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Vascular Calcification in Chronic Kidney Disease*

Kalcyfikacja naczyń w przewlekłej chorobie nerek

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Abstract

The aim of this review is to present current knowledge of vascular calcification in chronic kidney disease. Recent investigations point mainly at the disturbed balance between factors facilitating and protecting extraosseous mineralization as crucial in the creation of calcification of vessels. Chronic kidney disease is associated with multiple abnormalities potentially accelerating calcification, such as hypercalcemia, hyperphosphatemia, dyslipidemia, and inflammation. Their impact triggers the change of vascular smooth muscle cells into osteoblast- or chondrocyte-like cells expressing bone-associated proteins. This differentiation induces the production of apoptotic bodies that become nuclei of mineralization. The decreased capacity of calcification inhibitors, such as fetuin-A, osteoprotegerin, and osteopontin, additionally weakens the protection against excessive mineralization. These disturbances are of paramount importance in CKD patients because of the close connection between pathomorphology and clinical outcome. Both medial and intimal calcification affect the cardiovascular system due to the increased vascular stiffness and calcified atherosclerotic lesions, creating a vast clinical panorama of symptoms from stroke to heart failure. Therefore, clarifying the mechanisms responsible for vascular calcification in CKD patients seems to be a key target for effective future prevention (**Adv Clin Exp Med 2008, 17, 2, 227–235**).

Key words: calcium/phosphate metabolism, vascular smooth muscle cells, fetuin-A, osteopontin, osteoprotegerin.

Streszczenie

Celem pracy jest przedstawienie aktualnego stanu wiedzy na temat kalcyfikacji naczyń w przewlekłej chorobie nerek (p.ch.n.). Badania ostatnich lat wskazują przede wszystkim na rolę zaburzonej równowagi między czynnikami ułatwiającymi pozakostną mineralizację a chroniącymi przed nią, jako kluczowego czynnika w powstawaniu zwapnień naczyniowych. Przewlekłej chorobie nerek towarzyszy wiele nieprawidłowości potencjalnie przyspieszających proces kalcyfikacji, takich jak: hiperkalcemia, hiperfosfatemia, zaburzenia gospodarki lipidowej lub stan zapalny. Ich wpływ powoduje zmianę komórek mięśni gładkich naczyń w komórki osteoblasto- i chondrocytopodobne, z ekspresją białek kostnych na ich powierzchni. To różnicowanie komórek prowadzi do tworzenia ciałek apoptotycznych, stających się jądrami krystalizacji. Zaburzona funkcja inhibitorów krystalizacji, do których należą fetuina-A, osteoprotegeryna i osteopontyna, dodatkowo zmniejsza skuteczność ochrony przeciwko nadmiernej mineralizacji. Powyższe zaburzenia odgrywają zasadniczą rolę u pacjentów z p.ch.n., gdzie obraz patomorfologiczny bezpośrednio implikuje stan kliniczny. Zarówno kalcyfikacja błony wewnętrznej, jak i środkowej naczynia uszkadza układ krążenia, zwiększając sztywność naczyń i wywołując tworzenie zwapnień w obrębie blaszek miażdżycowych, co powoduje występowanie różnych objawów klinicznych – od udaru do niewydolności serca. Wyjaśnienie mechanizmów odpowiedzialnych za kalcyfikację naczyń u pacjentów z p.ch.n. ma więc zasadnicze znaczenie w skutecznym zapobieganiu w przyszłości (**Adv Clin Exp Med 2008, 17, 2, 227–235**).

Słowa kluczowe: gospodarka wapniowo-fosforanowa, komórki mięśni gładkich naczyń, fetuina A, osteopontyna, osteoprotegeryna.

Extraosseous calcification is a common process in patients with chronic kidney disease (CKD) [1]. It may involve various organs and tissues, including the heart, valves, vessels and soft tissues. Among theses, vascular calcifications are the most frequent and clinically significant. The calcification of vessels has long been regarded as a passive process, progressing with age and

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depending only on the calcium apatite accumulation within the vessel wall. Recent investigations have proven that vascular calcification should be considered a series of cell-mediated interactions between promoters and inhibitors of mineralization [1]. Dysregulated mechanisms of calcification affect both the intima and the media of the vessel, aggravating the progression of athero- and arteriosclerosis. The clinical picture of the abovementioned abnormalities covers a wide range of manifestations, from stroke through myocardial infarction to heart failure due to left ventricular dysfunction [2]. Therefore, a detailed analysis of the mechanisms underlying vascular calcification may indicate effective prevention against cardiovascular complications, the main cause of morbidity and mortality among CKD patients.

