

Arterial Calcification: A Review of Mechanisms, Animal Models, and the Prospects for Therapy

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Abstract: The causes of arterial calcification are beginning to be elucidated. Macrophages, mast cells, and smooth muscle cells are the primary cells implicated in this process. The roles of a variety of bone-related proteins including bone morphogenetic protein-2 (BMP-2), matrix Gla protein (MGP), osteoprotegerin (OPG), osteopontin, and osteonectin in regulating arterial calcification are reviewed. Animals lacking MGP, OPG, smad6, carbonic anhydrase isoenzyme II, fibrillin-1, and klotho gene product develop varying extents of arterial calcification. Hyperlipidemia, vitamin D, nicotine, and warfarin, alone or in various combinations, produce arterial calcification in animal models. MGP has recently been discovered to be an inhibitor of bone morphogenetic protein-2, the principal osteogenic growth factor. Many of the forces that induce arterial calcification may act by disrupting the essential post-translational modification of MGP, allowing BMP-2 to induce mineralization. MGP requires gamma-carboxylation before it is functional, and this process uses vitamin K as an essential cofactor. Vitamin K deficiency, drugs that act as vitamin K antagonists, and oxidant stress are forces that could prevent the formation of GLA residues on MGP. The potential role of arterial apoptosis in calcification is discussed. Potential therapeutic options to limit the rate of arterial calcification are summarized.

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1. INTRODUCTION

Although arterial calcification has been recognized for more than 200 years,^{1,2} our understanding of the basic pathogenesis of this disorder is still incomplete. Until recently, calcification was considered to be a passive process that occurred as a nonspecific response to tissue injury or necrosis. Now there is strong evidence to support the concept that arterial calcification is, at least in part, an

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active process that is associated with the expression of growth factors, matrix proteins, and other bone-related proteins.^{3–6} Along with this paradigm shift has been the development of mouse models lacking specific calcification inhibitors, demonstrating the complex regulation of the calcification process. A number of excellent reviews have recently been published on arterial calcification.^{7–9} In this review, we will examine the cellular and molecular mediators, review the important lessons learned from animal models, and then ask four questions: (Q1) Is arterial calcification solely a marker for vascular disease or does it also contribute to vascular disease? (Q2) Is arterial calcification a manifestation of vascular apoptosis? (Q3) Does oxidant stress contribute to arterial calcification? (Q4) Is arterial calcification a manifestation of vascular vitamin K deficiency? These questions are only beginning to be answered. Finally, we will examine the prospects for treating and preventing arterial calcification.

The clinical significance of arterial calcification is not fully understood, but it is well known that this process is common; 75–95% of men and women at autopsy have coronary artery calcification regardless of the cause of their death.¹⁰ Coronary artery calcification has been linked to the risk of myocardial infarction,¹¹ the risk of dissection during coronary angioplasty,¹² adverse events from and approaches for coronary artery bypass grafting,^{13–16} among a variety of other outcomes.¹⁷ Although a thorough discussion of the many clinical implications of arterial calcification and its detection are beyond the scope of this review, it is clear that the problem is enormous and will continue to grow as the population ages.

2. THE CELLULAR SOURCE OF CALCIFICATION

There is now considerable evidence that arterial calcification is an active, cell-controlled event, a conclusion that is supported by the expression of proteins that are involved in both the initiation and the inhibition of mineralization. The cells that participate in this process depend on whether calcification occurs primarily in intimal atherosclerotic plaques or in Mönckeberg's sclerosis, which is defined as calcification restricted to the arterial medium, occurring primarily in diabetics and patients with renal failure.

Macrophages, mast cells, and VSMC (vascular smooth muscle cells) in the plaque are believed to be the primary cells involved in atherosclerotic intimal calcification, with a smaller contribution from SMC.^{6,18} Macrophages release TNF- α in response to oxidized LDL,¹⁹ and TNF- α treatment of vascular SMC has been shown to enhance osteoblastic differentiation, alkaline phosphatase expression and mineralization.²⁰ Macrophages express a variety of bone-related proteins including matrix Gla protein, (MGP)⁴ osteopontin,⁴ alkaline phosphatase and bone sialoprotein.²¹

Extracellular mast cell tryptase is often seen at sites of early calcification and around small calcium deposits, but seldom seen around larger, solid calcifications.^{18,22} This finding suggests that mast cells may have a role in the initiation of calcification, by an unknown mechanism. It is known that mast cells release cytokines such as TNF α and IL-1^{23,24} which could influence calcification in a variety of ways, such as altering the balance between osteoprotegerin and its ligand.²⁵ Both macrophages and mast cells emphasize the connection between inflammation and intimal calcification. Both cells release metalloproteinases and therefore the presence of calcification may be indirectly linked to destabilizing forces.

In medial calcification, the source of bone-associated proteins alkaline phosphatase, bone sialoprotein, bone Gla protein, and others is almost exclusively the VSMC.²⁶ In the lesions of Mönckeberg's sclerosis, the cells associated with calcification were positive for α -SM actin and SM 22 α .²⁶ Primary cultures of VSMCs from normal vessels and from MS vessels expressed MGP, collagen types I and II, osteonectin, osteocalcin, alkaline phosphatase, bone sialoprotein, osteopontin, and BMP-2, indicating that VSMCs can express osteoblast-specific genes.²⁶

“Calcifying vascular cells” (CVCs) are a subpopulation of smooth muscle cells from the medium that exhibit osteoblastic characteristics and form calcified nodules *in vitro*.^{27,28} Calcifying vascular cells have features in common with pericytes including a stellate shape and positive staining with the 3G5 antibody as well as similarities to osteoblasts.²⁷ Some of the mechanisms for the differentiation of CVCs have been elucidated. Factors that increase intracellular cAMP levels have been shown to enhance osteoblastic morphological appearance of these cells and also to modulate bone-related gene expression, including up-regulating procollagen type I and MGP while down-regulating the expression of osteopontin.²⁷ Estrogen also appears to enhance the osteoblastic phenotype of these cells. When CVCs are exposed to 17 β -estradiol for 21 days, they have increased numbers of calcified nodules, alkaline phosphatase activity, and osteocalcin levels, suggesting that 17 β -estradiol promotes osteoblastic differentiation.²⁸ Additional factors that enhance calcification by CVCs include 25-hydroxycholesterol and TGF- β 1. Interestingly, MGP expression also increases in response to factors that promote CVC differentiation, perhaps as a mechanism for feedback inhibition of the process. The extracellular matrix is also an important regulator of calcification by cultured VSMCs.²⁹ Culturing VSMCs on collagen type I or fibronectin enhances, while collagen type IV inhibits mineralization by bovine aortic medial SMC.

