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Genetic Pathways of Vascular Calcification

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Abstract

Vascular calcification is an independent risk factor for cardiovascular disease. Arterial calcification of the aorta, coronary, carotid and peripheral arteries becomes more prevalent with age. Genomewide association studies have identified regions of the genome linked to vascular calcification, and these same regions are linked to myocardial infarction risk. The 9p21 region linked to vascular disease and inflammation also associates with vascular calcification. In addition to these common variants, rare genetic defects can serve as primary triggers of accelerated and premature calcification. Infancy-associated calcific disorders are caused by loss of function mutations in *ENPP1* an enzyme that produces extracellular pyrophosphate. Adult onset vascular calcification is linked to mutations *NTE5*, another enzyme that regulates extracellular phosphate metabolism. Common conditions that secondarily enhance vascular calcification include atherosclerosis, metabolic dysfunction, diabetes, and impaired renal clearance. Oxidative stress and vascular inflammation, along with biophysical properties, converge with these predisposing factors to promote soft tissue mineralization. Vascular calcification is accompanied by an osteogenic profile, and this osteogenic conversion is seen within the vascular smooth muscle itself as well as the matrix. Herein we will review the genetic causes of medial calcification in the smooth muscle layer, focusing on recent discoveries of gene mutations that regulate extracellular matrix phosphate production and the role of S100 proteins as promoters of vascular calcification.

Keywords

vascular smooth muscle; calcification; S100/calgranulin

Introduction

Vascular calcification is an actively regulated form of matrix mineral metabolism resulting in the deposition of calcium phosphate, primarily in the form of hydroxyapatite, in the vessel wall. Bone is largely composed of hydroxyapatite [Ca₁₀(PO₄)₆(OH)₂], and hydroxyapatite crystals grow from extruded matrix vesicles. Matrix vesicles are 100–200 nM particles that bud from the surface of osteoblasts, and contained within matrix vesicles are the beginnings of crystals. Matrix vesicles are the nidus around which bone formation ensues (Anderson 2003). Like bone, vascular smooth muscle can similarly exude matrix vesicles as a

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mechanism to promote vascular calcification, and some of the protein content is shared between bone and smooth muscle derived matrix vesicles (Figure 1) (Kapustin et al. 2011). Under normal vascular conditions, calcium phosphate deposition in the vessel wall is a thermodynamically unfavorable reaction, and pyrophosphate inhibits the formation of crystals.

Vascular calcification increases with age, and is dysregulated in diabetes, dyslipidemia, renal disease, and hypertension. Vascular calcification is readily detectable by multiple imaging modalities. CT scans of mummified ancient Egyptians have documented a high prevalence of calcification affecting the coronary, carotid, iliac, femoral, and peripheral leg arteries and extending to the aorta, indicating that calcification is not a modern disease (Figure 2), (Allam et al. 2011). Vascular calcification of the smooth muscle medial layer of arteries, termed Mönckeberg's calcification, is thought to be less directly associated with inflammation compared to calcification affecting the intimal layer because it is comparatively less associated with inflammatory cell infiltrate. Calcification of one arterial vascular bed may also be linked to calcification in other arteries, but is not necessarily linked to extra-vascular ectopic calcification. For instance, 50% of patients with severe aortic valve calcification and stenosis have no detectable obstructive coronary artery disease despite a similar shared profile of risk factors, suggesting that different vascular cells have different susceptibility to undergo calcification (Branch et al. 2002).

