Review Article

Molecular Mediators and Controlling Mechanism of Vascular Calcification

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Abstract

Bone formation involves hydroxyapatite crystals, whose development begins in matrix vesicles that bud from osteoblasts. Vascular smooth muscle cells that have undergone osteoblast differentiation are also able to release similar vesicles with shared protein content. Such differentiation is restrained or inhibited under normal conditions, and there is a balance with osteoclast differentiation experienced by monocytes and macrophages within the vascular wall. Moreover, the reaction which allows crystal growth is thermodynamically unfavorable and is inhibited by pyrophosphate. In some situations, physiological balance is broken and vascular calcification (VC) is able to progress. VC has traditionally been considered to be a passive process that was associated with advanced age, atherosclerosis, uncommon genetic diseases, and some metabolic alterations such as diabetes mellitus and end-stage kidney failure. However, in the last years, VC has been proven to be an active and regulated process, similar to bone mineralization, in which different bone-related proteins are involved. VCs are actively regulated biological processes associated with crystallization of hydroxyapatite in the extracellular matrix and in cells of the media or intima of the arterial wall. Both patterns of VC often coincide and occur in patients with type II diabetes, chronic kidney disease, and other less frequent disorders; VCs are also typical in senile degeneration. Recent results question the classic classification of VC into intimal and medial calcification, at least in capacitance arteries. Pro- and anti-calcifying mechanisms play an active role in calcium deposit ion in vascular cells, making this area an active focus of research.

Keywords: Controlling Mechanism, molecular mediators, vascular calcification

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Introduction

Bone formation involves hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] crystals, whose development begins in matrix vesicles (MVs) that bud from osteoblasts. Vascular smooth muscle cells (VSMCs) that have undergone osteoblast differentiation are also able to release similar vesicles with shared protein content.[1] Such differentiation is restrained or inhibited under normal conditions, and there is a balance with osteoclast differentiation experienced by monocytes and macrophages within the vascular wall. Moreover, the reaction which allows crystal growth is thermodynamically unfavorable and is inhibited by Calcification Inhibitors.[2] In some situations, physiological balance is broken and vascular calcification (VC) is able to progress. Occurrence of VC is not new. It has been discovered in the "Iceman" who lived 5000 years ago,[3] and thscientists had already paid attention to this phenomenon and to its relation with renal disease in the 19th century.[4] VC occurs when vessel and/or valvular tissue becomes mineralized. Conventionally, calcification has been classified depending on where the calcium was deposited. In this way, arterial calcification has been divided into intimal calcification (associated with atheromatous plaques[5]) and medial calcification (known as Mönckeberg's sclerosis) linked to vascular stiffness due to the mineralization of elastic fibers and atherosclerosis seen with age, diabetes, and chronic kidney disease (CKD).[6] Calcification of the intimal layer is reflective of atherosclerotic heart disease. Calcium deposition in the

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intimal layer of the coronary arteries (known as coronary artery calcification) can lead to vascular occlusion. It is detectable in ~30% of adults without clinical CVD[7-10] and is incrementally predictive of future cardiovascular events and overall mortality, independent of traditional CVD risk factors.[11-13] Certain patient groups, especially those with CKD, are at greater risk for coronary artery calcification.[14-16] In 2004, it was determined that 11% of the general population in the United States had CKD, translating into >19 million affected people.[17] CKD is defined as the presence of kidney damage with or without reduced kidney function.[18] The severity of CKD is determined by a staging process that is based on an estimated glomerular filtration rate. Moderate to severe CKD (Stages 3–5) is represented by an estimated glomerular filtration rate of < 60, <30, and <15 mL/min, respectively, and Stage 5b encompasses those individuals who require a form of kidney replacement therapy (hemodialysis [HD], peritoneal dialysis, or kidney transplant).[18]

At every stage of CKD, the leading cause of mortality is CVD and patients are more likely to die of a cardiac event than they are to ever require a form of kidney replacement therapy.[19] CKD patients are particularly prone to medial calcification (known as Mönckeberg's sclerosis), which leads to arterial stiffening, elevated systolic pressure, and increased cardiac workload.[20,21] Medial calcification is predictive of cardiovascular and all-cause mortality in CKD patients, independent of intimal calcification and CVD risk factors.[22,23] Calcific uremic arteriopathy, also known as calciphylaxis, classically manifests as calcification of cutaneous and subcutaneous arteries with occlusive intimal proliferation and subsequent fat necrosis.[24] VC increases with age and is notably dysregulated in diabetes, dyslipidemia, renal disease, and hypertension.[2] Although the cellular and molecular events leading to calcium deposition in vascular tissue continue to be explored, it is understood to be a highly regulated process.

