Vascular Calcification in Chronic Renal Failure
What Have We Learned From Animal Studies?

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Abstract: Accelerated atherosclerotic plaque calcification and extensive medial calcifications are common and highly detrimental complications of chronic kidney disease. Valid murine models have been developed to investigate both pathologically distinguishable complications, which allow for better insight into the cellular mechanisms underlying these vascular pathologies and evaluation of compounds that might prevent or retard the onset or progression of vascular calcification. This review describes various experimental models that have been used for the study of arterial intimal and/or medial calcification and discusses the extent to which this experimental research has contributed to our current understanding of vascular calcification, particularly in the setting of chronic renal failure. (Circ Res. 2011;108:249-264.)

Key Words: animal models ■ vascular calcification ■ chronic renal failure

Cardiovascular disease is the main cause of morbidity and mortality in patients with chronic kidney disease (CKD), accounting for approximately 50% of all deaths in patients on dialysis and in recipients of renal transplants.1 A large-scale prospective population-based study showed that renal function was inversely associated with cardiovascular and all-cause mortality.2 Vascular calcification is the major cause of cardiovascular disease in patients with CKD as it contributes to myocardial ischemia, impaired myocardial function, valvular insufficiency, arrhythmias and stroke and is shown to be a strong independent predictor of mortality in the general3,4 as well as in the dialysis population.5-7 In patients with end-stage renal disease, the risk of death increases with the number of vascular sites affected by calcification in the aorta and the carotid and femoral arteries.5 The high prevalence of vascular calcification is already reported in early stages of CKD8,9 and in young dialysis patients.10,11

Atherosclerotic plaque burden in the intimal layer is higher and these lesions are also more heavily calcified in CKD patients compared to the normal population.12-14 Although intimal calcification is associated with cardiovascular mortality,6 it remains controversial whether calcification of advanced atherosclerotic lesions stabilizes plaque vulnerability15-17 or rather contributes to rupture of the fibrous cap,18,19 leading to thrombotic events and myocardial infarction. Interestingly, Ehara et al20 nicely showed that the pattern of calcification in the intimal plaque is of importance: small, frequent, spotty calcifications are more often found in patients with an acute myocardial infarction or with unstable angina pectoris, whereas the presence of extensive calcifications was highest in those with stable angina pectoris. Recent investigations add new insights to this observation and indicate that, in contrast to spacious calcified areas, sparse microcalcifications produce local stress,21 activate macrophages to secrete inflammatory mediators22 and induce apoptosis of vascular smooth muscle cells,23 all factors that make the atherosclerotic plaque vulnerable and prone to rupture. Aside from intimal calcifications, patients experiencing CKD also develop typical calcifications in the medial layer of the vessel wall, independently of local inflammatory processes as seen in atherosclerotic calcification. Medial calcification also contributes to cardiovascular mortality in CKD patients6 because it causes loss of vessel elasticity, resulting in increased systolic blood pressure and left ventricle hypertrophy which may ultimately lead to arrhythmia and heart failure.24

Traditional risk factors alone, such as hypertension and dyslipidemia, do not fully account for the high calcification burden in the vasculature of the dialysis population.25,26 Hyperphosphatemia, caused by a reduced renal phosphate excretion, and an increased calcium × phosphate product level are identified as important culprits of accelerated arterial calcification in these patients.6,10,27-29 The disturbed mineral balance in CKD and the central role of phosphate herein is presented and explained in Figure 1. Moreover, particular therapies used to control phosphate homeostasis have been reported to negatively affect the extent of vessel calcification. Administration of calcium-containing phosphate binders, the most commonly used compounds to treat hyperphosphatemia and secondary hyperparathyroidism, often goes along with an excess calcium load and is found to be...
associated with the progression of vascular calcification, particularly when used in combination with vitamin D. In addition, several inhibitors of the calcification process are dysregulated in patients with CKD. Matrix Gla protein, fetuin-A, and pyrophosphate, either locally or systemically, prevent the formation of calcium phosphate crystals in the artery. However, plasma concentrations of the inactive un-carboxylated form of matrix Gla protein were markedly increased from CKD stage 4 onward and associated with aortic calcification. In dialysis patients, circulating fetuin-A and pyrophosphate are reduced compared to healthy subjects and are found to be negatively associated with vascular calcification. Another important factor one must take into account with regard to the onset and progression of arterial calcification, is the condition of the bone. The disturbed mineral metabolism in patients with CKD leads to renal osteodystrophy. Both high- and low-turnover bone diseases may contribute to the development of vascular calcification. An increased bone turnover provides a continuous supply of calcium and phosphate to the serum, whereas a reduced bone turnover results in an impaired capacity to incorporate these minerals into the bone. The link between the bone status and vascular calcification is also found in patients with osteoporosis: demineralization of the bone is associated with mineralization in the vessel wall.

Several decades ago, the prevalence of vascular calcification in dialysis patients was described in autopsy studies. The development of new noninvasive imaging techniques such as electron beam computed tomography and ultrasound allowed screening, quantification and follow-up of large study groups for the presence of arterial calcification. However, these techniques are not able to distinguish between intimal and medial calcification. As such, the availability of

Figure 1. Disturbed mineral metabolism in CKD patients and the central role of hyperphosphatemia. Renal failure leads to both impaired phosphate excretion and active vitamin D production. Increased serum phosphate levels may induce secretion of phosphatropic hormone FGF23 in osteocytes, which in turn further inhibits active vitamin D production, resulting in decreased intestinal calcium absorption. Low calcium and vitamin D concentrations, together with high phosphate levels, stimulate PTH production in an attempt to normalize calcium and phosphate by increasing renal phosphate excretion and calcium reabsorption. However, as in renal failure, the kidneys are not able to react appropriately, and PTH will recruit calcium from the bone, thereby also releasing phosphate. Hyperphosphatemia persists, creating a vicious cycle. Phosphate binders, vitamin D receptor activators, calcimimetics and bisphosphonates act on different targets within the disturbed mineral metabolism.
animal models that develop either calcified atherosclerotic plaques or medial calcification offers the advantage to study both pathological distinct entities separately.

Use of Animal Models to Study Vascular Calcification

Generally, animal models serve to mimic (patho)physiological states known to occur in humans and are used in basic as well as in applied research. The use of animal models allows us to study in detail the successive stages of a pathological process in function of time and to get new insights in the cellular mechanism and molecular pathways underlying these processes. Laboratory animals are also used for the development and evaluation of new therapeutics. Likewise, the isolated effect of particular drugs on pathological entities can be investigated under standardized conditions without the interference of other therapies that are often prescribed in patients.