Calcification of the Tunica Intima and Tunica Media

Intimal calcification is asymmetric and characteristic for atherosclerotic plaques. Initially, lipid-laden macrophages (foam cells) accumulate in the intima and form regions of thickening, called fatty streaks [3]. The simultaneous migration and proliferation of vascular smooth muscle cells (VSMCs) coexist with extracellular matrix synthesis and the deposition of mineralized proteins [3]. Continuous lipid accumulation leads to the formation of lipid cores, further encapsulated by collagen. VSMC proliferation may result in overproduction of collagen fibers and lipid core destruction. Additionally, proinflammatory cytokines, especially TNF-α, stimulate VSMC differentiation into a bone-like phenotype. Necrotic lipid cores are the initial localization of calcifications. Calcification of the intima is strictly connected with inflammation, the condition present in patients with CKD [4].

Medial calcification (mediasclerosis) is a concentric process within the adventitial layer of the vessel wall and is responsible for the increased vascular stiffness and the decreased arterial capacity [2]. The main changes concern adventitial pericytic myofibroblasts, pluripotent mesenchymal stem cells, ready to change their phenotype to the osteogenic lineage, thus initiating calcification. Their subsequent migration and proliferation causes the medial thickening and increased matrix turnover responsible for decreased distensibility in the elastic arteries and increased pulse wave velocity [2]. A subpopulation of proliferative VSMCs, called multipotent calcifying vascular cells (CVCs), seems to be a key player in the calcification game. Although CVCs are prone to oxidative stress, little is known about the inflammatory background of medial sclerosis.

In CKD patients both schemes of calcification are present, although none of the imaging techniques could so far distinguish between these two types of mineralization. Recent data concerning patients on hemodialysis have demonstrated that intimal calcification appears later than medial, especially in subjects with risk factors such as smoking or hyperlipidemia present prior to the start of dialysis [5]. Meanwhile, medial calcification is connected with a higher incidence of calcium/phosphate metabolism disturbances. While a preponderance of mediasclerosis is seen in children and young adults on hemodialysis when compared with adults and the elderly, adults without CKD present only with atherosclerosis [6]. Therefore, it may be hypothesized that medial calcification is more characteristic of CKD than intimal. Nonetheless, their overlap becomes evident with age, thus accounting for amplification and acceleration of cardiovascular complications in patients with renal insufficiency.

Theories for Vascular Calcification

Recent years have brought to light several mechanisms implicated in triggering vascular calcification. The complexity of mineralization may at least partly result from the fact that this is a multistep process regulated on different levels and depending on multiple factors [1]. However, there has been agreement that the main reasons for excessive calcification are the induction of bone formation strictly connected with the release of nucleational complexes from the actively remodeling bone and with the presence of apoptotic bodies and necrotic debris derived from the dead cells as well as the loss of factors inhibiting mineralization (Fig. 1). Besides, chronic kidney disease represents a set of complications, such as microinflammation, calcium/phosphate metabolism disturbances, enhanced oxidative stress, and carbonyl stress, that can additionally influence the vascular calcification process, accelerating its progression in the course of CKD [2].

VSMC Dedifferentiation

Vascular smooth muscle cells are a wall component of paramount importance for the calcification process [7]. They create a population of various subtypes that, despite phenotypic similarity, present different features depending on the stimuli