Mechanisms of Vascular Calcification

VC is a pathologic response to toxic stimuli involving metabolic substances and/or inflammatory cells.[25-29] Historically, VC was considered to be a passive process, the result of Ca^{2+} and P ions exceeding solubility in tissue fluid, thereby inducing the precipitation and deposition of hydroxyapatite crystals.[30] However, the current thinking has shifted away from this passive theory; VC formation is now considered a complex, actively controlled intracellular molecular process, involving the differentiation of macrophages and VSMCs into osteoclast-like cells, similar to that which occurs in bone formation.[26,31-33] The underlying pathophysiological mechanisms resulting in VC can be broadly described as (1) elevation in serum Ca^{2+} and P levels, (2) induction of osteogenesis, (3) inadequate inhibition of the mineralization process, and (4) migration and differentiation of macrophages and VSMCs into osteoclast-like cells.[26,31,32,34] Genetic predisposition certainly plays an important role in the genesis of this phenomenon.[30] According to Rutsch *et al*., 40%–50% of cases of coronary calcification can be attributed to genetics.[35] Genes ENPP1 and NT5E are, respectively, implicated in infancy and idiopathic VC. The first one encodes a protein which transforms ATP to adenosine and PPi (inhibitor of calcification) whereas the second one converts adenosine monophosphate into adenosine and inorganic phosphate (Pi, accelerator of mineralization).[36]

The VC phenotype caused by mutations in these genes underlines the role of PPi and Pi in pathogenesis.[2] Mutations in ABCC6, a gene encoding a nucleoside-sensitive transporter, have also been linked to hereditary calcification.[36] Alternative action of ABCC6 may include deficient hepatic production of inhibitory factor of matrix Gla protein (MGP), an important inhibitor of calcification.[2,37] Another major mechanism of development of VCs is similar to that of bone formation[36] [Figure 1]. First, VSMCs undergo osteogenic differentiation into phenotypically distinct osteoblast-like cells.[34,38] In case of renal failure, Pi plays a key role in this mechanism.[39,40] *In vitro*, high extracellular Pi concentrations induce a rise in intracellular Pi concentration which is actively mediated by Pit-1, a sodium dependent Pi cotransporter.[40,41] This increasing Pi concentration in the VSMC induces a phenotypic switch of VSMCs into osteoblast-like cells.[34,40,42] The protein core-binding factor subunit 1α/runt-related transcription factor 2 (Cfba1/Runx2) is a specific and indispensable transcriptional regulator for this osteoblastic differentiation.[36] Its expression is also enhanced with high extracellular Pi.[40,42,43] These new cells will express alkaline phosphatase (ALP), secrete, under the control of Cfba1, bone-associated proteins (such as osteopontin [OPN],[44] collagen type 1, osteoprotegerin [OPG], bone morphogenetic protein-2 [BMP-2] and osteocalcin [OC][40,45]), and release mineralization-competent MVs in the extracellular matrix.[39,40,46] VSMCs release MVs under normal physiological conditions and these MVs are protected from mineralization by the presence of calcification inhibitors.[36] Under pathological conditions, a combination of factors makes the MVs mineralization competent.[47] Moreover, an increase of intracellular Pi level mediated by Na/Pi transporter is thought to induce VSMC apoptosis through an unclear process that possibly involves a disruption in mitochondrial metabolism.[48] Some studies suggest that apoptosis leads to calcification.[49-51]

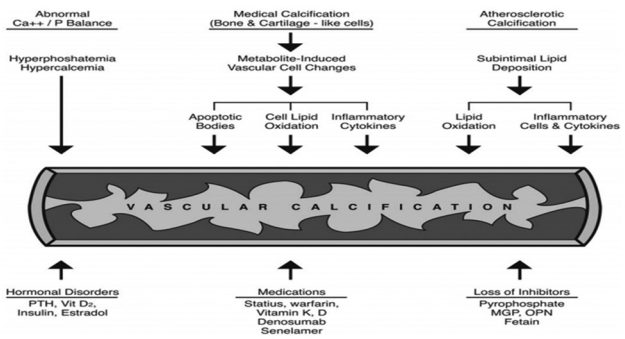

Figure 1: Schematic diagram depicting multiple mechanisms leading to vascular calcification

The MVs, in which pro-apoptotic factor BCL2-associated X protein has been identified,[52] may be remnants of apoptotic cells. As MVs have the capacity to concentrate and crystallize calcium, apoptosis could be a key regulator of VC.[48] More recently, a different point of view has emerged according to which phenotypically distinct osteoblast-like cells might originate from stem cells rather than VSMCs.[36] A new mechanism called "circulating cell theory," suggesting an active role for circulating cells arising from sources such as bone marrow, has been postulated to contribute toward VC.[36]