Population-based association studies for vascular calcification

Vascular calcification has a high heritable component. Forty to 50% of the variance of aortic and coronary calcification can be attributed to genetics (review in (Rutsch et al. 2011)). For example, a study of 698 asymptomatic white adults from 302 families estimates 42% of the variation in coronary artery calcification (CAC) was attributable to genetic factors after adjustment of traditional risk factors like age, sex, fasting glucose, systolic blood pressure, pack-years smoking, and LDL cholesterol (Peyser et al. 2002). Genomewide association studies have identified several loci linked to coronary artery calcification, and some of these same loci are shared with those for coronary atherosclerosis. An early genomewide study of sibships enriched for hypertension was able to identify loci at 6p21.3 and 10q21.3 associated with CAC (Lange et al. 2002). The 9p21 locus, which has been extensively linked to vascular disease, also associates with subclinical coronary atherosclerosis and calcification (Assimes et al. 2008). A recent meta-analysis of CAC and MI in nearly 10,000 subjects from five cohorts identified 48 single nucleotide polymorphisms (SNPs) at 9p21 near the cyclin genes, *CDKN2B* and *CDKN2AA*, that met significance for CAC (O'Donnell et al. 2011). These genes encode cyclins that may be broadly linked to cellular senescence and inflammation. This locus has remained enigmatic since it is inherently a gene poor area, and thus the causative DNA sequences have been debated (Holdt and Teupser 2012). The meta-analysis of CAC also found a single SNP in at 6p24 in the *PHACTR1* gene (O'Donnell et al. 2011), and this same locus has also been linked to myocardial infarction (Kathiresan et al. 2009). Cell-based studies using siRNA to reduce *PHACTR1* demonstrate that this VEGF-responsive gene regulates endothelial lamellipodia through its interaction with actin and protein phosphatase 1 (Allain et al. 2012).

Mendelian disorders of vascular calcification

Rusch et al. discovered *ENPP1* as the gene responsible for generalized arterial calcification in infancy or idiopathic infantile arterial calcification (Ruf et al. 2005; Rutsch et al. 2003). *ENPP1* encodes a cell surface protein that regulates extracellular phosphate (Figure 3). In this disorder, infants present with calcification of the internal elastic lamina of large and medium size arteries, which predisposes these arteries to stenosis from vascular smooth muscle proliferation. Recessive loss of function mutation in *ENPP1* leading to loss of

activity, and infants born with this recessive disease typically do not survive beyond six months. The *ENPP1* gene had been previously linked to ectopic calcification through a mouse model, known as the “tiptoe walking mouse” or *ttww* (Okawa et al. 1998). *Tww* mice have homozygous mutation causing a stop codon at amino acid position 568 (of 905 residues) leading to a truncation of the ecto-nucleotide pyrophosphatase/phosphodiesterase 1 NPP1 leading to ossification of the spine ligaments. Homozygous or heterozygous mutation in *ENPP1* in the ApoE background attenuates atherosclerosis but enhances medial calcification in mice (Nitschke et al. 2011). These studies highlight the important role of pyrophosphate and phosphate metabolism in the pathobiology of arterial calcification.

The NPP1 protein, encoded by *ENPP1*, is an enzyme that converts extracellular ATP to adenosine and thereby generates pyrophosphate, an important inhibitor of calcification (Figure 3). A second enzyme in this pathway, the cell surface protein CD73, encoded by the gene *NT5E*, catalyzes the conversion of AMP to adenosine and inorganic phosphate, an accelerator of calcification. The *NT5E* gene was recently linked to vascular calcification (St Hilaire et al. 2011). Adults from three different families were affected with calcification of the lower extremity arteries that also extended to hand and foot joint capsules, and these individuals had recessive loss of function mutations in *NT5E*. Fibroblasts carrying the *NT5E* mutations demonstrated reduced expression and enzymatic activity of CD73, as well as increased accumulation of calcium phosphate crystals (St Hilaire et al. 2011). *NT5E* encodes ecto-5'-nucleotidase which catalyzes the conversion of purine 5' mononucleotides to nucleosides, with the preferred substrate as AMP. The phenotype from *NT5E* mutations is comparatively milder and more restricted than the phenotype induced from *ENPP1* mutations, but nonetheless reinforces the critical role of ATP-derived pyrophosphate and inorganic phosphate. Although these are rare disorders, and the numbers of individuals with mutations is small, *NT5E* mutations appear to target vessels below the diaphragm muscle and spare the coronary and carotid vessels. It is possible that distinct arterial beds are more or less susceptible levels of pyrophosphate, inorganic phosphate and adenosine or that other proteins may supplement loss of *NT5E* affording greater protection to the coronary and carotid vasculature (Figure 3).

