Identifying early pathogenic events during vascular calcification in uremic rats

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Vascular calcification in chronic kidney disease is a very complex process traditionally explained in multifactorial terms. Here we sought to clarify relevance of the diverse agents acting on vascular calcification in uremic rats and distinguish between initiating and complicating factors. After 5/6 nephrectomy, rats were fed a 1.2% phosphorus diet and analyzed at different time points. The earliest changes observed in the aortic wall were noticed 11 weeks after nephrectomy: increased Wnt inhibitor Dkk1 mRNA expression and tissue non-specific alkaline phosphatase (TNAP) expression and activity. First deposits of aortic calcium were observed after 12 weeks in areas of TNAP expression. Increased mRNA expressions of Runx2, BMP2, Pit1, Pit2, HOXA10, PHOSPHO1, Fetuin-A, ANKH, OPN, Klotho, cathepsin S, MMP2, and ENPP1 were also found after TNAP changes. Increased plasma concentrations of activin A and FGF23 were observed already at 11 weeks post-nephrectomy, while plasma PTH and phosphorus only increased after 20 weeks. Plasma pyrophosphate decreased after 20 weeks, but aortic pyrophosphate was not modified, nor was the aortic expression of MGP, Msx2, several carbonic anhydrases, osteoprotegerin, parathyroid hormone receptor-1, annexins II and V, and CD39. Thus, increased TNAP and Dkk1 expression in the aorta precedes initial calcium deposition, and this increase is only preceded by elevations in circulating FGF23 and activin A. The expression of other agents involved in vascular calcification only changes at later stages of chronic kidney disease, in a complex branching pattern that requires further clarification.

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The ectopic calcification of large arteries is a common complication in chronic kidney disease (CKD), and in combination with abnormalities in circulating biomarkers and bone histomorphometry, it forms a part of CKD–mineral bone disorder (CKD-MBD).[1] Cardiovascular complications are the main cause of mortality in CKD patients, and medial vascular calcification (VC) is a significant contributing factor.[2]

Calcium phosphate deposition in large arteries ends in the form of hydroxyapatite deposits, which cause structural changes to the wall layers, accompanied by gene expression and osteochondrogenic phenotype transition of the vascular smooth muscle cells (VSMC).[3] Among the bone-forming genes, these cells overexpress phosphatases such as tissue nonspecific alkaline phosphatase[4] (TNAP) and PHOSPHO1,[5] which, among other activities, hydrolyze the calcification inhibitor pyrophosphate (PPi). Other suggested mechanisms of VSMC calcification include the formation of matrix vesicles, apoptotic bodies,[6] and exosomes[7] that serve as nucleation sites for calcium phosphate precipitation, plus the overexpression of Pi transporters,[8] altered extracellular matrix, elastin degradation,[9] etc. In addition, during the formation of CKD-MBD, hormonal network disorders such as the increased secretion of fibroblast growth factor 23 (FGF23) and a decreased abundance of soluble Klotho also seem to be involved in VC pathogenesis.[10] More recently, the deleterious effects on the vasculature caused by circulating Wnt inhibitors as a consequence of an injured kidney have been described, including Dkk1,[11] activin A,[12] and sclerostin.[13] Such effects could include dedifferentiation of VSMC toward osteoblastic transition and vascular calcification,[13] as well as endothelial-to-mesenchymal transition in the vasculature of CKD and atherogenic animal models.[11]

In this work, we attempt to clarify some aspects of this complex pathogenic scenario of vascular dysfunction and ectopic calcification during CKD. In the search for a less complex but reliable model, *in vitro* methods were discarded,[14] and we focused on the very early stages of aortic calcification using rats that have undergone 5/6 nephrectomy (Nx) and have been fed a Pi-rich diet. The aorta calcium content, specific gene expression changes, serum and urine parameters, and protein expression and activities were analyzed at different time points after nephrectomy. Our findings have allowed us to discriminate between the initiating and complicating pathogenic events of VC and to identify the changes that occur before the first calcium deposits are found.

RESULTS

Blood plasma analysis

One week after the 5/6 Nx, animals were given free access to a 1.2% Pi diet and water. Table 1 shows plasma biochemical parameters at each time point.

As expected, in the 5/6 Nx animals (a model of stages 3–4 CKD), the urea and creatinine concentrations had increased at all analyzed times. Calcium was not modified, and despite the Nx and the fact that the animals were eating food with Pi at twice the control concentration, the plasma Pi only increased at week 20, when the parathyroid hormone (PTH) level also increased. The low concentration of Pi at earlier times was most likely maintained by the phosphatonin FGF23, which was 13.5 times higher at week 11 and 22 times higher at week 12 than in control rats.

Interestingly, the concentrations of neither the cytokine tumor necrosis factor α (TNFα), the circulating Klotho, nor the Wnt inhibitor Dkk1 were altered in the plasma, but the VC inhibitor PPi diminished significantly after 20 weeks. Activin A, however, was statistically increased at all 4 time points, and sclerostin also increased, but the statistical significance was only observed at week 20 with analysis of variance.