A. Pericytes

As noted, calcifying vascular cells have characteristics of osteoblasts and pericytes and may represent a mesenchymal lineage that is intermediate between pericytes and osteoblasts.^{3,27} Pericytes also have a variety of phenotypic characteristics in common with SMC,^{30–32} resulting in their description as the “microvascular smooth muscle cell.”³⁰ The pleomorphic nature of pericytes could derive from the fact that it is one of the few adult cells that retains pluripotentiality.³²

Pericyte differentiation is associated with markers of osteoblast differentiation including osteopontin, osteonectin, bone sialoprotein, osteocalcin, and alkaline phosphatase.^{32–36} At high culture density they form nodules that mineralize with hydroxyapatite.³⁴ Furthermore, pericytes were capable of forming bone and cartilage in the diffusion chamber assay, an accepted model of *in vivo* osteogenesis.³³ Pericytes express MGP under conditions in which they undergo osteogenic differentiation and deposit a calcified matrix.³⁷ Microvessels are abundant in the intima of atherosclerotic plaques, thereby providing an avenue by which pericytes could contribute to arterial calcification.³⁸ Pericytes are therefore a potential cellular initiator of calcification in arteries, especially those with atherosclerosis and neovascularization. Although it is possible that calcifying vascular cells are derived from pericytes, CVCs have been shown to exist in normal arteries with no apparent neovascularization, raising doubt that these cells arise from microvascular pericytes.²⁷

B. Vascular Dendritic Cells

Vascular dendritic cells have been proposed to contribute to arterial calcification. These cells have been detected in atherosclerotic plaque in foci of calcification and have been demonstrated to express S-100, a calcium binding protein, deposits of which are detected in the ECM in foci of calcification.³⁹ Dendritic cells expressing S-100 have been detected in aortocoronary saphenous vein bypass grafts with high degree stenotic lesions and in areas of calcification. In contrast, no S-100 positive cells were seen in normal ungrafted saphenous veins.⁴⁰

A variety of other cells could be involved in arterial calcification. Platelets are known to contain several bone-related proteins in their α granules, including osteonectin,⁴¹ bone sialoprotein,⁴² and osteocalcin.⁴³ Platelets can also be carriers of tissue factor-containing microparticles that stimulate thrombus formation.⁴⁴ In addition to its role in thrombosis, the thrombin that is generated may have roles in stimulating calcification as well.⁴⁵

3. CELLULAR-INDEPENDENT ARTERIAL CALCIFICATION

Some sites of calcification appear to be acellular and may therefore occur independently of cellular mechanisms.⁴⁶ Calcification may be initiated at sites of deposits of acellular material. The presence of thrombus contributes to the development of calcification of biomaterials and calcification is known to occur in intravascular thrombus.^{47–50} Fibrin is found to co-localize frequently with noncollagenous bone matrix proteins in atherosclerotic carotid arteries.⁵¹ Ochronosis, a disorder caused by lack of the enzyme homogentisic acid oxidase, is accompanied by dystrophic calcification of ear cartilage, the intervertebral disks, and the aortic root and aortic valve. Deposits of polymerized homogentisic acid which disrupt collagen fibers and serve as a nucleus for calcification appear to be causes for the arterial and valvular calcification.^{52–54} Alternatively, acellular calcification may arise because hydroxyapatite persists long after the cell of origin is lost, thereby removing all evidence of the cell-associated origin of the calcification.³⁹

4. THE MOLECULAR MEDIATORS OF ARTERIAL CALCIFICATION

A. BMP-2

Bone morphogenetic proteins (BMPs) are a group of related growth factors including BMP 1–7.⁵⁵ BMP-2-through BMP-7 share homology with tissue-derived growth factor-beta (TGF- β) and therefore belong to the TGF- β super family of proteins.⁵⁶ When implanted in soft tissues, only recombinant BMP-2 and BMP-4 have been shown to induce osteoblast differentiation and the entire set of tissue events needed for bone formation.⁵⁷ Evidence is accumulating that BMP-2 by itself has the full potential to initiate bone formation.⁵⁸ BMP-2 is expressed in human atherosclerotic lesions.³ The expression of BMP-2 in both normal and MS vessels has been suggested as evidence that it is unlikely to induce medial ossification in Mönckeberg's sclerosis.²⁶ However, this analysis does not consider the possibility that in the normal artery, BMP-2 might exist in an inactive complex with MGP or another inhibitor. Signal transduction of BMPs involves SMAD proteins. SMAD 1 activates OPN expression in response to BMP-2 through an interaction with Hoxc-8, a homeodomain transcription factor. Stable expression of the aminoterminal region of Smad 1 in 2T3 osteoblast precursors induces osteoblast gene differentiation and bone matrix formation.⁵⁹

B. Matrix Gla protein (MGP)

Matrix Gla protein is a 14 kDa protein that is present in cartilage,⁶⁰ bone matrix,⁶¹ and the arterial wall.^{4,62} In the media of vessels, MGP is expressed predominantly by VSMC and in the intima by macrophages, VSMC, and endothelial cells.⁴ MGP contains 5 γ -carboxyglutamic acid residues⁶³ that are derived from specific glutamic acid residues by the action of γ -carboxylase using vitamin K, an essential cofactor for this enzyme.⁶⁴ Matrix Gla protein is unique among the vitamin K-dependent proteins in that it retains the propeptide sequence within the mature protein.⁶⁵ The propeptide is an anchor that mediates the binding of vitamin K-dependent proteins to the γ -carboxylase during post-translational modification in the endoplasmic reticulum. The functional significance of the preservation of the propeptide within the sequence of mature MGP is unknown. MGP knockout mice have extensive arterial calcification,⁶⁶ demonstrating that MGP acts as an inhibitor of calcification. Although the mechanism for this effect is under investigation, we have demonstrated that MGP binds to BMP-2⁶⁷ and Boström et al.⁶⁸ have presented data to suggest that MGP blocks the osteo-inductive properties of BMP-2. In further support of this inhibitory function,

MGP expression is lower in the media of arteries from diabetic patients with Mönckeberg's sclerosis than in normal vessels.²⁶ In addition to binding BMP-2, there are other potential mechanisms by which MGP could inhibit calcification. Antibodies to MGP were shown to inhibit the deposition of a calcified matrix by cultured pericytes. This evidence was felt to support the role of MGP in regulating the differentiation of pericytes into cells capable of calcification.³⁷ Two polymorphisms of MGP (A-7 and Ala 83) are more common in patients with arterial calcification.⁶⁹ Two human syndromes have been postulated to result from abnormalities in MGP. Singleton–Merten syndrome is characterized by aortic calcification, short stature, osteopenia, decreased muscle mass, and death within first year.⁷⁰ The Keutel syndrome is characterized by abnormal cartilage calcification involving the ears, nose, larynx, trachea, and ribs, but arterial calcification has not yet been reported.⁷¹ Mutations in MGP have been reported in the Keutel syndrome.⁷²

