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Coronary Arterial Calcification as an Active Process: A New Perspective on an Old Problem

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Abstract. The mechanism and purpose of coronary atherosclerotic calcification remain unknown. However, evidence reviewed here suggests that calcification is not passive precipitation or adsorption, but instead is organized and regulated. Gla containing proteins and other proteins normally associated with bone metabolism appear to play an important role in this process. A variety of studies are currently in progress in our laboratory which we hope will provide a more comprehensive understanding of processes leading to coronary calcification as well as prognostic data useful in clinical cardiologic practice. A clearer understanding of the nature and significance of coronary calcification may well pave the way toward new interventions to protect myocardium and minimize the morbidity and mortality associated with coronary artery disease.

Key words: Coronary calcification — Bone proteins

The First 250 Years

Just after the turn of the 18th century and within a few years of the first descriptions of coronary atherosclerosis, calcified coronary arterial lesions were recognized and described by Bellini and Thebesius [1, 2]. For over 200 years thereafter ". . . [Coronary arterial] calcification was considered to be the very essence of coronary sclerosis" [2]. Virchow noted that vascular calcification was similar to bone formation and described calcified atherosclerotic coronary lesions as "an ossification, and not a mere calcification" [3]. In the 20th century, however, coronary calcification was accorded a much less important role [4–6], as it was realized that cholesterol metabolism and other factors played a major role in atherogenesis. With the development of coronary angiographic techniques, coronary calcification was noted but was considered a manifestation of the later stages of the degenerative process of atherosclerosis [7–9]. This view resulted in part from the poor resolution of the radiographic imaging techniques of the time which had a low sensitivity for the detection of calcium.

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Thus, atherosclerotic calcification has come to be commonly regarded as a passive process of adsorption or precipitation, merely an ancillary effect of advanced atherosclerotic degenerative processes. However, recent evidence to be reviewed here challenges this view and suggests that Virchow's depiction of atherosclerotic calcification as ". . . an ossification, and not a mere calcification" [3] may have been much more accurate. Atherosclerotic calcification may be organized, regulated, and may even have purpose.

Coronary Calcification: Natural History

Stary has found that atherosclerotic calcification begins as early as the second decade of life just after fatty streak formation [10]. However, calcific deposits are found more frequently and in greater amounts in elderly individuals and in more complex lesions [10]. Stary's electron microscopic evidence supports the matrix vesicle theory according to which hydroxyapatite, the predominant crystalline form of calcium deposits [11], is formed primarily in vesicles that pinch off from arterial wall cells analogous to the way matrix vesicles pinch off from chondrocytes in developing bone [12–17].

However, early small calcium deposits are also observed within extracellular lipid-rich accumulations of debris whose origin is uncertain [10]. Hirsch et al. [18] used a fluorescent cholesterol probe, scanning electron microscopy, and energy-dispersive X-ray microanalysis to demonstrate a very close spatial association between unesterified cholesterol and hydroxyapatite. Thus, there may be more than one mechanism of calcium deposition in atherosclerosis.

Furthermore, calcification appears much earlier in atherosclerotic lesion pathogenesis than previously thought. High resolution noninvasive imaging studies using digital subtraction cinefluorescopy (DSC) [19–21] and ultrafast computerized tomography (UFCT) [6, 22–24] have lent support to this notion. These studies and autopsy data [10, 24] have demonstrated calcification just after the appearance of fatty streaks.

Biochemistry: The Gla Proteins

At present, the biochemical sequence of events leading to

Table 1. Known Gla proteins, site of synthesis, and known or proposed function

Protein	Site of Synthesis	Function
Prothrombin	Liver	Coagulation
Factor VII	Liver	Coagulation
Factor IX	Liver	Coagulation
Factor X	Liver	Coagulation
Protein C	Liver	Fibrinolysis; modulation of coagulation
Protein S	Liver	Fibrinolysis; modulation of coagulation
Protein Z	Liver	Coagulation
Bone Gla protein (BGP) osteocalcin	Bone; Atheroma ?	Bone metabolism; bone calcium transport ?
Plaque Gla protein (PGP)	Atheroma ?	Calcium transport in atheroma?
Matrix Gla protein (MGP)	Heart, epithelium, bone, & many tissues; ubiquitous	Bone metabolism, calcium transport in atheroma?

atherosclerotic calcification is not well understood. However, several poorly characterized proteolipids have been isolated from calcified atherosclerotic lesions [25–27], and recently attention has focused on a unique class of proteins known as Gla-containing proteins.