Under the influence of chemoattractants (released by damaged endothelium for instance), these bone marker-positive cells may home to diseased arteries. Under pathologic conditions such as an imbalance between promoters and inhibitors of VC, this population may further undergo osteogenic differentiation in the lesions, which could promote vessel mineralization.[36,53] Another recent study has also claimed that multipotent vascular stem cells present in the blood vessel wall might differentiate into osteoblast-like cells.[54] Nevertheless, this point of view is still very controversial. Although the role of Pi is well established in osteoblastic differentiation process, many other factors can influence this phenotype conversion and accurate causal mechanisms remained not completely understood.[36] Under normal conditions, VSMCs produce endogenous inhibitors of calcification such as MGP, OPN, OPG, and PPi.[29] A long-term exposure of VSMCs to a variety of stresses can overwhelm the action of these inhibitors and induce differentiation.[47] Among these chronic stresses, ionic disorders (especially hyperphosphatemia and hypercalcemia) are incriminated, but inflammation, hormonal perturbation, metabolic disorders, and oxidative stress can also lead to VC.[36] Oxidative stress in VSMCs, in particular generated by hyperlipidemia and oxidized lipoproteins or uremic milieu,[55] causes the expression of Runx2,[56] osterix and governs Wnt signaling,[57] leading to osteogenic differentiation. Inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), can also induce calcification through Msx2/Wnt/β-catenin pathway.[58] In support of that, calcium deposits colocalize with inflammatory cells *in vitro*[59,60] and *in vivo*.[61] Moreover, it has been suggested that mineral crystals may themselves be pro-inflammatory, creating a vicious cycle of inflammation and calcification.[62,63] The receptor for advanced glycation end products (RAGE) endogenously expressed in endothelial cells and its ligands (in which S100 family proteins are found) is also known to be involved in atherosclerotic formation and VC.[36] It has been suggested that galectin-3 and RAGE modulate vascular osteogenesis in part through Wnt/β-catenin signaling.[64] Several trials have shown a raise in serum levels of S100/calgranulins in vascular disease.[65,66] Thereby, S100 proteins could be a potential biomarker and therapeutic target to develop.[2] Involved in the control of both parathyroid hormone (PTH) and calcitonin secretion, the calcium-sensing receptor (CaSR) is a G protein–coupled cell surface receptor that is able to sense extracellular calcium ions. Evidence has been provided to demonstrate that a decrease in the CaSR protein expression in the vasculature is directly involved in the development of VC.[67,68]

It is of particular interest to note that calcimimetics, which are allosteric drug compounds that selectively target the CaSR, decrease VC at least in part through local control of the CaSR expression in VSMC.[69,70] However, so far, the mechanism whereby the CaSR exert its protective effect remains largely unknown. Hormones have pleiotropic effects on calcific vasculopathy. For example, the adipose-derived factor, leptin, promotes VC *in vitro*[71] and *in vivo*.[72] Adiponectin-deficient mice have increased VC.[73] The influence of PTH is a part of bone turnover process.[36] A disruption between promoters and inhibitors can also generate VC. Moreover, similar to bone formation, there might a balance between VC and its resorption. Indeed, monocytes and macrophages contained in the calcified wall can differentiate into an osteoclast-like phenotype and counteract the action of VSMCs that have undergone osteoblast differentiation.[74] Hyperphosphatemia would disadvantage osteoclast phenotype by downregulating receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL)-induced signaling,[75] but this is not clear whether osteoclast-like cells can really counteract VC or solely witness vascular remodeling process.[36] All these modifications will favor for an optimal microenvironment for hydroxyapatite formation and calcification. Similar osteogenic differentiation is also observed, *in vivo*, in animal and human uremic models.[38,42,76]

Biomarkers

Calcification inhibitors

Under normal conditions, blood vessel cells express mineralization-inhibiting molecules.[77] The loss of their expression, as happens in CKD, causes what is known as "loss of natural inhibition," giving rise to spontaneous calcification and increased mortality.[77] A list of these calcification-inhibiting molecules has been drawn up after mutation analysis on mice, including among others.

Fibroblast growth factor-23 and Klotho

Fibroblast growth factor-23 (FGF-23) is an approximately 30 kDA protein released by bone that requires the presence of the cofactor Klotho for its classical effects.[36] FGF-23 promotes Pi excretion by reducing its proximal reabsorption by reducing the expression of NPT2a and NPT2c mRNA, sodium/Pi transporters.[78] FGF-23 also decreases conversion of calcidiol into its active form by reducing 1α-hydroxylase activity.[79] Thereby, gastrointestinal absorption of calcium and Pi is reduced. In parathyroid glands, FGF-23 decreases PTH secretion and parathyroid cell proliferation.[80] FGF-23 null mice develop hypercalcitriolemia and VC.[36] Although the mechanistic link remains to be explained, FGF-23 may serve as a novel risk marker for the cardiovascular mortality in CKD.[79] In patients with coronary artery disease (CAD), the same independent link between FGF-23 and mortality has been demonstrated.[81] In contrast to FGF-23, Klotho excess has

never been shown to be noxious.[82] Interestingly, Klotho levels are upregulated by Vitamin D receptor agonists (calcitriol or paricalcitol) in CKD mice submitted to a high Pi diet. These mice show half less calcification than those who did not receive therapy. Phosphaturia is increased whereas phosphatemia and FGF-23 levels are lowered.[83] In contrast, vascular Klotho deficiency favors the development of arterial calcification and mediates resistance to beneficial vascular effects of FGF-23.[82]