C. Osteoprotegerin (OPG)

OPG is a secreted member of the tumor necrosis factor *receptor* gene superfamily, that acts as a natural *inhibitor* of the activity of osteoprotegerin ligand (OPG-L).²⁵ OPG-L has homology to members of the TNF ligand family and is also known as osteoclast differentiation factor (ODF), TNF-related activation-induced cytokine (TRANCE) and receptor-activator of nuclear factor (NF)- κ B ligand (RANKL). OPG-L exists in 2 biologically active forms, a cellular type II transmembrane form, and a soluble form that is released by proteolysis induced by a specific metalloprotease. The interaction of OPG-L with its receptor RANK (receptor-activator of NF- κ B) serves as the final common effector regulating the differentiation, activation, and apoptosis of osteoclasts. The promoter of the murine OPG-L gene has a response element for *Osf-2/Cbfa1*, a transcription factor that is essential for the commitment to the osteoblastic differentiation pathway.²⁵ Osteoprotegerin is a survival factor for endothelial cells in response to serum deprivation and appears to mediate the NF- κ B-dependent protective effects of α v β 3 ligation in endothelial cells.⁷³ OPG mRNA and protein are induced 5–7-fold following α v β 3 ligation with osteopontin.⁷³ The role of OPG, OPG-L, and RANK have been demonstrated in mice. Mice lacking OPG-L lack osteoclasts and develop severe osteopetrosis.⁷⁴ Transgenic mice that overexpress OPG and those lacking RANK also have defective osteoclastogenesis and an osteopetrosis phenotype.^{75,76} In contrast, mice lacking OPG have severe osteoporosis with high rates of pathological fractures.⁷⁷

D. Osteocalcin

Also known as bone Gla protein osteocalcin contains 3 Gla residues and is the most abundant non-collagenous bond extracellular matrix protein.⁷⁸ Osteocalcin is expressed only by osteoblasts and osteoblast-like calcifying vascular cells and may be useful as a marker of osteoblastic differentiation. Osteocalcin null mice have increased bone density⁷⁹ with normal resorption in females after ovariectomy⁸⁰ and normal arteries.

E. Osteopontin (OPN)

Osteopontin, an RGD containing phosphoprotein that can bind calcium and mediate cell adhesion and migration, was the first calcification-regulating protein to be discovered within calcified plaques.⁸¹ The expression of osteopontin is high in macrophages in calcified atherosclerotic lesions but low in medial VSMCs in both normal arteries and arteries with Mönckeberg's sclerosis.²⁶ Despite the overall low levels of expression, the protein accumulated at the boundary between VSMC and calcification. OPN is a potent inhibitor of hydroxyapatite deposition by cultured VSMC.⁸² Osteopontin, binding to α v β 3, protects endothelial cells from apoptosis induced by growth factor withdrawal.⁸³

OPN^{-/-} mice have normal development and fertility but have a higher bone volume at 4–6 months than OPN^{+/+} mice.⁸⁴ Estrogen deficiency induced by ovariectomy causes a 60% decrease in bone mass in OPN^{+/+} mice but only a 10% reduction in OPN^{-/-} mice.⁸⁵ This finding may be explained by the fact that OPN is a high-affinity ligand for $\alpha\nu\beta 3$ and the interaction between OPN and $\alpha\nu\beta 3$ may be required for maximal bone resorption by osteoclasts. Neither OPN^{-/-} mice nor transgenic mice overexpressing OPN have been reported to have arterial calcification.⁸⁶

F. Osteonectin

Osteonectin is a 43 kD ECM protein also known as SPARC (secreted protein acidic and rich in cysteine) that is expressed in areas of active remodeling in the skeleton and other tissues.^{87,88} Osteonectin binds to collagen and hydroxyapatite and can regulate cell proliferation and cell-matrix interactions.^{87,88} Osteonectin has roles in regulating angiogenesis and pericellular proteolysis.^{89,90} In vitro, osteonectin is one of the most potent inhibitors of hydroxyapatite crystal formation.^{91,92} VSMCs in normal arteries express osteonectin at high levels, and expression is significantly upregulated compared to that in veins,⁹³ but in Mönckeberg's sclerosis, osteonectin expression is significantly reduced or absent.²⁶

Null mice have decreased osteoblasts and osteoclasts as a result of decreased basic multicellular units, the functional units of osteoclasts and osteoblasts responsible for bone remodeling. This results in a significant reduction in the density of trabecular bone that becomes more severe with age.⁹⁴ They have a low-turnover osteopenia with trabecular bone having decreased mass, microarchitecture, and biomechanical properties.⁹⁴ Despite the reported role of osteonectin as an inhibitor of hydroxyapatite formation, osteonectin deficient mice have not been reported to have arterial calcification.

G. Biglycan

Biglycan is a member of the leucine-rich proteoglycans that also includes decorin, fibromodulin, lumican, epiphyhan, and karatocan.⁹⁵ Biglycan binds to collagen⁹⁶ fibrils and TGF- β .^{97,98} Biglycan is detected associated with collagen types I and III in both primary atherosclerotic and restenotic coronary artery tissues, whereas decorin is detected almost exclusively in atherosclerotic but not restenotic tissue.⁹⁹ Oxidized LDL specifically stimulates the expression of biglycan but not versican or decorin in monkey arterial VSMC cultures.¹⁰⁰ IL-1 induces the expression of decorin but not biglycan by cultured monkey arterial SMC.¹⁰¹ TGF- $\beta 1$ and PDGF increase the expression of biglycan but not decorin from arterial SMCs.¹⁰² Biglycan null mice are born without apparent defects and are normal until 3 months when their growth rate decreased.¹⁰³ These mice developed an osteoporosis phenotype with reduced cortical bone thickness, trabecular bone volume, and decreased bone strength.¹⁰³ Arterial calcification has not been reported in these mice, however.

H. Decorin

Decorin, a proteoglycan with chondroitin and dermatan sulfate glycosaminoglycans, forms part of the collagen network in human arteries. Decorin colocalizes with collagen, aids in assembly of collagen fibers, binding, and regulation of growth factor activity and control of cell growth. Vascular smooth muscle cells in culture express low levels of decorin^{104,105} and in human nonatherosclerotic arteries, prominent staining for decorin is detected only in the adventitia. In atherosclerotic arteries, decorin is detected mainly in the layer of intima closer to the media and less in the subendothelial part of the intima¹⁰⁶ and near the macrophage-rich lipid core.¹⁰⁷ Although its role in arterial calcification is unknown, decorin appears to be an inhibitor of calcification in bone, as primary calcification of bone matrix occurs after its removal from collagen fibrils.¹⁰⁸ The ability of collagen

to bind LDL is greatly enhanced by the presence of decorin,¹⁰⁹ a finding that could have important implications for the effects of oxidized lipids on the arterial wall and on bone.

I. Osteoglycin

Osteoglycin was originally described as a bone matrix protein.¹¹⁰ Later it was found to be highly expressed in the vasculature, uterus, and kidney as well.¹¹¹ It is a member of a family of related proteins characterized by tandem repeats of a leucine-rich sequence bounded by two conserved cysteine clusters that are involved in interactions with other proteins.¹¹² Osteoglycin was originally called osteoinductive factor, since it induced ectopic bone formation when injected into skeletal muscle, but this activity was later found to be due to contamination with BMP-2 and BMP-3 which co-purified with it and may associate with it in vivo.¹¹³ Osteoglycin is expressed in the media and adventitia of normal arteries but is down-regulated in atherosclerosis.¹¹¹ It is up-regulated at 14 days following balloon injury at a time when most VSMC proliferation has ceased. Studies of osteoglycin expressed by cultured VSMC have demonstrated that expression is decreased in proliferating cells. Thus, osteoglycin expression is characteristic of normal, differentiated, non-proliferating VSMC. Osteoglycin may be an essential component of the normal arterial matrix that is up-regulated after injury and potentially contributes to vascular remodeling.¹¹¹ Osteoglycin shares about 30% homology with decorin and biglycan. Like decorin, it may have a role in modulating matrix structure by interacting with collagen. Its role in arterial calcification, if any is unknown. It is possible that the down-regulation of osteoglycin in atherosclerotic arteries allows BMP-2 to be released from matrix depositories and consequently have greater activity in inducing calcification.