Gla (gamma carboxyglutamate) is an unusual amino acid residue whose only known function is to bind calcium [28–30]. *In vitro,* these residues strongly inhibit precipitation of calcium salts [31] and hydroxyapatite crystal growth [32]. Gla proteins bind weakly to calcium ions; the dissociation constant for bovine bone Gla protein (BGP), for example, is 2–3 mM [33, 34]. However, Gla proteins have a very high affinity for hydroxyapatite, with a dissociation constant for BGP of the order of 10^{-7} molar [35–37]. Gla proteins thus do not interfere with normal calcium homeostasis as they are not calcium chelators, but if precipitation of calcium occurs, available Gla-containing proteins would be expected to bind to the precipitate. Decarboxylation of Gla residues to glutamyl residues greatly diminishes the affinity of Gla-containing proteins for hydroxyapatite [36, 37].

Two classes of Gla-containing proteins are known to exist. One class is synthesized in the liver and circulates in plasma; a second class of Gla proteins is synthesized primarily in bone and to a lesser extent in soft tissues [28, 30]. The Gla proteins of hepatic origin participate in coagulation and include prothrombin, factor VII, factor IX, factor X, protein C, protein S [28], and protein Z [38, 39]. Two nonhepatic Gla proteins have been well-characterized thus far. Although their precise function is unclear, they both appear to be involved in calcium metabolism in bone [28, 30]. These two proteins have been designated bone Gla protein (BGP or osteocalcin) [40–44], and matrix Gla protein (MGP) [30, 43, 45]. A third nonhepatic Gla protein, structurally unrelated to any other known Gla protein, has been isolated from calcified atherosclerotic plaque, and has been designated plaque Gla protein (PGP) [46–48]. A fourth putative Gla protein called atherocalcin was isolated from calcified atheroma [49], but was later found to consist of BGP complexed to albumin [50] (Levy & Howard unpublished; cited in [50]). Table 1 shows the known Gla proteins, their location of synthesis, and known or proposed function.

Gla Proteins and Vitamin K Metabolism

Gla residues in Gla-containing proteins occur as a result of a posttranslational modification catalyzed by a vitamin K-dependent enzyme, gamma-glutamate carboxylase, which carboxylates the gamma carbon of specific glutamyl residues [28]. Vitamin K in its hydroquinone form participates as an essential cofactor in this reaction [51, 52], and is oxidized to an epoxide by an epoxidase. Carboxylase and epoxidase activity may be due to a single enzyme [53].

Vitamin K in its epoxide form is then reduced first to a quinone and then back to the hydroquinone by reductases [28]. The hydroquinone can then participate in another redox cycle with the carboxylase, and thus as long as redox cycling can continue, a supply of vitamin K quinone is not needed.

Coumarin derivatives such as warfarin inhibit the reductases which catalyze vitamin K quinone and epoxide reduction [54]. There is, however, an alternate source of vitamin K hydroquinone to drive the carboxylation reaction under conditions of warfarin inhibition. An NADH-dependent reductase can reduce vitamin K quinone, *but not the epoxide,* to vitamin K hydroquinone in a reaction that is not inhibited by warfarin [55], so that as long as there is a constant supply of vitamin K quinone, normal carboxylation proceeds and functional Gla containing coagulation factors are produced. If vitamin K is administered concomitantly with warfarin to rats, normally carboxylated Gla-containing coagulation proteins are produced and normal coagulation times are observed [30, 56] (Fig. 1).

Although vitamin K supplementation during anticoagulant therapy provides a cofactor for carboxylase-catalyzed synthesis of functional Gla-containing coagulation factors, no such effect has thus far been observed in nonhepatic tissues where the synthesis of other Gla proteins such as BGP takes place [28, 30, 57]. In both animals and humans, treatment with warfarin decreases plasma levels not only of Gla-containing coagulation factors but also of BGP [57]. However, carboxylated BGP is apparently not produced when vitamin K supplements are added to a warfarin regimen and bone levels of BGP drop precipitously [30, 56, 58, 59].

Function of Gla Proteins in Normal Arterial Wall

Gamma-glutamate carboxylase has also been identified in many nonhepatic tissues, including arterial intima [28, 60–62]. Vermeer's group has reported that carboxylase activity in normal arteries is three times that found in atherosclerotic arteries [61, 62].