Recently, mutations in the *ABCC6* gene encoding a transmembrane ATP-binding protein were described in 14 of 28 children with idiopathic infantile arterial calcification and pseudoxanthoma elasticum (Nitschke et al. 2012). The phenotypic spectrum of disease associated with *ABCC6* mutations was much broader than previously assumed, including severely affected children that died of myocardial infarction within the first three month of life. Mice lacking *Abcc6* develop ectopic calcification of the skin, arteries and retina (Klement et al. 2005), and as reported by another group, older *Abcc6* null mice develop calcification of medium sized arteries, the aorta and the vena cava (Gorgels et al. 2005). The *Abcc6* gene was also identified as responsible for the “Dyscalc 1” phenotype in mice (Meng et al. 2007). Dystrophic calcinosis is the deposition of calcium at the sites of inflammation and necrosis. In the mouse vascular calcification and disruption of the aorta elastic lamina share some genetic loci suggesting that medial disruption may be a precursor to calcification (Wang et al. 2009).

ABC proteins are transporters, and on the basis of clinical and histological similarities between idiopathic infantile arterial calcification and pseudoxanthoma elasticum, Markello postulated that *ABCC6* might be a transporter for adenosine to molecularly link these disorders (Markello et al. 2011). Alternative action of *ABCC6* may include deficient hepatic production of inhibitory factors, including a reduced γ -carboxylation of matrix Gla Protein (MGP), an important inhibitor of calcification (Li et al. 2007). While the biology of *ENPP1*, *NT5E* and *ABCC6* and their role in ectopic and vascular calcification is likely to expand, recent studies implicate RAGE, the receptor for advanced glycation endproducts, as one

pathway through which NPP1/ENPP1 may act (Cecil and Terkeltaub 2011). *ENPP1* null mice in combination with loss of RAGE developed less arterial calcification and intra-arterial chondrogenic differentiation compared to *ENPP1* null mice with intact RAGE signaling, indicating an important role for oxidative stress and chronic inflammation as modifier of phosphate-mediated calcification (Cecil and Terkeltaub 2011).

RAGE and its ligands in vascular calcification

RAGE is a receptor endogenously expressed in endothelial cells, macrophages, dendritic cells, smooth muscle cells and other vascular cells. RAGE is an immunoglobulin superfamily molecule whose multiple ligands have been shown to accumulate in disease condition, such as advanced glycation endproducts (AGE), the product of nonenzymatic glycation and oxidation of proteins, S100 proteins, high mobility group box 1 (HMGB1), amyloid β and others. Once activated, RAGE initiates a cascade of downstream signals including activation of transcription factor NF κ B, activation of ERK1/2, MAPK (Hofmann et al. 1999). There is substantial evidence for RAGE as a mediator of vascular disease (review in (Yan et al. 2010)), and importantly, mice lacking RAGE have attenuated atherosclerosis in apolipoprotein deficient mice (Harja et al. 2008). More recent work has identified members of the calcium-binding EF-hand family of S100 proteins, which are ligands for RAGE, as likely candidates involved in vascular disease and vascular calcification. S100A8 (also known as calgranulin A, or myeloid related protein 8, MRP8), S100A9 (also known as calgranulin B, or myeloid related protein 14, MRP14) and S100A12 (also known as calgranulin C, or extracellular newly identified RAGE-binding protein, EN-RAGE) share structural and functional similarities and often termed S100/calgranulin.

Although humans have an S100A12 gene, there is no orthologous gene in the mouse. To evaluate the role of S100A12, human S100A12 was expressed in murine vascular smooth muscle driven by the SM22 α promoter (Hofmann Bowman et al. 2010). On its own, expression of human S100A12 in mice promotes the formation of aortic aneurysm (Hofmann Bowman et al. 2010). The S100A12 transgene was backcrossed into the hyperlipidemic ApoE null mice, where it produced a 1.4 fold increase in atherosclerotic plaque size and more specifically a large increase in calcified plaque area and in medial calcification in the S100A12/ApoE null mice (Hofmann Bowman et al. 2011). The ApoE background on its own is not associated with aortic calcification; it is the combination of enhanced S100 signaling in the context of hyperlipidemia that promotes calcification. Figure 4 shows an example of the vascular pathology in S100A12tg/ApoE null mice with enhanced intimal calcification, a large necrotic core, and breakdown of elastic fibers. These features are similar to what is seen in human atherosclerosis and these features are associated with plaque rupture and atherothrombosis.