J. Collagen Type II

Type II collagen is the major matrix protein of cartilage cells and is a marker for cartilaginous metaplasia in arteries. Qiao et al.¹¹⁴ have shown that aortic cartilaginous metaplasia, which is genetically determined in certain mouse strains, is associated with the presence of arterial chondrocytes that express collagen type II.¹¹⁴ Collagen type II can bind annexin V, which is essential for matrix vesicle formation.¹¹⁵ Collagen type II expression is expressed in the arterial media of diabetic patients with Mönckeberg's sclerosis.²⁶

K. Bone Sialoprotein (BSP)

Bone sialoprotein is a 70 kDa sialic acid-rich acidic ECM glycoprotein synthesized by both osteoblasts and osteoclasts.^{116,117} BSP has an RGD (arginine-glycine-aspartic acid, or Arg-Gly-Asp) sequence near its carboxyterminal domain¹¹⁸ that is recognized by $\alpha v \beta 3$ integrin^{119,120} and may therefore be involved in regulating cell adhesion and migration. BSP represents 15% of the noncollagenous proteins found in the mineralized matrix.¹²¹ BSP binds collagen¹²² and is associated with the early phases of bone formation acting as a potent and specific nucleator of hydroxyapatite at the mineralization front of bone.^{123,124} In osteoblasts that produce BSP, expression is restricted to those cells that have secreted and are actively mineralizing a type I collagen matrix.¹²⁵ BSP mediates the attachment and migration of endothelial cells by an interaction between $\alpha v \beta 3$ and RGD. Furthermore, BSP is angiogenic in the chick chorioallantoic membrane assay, indicating that BSP could have an important role in the angiogenesis that occurs during bone formation.¹²⁶ BSP binds collagen and can nucleate hydroxyapatite. Although VSMC in culture express BSP¹²⁷ and BSP can be induced in VSMC by factors that promote their osteoblastic differentiation,¹²⁸ normal vessels express minimal or no BSP.²⁶ In contrast, expression of BSP is up-regulated in the media from patients with Mönckeberg's sclerosis.²⁶

L. Alkaline Phosphatase

Alkaline phosphatase is an essential component of matrix vesicles where it increases orthophosphates for the growing hydroxyapatite crystal. The essential role of alkaline phosphatase in bone formation is demonstrated by the disease hypophosphatasia, characterized by lack of functional alkaline phosphatase and defective bone mineralization.¹²⁹ Tissue nonspecific alkaline phosphatase is present in systemic arteries, arterioles, and some capillaries.¹³⁰ It is possible that the enzyme plays a role in arterial calcification by the same mechanism of action as in bone.¹³¹

M. Parathyroid Hormone-Related Peptide

Parathyroid hormone-related peptide is a 141 amino acid residue peptide that is homologous to the amino-terminus of PTH, binds to the same receptor and mimics the effect of PTH on calcium and phosphate homeostasis.^{132,133} Unlike PTH, PTHrP is expressed in many adult tissues including SMC.^{134,135} PTHrP is overexpressed in coronary atherosclerotic smooth muscle cells, although the expression in calcified lesions is significantly less than in noncalcified lesions.¹³⁶ The vascular calcifying effect of 1,25-dihydroxyvitamin D₃ may be mediated through suppression of PTHrP expression in VSMC.¹³⁷ There is an inverse relationship between PTHrP and calcification of VSMC in vitro and the addition of exogenous peptide inhibits calcification.¹³⁸ PTHrP appears to be an autocrine and/or paracrine factor that regulates vascular calcification.

5. MOUSE MODELS OF ARTERIAL CALCIFICATION

Several murine knockout models of genes that regulate bone formation have led to new insights into the pathogenesis of arterial calcification. Mice lacking matrix Gla protein have extensive calcification of the aorta, its branches, and all elastic and muscular arteries including the coronary arteries.⁶⁶ Arterioles, capillaries, and veins do not calcify. These mice die at about 2 months from rupture of a brittle, essentially ossified, aorta. In addition to the dystrophic arterial calcification, the MGP^{-/-} mice also have aortic valvular calcification, and calcified cartilage in the trachea and bone growth plates.⁶⁶

The MGP knockout mice prove that MGP has an important role in preventing arterial calcification. Since MGP has gamma-carboxyglutamic acid (Gla) residues, it has been postulated that MGP binds to hydroxyapatite, producing a protein layer that inhibits further mineralization.¹³⁹ We have recently proposed an alternative hypothesis for the inhibitory effect of MGP. MGP was shown to form a complex with BMP-2.⁶⁷ Boström et al.⁶⁸ have provided evidence that MGP neutralizes the osteo-inductive activity of BMP-2.

Osteoprotegerin knockout mice develop medial and subintimal calcification that is apparent at 2 weeks and is marked by 2 months.⁷⁷ These animals also develop severe osteoporosis and thus represent one of the few models of the “calcification paradox.” Overall, the calcification is less extensive than in the MGP^{-/-} mice, with the occurrence in only two-thirds of the null mice and with calcification being evident primarily in the aorta and renal arteries.⁷⁷ As was also demonstrated in the MGP null mice, dystrophic calcification is not observed in smaller arteries, capillaries or veins, suggesting that these vessels express additional unknown inhibitors of calcification. The arteries that exhibited calcification in the OPG^{-/-} mice are also sites of endogenous OPG expression, suggesting that OPG may have a specific role in protecting these arteries from calcification.⁷⁷ The OPG^{-/-} mice also develop partial aortic dissection and SMC proliferation in the intima and media suggesting additional regulatory roles for OPG.⁷⁷

Smad1, 4 and 5 mediate intracellular signaling of bone morphogenetic protein-2, while Smad6 and 7 inhibit signaling of the TGF- β superfamily.¹⁴⁰ There is a response element for BMP-2 in the smad6 promoter and the induction of smad6 by BMP-2 appears to be an inhibitory feedback signaling mechanism.¹⁴¹ Smad6 knockout mice develop ossification of the aorta around the outflow track of the heart.¹⁴² There was cartilaginous metaplasia and the presence of trabeculated bone structures, sometimes with bone marrow elements present in the aortic medium. These changes were restricted to areas where endothelial cells and VSMC expressed smad6.¹⁴² Thus, without its intracellular inhibitor, BMP-2 induces arterial calcification in only a short anatomical segment. A possible explanation for this observation is that another inhibitor, such as MGP, acts to inhibit BMP-2 activity, thereby preventing more widespread arterial calcification.

Mice lacking the carbonic anhydrase isoenzyme II gene develop vascular calcification of the media of small arteries and arterioles in a number of organs.¹⁴³ The calcification was age dependent and tended to be more severe in males than females. Veins were not involved, including those that bordered an affected artery.¹⁴³ The mechanism for the calcification is not known, but may relate to the metabolic acidosis that these mice develop or to alterations in cellular calcium influx or efflux.