The most useful, practical, and valid species for the study of cardiovascular disease are small rodents: rats and mice. These species are easy to manipulate and have a short lifespan of 1 to 2 years, which are time- and cost-saving advantages for experimental studies. Moreover, rats and mice resemble humans in metabolism and physiology. The rat and mouse genomes contain about the same number of genes as the human genome. Numerous human genes known to be involved in particular disorders have counterparts in the rat and mouse genome and appear highly conserved through mammalian evolution.

To examine the cellular and molecular mechanisms underlying the process of vascular calcification, a variety of rat and mouse models have been developed. Gene deletion in mice is a useful technique to unravel the specific role of a particular protein in the development of vascular calcification. This way, the importance of the phosphaturic hormone fibroblast growth factor (FGF) 23 and its necessary cofactor Klotho in the process of vascular calcification have been demonstrated: FGF23 deficient mice exhibit extensive vascular and soft tissue calcification. Several murine knockout models of genes that regulate bone formation have led to new insights into the pathogenesis of arterial calcification. Matrix Gla protein, osteoprotegerin (OPG), and fetuin A knockout mice display extensive aortic calcifications thereby demonstrating the preventive role of these proteins in the calcification process. Alternatively, to induce vascular calcification in rodents, various stimulants such as vitamin D, nicotine, warfarin or uremic toxins such as indoxyl sulfate have been administered.

This review focuses on animal models used to study uremia-related vascular calcification and discusses to what extent the use of these models have contributed to our current knowledge on this vascular pathology.

Animal Models of Chronic Renal Failure With Intimal/Medial Calcification

A drawback of using rodents for the study of atherosclerotic plaque calcification consists of the high resistance of mice and rats to the development of atherosclerosis, with exception of mice on a C57/BL6 genetic background. The lipid profile of rodents significantly differs from humans in that mice and rats carry most of their plasma cholesterol on high-density lipoprotein (HDL), which is known to protect against atherosclerosis, whereas humans carry ≈75% of their cholesterol on low-density lipoprotein (LDL). The high levels of antiatherosclerotic plasma HDL and low levels of proatherogenic LDL contribute to the resistance to atherosclerosis in rodents. Therefore, the current mouse models are based on interventions in lipoprotein metabolism through genetic and dietary manipulations.

Apolipoprotein E Knockout Mice

Feeding C57/BL6 mice an atherogenic diet (15% fat, 1.25% cholesterol, and 0.5% cholic acid) results in very small atherosclerotic lesions that are mainly limited to the fatty streak stage. A search for a mouse model that reflects the human pathology covering the entire spectrum of atherosclerotic plaque lesions led to the development of the apolipoprotein (apo)E knockout model. ApoE, a glycoprotein and constituent of all lipoproteins except LDL, serves as a ligand for receptors involved in the clearance of chylomicrons and very low-density lipoproteins (VLDL) remnants from the circulation. Deletion of the apoE gene in mice results in a pronounced increase in plasma VLDL and to a lesser extent in LDL concentrations as well as a 45% reduction of the normal HDL levels leading to the development of atherosclerotic lesions largely confined to the aortic root and the ascending aorta. These lesions resemble their human counterparts and develop over time from early fatty streaks comprised of foam cells and migrating smooth muscle cells over more advanced lesions with a necrotic core and proliferating smooth muscle cells surrounded with a fibrous cap to calcified plaques. The development of more widely distributed and more complex atherosclerotic plaques in the apoE knockout mouse requires a high-fat, high-cholesterol diet. When fed this diet, total plasma cholesterol levels are elevated 3-fold, lesion formation is significantly accelerated and lesion size is increased.

Superimposing chronic renal failure (CRF) on the apoE deletion increases the aortic plaque area and markedly accelerates atherosclerosis. Sixteen-week-old apoE knockout mice with normal renal function show moderate intimal calcification and only mild medial calcification (6.1 ± 0.03 and 1.0 ± 0.01 area %, respectively). Subtotal nephrectomy (removal of one kidney and partial reduction of the other by electrocautery) enhances aortic calcification at both the intimal plaque sites and the atherosclerosis-free tunica media after 6 weeks (17.8 ± 0.05 and 3.8 ± 0.01 area percentage, respectively).

The apoE knockout model combined with induction of renal failure involves the development of atherosclerosis and in particular develops atherosclerotic plaque calcifications at the intima. This model is less suitable to study medial calcification, but provides a useful tool to study the molecular and cellular mechanisms responsible for intimal calcifications associated with CRF and to evaluate new therapeutic strategies for this harmful complication.

Low-Density Lipoprotein Receptor Knockout Mice

The LDL receptor (LDLR) knockout mouse represents an apoE knockout model. ApoE, an apolipoprotein and constituent of all lipoproteins except LDL, serves as a ligand for receptors involved in the clearance of chylomicrons and very low-density lipoproteins (VLDL) remnants from the circulation. Deletion of the apoE gene in mice results in a pronounced increase in plasma VLDL and to a lesser extent in LDL concentrations as well as a 45% reduction of the normal HDL levels leading to the development of atherosclerotic lesions largely confined to the aortic root and the ascending aorta. These lesions resemble their human counterparts and develop over time from early fatty streaks comprised of foam cells and migrating smooth muscle cells over more advanced lesions with a necrotic core and proliferating smooth muscle cells surrounded with a fibrous cap to calcified plaques. The development of more widely distributed and more complex atherosclerotic plaques in the apoE knockout mouse requires a high-fat, high-cholesterol diet. When fed this diet, total plasma cholesterol levels are elevated 3-fold, lesion formation is significantly accelerated and lesion size is increased.

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cell-surface receptor responsible for the clearance of cholesterol-rich lipoproteins from the circulation through receptor-mediated endocytosis in hepatocytes. Loss-of-function mutations in the LDLR gene results in familial hypercholesterolemia characterized by dramatically elevated plasma LDL and is a serious risk for the development of atherosclerosis and coronary heart disease.

LDLR knockout mice show a modest, doubling of plasma cholesterol, mainly attributable to elevated LDL levels. The extent of atherosclerosis in these animals is minimal, with only small fat deposits in the aorta. However, when fed a 1.25% cholesterol-rich diet, LDLR knockout mice display an 8-fold increase in plasma cholesterol levels and show rapidly developing, large atherosclerotic lesions over the entire length of the aorta. Administration of a high-cholesterol, high-fat diet to LDLR knockout mice leads to a 2-fold increase in aortic calcification and a decreased bone formation rate. Vascular calcification in this model is largely associated with atherosclerotic lesions and only punctuate calcifications were observed in the tunica media.

Similar to the apoE knockout model, partial ablation of the right kidney followed by left nephrectomy in the high fat fed LDLR knockout model results in a 3-fold increase in aortic calcium content. In addition, superimposing renal failure caused low bone turnover disease as shown by significantly reduced osteoblast number and perimeter, mineralizing surfaces and bone formation rates.