The presence of gamma-glutamate carboxylase activity and MGP mRNA in normal vessel wall [60], and the high serum levels of PGP in normal subjects compared with patients with atherosclerosis [47] suggests that normal arteries produce Gla proteins. However, current techniques have not demonstrated these proteins in normal vessel wall. Vermeer has speculated that these proteins are produced in normal intima where they prevent hydroxyapatite precipitation. As no hydroxyapatite is present, Gla proteins do not bind to the wall and instead diffuse into the vascular space and are excreted [28, 46–48]. It should be noted that this notion is based on *in vitro* observations of the behavior of Gla proteins. How these proteins actually behave *in vivo* is unknown, and their physiological function *in vivo* in atherosclerotic lesions is also a matter of speculation.

Atheroma, Atherosclerotic Calcification, and Gla Proteins

We suggest that Gla proteins may be actively related to ath-

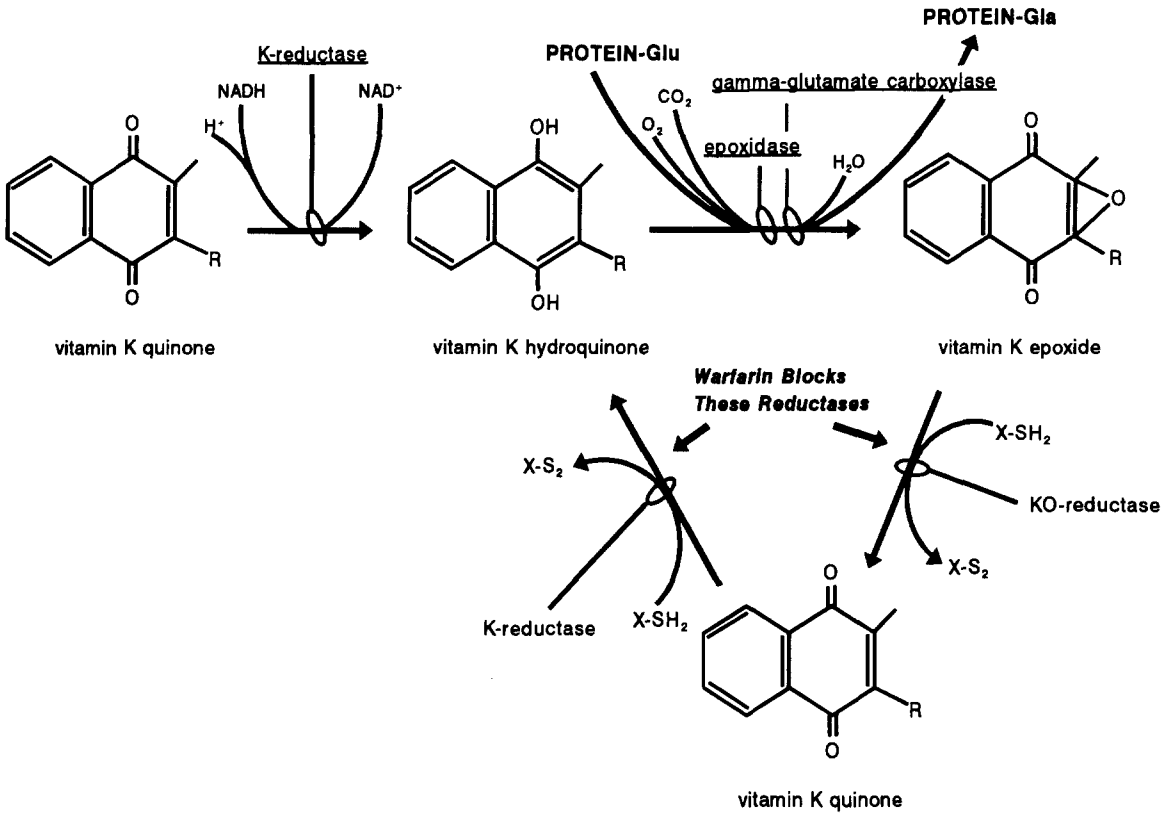

Fig. 1. The gamma-glutamyl carboxylase reaction and the vitamin K redox cycle. Vitamin K quinone is the form of vitamin K that is absorbed from the blood and circulates in plasma. NADH-dependent K-reductase is present in liver but has not been found in nonhepatic tissue. K-reductase and KO-reductase are inhibited by coumarin derivatives such as warfarin; they may be the same enzyme. The NADH-dependent K-reductase is not inhibited by warfarin. The identity of the thiol reductant is unknown, but may be cysteine residues or dithiothreitol [28].