Fibrillin-1 is the major structural component of microfibrils and the defective gene product in Marfan's syndrome. Mice with a homozygous hypomorphic mutation of the fibrillin-1 gene develop features of Marfan's syndrome and also have medial calcification as an early pathological finding.¹⁴⁴ Calcification is confined to the arterial elastic lamellae.¹⁴⁴ Recent studies have shown that Marfan's syndrome is accompanied by calcium deposition in the elastic fibers of the media in multiple arteries, demonstrating concordance with the mouse model.¹⁴⁵

The klothe gene encodes a membrane protein that shares sequence similarity with the enzyme β -glucosidase.¹⁴⁶ Mice that lack the gene product develop a syndrome that resembles normal human aging with a decreased lifespan, infertility, and skin atrophy.¹⁴⁶ These mice also have ectopic calcification in the media or arteries and arterioles and also in the parenchyma of several organs. The klothe deficient mice also developed osteoporosis.¹⁴⁶

Studies in inbred strains of mice have demonstrated that there is a genetic component to arterial calcification.^{114,147} DBA/2J mice are susceptible to coronary and aortic calcification, even when they are fed a chow diet. When they are fed an atherogenic diet for 15 weeks, 100% of the animals develop calcification of the aorta. In contrast, MRL substrain mice are resistant to aortic and coronary artery calcification even when fed an atherogenic diet.¹⁴⁷ Interestingly, some MRL substrains are known to express high levels of osteopontin,¹⁴⁸ an inhibitor of mineralization by VSMCs in culture.⁸²

Studies with inbred strains of mice have also shown that certain arteries are more prone to atherosclerosis than others. The C3H/HeJ mice show susceptibility to calcification of the coronaries, for example Ref. [147]. In most mice, however, there is a relatively poor correlation between calcification in the aorta and the coronary.¹⁴⁷

High fat high cholesterol diets promoted the development of atherosclerosis with the primary site of predilection in all strains being at the base of the coronary sinus. The development of aortic calcification was often associated with atherosclerosis, but the atherogenic diet also promoted the development of coronary calcification independently of atherosclerosis, especially in certain strains (C3H/HeJ; Ref. [147]).

The occurrence of arterial calcification in transgenic mice overexpressing human apo AII and fed a chow diet was not significantly different from the background strain, with only 9% of mice having calcification of the aorta and none having coronary calcification.¹⁴⁷ In contrast, two-thirds of apoE knockout mice fed a chow diet had calcification of both the aorta and coronary arteries.¹⁴⁷ This difference could be due to the more severe atherosclerosis in the apoE knockout mice. When fed an atherogenic diet, apoE knockout mice also have more extensive atherosclerosis and calcification than LDL receptor knockout mice.¹⁴⁹ It is possible that apoE has an independent effect on calcification.¹⁵⁰ Interestingly, apoE has been proposed to have a role in plasma transport of vitamin

K,⁶⁴ so that it is possible that apoE knockouts have mild vitamin K deficiency with inadequate carboxylation of MGP.

In inbred strains of mice, a genetic predisposition has been described for arterial cartilage metaplasia, which is one of the pathways for the development of arterial calcification.¹¹⁴ Cartilage metaplasia often occurs in the aortic root, where calcification also predominates.¹¹⁴ A significant correlation between the occurrence of arterial cartilage metaplasia and aortic calcification has been described in inbred strains of mice. An atherogenic diet promoted the development of cartilage metaplasia in susceptible strains of mice. The extreme hypercholesterolemia in apoE knockout mice is associated with more extensive cartilage metaplasia that involves the aortic arch as well as the aortic root.¹¹⁴

6. OTHER ANIMAL MODELS

A variety of stimulants, such as hypercholesterolemia, vitamin D, and nicotine, alone or in combination have been used to induce arterial calcification in a variety of different animals.^{151–156} The effect of nicotine has been postulated to be related to its ability to release catecholamines or to its direct vasoconstrictive effect of arteries.¹⁵¹ The combination of vitamin D₃ and nicotine (VDN) treatment of rats leads to a 20–35-fold increase in the calcium content of the aorta, which is accompanied by systolic hypertension and increased aortic wall stiffness.^{155,156} The mechanism for vitamin D-induced augmentation of arterial calcification may be related to its ability to stimulate alkaline phosphatase expression while suppressing expression of PTHrP.^{137,138} Interestingly, supplemental vitamin K₂ has been reported to suppress the induction of calcinosis by vitamin D₂.¹⁵⁷ Although not studied, it is possible that the supplemental vitamin K₂ augments the Gla modification of MGP, rendering it a better inhibitor of BMP-2 in the vessel wall.

The deposition of calcium in the VDN model occurs preferentially on the internal elastic lamina¹⁵⁴ and other elastic fibers and leads to the destruction of the elastic network of the vessel. There is a decrease in the elastin-specific amino acids desmosine and isodesmosine with VDN treatment, and the content of these amino acids is inversely correlated with the calcium content.¹⁵⁴ This suggests that the calcium deposition on the elastic lamellae induced by VDN treatment leads to proteolysis of elastic fibers (elastocalcinosis) with loss of arterial compliance.

Exposing rats to warfarin leads to increased levels of calcification as detected by von Kossa staining within the aortic media as well as the media of smaller arteries.^{139,158} Cerebral arteries, veins, and capillaries are not affected by warfarin-induced calcification.¹³⁹ The mechanism for this effect is presumably by the ability of warfarin to block the γ -carboxylation of matrix Gla protein. We have found that the N-terminus of MGP, containing its 5 Gla residues, is essential for MGP to bind to BMP-2.⁶⁷ Therefore, MGP that is synthesized in the presence of warfarin is inactive because of the lack of these vitamin K-dependent modifications. In a recent modification of this model, warfarin was shown to induce even more extensive arterial calcification in the younger animals, a finding felt to be related to their higher levels of serum phosphate.¹⁵⁹ Furthermore, warfarin and vitamin D in young animals were found to be highly synergistic stimuli for arterial calcification.¹⁵⁹

7. GENERAL OBSERVATIONS FROM THE ANIMAL MODELS

The distribution of calcification varies considerably in the models. Thus far, the MGP null mouse has produced the most extensive arterial calcification. Arterial calcification in the osteoprotegerin

knockout mouse is more limited and is extremely localized in the *smad6* knockout mouse. Small arteries and arterioles are involved with carbonic anhydrase isoenzyme II null mice, while *klotho* knockouts have involvement of arteries (large and small) and arterioles. The MGP null model has involvement of some small (e.g., coronary) arteries, while the remaining models involve primarily the aorta. Although the reported extent of vascular calcification in these models may be, to some degree, a function of the rigor with which it is searched for, there are nevertheless obvious differences in the distributions of the pathology.