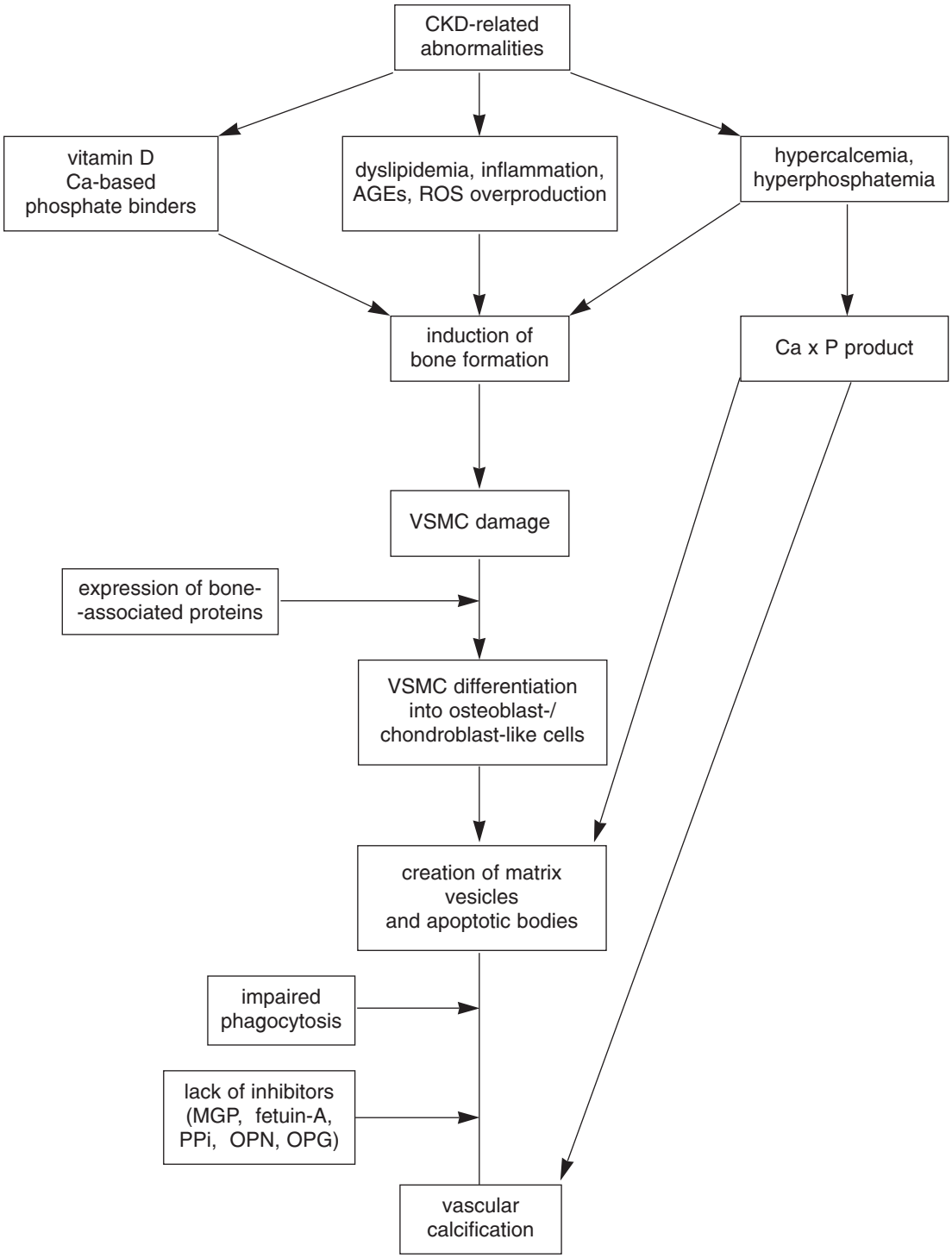

Fig. 1. The mechanisms responsible for vascular calcification in chronic kidney disease (CKD).
AGEs – advanced glycation end-products, ROS – reactive oxygen species, VSMC – vascular smooth muscle cell, MGP – matrix Gla protein, PPi – pyrophosphates, OPN – osteopontin, OPG – osteoprotegerin

Ryc. 1. Mechanizmy odpowiedzialne za kalcyfikację naczyń w przewlekłej chorobie nerek (p.ch.n.).
AGEs – końcowe produkty glikozylacji, ROS – wolne rodniki tlenowe, VSMC – komórki mięśniówki gładkiej naczyń, MGP – białko macierzy Gla, PPi – pirofosforany, OPN – osteopontyna, OPG – osteoprotegeryna

currently influencing them. Microvascular myofibroblasts, called pericytes, as well as adventitial myofibroblasts are multipotent mesenchymal stem cells that can be recruited to osteogenic, chondrogenic, or adipogenic lineages during vascular calcification [7]. Within the proliferative mural VSMCs there is a subpopulation of calcifying vascular cells (CVCs) ready to differentiate into osteogenic or chondrogenic phenotypes, but unable to reach the adipogenic pathway.