Overall, the LDLR knockout mouse represents a more moderate model for atherosclerosis and associated intimal plaque calcification as compared to the apoE knockout mouse, which is mainly attributed to a lower degree of hypercholesterolemia. However, the plasma lipid profile of LDLR knockout mice fed a cholesterol-rich diet resembles more that of humans as the largest proportion of the plasma cholesterol levels consists of LDL, whereas most plasma cholesterol in apoE knockout mice is carried by VLDL.

**Animal Models of Chronic Renal Failure With Medial Calcification**

Recently, the first uremic mouse model with isolated medial calcification has been described. Electrocautery of one kidney followed by nephrectomy of the other in calcification-prone DBA/2 mice on a 0.9% high P diet led to widespread medial calcification in the vascular tree after 14 to 15 weeks at levels comparable to those observed in humans, without interference of atherosclerosis and in the absence of inflammation. In agreement with clinical observations, the extent of arterial medial calcification shows uremia severity and phosphate dependence and correlation with serum FGF23. Furthermore, uremia increased serum PTH and alkaline phosphatase levels that were similar to changes in CKD patients. When applied in knockout mice, this mouse model opens new opportunities for clarifying the role of molecules in uremia-related medial calcification.

Because rats are highly resistant to atherosclerosis, these animals form an appropriate tool to induce and study the process of calcifications in the tunica media without interference of intimal calcifications associated with the inflammatory and lipid involved atherosclerotic process. To examine uremia-related medial calcifications in rats, two CRF models are most commonly used, ie, the remnant kidney model and the adenine model. The remnant kidney model comprises the surgical reduction of the renal mass, whereas in the adenine model, more severe renal impairment is achieved by feeding the rats an adenine-rich diet.

**Remnant Kidney Rat**

The remnant kidney or 5/6th nephrectomy rat model reproduces experimental CRF by mimicking the progressive nephron loss that occurs in human CRF. Reduction in renal mass is usually installed by a two-stage surgical procedure: partial nephrectomy of one kidney and total nephrectomy of the contralateral kidney. Partial nephrectomy can be performed with different methods, ie, resection or ligation of the upper and lower poles of the kidney, catherization or electrocoagulation of the renal cortex and arterial ligation. Arterial ligation, ie, ligation of 2/3 of the extrarenal branches of the renal artery of the kidney, performed on one kidney combined with resection of the other kidney is the most common used 5/6th nephrectomy rat model. A mild to moderate degree of CRF is induced with a 2 to 3-fold increase in serum creatinine values. Administration of a high phosphorus diet to remnant kidney rats induces hyperphosphatemia and high parathyroid hormone (PTH) concentrations with the development of secondary hyperparathyroid bone disease, typical hallmarks that occur in CKD patients.

The 5/6th nephrectomy rat model is also used to evaluate uremia-related vascular calcifications. Follow-up of 10 weeks of nephrectomized rats fed a standard rodent diet (0.8% P and 1.1% Ca) reveals that no calcification develops in the aorta, except for the aortic arch, which is the site most prone for the onset of calcification. Increasing the dietary phosphorus concentration to 0.9% P in 5/6th nephrectomized rats did not induce medial calcifications after a short-term follow-up of 6 to 8 weeks. Even after a long-term follow-up of 6 months, only a small increase in aortic calcium content was noted (2.15 mg/g wet tissue in remnant kidney rats versus 0.39 mg/g wet tissue in control rats). Only a high 1.2% phosphorus and a low 0.4% calcium content in the diet to induce severe secondary hyperparathyroidism resulted in severe medial calcification in the abdominal aorta in 5 of 8 nephrectomized rats after a 10-week observation period.

To obtain manifest medial calcifications in this uremic rat model, 1,25(OH)2 vitamin D3 was administered orally, subcutaneously, or intraperitoneally. As such, an aortic calcium content up to an average 35 mg/g wet tissue was established. Diffuse, massive calcifications of the entire aortic wall, involving both the thoracic and abdominal part, develop in 1,25(OH)2 vitamin D3-treated 5/6th nephrectomized rats with a mean calcified area of more than 50%. This rat model for uremia-related arterial calcification shows a reproducible and comparable severe degree of medial calcifications in all rats.

Recently, a new remnant kidney model for medial calcification has been generated in which 5/6th nephrectomized rats are fed a high-phosphorus, high-lactose diet (1.2% P, 1% Ca, and 20% lactose). Lactose has been shown to increase calcium and phosphorus absorption in the intestine.
Medial calcifications were reported with aortic calcium concentrations of approximately 25 mg/g dry tissue. However, the biological variability and reproducibility of the development of arterial calcification in this animal model could not be deduced from the study report.

Another method to induce vascular calcification in subtotally nephrectomized rats is introduced by Neves et al. Parathyroid glands were removed and replaced by a subcutaneously implanted mini-pump that continuously infuses 1–34 rat PTH fragments at a supraphysiological rate. Regardless of the dietary phosphorus content (1.2% versus 0.2%), all remnant kidney rats on PTH replacement developed intense, comparable arterial medial calcifications. This experimental model is interesting to investigate the isolated effect of PTH on cardiovascular calcifications in the setting of CRF.

**The Cy/+ Rat With Autosomal Dominant Polycystic Kidney Disease**

The heterozygous Cy/+ rat develops polycystic kidney disease and progressive CRF attributable to a spontaneous genetic mutation (Cy) encoding for a protein with an unknown function. Persistent azotemia is seen from 10 weeks of age onwards and slowly progresses to uremia around 40 weeks, accompanied by anemia, hypertension, hyperlipidemia, and secondary hyperparathyroidism. Recently, Moe et al reported that in this rat model of slowly progressive kidney disease all 3 components of Chronic Kidney Disease - Mineral and Bone Disorder are induced when fed a normal 0.7% phosphorus diet: (1) abnormal serum biochemistries, ie, a significant rise in serum phosphate and PTH between 20 and 38 weeks of age, (2) abnormal bone remodeling, ie, secondary hyperparathyroid bone disease with fibrosis, and increased osteoblasts and osteoclasts, and (3) calcification in the tunica media of the aorta. In this rat model, 60% of the animals developed mild to severe aortic calcifications after 38 weeks of age, although no histological evidence for calcification was found at 34 weeks. The advantage of this rat model for uremia-related vascular calcifications consists of the spontaneous development of hyperphosphatemia, secondary hyperparathyroidism and calcified lesions in the aorta without manipulation of the dietary phosphorus content. The main disadvantage however, particularly for the use of interventional studies, is the slow progression of CRF and as a consequence the late onset of the first calcified spots in the arteries (between 34 to 38 weeks of age) and the limited reproducibility.