Fetuin-A

Fetuin-A (Fet-A) is a serum 59-kDa glycoprotein that inhibits ectopic VC,[77] produced by the liver that possess a systemic action.[84,85]

It is a powerful inhibitor of hydroxyapatite formation, reducing the formation of crystals in *in vitro* solutions containing calcium and phosphorus without affecting those that are already formed.[77,86] Mice that are deficient in this protein develop extensive calcifications in soft tissue such as the myocardium, kidneys, tongue, and skin.[87] Fet-A is thought to inhibit calcification by binding early calcium Pi crystals and by inhibiting crystal growth and mineral deposition.[36] This could be facilitated by the formation of large calciprotein particles (CPPs).[86,88] Indeed, the accumulation of naked calcium Pi crystals is responsible for extraosseous calcification and causes inflammation. These crystals are usually digested by the cells of the reticuloendothelial system such as macrophages. In contact with the crystals, macrophages secrete pro-inflammatory cytokines and undergo more apoptosis.[36] The formation of Fet-A CPP facilitates the clearance of these crystals and thereby reduces their negative impact. Fet-A likely plays a very important role in the stabilization of these complexes and reduces the inflammatory response.[36] Fet-A binds and sequesters insoluble mineral nuclei, forming soluble colloidal CPP, thereby inhibiting crystal growth and aggregation.[36] Macrophages secrete less cytokines and undergo less apoptosis phenomenon as compared to reactions caused by naked crystals. This property of Fet-A to decrease inflammation may be influenced by the phosphorylation degree of the glycoprotein.[89] In these studies, lower serum Fet-A concentrations have been associated with increases in calcification scores, arterial stiffness, mortality, and incidence of cardiovascular events.[90-94]

Osteopontin

OPN is a phosphoprotein that is usually found in mineralized tissue such as bones and teeth.[77,95] It inhibits mineralization by blocking hydroxyapatite formation and activating osteoclast function.[96] Although it is not found in normal arteries, its expression is detected in atherosclerotic plaques and calcified vessels. OPN knock-out mice do not develop VC, but when these mice are bred with MGP knock-out mice, the VCs are more important than in simple MGP knock-out mice.[97] OPN must be phosphorylated to act as a calcification inhibitor.[36,98] OPN inhibits mineralization of VSMC by binding to the mineralized crystal surface.[99] On the contrary to the fully phosphorylated OPN, cleaved OPN could act as a pro-inflammatory cytokine and a pro-angiogenic factor facilitating vascular mineralization.[96,100]

The possibility that OPN could serve as a calcification serum marker is controversial.[36] Berezin and Kremzer showed that OPN was a good predictor of coronary calcification in type II diabetes mellitus patients.[101] Tousoulis *et al*. found a positive association between OPN and arterial stiffness in CAD.[102] Indeed, the discrepancy between the different studies may perhaps be explained by the differences in patient populations. It is thought that OPN plays a key role in inflammatory process.[36] Its relation with diseases related to inflammation such as atherosclerosis, obesity and autoimmune diseases has already been shown.[96,103-106] It has also been suggested that hyperglycemia could upregulate OPN and thereby lead to VSMCs proliferation.[107]

Osteoprotegerin

OPG is a member of the TNF receptor family that has been identified as a regulator of bone resorption.[108] OPG is produced by many tissues, including cardiovascular system, lungs, kidney, and immune system.[109] OPG is a regulatory factor produced by bone marrow-derived stromal cells.[36] OPG plays a pivotal role in the regulation of the bone turnover, inhibiting osteoclast differentiation and acting as a decoy receptor for the RANKL system.[110] It interferes with the interaction between RANK (expressed by osteoclast-like cells) and RANKL (expressed by osteoblast-like cells). OPG is also thought to inhibit ALP activity.[111] OPG levels are significantly higher in CKD patients, in relation to the severity of renal failure. Although OPG is known to impede osteoclast differentiation in bone, OPG is usually considered as a protective factor against VC as it blocks the bone remodeling process in the vascular tissue.[36] OPG is also a neutralizer of the pro-apoptotic actions of TNF-related apoptosis-inducing ligand, which strongly activates vascular cells apoptosis.[112] Apoptotic bodies can also lead to mineralization. In support of that, it has been observed that OPG-deficient mice do develop both severe aortic calcifications and osteoporosis.[113,114] Interestingly, OPG seems to be a marker of VC onset rather than a severity or progression predictor.[36,115]

Osteocalcin

OC, a Vitamin-K-dependent matrix protein that inhibits calcium salt precipitation *in vitro*,[116] shows a strong affinity for hydroxyapatite.[36] OC has been found in calcified atherosclerotic plaques and calcified aortic valves.[117] It was generally thought that OC inhibits crystal growth[118] and limits bone formation.[119] Nonetheless, its utility as serum marker is still discussed in conflicting studies. Aoki *et al*.[120] did not show any relationship between OC and VC in type II diabetes mellitus patients whereas Kim *et al*.[121] found an inverse correlation between OC and Agatston calcification score in Asian women, even after adjusting for age.[36] To define if OC can be used as a diagnostic or a screening tool, the role of OC in the pathogenesis of VC clearly remains to be clarified.