One of the more interesting features of animal models of vascular calcification is that, although the involvement of arteries and arterioles is variable, the veins are uniformly spared. Even in the most dramatic model of arterial calcification, the matrix Gla protein null mouse, there has been no report of venous calcification. This could be due to the fact that arterial smooth muscle cells express a distinct complement of genes from venous SMC⁹³ that are able to induce calcification; alternatively, venous SMC may express unique inhibitors. Veins also have a relative paucity of elastic lamellae, which often serve as an initial nidus for medial calcification. Furthermore, veins are relatively resistant to the development of atherosclerosis. Even though vascular calcification is overwhelmingly a disease of arteries, venous calcification has been reported. Perhaps the most common manifestation of venous calcification are phleboliths, often detected on routine radiographs.¹⁶⁰ Cases of extensive venous calcification are rare and usually occur in the setting of venous thrombosis.¹⁶¹ Saphenous veins, important conduits for bypassing coronary arterial as well as peripheral arterial obstructive disease have been reported to have a low prevalence of calcification. Prior to their use as arterial bypass conduits, medial sclerosis is detected in 36% of long saphenous veins but only 1% have evidence of calcification.¹⁶² Regions of vein wall calcification in association with a thickened intima are also occasionally seen in vein remnants from patients undergoing infrainguinal arterial reconstruction.^{163,164} Thus, veins can be induced to calcify but the stimulus for this response has not been identified.

Another observation from the animal models is that calcification inhibitors during development may differ from post-natal inhibitors. MGP null mice were not different from normal mice at 1 week, but arterial calcification is evident at 2 weeks after birth,⁶⁶ suggesting that different genes may prevent arterial calcification during embryogenesis and early post-natal life. There are other inhibitors of BMP-2 that could be more important inhibitors during development such as noggin,¹⁶⁵ chordin,¹⁶⁶ gremlin, cerebrus, and DAN.¹⁶⁷

There are only two models for the “calcification paradox”, i.e., the occurrence of arterial calcification and osteoporosis in the same animal. This phenomenon occurs frequently in elderly patients, especially women,¹⁶⁸ and has been suggested to be due to the differential effects of oxidized lipids on artery and bone.¹⁶⁹ It is notable that osteoporosis in animal models has not yet been reported from hyperlipidemia, either with or without a genetic background predisposing to high lipid levels. Osteoprotegerin and *klotho* knockout animals both exhibit the calcification paradox, although *klotho* animals also demonstrate ectopic calcification in organ parenchyma as well as in arteries. It is also notable that the disruption of at least two bone-related proteins (osteonectin and biglycan) produces an osteoporotic phenotype without arterial calcification.

It is not known whether warfarin therapy accelerates the calcification process in patients as it does in the rat model. The rats were given high doses of warfarin combined with vitamin K, allowing the artery wall to be “anticoagulated” without systemic bleeding. Nevertheless, patients may be exposed to warfarin for many years, so that the effects of a small rate of calcification could be magnified by chronic exposure. One report has suggested that warfarin may increase the risk of calcific uremic arteriopathy (CUA), a syndrome of progressive cutaneous necrosis in patients with chronic renal failure.¹⁷⁰ Patients develop livido reticularis followed by skin ulceration and occasional ischemic infarction of visceral organs. Skin biopsies

show evidence of small vascular intimal fibrosis with calcification and some vascular thrombosis. Coates et al.¹⁷⁰ reported a series of 16 patients with CUA, of whom 8 were being treated with warfarin. Warfarin probably exerts its effects by preventing the Gla modification of MGP, thereby preventing MGP from adequately inhibiting BMP-2. Further studies are warranted to examine the potential for chronic warfarin to exacerbate the process of arterial calcification in patients.

A. Question 1: Is Arterial Calcification Solely a Marker for Vascular Disease or Does it Also Contribute to Vascular Disease?

Arterial calcification is usually considered a marker for atherosclerosis or, in the case of Mönckeberg's sclerosis, other disease processes such as diabetes mellitus or renal failure. However, it is also possible that arterial calcification exacerbates or inhibits the progression of the disease process. Calcification of the arterial media is first observed at elastic lamellae, and intense calcification is often accompanied by elastic fiber fragmentation.¹⁵⁴ Although it is possible that elastic fiber degradation and calcification are caused by related but independent mechanisms, it is also conceivable that the deposition of hydroxyapatite directly activates proteases or induces protease production by neighboring cells. Studies from the vitamin D plus nicotine (VDN) models support a role for calcification alone, in the absence of hyperlipidemia or other perturbations, in disrupting elastic fibers.¹⁵⁴ It should be noted that calcification and degradation of elastic fibers with increased arterial stiffness is a common feature of aging.¹⁷¹

It is interesting to note that MGP is detected along the elastic lamellae,^{26,62} suggesting that this is an important site for defense against calcification. The destruction of elastic lamellae could be a mechanism by which calcification accelerates the rate of progression of atherosclerosis, since elastin is an important regulator of VSMC proliferation and migration. Mice lacking elastin develop severe intimal hyperplasia.^{172,173} It is interesting in this regard to note that two human diseases (Marfan's syndrome and pseudoxanthoma elasticum) are accompanied by elastic fiber calcification and degradation along with intimal hyperplasia.^{144,174}

Since Mönckeberg's sclerosis affects the media, it would be predicted to affect vasomotor function, but not have a significant effect on luminal dimensions. However, two types of medial calcifications have been described¹⁷⁵: (i) a benign slowly progressive form with thin medial calcifications and little or no luminal narrowing, and (ii) a malignant, progressive form in which massive medial calcifications displace the internal elastica lamina toward the lumen, even penetrating into the intima and cause luminal narrowing. It is possible that medial calcification in diabetes and renal failure leads to elastin fiber degradation, which allows increased medial VSMC migration and proliferation, culminating in an encroachment of the vascular lumen.

Several roles, often contradictory, have been offered for the effect of intimal, atherosclerosis-related calcification. Fisher et al.¹⁷⁶ concluded that calcification *limits* the growth of carotid plaque. They found that the thickest plaques were not usually calcified, whereas adjacent thin rims of atherosclerosis often were.¹⁷⁶ Clinical studies have reported both association¹¹ and lack of association¹⁷⁷ between coronary artery calcification and acute coronary ischemic events, with the discrepancy possibly due to differences in the characteristics of the study populations.¹⁷⁸ It has been postulated that coronary artery calcification could protect against plaque rupture.¹⁷⁹ By biophysical analysis, calcified areas have been predicted to be less likely to undergo plaque rupture.¹⁸⁰ A plaque with a heavily calcified cap is stiffer and predicted to be more resistant to rupture.^{181,182} One clinical study has supported a stabilizing role for calcification in aortic plaque. In the French aortic plaque in Stroke study (FAPS), 334 patients > 60 years old with stroke were evaluated by TEE and were followed for 2–4 years.¹⁸³ The absence of calcification in the plaques by transesophageal echocardiography was associated with an increased risk

of embolic stroke.¹⁸³ However, recent studies have suggested that calcification has little if any effect on plaque stability. Huang et al.¹⁸⁴ studied 10 ruptured and 10 stable plaques using large strain finite element analysis. In contrast to lipid pools, which were found to dramatically increase stress, calcification did not decrease the mechanical stability of the plaque as modeled in this system.¹⁸⁴ Whether calcification promotes or protects against plaque rupture could depend on both the extent and the distribution of calcification relative to important anatomical landmarks and the presence of inflammatory cells that lead both to calcification and produce destabilizing enzymes.