Isolated smooth muscle cells from S100A12 transgenic mice showed an increase in osteoblastic gene expression and calcification in a RAGE and oxidative stress dependent manner (Hofmann Bowman et al. 2011). Moreover, experiments utilizing knockdown of NADPH-oxidase Nox-1 showed that NADPH-derived reactive oxygen species is one mechanism by which S100A12 mediates calcification in smooth muscle cells (Gawdzik et al. 2011). Although these studies focused on S100A12 produced from vascular smooth muscle, S100A12 is secreted and therefore may derive from multiple sources to mediated vascular disease. It is tempting to speculate, that the pathological role of S100A12 in vascular smooth muscle is derived from its physiological role in neutrophils, where S100A12 is highly endogenously expressed, and implicated as a protein linked to host defense with antiparasitic and antifungal ability (Gottsch et al. 1999).

More recent studies showed that homolog S100A8/9 interacts with Nox2/gp91phox in phagocytes (Berthier et al. 2003) resulting in ROS production, also known as respiratory

burst, which is an important feature of the innate immune response to mediate microbial killing. Oxidative stress has many detrimental effects on vascular cells, including activation of RUNX-2 in smooth muscle cells, a master regulator of osteoblastic gene activation (Byon et al. 2008). Furthermore, S100A8/9 and S100A12 induce cell death in a variety of cell types, and it is possible that through pro-apoptotic and cell death-mediating functions, the S100 proteins indirectly promote vascular calcification by providing necrotic bodies as calcification nidus. Reduction of S100A12 in aortic smooth muscle cells harvested from patients with aortic aneurysms attenuated expression of death receptor FAS, and attenuated activation of caspase 3, and cell death (Das et al. 2012). The findings of enhanced atherosclerosis and vascular calcification in transgenic mice with smooth muscle targeted expression of S100A12 is in agreement with other animal models, including the findings by Croce and colleagues demonstrating attenuated atherosclerosis in apolipoprotein E deficient mice that also lacking S100A9 (Croce et al. 2009), or by reduction of S100A9 with neutralizing antibodies (Maiseyeu et al. 2012). Taken together, these findings suggest an important role for S100/calgranulins to directly participate in biological functions leading to vascular disease.

RAGE, S100A12 and human vascular disease

A number of clinical studies have shown increased S100/calgranulins-serum levels or pathological expression in human vascular disease (reviewed in (Averill et al. 2012; Hofmann Bowman and Schmidt 2011), thus providing a rationale to develop S100 proteins as biomarker and potential therapeutic target for cardiovascular diseases, including vascular calcification. In an attempt to develop a peripheral-blood-cell-derived gene expression signature that correlates with the presence and severity of coronary atherosclerosis, recent work has identified S100A12, S100A8 and S100A9 among 27 genes that were found to be significantly up-regulated in two independent cohorts of angiographically confirmed obstructive CAD (either greater than 70% stenosis in one vessel, or >50% stenosis in two vessel) in clinically stable patients (Wingrove et al. 2008). Although coronary calcification was not specifically studied as phenotype in these peripheral-blood gene expression studies, a calcific component can be assumed given the relationship between vessel calcification and degree of obstruction, with heavy calcified vessels being more likely to exhibit luminal obstruction.

