-6[a]	[83]	Uremic serum[b]	[77]	Parathyroid-related protein[b]	[73,85]
Leptin[a]	[86]	vitamin D3[b]	[87]	Phosphonoformic acid (PFA)[b]	[67]
Minimally modified LDL[a]	[88]				

[a] Calcifying vascular cells.
[b] Smooth muscle cells.
[c] Pericytes.

derived from cultured SMCs can act as initiating and nucleating sites for calcium phosphate deposition. The link between apoptotic bodies and matrix vesicles, however, has not been established.

4. Conclusions

It has become clear that elaborate and highly regulated mechanisms exist for controlling vascular calcification. In normal vessels, inhibitory mechanisms appear to outweigh inductive mechanisms thereby preventing mineralization. Under pathological conditions, inductive mechanisms appear to override inhibitory mechanisms. Increasing our understanding of inhibitory and stimulatory pathways will aid in developing therapeutic strategies to prevent vascular calcification, an urgent need in the CKD population.

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