Pyrophosphate

PPi is a small molecule made of two Pi ions.[36] It acts as a calcification inhibitor by inhibiting hydroxyapatite crystal formation.[122] Once again, knock-out mice (in fact, knock-out mice for a precursor) develop VCs.[123] Absence of PPi would promote VSMC differentiation, but the mechanism is not fully understood.[124,125] O'Neill *et al*. demonstrated the negative association between PPi and VC in CKD.[126] Although the short half-life of PPi limits the possibility for improving VC by bolus injections, daily peritoneal dialysis achieved with a solution which contains PPi in CKD mouse model do succeed in inhibiting calcification.[127] O'Neill *et al*. demonstrated that daily intraperitoneal injections in rats could also reduce both incidence and amount of calcification.[128] PPi has been shown to inhibit mineralization on rat aortic VSMCs cultures too.[129] Furthermore, bisphosphonates, nonhydrolysable analogs of PPi, have also proved their ability to inhibit aortic calcifications in experimental renal failure rats. Calcification was stopped in cultures of rat aortas as well as *in vivo* model.[36] It supports the idea that bisphosphonates have direct effects on VC, independent of bone,[130] maybe through a downregulation of Notch1-RBP-Jκ signaling pathway and MsX2 gene induction.[131] ATP, which is a polyphosphate associated with nucleoside, might also act as calcium Pi deposition inhibitor, not only as the source of PPi but also as a direct inhibitor.[132] Even if PPi seems to be a promising marker, its determination has been performed in a single center only and the transferability to other centers should be validated.

Matrix Gla protein

MGP is a Vitamin K, 14-kDa γ-carboxylated protein expressed by chondrocytes, VSMCs, endothelial cells, and fibroblasts.[36] Its role as a calcification inhibitor has been illustrated by MGP knock-out mice that develop extensive arterial calcifications.[133,134] In 2002, Moe *et al*. demonstrated a correlation between vascular MGP expression and the calcification of epigastric arteries in dialysis patients.[76,135] MGP-deficiency in humans leads to Keutel syndrome, a rare genetic disease hallmarked by abnormal soft tissue calcification.[134] MGP binds calcium crystals, inhibits crystal growth, and plays a role in the normal phenotype of VSMCs in preventing the osteoblastic differentiation.[43,136] MGP also binds and inactivates a pro-mineralization factor, BMP-2.[137] Among other effects, BMP-2 promotes osteogenic conversion of VSMCs through MSX2 transcription factor.[36] MGP could also protect mineral nucleation sites on elastin and thereby prevent spontaneous calcification of the elastic laminae.[36] In support of that, the irregular calcification of the thoracic and abdominal aorta segments in MGP −/− mice correlates with the local variations of the elastin content.[134] Parallel to this study, other authors hypothesized a mineralization process by size exclusion, in which MGP proves to be essential to prevent mineralization within fibrils.[36]

Calcification activators

There are studies that speculate that, as well as hyperphosphatemia and hypercalcemia, there are substances present in the blood serum of patients with CKD capable of stimulating calcification.[38] Bovine VSMC in the presence of uremic serum increases the expression of calcification-related proteins. A large number of uremic factors have been identified that are capable of inducing osteogenic genes, transforming osteoblasts, and secreting some bone matrix proteins in the walls of blood vessels and soft tissue. Some of these factors are TNF,[59] inflammatory cytokines,[138] fibronectin,[139] type-I collagen,[139] and 25-hydroxycholesterol.[140] These uremic serum substances stimulate the expression of molecules essential to vesicular calcification.