B. Question 2: Is Arterial Calcification a Manifestation of Apoptosis?

Cellular degradation products in the media of arteries have long been considered as a potential nidus for calcification.^{185–187} Cells that undergo apoptosis or necrosis may provide membrane fragments that play a role in initiating calcification.¹⁸⁸ Apoptosis is a relatively common event in atherosclerotic arteries¹⁸⁹ and after arterial injury.¹⁹⁰ Cytoplasmic vesiculation is a recognized feature of both apoptosis, and the formation of matrix vesicles may therefore represent a manifestation of programmed cell death.

Matrix vesicles are extracellular membrane invested particles of 100–200 nm in diameter. These particles are produced by polarized budding and pinching off of membrane segments from chondrocytes, osteoblasts, and odontoblasts.¹¹⁵ They serve as the initial site of calcification of all skeletal tissues. Several components of the matrix vesicles are thought to be involved in initiating calcification including: calcium binding proteins and phospholipids, annexins (II, V, and VI that act as a calcium ion channels), and alkaline phosphatase that increases orthophosphate concentration.^{115,191} Collagen types II and X, which are associated with matrix vesicles can facilitate calcification by binding to both annexin V and alkaline phosphatase.¹¹⁵ The interactions between collagen type II and X in the extracellular matrix with annexin V stimulates its calcium channel activities, concentrating calcium in the matrix vesicle as the first step in biomineralization.¹¹⁵

Matrix vesicles have also been found in intimal atherosclerotic plaques and in regions of medial calcification.^{192–194} Matrix vesicles can be isolated from human atherosclerotic plaque by collagenase digestion and differential ultracentrifugation. When exposed to a medium containing physiological levels of calcium, phosphate, and 1 mM ATP, these vesicles accumulate hydroxyapatite.¹⁹⁴ Matrix vesicles from atherosclerotic plaques had higher extents of calcifiability compared to those isolated from nonatherosclerotic vessels.¹⁹⁴ Furthermore, membrane-bound matrix vesicles can be seen in the ECM (extracellular matrix) of cultured VSMC that are participating in the deposition of hydroxyapatite crystals.⁸² VSMC have recently been shown to release tissue factor-rich microparticles of a size consistent with matrix vesicles.¹⁹⁵ Currently, it is unknown whether these tissue factor-containing microparticles also function as matrix vesicles that initiate calcification.

In some animal models, cell death appears to be the principal stimulus for calcification. Gadeau et al.¹⁹⁶ found that arterial calcification in the balloon-injured rabbit aorta occurred early (at 2–4 days) in areas of the media where cells had been killed by necrosis or apoptosis. Calcification occurred before the matrix proteins osteopontin or osteocalcin were detected, indicating that the early calcification process in this model was more closely tied to cell death than to the expression or deposition of bone-associated proteins.¹⁹⁶ Further studies are warranted to examine the links between apoptosis, necrosis, and arterial calcification.

C. Question 3: Does Oxidant Stress Contribute to Arterial Calcification?

Many factors that have been linked with increased prevalence of arterial calcification (hypercholesterolemia, hypertension, diabetes, and dialysis-dependent end stage renal disease)

are associated with increased levels of oxidative stress.^{197–200} We postulate that the generation of oxidant stress could be the common mechanism by which any of these factors predisposes to arterial calcification.

There are a variety of potential mechanisms by which oxidants could enhance calcification. Lipid oxidation products (minimally oxidized LDL, but not LDL itself) induce CVC to undergo calcification.¹⁶⁹ 25-Hydroxycholesterol enhances calcification by calcifying vascular cells in vitro.¹⁶⁹ It has been postulated that oxidized lipids might mimic the effect of vitamin D₃ in the vessel wall, or that these lipids might act as transport vehicles to deliver vitamin D₃ into the vessel wall.¹⁶⁹

As noted above, matrix Gla protein is an inhibitor of arterial calcification that is dependent on the presence of Gla residues for its activity. It is possible that oxidant stress could produce a functional vitamin K deficiency in the arterial wall. Vitamin KH₂ is an essential requirement for the carboxylation of certain target glutamic acid residues that are modified to Gla residues by the enzyme gamma carboxylase.⁶⁴ Vitamin KH₂ has antioxidant activity and is itself a target of certain oxidants.^{201,202} The vitamin K cycle, which exists in many tissues including arteries and cultured vascular smooth muscle cells,⁶² is essentially a redox cycle that maintains reduced vitamin K.⁶⁴ The vitamin K cycle is embedded in the interior of the rough endoplasmic reticulum.²⁰² Vitamin K epoxide reductase (VKOR) is sensitive to lipid peroxidation, perhaps because these agents disrupt the essential membranous lipid environment that is required for VKOR activity or oxidize critical thiol residues.²⁰² Finally, gamma glutamate carboxylase activity has been reported to be three-fold higher in normal arteries compared to atherosclerotic arteries.^{203,204}

In summary, there are many factors that induce calcification that are also accompanied by oxidant stress. The disruption of the ability to generate the reduced form of vitamin K, the direct oxidation of vitamin KH₂, or the inability to effectively use the vitamin KH₂ co-factor could lead to the inability to adequately modify MGP to form its 5 Gla residues.

D. Question 4: Is Arterial Calcification a Manifestation of Vascular Vitamin K Deficiency?

As noted, matrix Gla protein, an inhibitor of BMP-2, requires vitamin K-dependent carboxylation for full activity. Oxidant stress and medications such as warfarin and diphenylhydantoin²⁰⁵ may antagonize the activity of vitamin K in the vessel wall. A finding with widespread public health implications is that a significant part of the population may have a mild deficiency of vitamin K. This deficiency may contribute both to arterial calcification and osteoporosis in the elderly.^{206–209} It has been suggested that mild vitamin K deficiency be defined on the basis of an elevation in circulating levels of osteocalcin.

8. PROSPECTS FOR THERAPY OF ARTERIAL CALCIFICATION

Hydroxyapatite [Ca₅(PO₄)₃OH] is a highly insoluble material.²¹⁰ Removing hydroxyapatite from arteries would be the equivalent of demineralizing bone, a very daunting challenge indeed. Most therapeutic approaches are therefore directed at preventing the progression of arterial calcification.

A. Chelation and Demineralization Therapy

Chelation therapy might theoretically have benefit in removing calcium as well as via an antioxidant effect.^{211,212} However, benefits from this therapy are unproved and there are significant potential side effects.²¹¹ One company is developing a proprietary demineralization therapy based on the

bone-dissolving activity of osteoclasts that can be delivered by a catheter-based system.²¹³ However, there are currently no clinical trials that support the use of this therapy.

B. Vitamin K

If a vitamin K deficiency in the arterial wall is demonstrated as a common cause of arterial calcification, then vitamin K supplementation would appear to be ideal therapy. There have been no clinical trials to examine this issue, however. Vitamin K can reduce the occurrence of osteoporosis in selected populations^{214,215} and is well tolerated. Vitamin K therapy does not carry the risk of causing hypercoagulable state as does other potential forms of therapy to prevent arterial calcification (estrogen and SERMs).