Aorta calcification

Aorta calcification was studied in several ways. First, the traditional stainings of calcium phosphates using alizarin red and von Kossa only yielded positive results after 20 weeks in 1 of the animals (Figure 1a for weeks 12 and 20). The staining is shown as a positive control and an example of intense calcification in the aortic arch, but in most of the animals positive staining results were still not observed. However, the study was stopped at this point to focus on the early steps. To confirm these findings, the calcium content was colorimetrically quantified, and based on the higher sensitivity of colorimetry over histological staining, the results showed a significant increase of calcium in the aorta already at 12 weeks after Nx (Figure 1b). This finding was corroborated in a confirmatory experiment that also included nephrectomized animals eating food containing 0.6% Pi and sham-operated animals that ate 0.6% and 1.2% Pi for the same period of time (Figure 1b, inset; 3 animals per group). Only the nephrectomized animals eating the 1.2% Pi diet showed a calcium content increase at week 12. The differences in calcium content between both experiments reveal the variability and complexity of the pathogenic process, but they still emphasize a significant increase after 12 weeks of Nx and a Pi-rich diet.

To clarify these divergent results between calcium staining and colorimetric determination, aorta sections were studied by electron microscopy. At week 11, no calcium deposits were observed by scanning electron microscope (SEM) analysis. However, the first calcium deposits of <1 μm were observed after 12 weeks (Figure 1c). Field emission SEM revealed that there were 2 types of deposits from the 12-week Nx animals according to the Ca/P ratios (Figure 1d): the most frequent deposits had a ratio of 1:35, compatible with amorphous calcium phosphate, whereas a few of the deposits had a ratio of 1:65, compatible with hydroxyapatite (i.e., a more crystalline form of calcium phosphate). Aortas from sham-operated rats eating a 1.2% Pi diet and from 5/6 Nx rats eating a 0.6% Pi diet yielded negative results in analyses for the presence of calcium deposits.

Gene expression analysis

Genes likely to be involved in vascular calcification were analyzed using quantitative real-time polymerase chain reaction. The genes that were not modified even after 20 weeks were Msx2; carbonic anhydrases III, IV, VIII, IX, and XIII; MGP; osteoprotegerin; PTH receptor-1; annexins II and V; CD39; metalloproteinase 2 (MM2); and type I collagen. The expressions of MM9 and the cell cycle regulator and senescence marker p16INK4a (Cdkn2a) were almost residual up to 20 weeks.

Despite the absence of expression changes in the previous genes, many other genes were overexpressed at week 20, as shown in Figure 2, thereby reflecting the classical complexity traditionally described for VC. This complexity forced us to

Table 1 | Blood plasma parameters in control and uremic rats after the indicated weeks since nephrectomy

Parameter	Control	11 wk	12 wk	16 wk	20 wk	*P* ANOVA
Urea (mg/dl)	41.0 ± 1.3	57.1 ± 0.9	59.3 ± 5.5[a]	66.0 ± 8.5[a]	77.3 ± 10.0[a]	0.0003
Creatinine (mg/dl)	0.21 ± 0.02	0.46 ± 0.10[a]	0.58 ± 0.06[a]	0.63 ± 0.09[a]	0.76 ± 0.10[a]	<0.0001
Ca^{2+} (mg/dl)	1.89 ± 0.02	2.05 ± 0.07	1.89 ± 0.04	1.78 ± 0.12	1.81 ± 0.03	0.0589
Pi (mg/dl)	11.9 ± 0.4	14.0 ± 0.5	13.7 ± 1.1	14.5 ± 1.1	33.4 ± 5.7[a]	<0.0001
FGF23 (ng/ml)	0.11 ± 0.05	1.48 ± 0.26	2.50 ± 0.58[a]	2.76 ± 0.74[a]	5.04 ± 1.17[a]	<0.0001
PTH (pg/ml)	0.88 ± 0.20	0.50 ± 0.04	0.40 ± 0.06	0.98 ± 0.26	17.0 ± 3.6[a]	<0.0001
TNFα (ng/ml)	7.22 ± 0.09	7.18 ± 0.08	7.09 ± 0.10	7.33 ± 0.13	7.45 ± 0.18	0.4465
PPi (μmol/l)	2.12 ± 0.19	2.08 ± 0.17	2.00 ± 0.37	1.29 ± 0.51	0.63 ± 0.37	0.0305
Klotho (ng/ml)	0.36 ± 0.05	0.61 ± 0.10	0.35 ± 0.04	0.46 ± 0.81	0.42 ± 0.05	0.6958
Activin A (pg/ml)	6.04 ± 0.82	26.2 ± 8.5[a]	33.6 ± 7.3[a]	32.2 ± 5.8[a]	22.8 ± 2.0[a]	<0.0001
Dkk1 (pg/ml)	73.9 ± 18.2	62.4 ± 9.6	76.2 ± 8.6	92.3 ± 22.3	80.7 ± 11.2	0.9553
Sclerostin (ng/ml)	1.05 ± 0.06	1.76 ± 0.05	2.33 ± 0.34	1.71 ± 0.17	3.77 ± 1.10[a]	0.0017

ANOVA, analysis of variance; FGF23, fibroblast growth factor 23; PTH, parathyroid hormone; TNFα, tumor necrosis factor α.
[a]Statistically different from control values with ANOVA and Dunnett's posttest.
Control data are the pool of all control animals from the 4 time points because they were similar and statistically not different. The rest of values are the mean ± SEM of 5 animals.