Alkaline phosphatase

ALP is one of the osteoblastic phenotype markers and is considered essential in the VC process.[77] It has been detected in vascular and heart valve calcifications. ALP expressed on the surface of cells can act on Pi liberators, releasing inorganic Pi.[141] Inflammatory cytokines and Vitamin D induce its upregulation and mineralization.[141,142]

Core-binding factor alpha 1

Core-binding factor alpha 1 (Cbfa1) is the main regulator of bone cell differentiation.[77] Cbfa1-deficient mice have problems with cartilage formation and bone mineralization.[143] It acts as a transcription factor that accelerates the expression of important osteoblast lineage genes such as OC, OPN, ALP, or type-I collagen.[144] Its expression is upregulated by Pi43 and uremic toxins.[38]

Bone morphogenetic protein-2

BMP are a group of, at least, 30 proteins that receive their name from their osteoinductive properties.[77] BMPs belong to a subdivision of TGF-β-like growth factors family. BMPs regulate growth, differentiation, and development in the embryo as well as during tissue remodeling processes in the adult organism. BMP-2 is an important molecule in the regulation of bone formation as well as in VC.[36,77] In bone, it promotes osteoblast differentiation and mineralization.[145] Inhibition of BMP-2 inhibits osteoblast differentiation and bone formation *in vivo* and *in vitro*[146] and protects against atherosclerosis and VC.[147] They act by binding to a heterodimeric system of transmembrane receptors (BMP-1 and BMP-2 receptor) that trimerises upon binding. The binding of a BMP to its specific type II receptor results in the type 1 receptor being activated. This causes phosphorylation and nuclear translocation of the Smad transcription factors, thus modifying the transcription rate of target genes.[148] They then induce ectopic bone formation.[149]

Sclerostin

Sclerostin is an osteocyte-specific glycoprotein and is considered as a potent inhibitor of bone formation.[150,151] It inhibits specific coreceptors needed for β-catenin-dependant signaling activation.[152] This pathway is involved in osteoblast-mediated bone formation.[153] It is thought that sclerostin plays a role in bone mechanosensibilization.[36] When bone undergoes a substantial strain, sclerostin production would be decreased and bone could thus increase its formation in response to

mechanical stress.[154] As β-catenin belongs to Wnt cascade signaling and as Wnt pathway is thought to be implicated in development of VC, it is interesting to investigate a potential association between sclerostin levels and VCs.[36] In non-CKD patients, some studies have demonstrated a positive association between sclerostin levels and VC,[155,156] whereas in other ones, there was not a significant correlation between the two parameters.[157,158]

Receptor activator of nuclear factor kappa-B ligand

RANKL (also known as osteoprotegerin ligand) is a protein consisting of 316 amino acids with a molecular weight of 38 kD. Its expression is also modulated by several cytokines, glucocorticoids, and PTH.[77,159] RANKL is produced by osteoblast lineage cells and activated T-cells. It promotes osteoclast formation, fusion, differentiation, activation, and survival, leading to increased bone resorption and bone loss.[160] RANKL stimulates its specific receptor RANK, which is expressed in fewer cells such as progenitor cells and mature osteoclasts, activated T-cells, and dendritic cells.[161-163] The activation of RANK by RANKL triggers the NF-κB intracellular signaling cascade. The final stage of RANK activation is the NK-κB translocation into the nucleus, which can take place by the classical or alternative pathway.[77] Both pathways are regulated by their kinases which are, respectively, IKK and IKKα. The NK-κB translocation to the nucleus modulates the expression of different genes, for example, BMP4.[164] The biological effects of OPG are the opposite of RANKL-mediated effects because OPG acts as a soluble inhibitor that prevents RANKL interaction and the subsequent stimulation of its RANK receptor.[165] Many trials have shown that VC as well as arterial stiffness and cardiovascular events are inversely related to serum RANKL[166-168] and positively related to serum OPG.[90,102,103,114,120,168-174]

Strategies to Reduce Vascular Calcifications

Any strategy designed to reduce the impact of VCs has to begin with primary prevention measures to control cardiovascular risk factors. In the particular case of CKD, it is imperative to avoid further kidney damage. In this respect, it is crucial to promote a healthy lifestyle, with a balanced diet, regular physical exercise, smoking abstinence, and low alcohol intake. Once VCs appear, secondary prevention must aim to reduce their complications, intensifying previous measures, and initiating the appropriate drug therapy. Theoretically, any kind of intervention aiming to reduce VC should curtail the influence of factors that promote calcifications and/or augment the effects of factors that may inhibit calcifications.[16] Most strategies to reduce VCs have focused on the most common modifiable risk factors such as hyperphosphatemia, hypercalcemia, the CaxP product, hyperparathyroidism, smoking, hyperlipidemia, and hypertension.

Control of hyperphosphatemia, hypercalcemia, and CaxP product

Disturbances in serum phosphorus, calcium, and calcium-phosphorus product are frequently seen in CKD patients and are implicated in the promotion of VC as well as in an increased death risk.[16] Due to the fact that dietary restriction of phosphorus and intermittent dialysis are not usually effective in controlling serum phosphorus, most patients with CKD Stage 5 show a high prevalence of hyperphosphatemia with its known implications in the pathogenesis of secondary hyperparathyroidism, cardiovascular alterations, and mortality. As mentioned before, *in vivo* and *in vitro* studies shed light on the role of phosphorus as promoter of VC, demonstrating that the control of phosphorus should be a priority in clinical practice. Calcium Pi binders such as calcium acetate and calcium carbonate have replaced aluminum hydroxide as the most widely prescribed Pi binders. The possible negative role of calcium loading from these binders on the progression of VCs has led to the abandonment of calcium- and aluminum-based Pi-binders in favor of new calcium- or aluminum-free Pi binders (sevelamer hydrochloride and lanthanum carbonate).