**The Adenine-Induced Chronic Renal Failure Rat**

More severe chronic renal impairment in the rat is established by the administration of an adenine-rich diet. Normally, adenine is efficiently salvaged by adenine phosphoribosyltransferase (APRT) and is present at very low levels in blood and urine. APRT is an ubiquitously expressed enzyme present in all mammalian cells and is involved in the purine nucleotide salvage pathway used to recover bases and nucleosides from RNA and DNA degradation. This enzyme represents the rate-limiting step in the conversion of adenine and 5-phosphoribosyl-1-pyrophosphate to adenosine monophosphate. When adenine is administered in excess, APRT activity is saturated and adenine is oxidized by xanthine dehydrogenase to 2,8 dihydroxyadenine via an 8-hydroxyadenine intermediate. Adenine and 2,8 dihydroxyadenine are excreted in the urine. However, the very low solubility of 2,8 dihydroxyadenine results in its precipitation in the kidney. The autosomal recessive metabolic disorder APRT deficiency represents the human counterpart in which the accumulation of insoluble 2,8 dihydroxyadenine results in nephrolithiasis and renal failure with permanent kidney damage. High-adenine feeding in rats results in the formation of crystals in the renal tubules, with subsequent tubular injury and inflammation, obstruction, and marked fibrosis. This severe kidney damage goes along with biochemical changes characteristic for the presence of CRF, such as significant elevated serum creatinine, blood urea nitrogen, and phosphorus. Okada et al have optimized the adenine-induced CRF model in the rat closely inducing the clinical condition of human CRF so that it has become a useful model for the study of complications of CKD patients.

Induction of CRF in rats by dietary administration of 0.75% adenine for 4 weeks results in irreversible renal dysfunction and when maintained on a high phosphorus diet (1.03% P and 1.06% Ca) for the next 4 weeks severe calcifications of the aortic media develop. An aortic calcium content of approximately 20 mg/g dry tissue on average (versus ±1.7 mg/g in control animals) was noted, and 8 out of 10 adenine-treated rats showed moderate to severe medial calcifications in the aorta. Renal pathology of the adenine rat model mimics the clinical situation with development of hyperphosphatemia, hypocalcemia, severe secondary hyperparathyroidism characterized by high PTH levels, hypovitaminosis D, and renal osteodystrophy (mainly high bone turnover disease).

Over the last 5 years, several research groups made use of the adenine model to evaluate therapeutic agents in the prevention of medial calcification. From these studies, it became clear that up to 50% of the adenine rats are not prone to aortic medial calcification despite the installation of a comparable, stable moderate-to-severe CRF. Although this model reflects the high biological variability with regard to the development of vascular calcifications in humans and is appropriate to study the mechanism of vascular calcifications and the factors involved in this process, it is less suitable to compare the effect of numerous treatment regimens on arterial calcification in the context of CRF. Because only approximately half of CRF animals develop medial calcifications, a group with an unacceptably high amount of animals would be required to investigate and compare with an acceptable statistical power the effect of different therapeutic compounds on the development of vascular calcification. Interestingly, the group of Price introduced a variant on this experimental model by administration of an adenine-rich diet with a low 2.5% protein content to the animals for 4 weeks. This combination resulted in the development of severe, uniform medial calcification in the entire aorta of all rats 4 weeks after the induction of CRF. The consistent onset and progression of aortic medial calcification is most likely caused by the more severe hyperphosphatemia, because in this synthetic diet, less protein-bound phosphate and more
anorganic phosphate is present, with the latter being much more bioavailable. Another promising alternative was recently explored by Terai et al.\textsuperscript{93} dosing rats orally through gavage with adenine for 10 days. Unfortunately, only about one third of the animals presented aortic calcification after 15 weeks. However, subsequent treatment with a low dose of vitamin D\textsubscript{3} (100 ng/kg PO, 3 to 4 times/wk) for 18 days caused vascular calcification in all rats. Both variants on the adenine model represent valuable, improved uremic models for future therapeutic follow-up studies evaluating the prevention and regression of medial calcification by particular compounds.

In general, with regard to the comparability with human vascular pathology in the setting of renal disease, minimal requirements should be satisfied on rodent models for uremia-related vascular calcification. First, within each animal model a comparable and stable degree of CRF, established by increased serum creatinine or urea, needs to be induced as well as the concomitant biochemical changes inherent to CKD: (1) hyperphosphatemia, (2) secondary hyperparathyroidism, and (3) hypovitaminosis D. Regardless of the method to induce CRF in mice and rats, either through surgery or through the diet, these biochemical abnormalities are to be met. Taking into account the high incidence of renal osteodystrophy in CKD and being an important determinant for vascular calcification, animal models for CRF should display bone abnormalities similar to those seen in CKD patients. In this context, it is worth mentioning that high-turnover bone disease, as observed in most uremic patients, develops in subtotal nephrectomized apoE knockout mice,\textsuperscript{94} remnant kidney rats on a high phosphorus diet,\textsuperscript{69} and adenine-induced CRF rats.\textsuperscript{95} Until now, the uremic LDLR knockout mouse is the only experimental model available for studies on the development of vascular calcification in the setting of low-turnover bone disease,\textsuperscript{60} which is recognized with increasing frequency in CKD patients because of changed strategies to control secondary hyperparathyroidism. Criteria for vascular calcification (localization, extent, and consistency) in CRF animals depend largely on the aim of the study. Both apoE and LDLR knockout mice have shown to develop the successive stages of atherosclerotic plaque progression finally leading to calcified lesions which are accelerated by uremia and show striking similarities to human plaque progression.\textsuperscript{54,58} In uremic and nonuremic apoE and LDLR knockout mice, intimal plaque calcifications are characterized by loss of the vascular smooth muscle type structure and changes of vascular cells toward osteochondrogenic phenotypes with expression of bone associated proteins such as OPN, cbfa1, osterix, osteocalcin, BMP-2, collagen II and X, or sox9,\textsuperscript{64,96–98} which is in line with observations in human arteries affected by intimal plaque calcification.\textsuperscript{99,100} These experimental models are thus particularly useful for studies in the context of (uremic) intimal calcification. Despite the development of discrete medial calcifications in these uremic mouse models, the remnant kidney and adenine induced-CRF rat models or the calcification-prone mouse model described by El-Abbadi et al\textsuperscript{102} are preferentially used to study isolated uremic medial calcification. In accordance with medial calcification in human vessels,\textsuperscript{101,102} no local vascular inflammation is detected, the media thickness is increased and osteochondrogenic cells around calcified foci are observed in uremic rat models.\textsuperscript{70,77,102} Experiments aiming to investigate the efficiency of therapies on medial calcification should optimally use rodent models that develop consistent, robust medial calcification in all animals within a relative short time period as seen in the adenine-induced CRF rat model either on a low protein diet or treated with low dose vitamin D\textsubscript{3}, or 5/6th nephrectomized rats administered high doses of vitamin D\textsubscript{3}. Alternatively, experimental models showing a high biological variability with respect to the severity of arterial calcification might be applied to define and analyze determinants of vascular calcification and to get a better insight into the molecular evolution of the calcification process.