erosclerotic calcification. Although the function of the three nonhepatic Gla proteins is unknown, Vermeer and coworkers [28, 46–48] have suggested that PGP may be synthesized continuously and may transport calcium out of the vessel wall, thus preventing calcium deposition. The finding that gamma-glutamate carboxylase activity in normal arteries is three times higher than in atherosclerotic arteries [61, 62] is consistent with this suggestion. Decreased gamma-glutamate carboxylase activity in diseased arteries would result in decreased synthesis of functional Gla-containing proteins and a corresponding decrease in the capacity to transport calcium out of the vessel wall, thus resulting in increased calcium deposition. If for any reason calcification should occur, locally available Gla proteins would bind to the resulting hydroxyapatite. This could explain why Gla proteins can be isolated from calcified lesions [29, 46–48, 60] but not from normal vessel wall, whereas carboxylase activity, and by inference Gla production, is higher in normal than in diseased arteries.

Although adsorption of Gla-containing proteins from serum cannot be entirely excluded, this seems unlikely given the presence of MGP mRNA [60] and gamma glutamate carboxylase activity [28, 61, 62] in the vessel wall. Moreover, adsorption of serum Gla-containing coagulation proteins of hepatic origin to calcified atherosclerotic lesions does not occur [47, 48]. Finally, levels of PGP in the serum of patients with coronary artery disease or atherosclerotic peripheral vascular disease are very low (or even nondetectable) compared with normal subjects [63]. As coronary arterial calcification seems to occur exclusively in diseased arteries and is absent in normal vessel wall [1, 9, 64–67], there may be a mechanistic link between pathological processes leading to calcification and those leading to atherosclerosis. It is conceivable, for example, that atherosclerotic processes inhibit the synthesis and/or activity of gamma-glutamate carboxylase, thus perhaps explaining why atherosclerotic arteries contain only about 30% of the carboxylase activity found in normal arterial segments [62]. Alternatively, it is also conceivable that cells in atherosclerotic lesions synthesize less carboxylase.

Atheroma and Bone: A New Twist on an Old Idea

Recent autopsy data have demonstrated the presence of calcification in all of 34 atherosclerotic, and in none of 14 normal arterial segments [24, 68]. Energy-dispersive X-ray microanalysis revealed that this calcium was in the form of hydroxyapatite [11, 68]. Immunohistochemical staining for osteopontin (a phosphorylated calcium-binding glycoprotein involved with bone matrix metabolism [69, 70]) demonstrated excellent colocalization of osteopontin with calcification [24, 68]. Diffuse calcium and osteopontin staining was seen throughout each plaque, with more intense staining at the outer perimeters. There was no osteopontin or calcium staining observed in any of the normal segments. Similar results were seen [71] when these sections were also stained for bone sialoprotein, another bone matrix protein. Although

adsorption from circulating proteins cannot be completely ruled out, it is thought that these proteins are most likely produced locally, and recently this same group has induced cultured smooth muscle cells from porcine carotid arteries to synthesize large amounts of osteonectin and procollagen type I, moderate amounts of bone sialoprotein, and smaller amounts of BGP and osteopontin [72]. Cultured human aortic smooth muscle cells demonstrated somewhat different immunohistochemical results, staining positively for bone sialoprotein and osteopontin, moderately for the matrix protein osteonectin, and minimally for BGP and the proteoglycans biglycan and decorin [73]. Though most of this data must at this point be considered preliminary, these results are nevertheless intriguing. Other investigators have found osteopontin mRNA expression in newborn rat aorta [74] and injured rat carotid artery, and it has recently been shown that osteopontin mRNA expression is greatly increased in proliferating vascular smooth muscle cells in culture [75]. Giachelli et al. have also noted a striking association of osteopontin with calcific deposits in human coronary artery lesions obtained post mortem as well as in human carotid endarterectomy specimens [76].