These changes in the treatment have reduced hypercalcemic adverse events in comparison to calcium-based binders.[175] An experimental study demonstrated that treatment with sevelamer in rats decreased renal calcification as compared to rats that received calcium carbonate or untreated rats.[176] In addition, a clinical trial showed that sevelamer reduced the progression of both coronary and aortic calcifications compared to calcium carbonate.[177] However, the mechanism of the beneficial effect of sevelamer on the progression of calcification is still not fully understood. One possible mechanism is based on the reduction of the calcium load; however, reduced VCs may also result from reductions in total and low-density lipoprotein (LDL) cholesterol, which occur during treatment with sevelamer.[175]

Control of secondary hyperparathyroidism

The use of Vitamin D metabolites is a challenging subject that still remains controversial. The current treatment of secondary hyperparathyroidism in dialysis patients includes suppression of PTH with supraphysiologic doses of Vitamin D or its analogs. Although it is widely known that a high dosage of Vitamin D metabolites favors the onset and progression of VCs, several studies have paradoxically demonstrated a long-term beneficial effect of Vitamin D on VCs. Low Vitamin D status is associated with a higher prevalence of VCs, bone and mineral disturbances, susceptibility to some infections, higher risk of autoimmune diseases, some malignancies, and many other complications.[178] Observational studies in patients on HD and in the general population have also demonstrated a lower morbidity and a cardiovascular survival advantage in patients who are treated with Vitamin D receptor activators.[179,180] A major breakthrough in the management of the calcium Pi metabolism of dialysis patients was achieved recently with the introduction of calcimimetics. These compounds were the first agents introduced to lower PTH with advantageous effects on serum calcium and Pi. It has been demonstrated experimentally that the calcimimetic R568 reduces aortic calcifications and mortality in rats, in which aortic calcifications were induced using a high dose of calcitriol.[181] Moreover, another

experimental study showed that calcimimetics may even favor the regression of VC.[182]

Control of dyslipidemia

Hyperlipidemia, particularly increased LDL cholesterol, has been implicated in the progression of VCs. In addition, in the general population, the beneficial effect of lowering LDL cholesterol levels on the progression of calcification has been reported by several groups.[183,184] As mentioned previously, patients who were treated with sevelamer showed a significant decrease in LDL cholesterol levels,[177] which may explain the beneficial effects in the progression of cardiovascular calcification. It is known that the rapid progression of coronary arterial calcification in HD patients is associated with higher triglycerides and lower high-density lipoprotein cholesterol levels.[185]

Control of blood pressure

Hypertension is a modifiable risk factor for VCs in both general population and CKD patients. Several studies in ESRD and essential hypertension have shown that arterial stiffening is an independent predictor of mortality. As arteries become stiffer, the pulse wave velocity increases and it is responsible for a rapid return of wave reflections from the periphery to the ascending aorta during systole, which causes an abnormal rise of aortic systolic blood pressure with decreased diastolic blood pressure and high pulse pressure. Increased wave reflections and high pulse pressure are the independent risk factors for mortality of ESRD patients.[22]

Diabetes

Diabetes is a disease that is known to be complicated by heterogeneous metabolic risk factors, such as hyperglycemia, hyperlipidemia, insulin resistance, glycation, oxidative and carbonic stress, and tissue hypoxia. In the nonuremic population, VC occurs more frequently in diabetics. In CKD patients, VC in diabetics has been reported to be more prevalent and more advanced than in nondiabetics.[186] Several studies emphasize the importance of glycemic control in the prevention of the development and progression of VC in diabetic CKD patients.[187]