**Experimental Evaluation of Therapies for the Prevention of Vascular Calcification**

**Phosphate Binding Agents**

In end-stage renal disease, therapies mainly focus on controlling mineral homeostasis by targeting hyperphosphatemia and ensuing secondary hyperparathyroidism with phosphate binding agents and/or vitamin D receptor activators or calcimimetics (Figure 1). By doing so, one of the best known inducers of vascular calcification can be controlled: phosphate. A handful of clinical trials have studied the effect of phosphate binding agents on the progression of vascular calcification in predialysis,\textsuperscript{103} prevalent,\textsuperscript{29,104} and incident hemodialysis patients\textsuperscript{105} whereby the interest is mainly focused on comparing calcium phosphate binders (calcium carbonate and calcium acetate) with one of the noncalcium phosphate binding agents, sevelamer. However, these studies report conflicting results as some studies show that sevelamer attenuates the progression of arterial calcification in comparison with calcium-containing phosphate binders,\textsuperscript{29,104,105} whereas others did not find a difference between both treatments on the outcome of this disease process.\textsuperscript{106,107} Confounding factors in comparative clinical studies investigating the effect of different phosphate binders on vascular calcification may be (1) the concomitant use of other medications, such as 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3}; (2) the presence of comorbidity factors such as diabetes or smoking; and (3) variations in the dialysate calcium concentrations, thereby abrogating differences between phosphate binder use. Moreover, within a patient population, the degree of vascular calcification also differs over a wide range, and it has been shown that the presence or absence of arterial calcification at the start of phosphate binder treatment is an important determinant for the effect of calcium- versus non–calcium-containing phosphate binders on the progression of the calcification process. Indeed, when calcifications were not present at the moment of initiation of phosphate binder treatment, no difference in the development and progression of arterial calcification between calcium carbonate and sevelamer-treated patients was found, whereas a higher calcification score was noted after treatment with calcium-containing phosphate binders versus sevelamer when calcifications were already present before the start of treatment.\textsuperscript{103,105} Finally, ethical and practical considerations preclude histomorphometric
analysis of bone parameters in large comparative clinical studies which is recommended as it has been shown that bone metabolism plays an important role in the development of vascular calcification. Administration of exogenous calcium in end-stage renal failure patients with low turnover bone disease indeed holds a risk for vascular calcification as the excess calcium cannot be excreted through the urine nor be built into the bone. This hypothesis is supported by the work of London reporting a clear association between daily calcium carbonate dosage and aortic calcification and pulse wave velocity in hemodialysis patients with adynamic bone disease, whereas no such association was found in patients with intact bone turnover.108

To overcome these possible confounders in patient populations and to pursue a veracious comparison between phosphate binders under more standardized conditions, validated animal models with uremia-induced vascular calcifications are indispensable to add new perspectives on this debate. As shown in the Table, the effect of phosphate binding agents most commonly used in the clinic to date, ie, calcium carbonate or acetate, sevelamer and lanthanum carbonate, is investigated in different uremic murine models. Taken together, calcium carbonate as well as noncalcium phosphate binders seem to reduce the development of aortic calcification, either vascular medial calcification in adenine 5/6th nephrectomized rats or atherosclerotic plaque-related calcification in LDLR or apoE knockout mice. Yet, both studies of Phan et al109,110 demonstrated that sevelamer but not calcium carbonate as well as non-calcium phosphate binders seem to reduce the development of aortic calcification, either vascular medial calcification in adenine 5/6th nephrectomized rats or atherosclerotic plaque-related calcification in LDLR or apoE knockout mice. Yet, both studies of Phan et al109,110 demonstrated that sevelamer but not calcium carbonate reduced the severity of atherosclerotic lesions. Rather unexpected in light of the clinical trials pointing toward a detrimental effect of calcium phosphate binders on ectopic mineralization, dietary treatment with calcium carbonate decreased both plaque and non–plaque-associated calcifications in the aorta versus nontreated animals.109 Thus, by including a vehicle-treated group, which is ethically impossible in clinical trials, it could be demonstrated that, despite the risk for an excess calcium load, phosphate control using calcium-based agents has yet a more favorable effect on the prevention of vascular calcification compared to animals left untreated. Only few experimental studies made a direct comparison between calcium and noncalcium phosphate binders on the development of uremia-related arterial calcifications. Recently, Terai et al99 nicely showed that when serum phosphate and PTH were equally controlled by sevelamer and calcium carbonate, both agents significantly reduced the development of medial calcifications, although a slightly better outcome was seen with sevelamer which was probably attributable to lower serum calcium levels. These experimental data suggest that the traditional calcium containing phosphate binders are not necessarily harmful in CKD patients, particularly not in those without vascular calcification at the start of treatment, provided the calcium balance is regularly monitored and no adynamic bone disease is diagnosed. Taking into account the high costs associated with the newer noncalcium phosphate binders, the poorer patient compliance seen with sevelamer because of the high pill burden, and the lack of conclusive clinical results whether or not calcium-containing phosphate binders aggravate arterial calcification, calcium carbonate and acetate remain useful compounds in the management of hyperphosphatemia.

Calcimimetics
Calcimimetics are among the newest alternatives used to treat secondary hyperparathyroidism. These small molecules bind to the calcium sensing receptor of the parathyroid gland and mimic the effect of an elevated extracellular ionized calcium concentration. In this way, low serum calcium concentrations which are typically present in CKD patients not yet in dialysis less likely trigger PTH secretion. Cinacalcet (Sensipar) is currently the only clinically available calcimimetic agent. In addition to effective control of secondary hyperparathyroidism, treatment with cinacalcet may improve the mineral balance as dialysis patients show reduced serum calcium and phosphate concentrations when administered a daily 25 to 100 mg dose.111 The inhibition of continuous, excessive PTH production together with the reduction in serum calcium and phosphate concentrations offers hopeful perspectives for calcimimetics in the treatment of vascular calcification in CKD patients. Moreover, calcimimetics may directly affect the calcification process in vascular smooth muscle cells as the calcium sensing receptor is present in this cell type.112,113 Also important to notice is that administration of daily doses of cinacalcet initially lowers serum PTH levels with an increase of these values toward predose concentrations after 12 to 24 hours thereby inducing oscillating serum PTH levels.114 Daily intermittent decreases in serum PTH levels increase bone volume and density and thus have an anabolic-like effect on bone,115 which may also reduce the risk for vascular calcification. The beneficial effect of calcimimetics on bone is also found in experimental studies which revealed that R-568, a calcimimetic used for experimental purposes only, reverses osteitis fibrosis and restores the loss of cortical bone strength in uremic rats with high bone turnover disease.116 In rats exhibiting a low-turnover bone lesion and osteomalacia, daily gavage of R-568 exerted anabolic-like actions on the bone, manifested by an increased trabecular bone volume and bone mineral density.115 Most experimental data regarding the effect of calcimimetics on the development of vascular calcification point toward a suppressive effect on aortic calcification (Table). It seems that calcimimetics retard both intimal and medial calcification and even halt the progression of atherosclerosis.117 These experimental observations were the impetus for clinical trials on this subject. At present, an ongoing randomized, multicenter phase IV study (ADVANCE) evaluates the effect of cinacalcet combined with low dose vitamin D versus flexible vitamin D dosing on the progression of coronary artery calcification in hemodialysis patients.118 One study investigating the effect of cinacalcet on cardiovascular morbidity and mortality showed a significant reduction in hospitalization and a trend toward reduced mortality among patients treated with cinacalcet versus placebo.119