Arterial smooth muscle cells of CKD patients are constantly under the influence of a uremic milieu and inflammatory and oxidative stress conditions [4]. Oxidized lipid products facilitate the recruitment of inflammatory cells. Migrating monocytes, as well as macrophages *in situ*, are a source of chemotactic agents and proinflammatory cytokines which react with VSMCs. Experimental data have shown that, for example, tumor necrosis factor (TNF)-α, interleukin (IL)-1, advanced glycation end products (AGEs), and oxidized low-density lipoprotein (oxLDL) can stimulate the change of VSMCs to an osteoblastic phenotype. Due to this change, adventitial pericytic myofibroblasts start expressing bone morphogenetic protein (BMP)-2 and transcriptional regulator Msx2 [2]. BMP2--Msx2 signaling initiates osteogenic differentiation of VSMCs, whereas the BMP2-Runx2/core binding factor α (Cbfa)-1 pathway promotes chondrogenic differentiation. Adventitial myofibroblasts can also differentiate into an adipogenic lineage through peroxisome proliferator-activated receptor (PPAR)-γ. All these vascular osteoprogenitors have pleiotropic effects. Mxs2 promotes osteogenesis, but suppresses chondro- and adipogenesis [1]. Cbfa1 promotes chondrogenesis, but suppresses the others, and PPAR-γ activates adipogenesis while suppressing osteogenesis. Additionally, macrophages themselves can produce BMP2, and TNF-α may stimulate mesenchymal BMP2 expression. The whole process acts as a vicious circle in which all factors influence the others.

The exposure of modified VSMCs to calcium and phosphate results in the release of either apoptotic bodies from dead cells or matrix vesicles from living ones [7]. Both of them can accumulate calcium, thus acting as nuclei of mineralization and forming apatite crystals. Normally, apoptotic bodies undergo phagocytosis. Experimental data have shown that modified lipids inhibit this process and allow these bodies to calcify within the vascular matrix.

Factors Promoting Calcification

Hypercholesterolemia and Hyperglycemia

The role of hypercholesterolemia, present in CKD, in atherosclerosis has been established for years. Diabetes is also a common feature in CKD patients and also acts as one of the main factors predisposing to medial calcification. The most important role in mineralization is that of minimally oxidized, oxidized, and glycoxidized lipids.

Major lipid changes in atherosclerosis result from disturbed lipoprotein transport, mainly involving LDL (low-density lipoprotein). Under normal conditions, intra- and extracellular concentrations of LDL are balanced by the LDL receptor density and enzymatic reactions [8]. When cholesterol levels in cells are high, the synthesis of new cholesterol and proteins for LDL receptors is stopped. In the case of intracellular cholesterol deficiency, its synthesis is initiated, the LDL receptor density augments, and LDL transport from outside the cell is intensified [8]. Moreover, cholesterol increase from extracellular LDL is preferred to synthesis *in situ*. This preference is of paramount importance when LDL concentrations in the blood are increased. In such a situation, intracellular cholesterol synthesis is blocked and only extracellular transport takes place, thus playing a defensive role against hyperlipidemia [8]. Additional protection results from HDL-related cholesterol efflux. However, even slight modifications in LDL structure, for example that caused by oxidative stress or hyperglycemia, render LDL receptors on the endothelium unable to recognize the lipoprotein particles. Therefore, oxidized, glycated, or glycoxidized LDLs are caught by scavenger receptors present on endothelial cells and macrophages. The density of these receptors is independent of intracellular cholesterol concentrations. Moreover, glycoxidized LDLs induce their specific class A scavenger receptors (SR-A) and suppress class B type I scavenger receptors (SR-BI) for HDL, thus acting pro-atherogenically [9].

Due to these facts, macrophages and endothelial cells can be easily laden with cholesterol, thus transforming them into foam cells and initiating early atherosclerotic lesions. Finally, hypercholesterolemia up-regulates the proliferation and differentiation of calcifying vascular cells (CVCs), although this is possible only in the presence of macrophages [10]. Meanwhile, hyperglycemia promotes the osteogenic cascade through the BMP-2/Msx2 pathway.