Conclusion

At present, the ideal marker of VC does not exist. The pathophysiological mechanisms underlying this phenomenon are still poorly understood. As explained in the introduction, calcification can be induced by various situations. Etiologies that induce VC in diabetes mellitus patients are likely different from those which lead to the same result in CKD patients or postmenopausal women. Signaling pathways that are involved in VC may then depend on patient's status. A perfect marker would be ideally located on a hypothetical convergence point of all these pathological conditions. Thus, it could reflect reliably calcification emergence and progression in any situation. However, this view is maybe too utopian and simplistic. Over the years, study of biomarkers showed a large variety of conditions that can modulate vascular microenvironment composition, such as bone turnover, inflammation, Vitamin D status, or even oxidative stress. Within this vascular microenvironment itself, a dense and interconnected network of calcification inhibitors and promoters was highlighted as shown in Figure 1. (i) VSMCs undergo differentiation into osteoblast-like cells, in great part because of an intracellular Pi increased concentration, likely mediated by the co-transporter Pit-1, in response to extracellular hyperphosphatemia. (ii) Renal failure is one of the major hyperphosphatemia origin whereas (iii) FGF-23 is a factor which tend to moderate it by increasing Pi renal excretion. This FGF-23 action is achieved with Klotho's help. FGF-23 has other effects described, among which noxious ones are also suspected. (iv) Other factors such as BMP-2, absence of PPi (in part, due to ALP activity), oxidative stress, inflammatory process, or metabolic disorders are also known to be responsive to VSMCs conversion. (v) Calcium enhancement also proved to be deleterious, especially by its ability to induce Pit-1 overexpression and also by alteration of MGP and Fet-A actions, two VC inhibitors. (vi) The VSMCs conversion will favor excretion of bone-associated proteins, such as OPN, collagen type 1, BMP-2, and OC and (vii) VSMCs will release mineralization-competent MVs. In turn, (viii) BMP-2 can promote osteoblast differentiation and is a potent calcification inducer. (ix) MGP, expressed by chondrocytes and VSMCs under normal conditions, inactivates BMP-2. It also binds calcium crystals and inhibits crystal growth. Finally, it prevents osteoblastic differentiation too. As OC, its activity is Vitamin-K dependent and can be countered by Vitamin-K antagonists. (x) OC and (xi) osteonectin are known to bind calcium/Pi crystal but their accurate actions as inducers or inhibitors need to be specified, whereas the (xii) PPi inhibiting effect on crystal formation is well known. The recent discovery of OC metabolic effects might suggest OC is a promoter of VSMCs differentiation into osteoblast-like cells. (xiii) OPN activity would depend on its phosphorylation state. Fully phosphorylated OPN would inhibit mineralization by blocking hydroxyapatite formation and activating osteoclast function while the cleaved one could act as a pro-inflammatory cytokine and pro-angiogenic factor facilitating vascular mineralization. (xiv) OPG is considered as a regulatory factor. On one hand, it can prevent VC by blocking bone remodeling process in vascular tissue and by neutralizing the pro-apoptotic actions of TRAIL. It might also inhibit ALP activity. On the other hand, the inhibition of bone remodeling process by OPG could induce a calcium shift into vascular cells. (xv) Fet-A released by the liver inhibits mineralization perhaps through CPP formation while (xvi) PTH secreted by parathyroid enhances calcification phenomenon, as both low and high bone turnover might lead to VC. Under normal conditions, there is a balance between all these parameters. It is possible that each pathological condition disrupts the balance with its own approach. Nevertheless, it seems that all the calcification inhibitors do not possess the same potential. OPG serum levels appear to be correlated repeatedly to calcification in many pathological conditions. However, OPG would not reflect the severity of damages as would FGF-23 do. FGF-23 levels

should be followed up, either upwards or downwards, given the suspected duality of the effects of FGF-23. Low serum Fet-A levels are usually associated with VC but an increased CPP fraction of Fet-A would also be useful to reflect a procalcific milieu. Nonfully γ-carboxylated MGP is associated with VC too and might be an interesting marker to monitor patients under AVK treatment. OPN would be an attractive marker in diseases with inflammatory component such as diabetes or autoimmune diseases. Within this category of disease, it might help to reclassify asymptomatic subjects with classical risk factors into high-risk group for further examination. In population with renal deficiency, particularly susceptible to develop VC, the problem becomes even more complex. Whether patients undergo dialysis or not, reference values will need to be adapted, depending on renal failure severity and marker ability to be removed by dialysis. In addition, as described earlier, complex interactions exist between different actors (pro or anti-calcifications) such as PTH, Vitamin D, FGF-23, OPG, sclerostin, acting sometimes through redundant signaling pathways such as Wnt/β-catenin, Runx2/Cbfa1, and Notch1-RBP-Jκ. Furthermore, these interactions could be different according to the stage of CKD.[188] Thus, the stage of disease should be taken into account in the interpretation of biomarker and/or the combination of biomarkers. As evidenced by the present discussion, VC physiopathology is still far from being fully elucidated. The role of each biomarker needs to be clarified and many studies are still leading to contradictory results. *In vitro* observations are sometimes very different from conclusions observed in *in vivo* studies. Direct effects on vasculature and indirect effects mediated by bone turnover are not easy to discriminate. When a correlation between the serum levels of a calcification marker and calcification is clearly showing up, it still remains to determinate whether level fluctuations attest a noxious effect of the biomarker or if they highlight a compensatory process or even solely reflect phenomenon as bystander. Qualities that would be appreciated for selecting a good marker depend on its capacities to achieve clinical goals, particularly its ability to select high-risk patients for further investigation, to make a reliable calcification assessment, to provide a prognostic, to help in treatment choice, or to follow-up the treatment efficiency. Given importance to assess and control mineralization process, it is essential to keep going on building up more and more knowledge.

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Conflicts of interest

There are no conflicts of interest.

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