Bisphosphonates
Bisphosphonates, worldwide prescribed for the treatment of osteoporosis because of their inhibitory effect on osteoclast differentiation, recruitment, and activity, have also been shown to reduce the development of experimentally induced medial calcification (Table). The first-generation bisphosphonate, etidronate, as well as the more potent ibandronate,
<table>
<thead>
<tr>
<th>Uremic Murine Model and Treatment Groups*</th>
<th>Duration of Treatment</th>
<th>Effect on VC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phosphate binding agents</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/6 Nx</td>
<td>6 months</td>
<td>↓</td>
<td>Cozzolino et al 73</td>
</tr>
<tr>
<td>CaCO₃ 3%</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>Sevelamer 3%</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>Adenine rat (0.75% dietary adenine 4 wk)</td>
<td>5 weeks</td>
<td>↓↓</td>
<td>Katsumata et al 80</td>
</tr>
<tr>
<td>Sevelamer 1%</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>Sevelamer 2%</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>ApoE⁻/⁻ mouse</td>
<td>8 weeks</td>
<td>↓</td>
<td>Phan et al 109</td>
</tr>
<tr>
<td>Sevelamer 3%</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>LDLR⁻/⁻ mouse</td>
<td>14 weeks</td>
<td>↓</td>
<td>Davies et al 86</td>
</tr>
<tr>
<td>CaCO₃ 1%</td>
<td></td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>LDLR⁻/⁻ mouse</td>
<td>6 weeks</td>
<td>↓↓</td>
<td>Mathew et al 150</td>
</tr>
<tr>
<td>CaCO₃ 3%</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>LDLR⁻/⁻ mouse</td>
<td></td>
<td>↓</td>
<td>Mathew et al 151</td>
</tr>
<tr>
<td>LDLR⁻/⁻ mouse</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>LDLR⁻/⁻ mouse</td>
<td></td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Adenine rat (600 mg/kg adenine orally gavaged 10 days followed by 100 ng/kg vitD PO, 3–4×/wk for 18 days)</td>
<td>18 days</td>
<td>↓↓</td>
<td>Terai et al 93</td>
</tr>
<tr>
<td>CaCO₃ 3%</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>Sevelamer 1%</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>Sevelamer 3%</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>Adenine rat (0.75% dietary adenine 4 wk)</td>
<td>6 weeks</td>
<td>↓↓</td>
<td>Neven et al 85</td>
</tr>
<tr>
<td>LaCO₃ 1%</td>
<td></td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>LaCO₃ 2%</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td><strong>Calcimimetics</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>5/6 Nx (+0.1 μg/kg per day vitD SC)</td>
<td>26 days</td>
<td>↓↓</td>
<td>Henley et al 153</td>
</tr>
<tr>
<td>Cinacalcet (10 mg/kg per day PO)</td>
<td></td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>5/6 Nx (+80 ng/kg/48 hours vitD IP)</td>
<td>2 weeks</td>
<td>↓↓</td>
<td>Lopez et al 71</td>
</tr>
<tr>
<td>R-568 (1.5 mg/kg per day SC)</td>
<td>8 weeks</td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>5/6 Nx (+80 ng/kg/48 hours vitD IP)</td>
<td></td>
<td>↓↓</td>
<td>Lopez et al 75</td>
</tr>
<tr>
<td>AMG 641 (1.5 mg/kg per day SC)</td>
<td>2 weeks</td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>5/6 Nx (+600 IE/kg vitD in diet)</td>
<td>12 weeks</td>
<td>↓↓</td>
<td>Koleganova et al 20</td>
</tr>
<tr>
<td>R-568 (20 mg/kg per day SC)</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>5/6 Nx (+20% lactose in diet)</td>
<td>41 days</td>
<td>↓↓</td>
<td>Kawata et al 27</td>
</tr>
<tr>
<td>Cinacalcet</td>
<td>5 mg/kg per day PO</td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>15 mg/kg per day PO</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>ApoE⁻/⁻ mouse</td>
<td>2 months</td>
<td>↓</td>
<td>Joki et al 117</td>
</tr>
<tr>
<td>R-568 PO (dose not reported)</td>
<td></td>
<td>↓↓</td>
<td></td>
</tr>
<tr>
<td>Adenine rat (0.75% dietary adenine 4 wk)</td>
<td>4 weeks</td>
<td>↓</td>
<td>Henley et al 152</td>
</tr>
<tr>
<td>AMG 641 (3 mg/kg per day PO)</td>
<td></td>
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</table>

(Continued)
inhibits uremia-related vascular calcifications.\textsuperscript{91,120} In hemodialysis patients, etidronate also halted the progression of coronary and aortic calcification, however the studied population was small.\textsuperscript{121,122} Despite the fact that bisphosphonates nowadays are put forward in the treatment of vascular calcification, caution is warranted in dialysis patients, particularly with regard to the bone. Importantly, experimental studies suggested that the dose required for inhibition of arterial calcification also reduces bone mineralization\textsuperscript{120,123} by physicochemical inhibition of hydroxyapatite formation. More recently developed bisphosphonates such as risedronate and zoledronate, with a 1000 to 10,000 times greater potency than etidronate, are used at lower doses and might less likely reduce bone mineralization. Therefore, it would be meaningful to investigate in experimental studies whether administration of more potent bisphosphonates can inhibit vascular calcification without reducing bone mineralization.