Inflammation

The sustained release of pro-inflammatory cytokines observed in the course of CKD induces atherosclerotic (intimal) calcification. The migration of monocytes has been implicated as a key player in atherogenesis. Their recruitment is mainly governed by monocyte chemoattractant protein (MCP)-1, a member of the CC family of chemokines. The pro-atherogenic activity of MCP-1 has been observed in apoE$^{-/-}$ mice [11], whereas an investigation on rats revealed a growth-promoting effect of MCP-1 on VSMCs, which may suggest

a potent role of this chemokine in vessel wall remodeling [12]. Macrophage colony-stimulating factor (MCSF) is also a key regulator of monocyte and macrophage activation and correlated positively with aortic calcification in subjects on hemodialysis [13]. Another potent chemoattractant for monocytes is the soluble form of fractalkine (FK), the only member of the CX3C chemokine family, found in atherosclerotic plaques [14]. The next chemokine with well-established pro-atherogenic activity is IL-8. Its expression is stimulated by oxLDL and its chemoattractant action mainly concerns monocytes [15].

The pro-inflammatory factors may also influence, albeit indirectly, the process of medial calcification. A few experiments showed that TNF-α can stimulate CVC mineralization *in vitro*, although, as in the case of oxidized lipid impact, the presence of macrophages is needed [16]. The CRP elevation typical of CKD is another potent factor probably modifying mineralization. Its presence was described within atherosclerotic lesions and it is associated with coronary artery calcification score [17].

Oxidative Stress

Reactive oxygen species (ROS) overproduction and impairment of anti-oxidative mechanisms are hallmarks of CKD. The subsequent excessive lipid oxidation results in the above-mentioned cascade of interactions leading to the progression of atherosclerosis and triggers calcification.

Oxidized lipids can be generated in the vasculature by several enzymatic and non-enzymatic mechanisms. Animal experiments have provided evidence that lipoxygenases (LOXs) play a major role in atherosclerosis progression [18]. Enzyme-catalyzed oxidation takes place in leukocytes (isoform 5-LOX), platelets (isoform 12-LOX), and reticulocytes (isoform 15-LOX). During these reactions, enzyme-bound radical intermediates are generated, such as lipid alkyl ($L^{\cdot}$), alkoxyl ($LO^{\cdot}$), and peroxyl ($LOO^{\cdot}$). All of these react with $NO^{\cdot}$ and decrease its bioavailability. Moreover, 15-LOX is cytokine inducible and may show pro-atherogenic activity [19]. Another enzyme prone to cytokine stimulation is prostaglandin endoperoxide H synthase (PGHS), the expression of which is raised in the presence of IL-1 and TNF-α. It acts similarly to LOX, generating lipid radicals reacting with $NO^{\cdot}$ [20]. Increased activity of myeloperoxidase (MPO), the protease which catalyzes hypochlorous acid (HOCl) synthesis, may also play a causative role in lipid oxidation. MPO is produced by foam cells and its expression has been demonstrated in human atherosclerotic plaques. Nitrating and chlorinating products of reactions catalyzed by MPO damage tissues due to their toxic properties [21]. Sub-endothelial matrix destruction further increases ROS production and initiates smooth muscle cell migration, proliferation, and phenotypic changes, thus propagating the formation of atherosclerotic lesions, vascular remodeling, and calcification.

Oxidative stress is also responsible for impaired macrophage phagocytosis of apoptotic cells in atherosclerotic plaques and thus facilitates intracellular calcification. Apart from the already mentioned LOX and MPO, there are other macrophage-derived enzymes and substances influencing atherogenesis. Recently developed animal models revealed pro-atherogenic features of lipoprotein lipase (LPL) and anti-atherogenic activities of lysophospholipase 3 and lysophosphatidylcholine [22].