To date there are no described loss of function “Mendelian” mutations in the genes encoding RAGE and S100A12 in humans vascular disease. However, a number of polymorphisms have been described, particularly in RAGE, that have been linked to inflammatory states especially diabetes. The CODAM (Cohort Of Diabetes and Atherosclerosis in Maastricht) study linked the rs3134945 single nucleotide polymorphism (SNP) to higher glucose levels in diabetics (Gaens et al. 2008). A Finnish study associated a RAGE promoter polymorphism with protection in diabetic patients from coronary artery disease, myocardial infarction and peripheral vascular disease (Pettersson-Fernholm et al. 2003). A coding polymorphism G28S in RAGE was linked to diabetic nephropathy (Prevost et al. 2005) was although this same polymorphism was not associated with cardiovascular events in the general Framingham population (Hofmann et al. 2005). Together these data suggest that the RAGE pathway mediates its effect most strongly in the diabetic population. A meta-analysis further reinforces the link between RAGE promoter polymorphism and protection from vascular complications in the type 2 diabetic population (Lu and Feng 2010). Protein levels of soluble RAGE (also known as sRAGE) independently predict cardiovascular complications in type 1 diabetes, potentially reflecting the activation and production of RAGE in the context of accelerated vascular disease (Thomas et al. 2011). Genetic control is only one means of regulate RAGE levels, and therefore protein levels may be more predictive. The PREDICT (Personalized Risk Evaluation and Diagnosis in the Coronary

Tree) study measured the blood expression levels of 23 different mRNAs, including S100A12, finding that this combination was effective at helping diagnose obstructive coronary artery disease in nondiabetic patients (Rosenberg et al. 2010). Thus RAGE and S100A12 pathway are broadly tied to inflammation and vascular disease. Further studies are needed to demonstrate whether genes encoding these proteins are specifically linked to vascular calcification as is suggested by the animal models.

Summary

In complex disorders, such as arterial calcification, there are multiple contributing genes as well as environmental exposures that mediate outcome. Single gene Mendelian disorders are often useful for highlighting pathways that may not have been previously appreciated. For vascular calcification, mechanisms affecting smooth muscle proliferation, endothelial function, response to reactive oxygen species, vitamin K metabolism, osteochondral differentiation, together with a growing list of physiologic modulator molecules and environmental factors likely contribute to intimal calcification in atherosclerosis and for calcification of the tunica media of the arteries. The genetic findings now place focus on genes regulating extracellular phosphate metabolism, and one mechanism through which these genes exert their effect is through RAGE and S100 proteins.

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Figure 1.

Smooth muscle cells contribute to ectopic calcification. Vascular calcification within the medial wall limits vessel extensibility and is associated with increased cardiovascular events. In bone, calcification initiates through matrix vesicles that extrude from cells. Vascular smooth muscle cells (VSMCs, pink) also form matrix vesicles.

Figure 2. Calcified vessels in a female mummy, estimated age 40-45 years old, a princess living in the 17th Dynasty (1580 to 1550 BCE) of the Second Intermediate Period. Extensive calcification of the aortic and coronary vessels was detected using CT scanning. Reproduced from the Horus Study as published in JACC Cardiovascular Imaging with permission from Elsevier (Allam et al. 2011).

Figure 3.

Mutations that disrupt *ENNP1* and *NT5E* cause inherited forms of infantile and adult onset vascular calcification, respectively. *ENNP1* encodes NPP1, which hydrolyzes extracellular ATP to generate pyrophosphate (PP_i), an inhibitor of hydroxyapatite formation and calcification. *NT5E* catalyzes the production of inorganic phosphate (P_i), an accelerator of mineralization. Tissue neutral alkaline phosphatase (TNAP) also generates inorganic phosphate. An imbalance of PP_i, inorganic phosphate and calcium occurs in inherited vascular calcification. Mutations in *ABCC6*, a gene encoding a nucleotide sensitive transporter, have also been linked to inherited calcification in the form of infantile calcification and pseudoxanthoma elasticum.

Figure 4.

Advanced remodeling with calcification, necrotic core and elastic fiber degradation of atherosclerotic plaques in ApoE null mice that express human S100A12 in the smooth muscle (a-c) compared to WT/ApoE littermate mice (d-f). Alizarin Red stain for calcium (a,d), H&E stain (b,e) and Verhoeff van Giessen (VVG) stain for elastic fibers (c,f). L-lumen, FFN-fibrofatty nodule, *** osteoblast like cells. S100A12 is expressed in human smooth muscle atherosclerotic aorta (Image from (Hofmann Bowman et al. 2011)).