### Calcification Inhibitors

Today, the focus for treatment is shifting toward intervening on the circulating concentration of calcification inhibitors, that is often found to be disturbed in CKD patients. Pyrophosphate is one of the candidates. This molecule binds to hydroxyapatite crystals and prevents further incorporation of inorganic phosphate into these crystals.\textsuperscript{124} The protective role of pyrophosphate was demonstrated in cultured rat aortas exposed to high phosphate levels.\textsuperscript{125} An article published several decades ago showed that pyrophosphate inhibits vitamin D–induced aortic calcification in rats.\textsuperscript{126} Last year, at the American Society of Nephrology Renal Week, O’Neill et al\textsuperscript{127} presented a 78% reduction of aortic calcification in pyrophosphate-treated, adenine-induced uremic rats. Interestingly, pyrophosphate did not adversely affect bone formation or mineralization because these molecules, which, unlike bisphosphonates, are nonhydrolyzable pyrophosphate analogs, are degraded by bone alkaline phosphatase. Another promising therapy in the prevention of vascular calcification might be vitamin K, a cofactor responsible for the $\gamma$-carboxylation of the Gla residues of matrix Gla protein and thus for biological activation of this potent calcification inhibitor. Vitamin K deficiency,\textsuperscript{128} as well as increased circulating inactive matrix Gla protein levels,\textsuperscript{31} has been reported in CKD patients and are associated with a higher degree of vascular calcification.\textsuperscript{31,129} Prevention of warfarin-induced medial calcification in rats could be obtained by vitamin K2,\textsuperscript{130} and regression of this vascular pathology in this rat model was found under high intake of both vitamin K1 and K2.\textsuperscript{131} The effect of modifying calcification inhibitors or their regulators on vascular calcification in the setting of

<table>
<thead>
<tr>
<th>Uremic Murine Model and Treatment Groups*</th>
<th>Duration of Treatment</th>
<th>Effect on VC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisphosphonates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5/6 Nx (+$\mu$g/kg per day vitD PO)</td>
<td>3 weeks</td>
<td>Tamura et al\textsuperscript{153}</td>
<td></td>
</tr>
<tr>
<td>Etidronate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mg/kg per day SC</td>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>10 mg/kg per day SC</td>
<td></td>
<td>↓ ↓</td>
<td></td>
</tr>
<tr>
<td>Adenine rat (0.75% dietary adenine + 2.5% protein 4 wk)</td>
<td>16 days</td>
<td>Price et al\textsuperscript{91}</td>
<td></td>
</tr>
<tr>
<td>Ibandronate (0.25 mg/kg per day SC)</td>
<td></td>
<td>↓ ↓</td>
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<tr>
<td>5/6 Nx (+$\mu$g/kg per day vitD PO)</td>
<td>3 weeks</td>
<td>Tamura et al\textsuperscript{120}</td>
<td></td>
</tr>
<tr>
<td>Etidronate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg per day SC</td>
<td></td>
<td>↓ ↓</td>
<td></td>
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<tr>
<td>10 mg/kg per day SC</td>
<td></td>
<td>↓ ↓</td>
<td></td>
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<tr>
<td>Alendronate (200 $\mu$g/kg per day SC)</td>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Adenine rat (0.75% dietary adenine 5 wk)</td>
<td>4 weeks</td>
<td>Matsui et al\textsuperscript{154}</td>
<td></td>
</tr>
<tr>
<td>Alendronate (700 $\mu$g/kg per wk SC)</td>
<td></td>
<td>↓ ↓</td>
<td></td>
</tr>
<tr>
<td>Adenine rat (0.75% dietary adenine 7 days followed by 0.5% dietary adenine 21 days + 2.5% protein)</td>
<td>4 weeks</td>
<td>Lomashvili et al\textsuperscript{123}</td>
<td></td>
</tr>
<tr>
<td>Etidronate</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0.17 mg/kg per day SC</td>
<td></td>
<td>None</td>
<td></td>
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<tr>
<td>1.7 mg/kg per day SC</td>
<td></td>
<td>↓ ↓</td>
<td></td>
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<tr>
<td>17 mg/kg per day SC</td>
<td></td>
<td>↓ ↓</td>
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<tr>
<td>Pamidronate</td>
<td></td>
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<tr>
<td>0.03 mg/kg per day SC</td>
<td></td>
<td>↓ ↓</td>
<td></td>
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<tr>
<td>0.3 mg/kg per day SC</td>
<td></td>
<td>↓ ↓</td>
<td></td>
</tr>
<tr>
<td>3 mg/kg per day SC</td>
<td></td>
<td>↓ ↓</td>
<td></td>
</tr>
</tbody>
</table>

5/6 Nx indicates 5/6th nephrectomized rats. *Few studies included other treatment groups, but only those concerning the discussed treatments in this review are listed in the table. ↓ ↓, treatment causes a significant reduction of vascular calcification; ↓ , treatment causes a decreasing trend of vascular calcification.
CKD is still in its infancy, but initial experimental studies await confirmation in clinical trials.

Strikingly, animal models are almost always used to evaluate the effect of different therapies on the onset of vascular calcification and thus investigate the preventive capacity of the particular agent. However, a significant proportion of the CKD population displays already manifested calcification in the arteries before the start of dialysis. Therefore, animal models with uremia-related vascular calcification should be further used to examine whether the therapy under study is able to halt or retard the progression of existing calcified areas or, even more important, to regress vascular calcification. The latter is a challenging target as adverse effects on the bone should be avoided.