The Role of Calcium and Phosphate

Calcium/phosphate disturbances in CKD patients, resulting from the vitamin D metabolism disturbances and secondary hyperparathyroidism, are important factors facilitating extraosseous mineralization in these subjects. Moreover, the use of calcium-containing phosphate binders as well as the potential over-dosage of active vitamin D metabolites may aggravate the above symptoms. Both calcium and phosphate elevation act synergistically and accelerate calcification [23]. Elevated Ca and P levels activate the sodium-dependent phosphate co-transporter Pit-1 in two different ways: through hypercalcemia by inducing the expression of Pit-1 mRNA and through hyperphosphatemia by increasing P uptake via Pit-1 [24]. Consequently, there is both intracellular and intravesical P elevation. High intracellular phosphate triggers VSMC phenotypic changes such as upregulation of osteogenic genes encoding Cbfa-1 or alkaline phosphatase. Vesicles released by viable VSMCs in response to high extracellular calcium, together with high P concentration, contain preformed Ca-P apatite and are prone to calcification. Moreover, due to the elevation of Ca and P concentration (also because of the usage of calcium-based phosphate binders), the Ca x P ion product is increased and apatite crystal growth is accelerated by passive precipitation [25]. However, although VSMCs cultured *in vitro* in the presence of high Ca or P underwent apoptosis and calcification, the addition of normal human serum nearly neglected this impact and reduced the extent of mineralization. However, there should be factors in the serum, capable of inhibiting calcifi-

cation. Indeed, experiments performed on knockout mice have proven that inhibitors of calcification exist.

Inhibitors of Calcification

Matrix Gla Protein

Matrix γ-carboxyglutamic acid protein (matrix Gla protein, MGP) resides in the matrix of cartilage and of VSMCs in the vascular wall as well as in VSMC vesicles. MGP acts *in situ* and directly inhibits the formation of calcium crystals. Its activity strictly depends on vitamin K, which is necessary for γ-carboxylation and biological activation of MGP. Hypercalcemia can increase the expression of MGP through a calcium-sensing receptor. Elevated concentrations of MGP inhibit BMP-2 and thus modulate VSMCs dedifferentiation.

MGP-deficient mice develop widespread medial calcification of the aorta. Moreover, they die within a few weeks due to massive ruptures of the vessel, which looks like a bone [26]. That experiment clearly shows that MGP is essential for the inhibition of mineralization. Interestingly, systemic MGP overexpression had no influence on calcification, whereas local intervention prevented it completely. Thus MGP activity can be seen only locally. Studies on humans have shown high MGP expression in atherosclerotic plaques, possibly a counter-reaction to *in situ* calcification [27]. However, its expression significantly decreased in already calcified arteries. Patients on hemodialysis have shown elevated serum concentrations of MGP, probably resulting from its retention due to renal failure and having no protective effect on the progression of vessel calcification [28].

Fetuin-A

Fetuin-A (α-2-Heremans-Schmid glycoprotein), a protease inhibitor of the cystatin family, is a negative acute-phase protein produced mainly by the liver. It acts as a circulating inhibitor of hydroxyapatite crystal formation, mainly by creating calciprotein particles containing fetuin-A, calcium, and phosphate [29]. These colloidal spheres transport insoluble calcium crystals from the places of extraosseus calcification to the bones. Thus they clarify them. Fetuin-A can also induce phagocytosis and inhibit apoptosis [30]. It is also a component of the VSMC cytoplasm and VSMC-derived vesicles, where it can inhibit intracellular and intravesical calcification.

Fetuin-A deficient mice show severe calcifications in the heart, lungs, kidneys, and skin, but arteries stay intact, possibly due to the counter-regulation by MGP or osteopontin [31]. In humans, patients on hemodialysis have been the population of special interest. Within this group, low fetuin-A concentrations correlated with coronary and aortic calcifications, higher cardiovascular mortality, and hypoalbuminemia [32]. The sera from those patients could inhibit calcium/phosphate precipitation to a lesser extent than the sera from subjects with normal fetuin-A levels. In subjects on peritoneal dialysis there was a clear association with malnutrition-inflammation-atherosclerosis (MIA) syndrome.