Further evidence that bone proteins are involved in atherosclerotic calcification has recently been provided by Bostrom et al. [77] and Watson et al. [78] at UCLA. Calcified human carotid endarterectomy specimens obtained during surgery were found to express bone morphogenetic protein 2a (BMP-2a), an osteoblastic differentiation factor [77]. Calcification was primarily located at the base of the plaque specimens, but was also present in scattered locations throughout each lesion. Cells isolated from these lesions and grown in culture formed nodules, generated matrix material, formed multifocal calcifications (demonstrated by Alizarin red S and von Kossa histochemical staining techniques), and expressed BMP-2a. Electron microprobe analysis demonstrated calcifications to be composed of calcium and phosphate in a molar ratio similar to that of hydroxyapatite. Immunofluorescent and immunohistochemical techniques determined that the cells were neither endothelium nor smooth muscle cells, but had many of the characteristics of pericytes [77]. This same group has also reported preliminary results suggesting that these cells are present within the intima of normal bovine and human arteries and seem to increase in number as the arteries become atherosclerotic [78]. Moreover, they appear to function in many ways like osteoprogenitor cells: they produce alkaline phosphatase, express BMP-2a mRNA, and secrete BGP. These findings lend credence to the idea that atherosclerotic calcification is not merely passive adsorption but instead is an organized, regulated process similar in many respects to bone formation.

Does Atherosclerotic Calcification Have Purpose?

Why do atherosclerotic arteries become calcified? Though coronary calcification has in the past been regarded as a passive process of adsorption or precipitation, evidence reviewed here suggests that this may not be the case. If it is true that coronary calcification is an organized, regulated process, then to what end is this organization and regulation directed? Does calcification serve some functional role?

The mechanism of myocardial infarction is thought to involve sudden rupture of weakened atheromatous plaque followed by acute thrombosis [79–81]. Although the mechanism of plaque rupture is incompletely understood, a variety of biomechanical forces are thought to play a major role [82–90]. Though fracture mechanics, particularly in morphologically heterogeneous materials such as atherosclerotic plaque, is a subject of daunting complexity, tensile strength seems to be one important factor, and it has recently been shown that local variations in circumferential stress in atherosclerotic arteries are associated with the location of plaque rupture [82]. The angiographic severity of a lesion has been shown to be a poor predictor of its likelihood to rupture [91, 92], and, consistent with this, increasing stenosis severity of itself does not significantly increase circumferential stress [93].

A phenomenon familiar to angiographers is the formation of coronary collateral vessels [94–96]. As the blood supply through a major epicardial coronary artery becomes compromised by atherosclerotic obstruction, other coronary arteries often form small vessels which feed the threatened myocardium. If the obstruction becomes complete, the magnitude of collateral flow may even be sufficient to prevent infarction. The formation of coronary collaterals thus serves a protective function and has survival value.

Hypotheses

We speculate that coronary arterial calcification may, in a manner analogous to collateral formation, represent an attempt to protect threatened myocardium by strengthening weakened atherosclerotic plaque prone to rupture. Calcified lesions and fibrotic hypocellular lesions are much stiffer than cellular lesions [90], and biomechanical data suggests that calcified areas are unlikely to be associated with sites of plaque rupture [82]. *In vivo* evidence of the relative stability of calcified lesions has been obtained using intravascular ultrasound [97]. Thus, perhaps coronary calcification might represent an attempt by the arterial wall to stabilize and strengthen itself, thereby minimizing the risk of plaque rupture. The strategy of collateralization may be successful provided there is sufficient time for extensive collateral growth to occur prior to total occlusion of the vessel. The same may be true for calcification. For example, if a plaque develops a heavily calcified cap, it is about five times stiffer than a cellular lesion or normal vessel wall and very resistant to rupture [84, 90]. In the short term this may lead to increased stress near the junction of the cap with the adjacent intima, and it is here where plaque rupture often occurs. However, with more extensive calcification and fibrosis of the vessel, these weak points may be eliminated and risk of rupture correspondingly decreased. This may in part explain the high frequency of calcification in older populations [9, 19, 98,99]; that is, extensive atherosclerotic calcification may actually have survival value. This may also help explain why the presence of calcification is not an ideal prognostic indicator in a heterogeneous population (Detrano et al., in preparation). Only when extensive calcification has occurred is the vessel rendered resistant to rupture, and early or intermediate stages of calcification may actually enhance plaque vulnerability to rupture.

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