**Search for Mechanisms of Vascular Calcification**

Over the last decade, it became clear that the onset and development of calcification in the arterial wall follows an active, tightly cell-mediated mechanism, triggered by an imbalance between inducers and inhibitors, which at least in part mimics bone formation. A large body of in vitro and ex vivo research on cultured vascular smooth muscle cells and rat aortas has converged to a better understanding of the cellular and molecular mechanisms underlying vascular calcification, including some of the intracellular pathways involved. In addition, a growing number of studies use rodent models to further explore the cellular and molecular events regulating the calcification process in the arterial wall. As touched on before, several calcification inhibitors are discovered by their deletion in mice which resulted in distinct medial calcification. Knocking out specific genes in uremic apoe knockout mice has added new insights into the pathophysiology of atherosclerotic plaque calcification. In this murine model, fetuin A deficiency enhanced aortic intimal calcification without affecting the extent of atherosclerosis confirming the direct protective effect of this protein. On the other hand, deletion of cathepsin S in uremic apoe knockout mice led to significant less aortic calcification, with aorta medial calcification in matrix Gla protein knockout mice. Furthermore, studies performed in our laboratory with aorta medial calcification in matrix Gla protein knockout model,140 medial calcification was associated with aorta medial calcification in matrix Gla protein knockout mice.42 Furthermore, studies performed in our laboratory revealed that cells morphologically resembling hypertrophic chondrocytes, characterized by their round shape and the presence of a lacuna, were present adjacent to calcified areas in the media of adenine-induced uremic rats.102 Expression of the chondrocyte markers sox9, collagen II, and aggrecan confirmed the involvement of chondrocytes and cartilage tissue in the development of medial calcification.92,102 Additionally, we found that molecules involved in the osteoblast differentiation pathway were suppressed in the calcified vascular media of uremic rats.92 The expression of the osteoblast transcription factor, osterix as well as β-catenin and LDLR-related protein 6, molecules regulating the wnt/β-catenin pathway, were significantly downregulated just before and during the initial phase of the calcification process in the aorta. Both osterix and the wnt/β-catenin signaling pathway are responsible for the segregation of common osteochondroprogenitor cells toward osteoblasts, whereas suppression of the latter pathway and induction of the transcription factor sox9 guide these progenitor cells to chondrocytes.136,137 The simultaneous upregulation of chondrocyte-specific markers and suppression of the osteoblast differentiation pathway led us to the hypothesis that medial smooth muscle cells more likely transdifferentiate into chondrocyte-like cells than having an osteoblast-like fate during uremia-related medial calcification.92 Evidence for the expression of a chondrogenic phenotype in intimal and medial calcified lesions is also shown in arteries from diabetics138,139 and transplant donors.102 However, cartilage metaplasia might be just one of the pathological pathways causing vascular calcification. Indeed, in the LDLR knockout model,140 medial calcification was associated with upregula-
tion of bone morphogenetic protein-2, msx2, and the wnt signaling pathway, pointing toward the direct transdifferentiation of vascular cells to osteoblasts and suggesting that a cartilage intermediate is not required to form bone-like structures in arteries. Interestingly, treatment of LDLR knockout mice with antitumor necrosis factor-α therapy prevented the induction of these osteoblast markers and significantly reduced aortic calcium content thereby highlighting the crucial role of inflammation in phenotypic changes of medial smooth muscle cells. It is plausible to assume that the segregation between osteoblast or chondrocyte transdifferentiation in the vessel wall is determined by environmental factors, such as inflammatory mediators and/or uremic toxins.

Recent well-performed studies regarding the origin of chondrocytes in calcified arteries are of particular interest (Figure 2). A cell lineage–mapping study in a mouse model with distinctive arterial medial calcification demonstrated that medial smooth muscle cells underwent reprogramming toward an osteochondrogenic phenotype. Conversely, using bone marrow transplantation in LDLR knockout mice, 90% of chondrocyte-like cells in atherosclerotic intimal calcification were shown to originate from hematopoietic, particularly myeloid, precursors in the bone marrow. However, these findings need to be confirmed by a rigorous lineage tracing study and do not rule out the contribution of local chondrocyte transdifferentiation from vascular cells to chondrocartilaginous metaplasia during intimal calcification which is also a plausible mechanism as smooth muscle actin positive cells in atherosclerotic plaques show positivity for the chondrocyte marker sox9. These reports suggest that distinct mechanisms might be involved in intimal plaque mineralization and vascular medial calcification, the latter being much less associated with local inflammation.

Osteoclast-like cells have also been reported in calcified vessels. In calcified lesions of OPG knockout mice, multinucleated cells were associated with the expression of receptor activator of nuclear factor κB (RANK), an osteoclast cell surface receptor, and stained positive for the osteoclast-specific proteins cathepsin K and tartrate-resistant acid phosphatase. In human calcified atherosclerotic plaques, TRAP-positive cells were also found. Osteoclasts are derived from the monocyte/macrophage hematopoietic lineage and require macrophage colony-stimulating factor (M-CSF) and RANK ligand–mediated signaling for differentiation and maturation. As endothelial cells and vascular smooth muscle cells in the normal vessel wall produce M-CSF, and RANK ligand expression is induced in calcified vessels, these factors possibly may mediate the differentiation of osteoclast precursors such as monocytes/macrophages after being recruited to the vessel wall, eg, during atherosclerosis. However, despite little evidence for osteoclast-like cells to be present in calcified arteries, it is still unclear whether regression of vascular calcification takes place and whether these cells have the capacity to resorb pathological calcifications. One study mentioned a 75% regression of medial calcium 9 weeks after cessation of vitamin D treatment in rats. Macrophages, identified as CD68 cells, were attached to the aortic endothelium and located around vascular calcium deposits suggesting an active resorption process. A recent study reported that bone marrow-derived osteoclasts reduced the mineral content of calcified elastin fibers in vitro by 80%, and in vivo, subdermal implantation of osteoclasts in the vicinity of calcified elastin, diminished elastin mineralization by 50%. These observations suggest a potential mechanism by which osteoclast-like cells in the vessel wall could induce regression of arterial calcification.

At the molecular level, the role of OPG and RANK ligand in vascular calcification has gained more interest through animal studies. Both molecules are important for the coupled regulation of bone formation and resorption. Binding of RANK ligand, expressed by osteoblasts, with its receptor RANK, located at the membrane surface of the osteoclast precursors, induces osteoclast activation, differentiation, and

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**Figure 2.** Current hypothesis of the origin of chondrocyte-like cells involved in arterial intimal versus medial calcification. HA indicates hydroxyapatite; VSMC, vascular smooth muscle cell.
maturation, thereby starting the process of bone resorption. The effects of RANK ligand are blocked by OPG, a soluble decoy receptor for RANK ligand, that in this way regulates bone resorption by inhibiting the final differentiation and activation of osteoclasts. OPG treatment reduced both intimal and medial calcification in LDLR knockout mice and rats receiving vitamin D and warfarin treatment, respectively. Whether the preventive effect of OPG is ascribed to direct actions on the vessel wall or occurred secondary through targeting the bone by inhibiting bone resorption and limiting release of calcium and phosphorus from the bone needs further investigation. The latter hypothesis is supported by Helas et al., demonstrating that RANK ligand inhibition by denosumab inhibits glucocorticoid-induced bone resorption with a concomitant reduction in vascular calcium content by 50%. Clearly, the OPG/RANK/RANK ligand triad may serve as a promising new target for therapy in the prevention of vascular calcification, particularly in patients with a low bone mineral density.

Conclusion

Complementary to clinical and in vitro studies, various valuable animal models have added new insights into the cellular and molecular processes mediating intimal and medial calcification whether or not in the context of CRF. Using uremic experimental models has allowed evaluation of the preventive effect of therapeutics, intervening in the dysregulated mineral metabolism related to CKD, on the onset of vascular calcification. In view of new strategies for treatment of the high vascular calcification burden in CKD, animal models of vascular calcification are currently being used to evaluate therapies targeting the disturbed concentrations of calcification inhibitors present in CKD patients. In the future, uremic animal models could further improve their value in this research field as they serve as an excellent tool to study the regression of arterial calcification under treatment, which undoubtedly is of great clinical importance in the dialysis population. With regard to future studies aiming to investigate the reversal of vascular calcification, in vivo microtomography scanning has been shown to be a sensitive tool for the follow-up of aortic calcification in living rats.

Acknowledgments

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Disclosures

None.

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