Pyrophosphates

Pyrophosphates (PPi) are other local inhibitors of mineralization, produced by the vascular wall and protecting against calcification after injury [33]. The regulation of PPi activity depends on three factors. The first is ENPP-1 (ecto-nucleotide pyrophosphatase phosphodiesterase-1), the rate-limiting enzyme of intracellular pyrophosphate synthesis. The transmembrane protein encoded by the locus of progressive ankylosis (ANK) controls the transport of PPi to the extracellular compartment. They both cause the *in situ* elevation of PPi concentrations, whereas the tissue-nonspecific alkaline phosphatase (TNAP) is responsible for PPi degradation to phosphate. ENPP1-deficient mice present with soft-tissue calcification, localized mainly in tendons and ligaments. In humans, mutations in ENPP1 gene have been associated with idiopathic infantile arterial calcification. Mice deficient in ANK show calcifications of the periarticular areas and develop arthritis. In none of the above cases is vascular calcification seen. The decreased plasma PPi concentrations in patients on hemodialysis probably result from their clearance during the HD session [34].

Osteopontin

Osteopontin (OPN) is a phosphorylated glycoprotein found mainly in bones, but also in teeth, kidney, and on epithelial cells and macrophages. It has also been found in calcified arteries. OPN is capable of inhibiting calcification in many ways [35]. First, it acts directly by self-aggregation and adhesion to calcium/phosphate apatite crystals. Second, it may stimulate osteoclasts by binding to integrins on their surface and promoting the resorptive phenotype. Third, phosphorylated OPN may inhibit *in vitro* BMP-2/Msx2-dependent calcification. OPN is also responsible for matrix metalloproteinase-dependent vascular remodeling.

OPN-deficient mice develop widespread medial vascular calcification, vanishing after the administration of exogenous OPN. In patients on dialysis, there is a positive correlation between OPN levels and aortic calcification score [36].

Osteoprotegerin

Osteoprotegerin (OPG) is a member of the TNF receptor family, produced by various tissues, such as the immune system, kidneys, lungs, and the cardiovascular system. Its main role is to regulate bone resorption. OPG acts as a soluble decoy receptor for RANKL (receptor activator of NF-κB-ligand). Scavenging RANKL disables its binding to the receptor RANK. The blockage of RANK prevents osteoclast activation and inhibits osteoclastogenesis [37]. Additionally, *in vitro* studies have shown that OPG promotes the apoptosis of osteoclasts. OPG-deficient mice develop calcifications of the aorta and renal arteries as well as osteoporosis. OPG expression is also observed in calcified areas of the vessels. In humans, OPG has been identified as an independent risk factor for the progression of atherosclerotic lesions and correlated with the intensity of coronary artery disease. Surprisingly, OPG concentrations are increased in patients on hemodialysis compared with healthy controls and correlate with aortic calcification [38]. A study performed in children on hemodialysis and peritoneal dialysis confirmed the above increase in OPG levels [39]. Whether this elevation acts as a marker of cardiovascular damage or is a protective mechanism remains an unexplained paradox that needs further investigation.

Other Inhibitors of Calcification

The list of potential inhibitors of calcification is growing constantly. Among many others, fibroblast growth factor (FGF)-23 may be another key player protecting vessels against calcification. Although preliminary studies have shown massive calcification in FGF23-deficient mice, little is still known about its role in humans. Recently, special interest has also been focused on the product of the *klotho* gene, which may act pleiotropically, as a soluble cofactor essential for the stimulation of FGF23 and as a β-glucuronidase deglycosylating the calcium-channel TRPV5 (transient receptor potential vallinoid-5), a modulator of osteoclast function [40].

The authors concluded that vascular calcification is a finely tuned process which depends on the balance between agents promoting and inhibiting mineralization. The factors facilitating extraosseous calcification in CKD patients, such as inflammation, increased oxidative stress, hyperlipidemia, hypercalcemia, and hyperphosphatemia, result mainly from the CKD pathomechanism. However, the treatment of hyperparathyroidism may accelerate the processes triggered by CKD per se. The accessory insufficiency of protective mechanisms, such as local and systemic inhibitors of calcification, additionally pushes the balance towards the creation of osteo- and chondroblastic phenotypes of VSMCs, thus promoting further calcification of the vessels. Nevertheless, the fact that vascular calcification is so complex and requires the regulation on various levels creates multiple potential points at which interventional procedures could be tested. Moreover, the new therapeutic possibilities give an optimistic perspective and may enlighten the future search for effective prevention against extraosseous mineralization.

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