C. Statins

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, commonly known as “statins” may have the greatest potential of all currently available drugs in retarding the rate of arterial calcification. In one animal study, pig aortic intimal and medial calcification and atherosclerotic burden were found to decrease concomitantly following the removal of the dietary stimulus.²¹⁶ Thus far, only one study has reported evidence for regression of arterial calcification in humans. A retrospective study of 149 patients who underwent a baseline and follow-up EBCT scan at 12–15 months demonstrated that the volumetric calcium score decreased only in patients who are treated with HMG-CoA reductase inhibitors and who achieve an LDL cholesterol of 120 mg/dl or less.²¹⁷ Patients who are not treated with HMG-CoA reductase inhibitors, or who are treated but whose final LDL-cholesterol remains greater than 120 mg/dl, have a significant increase in their calcium score.²¹⁷ All statins may not be equally effective in reducing arterial calcification. In 24 patients with and 40 without CAD, the administration of fluvastatin for 1 year resulted in improved aortic compliance, increased HDL, decreased LDL: HDL ratio, and a decrease in carotid intimal-medial thickness, but there was a small rise in calcification score during the 1 year treatment.²¹⁸ It appears that calcification progresses most rapidly when volumetric scores are low;²¹⁹ therefore, the best prospect for retarding the rate of calcification or effecting its regression with any therapy including statins may be in the early stages of the disease process.

Statins may have particular benefit in patients with the “calcification paradox,” i.e., both arterial calcification and osteoporosis.²²⁰ Statins are able to inhibit osteoclast formation and bone resorption²²¹ while also promoting new bone formation.²²² Some observational studies suggest a benefit of statins in reducing the risk of fractures,^{223,224} but this finding has thus far not been supported in secondary analyses of randomized trials of statins for coronary heart disease.^{222,226}

Statins exert effects other than lowering cholesterol that could also be beneficial in reducing calcium accumulation. Kwak et al.²²⁷ have demonstrated that statins inhibit the interferon- γ -induced expression of class II major histocompatibility complexes on antigen presenting cells. Thus, statins decrease T cells responses that lead to the pro-inflammatory state of atherosclerosis and other diseases.²²⁷ Statins have a variety of other effects that could be beneficial, such as increasing nitric oxide production and preventing leukocyte adhesion.²²⁸ Determining whether these additional effects of statins are useful in reducing atherogenesis and arterial calcification will require further investigation.

D. Estrogen

Elderly post-menopausal women seem to be especially prone to developing osteoporosis and arterial calcification. An inverse association between the progression of calcification of the abdominal aorta and loss of bone mass and density in the metacarpals has been demonstrated.¹⁶⁸ Nakao et al.²²⁹

found that both males and post-menopausal females with arterial calcification had lower serum estradiol levels than same gender controls.²²⁹ Several studies have reported lower coronary artery calcium scores in post-menopausal women taking hormone replacement therapy than those who did not. McLaughlin et al.²³⁰ studied 914 self-referred post-menopausal women older than 50 who underwent EBCT. The mean total coronary artery scores for women receiving HRT was 54.2 and for women not receiving HRT was 86.2 ($P = 0.02$).²³⁰ Vogt et al.²³¹ found that current estrogen use was protective for the occurrence of aortic calcification. Shemesh et al.²³² used double helical computed tomography to compare the prevalence and extent of coronary artery calcification in 41 women who were on HRT from the first year of menopause to 37 women who had never taken HRT. The prevalence of CAC in the group taking HRT was 14.6% versus 43.2% in the nonusers ($P < 0.01$).²³² However, other groups have found no effect of hormone replacement therapy on calcification scores.^{233,234}

There is now considerable doubt as to whether hormone replacement therapy is cardioprotective. The HERS trial is the only prospective, randomized controlled trial to-date to determine if estrogen replacement therapy has a beneficial effect on coronary events.²³⁵ Participants were post-menopausal women with coronary artery disease and treatment was conjugated equine estrogen 0.625 mg plus medroxyprogesterone acetate 2.5 mg versus placebo. After 4.1 years of follow-up, there was no difference in the groups in major cardiovascular outcomes.²³⁵ In fact, the estrogen-treatment group had more events in the first year, but fewer in years 4 and 5.²³⁵ Additional trials are in progress to examine the cardioprotective effects of estrogen.

E. Serms

Selective estrogen receptor modulators (SERMs) bind to both the alpha and beta estrogen receptors with high affinity.^{236,237} They exhibit estrogen-like effects on the skeleton, cardiovascular system, and blood lipid levels, but anti-estrogen effects on the breast and endometrium. The most extensively studied SERM, raloxifene, protects the skeleton from osteoporosis.^{238,239} Raloxifene has favorable effects on lipids (reduces LDL, but does not increase HDL or triglycerides) and lowers fibrinogen.²⁴⁰ Interestingly, there is a fairly good correlation between plasma fibrinogen level and coronary artery calcification,²⁴¹ so this effect could provide protection against progression of mineralization. The effect of raloxifene on coronary heart disease is being evaluated in the Raloxifene Use for The Heart (RUTH) trial.²⁴²

F. Calcium Channel Blockers (CCBs)

Several studies in animals have demonstrated that calcium channel antagonists are effective in reducing arterial calcium deposition. This effect has been observed using a variety of stimuli to induce arterial calcification, including spontaneous hypertension, vitamin D₃ intoxication, advanced age, alloxan-induced diabetes, administration of nicotine, and hyperlipidemic diets.^{243,244}

These animal models produce arterial calcification that differs in its regional distribution and in responding to calcium antagonists. For example, SHR and salt-sensitive hypertensive Dahl rats fed a high salt diet have vascular calcium overload that is most evident in the distal segments of the arterial tree. In contrast, normotensive Wistar rats given a high dose of vitamin D₃ have calcification predominantly of the proximal arterial trunks of larger arteries such as the aorta, coronary, mesenteric and renal arteries.²⁴³ Verpamil and diltiazem are effective uniformly throughout the arterial tree, whereas nifedipine and its derivatives are more effective in preventing peripheral arterial calcification.²⁴³

Many of the models (such as vitamin D₃ overdose) that have been used to examine the inhibitory effect of calcium antagonists produce a disease that most closely resembles Mönckeberg's sclerosis. Studies with atherosclerotic animal models have also demonstrated a beneficial effect of

calcium antagonists. These drugs have a variety of anti-atherogenic effects,^{245–248} including an anti-oxidant effect of some CCBs that prevents LDL oxidation.^{249–250} Thus, it is possible that the reduction in arterial calcium content is explained by the reduction in atherosclerosis burden that is achieved by treatment with CCBs. However, the anti-atherogenic properties and the reduction in calcification are not always parallel. For example, although nilvadipine reduced calcium content in the aorta of rabbits fed a high cholesterol diet, this agent did not reduce lipid deposition.²⁵¹

CCBs could prevent the calcium overload of cells that occurs during atherogenesis.²⁵² As previously noted, matrix vesicles are enriched with calcium channels¹¹⁵ that increase the intravesicular calcium content which then combines with phosphate released by alkaline phosphatase to produce hydroxyapatite. However, these calcium channels are formed by annexins, and a specific inhibitor of calcium uptake by matrix vesicles has not yet been developed.

The effect of calcium channel blockers on human arterial calcification is unknown. The PREVENT trial found no benefit of amlodipine on the progression of arterial atherosclerotic lesions,²⁵³ but arterial calcification was not examined.

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