Early chronic kidney disease–mineral bone disorder stimulates vascular calcification

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The chronic kidney disease–mineral and bone disorder (CKD–MBD) syndrome is an extremely important complication of kidney diseases. Here we tested whether CKD–MBD causes vascular calcification in early kidney failure by developing a mouse model of early CKD in a background of atherosclerosis-stimulated arterial calcification. CKD equivalent in glomerular filtration reduction to human CKD stage 2 stimulated early vascular calcification and inhibited the tissue expression of α-klotho (klotho) in the aorta. In addition, osteoblast transition in the aorta was stimulated by early CKD as shown by the expression of the critical transcription factor Runx2. The ligand associated with the klotho-fibroblast growth factor receptor complex, FGF23, was found to be expressed in the vascular media of sham-operated mice. Its expression was decreased in early CKD. Increased circulating levels of the osteocyte-secreted proteins, FGF23, and sclerostin may have been related to increased circulating klotho levels. Finally, we observed low-turnover bone disease with a reduction in bone formation rates more than bone resorption. Thus, the CKD–MBD, characterized by cardiovascular risk factors, vascular calcification, increased circulating klotho, FGF23 and sclerostin levels, and low-turnover renal osteodystrophy, was established in early CKD. Early CKD caused a reduction of vascular klotho, stimulated vascular osteoblastic transition, increased osteocytic secreted proteins, and inhibited skeletal modeling producing the CKD–MBD.

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The chronic kidney disease–mineral and bone disorder (CKD–MBD) syndrome is an extremely important complication of kidney diseases. The CKD–MBD was named in 2006,[1] following the realization that the mineral and skeletal disorders accompanying kidney failure are important contributors to the CKD-associated cardiovascular disease and high mortality rates.[2–5] Recent studies have suggested that the CKD–MBD, defined as biochemical abnormalities in mineral metabolism, abnormalities in skeletal remodeling, and extraskeletal calcification, is present when the glomerular filtration is reduced by more than 40%.[6] Within the concept of the CKD–MBD, recent progress related to cardiovascular disease risk factors stimulated by kidney disease has uncovered three: vascular calcification, phosphorus, and fibroblast growth factor 23 (FGF23).[7–11] Vascular calcification particularly poses an increased risk of cardiovascular and all-cause mortality,[7,12] and of the clinical conditions associated with vascular calcification, the most extensive calcifications occur in CKD.[13] In CKD, vascular calcification is stimulated by hyperphosphatemia and positive calcium balance.[14–18]

Emerging data indicate that the CKD–MBD syndrome may begin early in the course of kidney disease and precede the development of clinically detectable abnormalities in plasma phosphorus (Pi), calcium (Ca), parathyroid hormone (PTH), and calcitriol, which are the hallmarks of the established CKD–MBD. Biomarkers of skeletal osteocyte function have been found to be abnormal early in kidney disease, both clinically and in translational models,[6,19–23] indicating that kidney injury had affected the skeleton, in other words, the CKD–MBD had begun. Pereira *et al.*[19] found by skeletal immunocytochemistry and plasma levels that osteocyte production of FGF23 and dentin matrix protein-1 was increased in stage 2 CKD. These results were confirmed by Sabbagh *et al.*[21] and Oliveira *et al.*,[22] who also found that osteocyte sclerostin was increased in early CKD, and that osteocyte nuclear β-catenin was decreased, indicating decreased osteocyte Wnt activity in early CKD. As Wnt activity is the major skeletal anabolic principle of the postnatal skeleton,[24,25] these results indicate that kidney disease signals a decrease in bone formation. Fang *et al.*[20] showed, in a translational model of early CKD, elevated

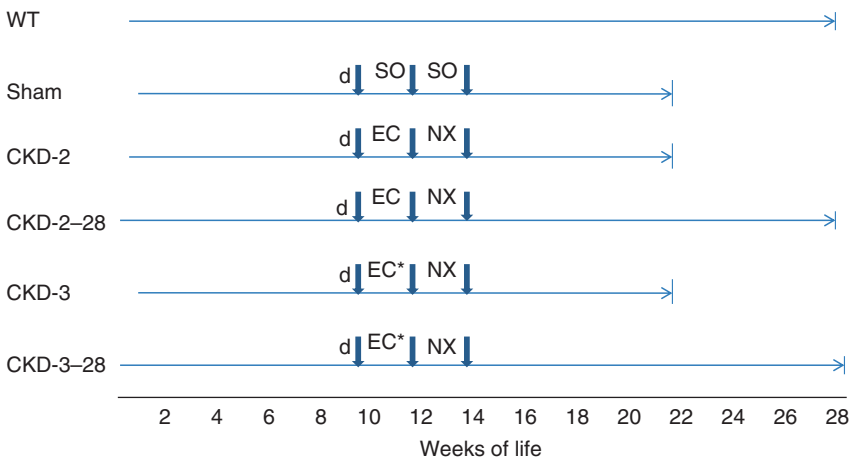

Figure 1 | A schematic drawing of the experimental design defining the various animal groups. WT, wild-type C57B6J mice were used to establish normative parameters; Sham, *low-density lipoprotein–deficient* (*ldlr−/−*) mice on a high-fat diet (d) undergoing sham operations (SO) are the control mice for the effects of chronic kidney disease (CKD); CKD-2, *ldlr−/−* high fat–fed mice undergoing mild renal injury (EC) and contralateral nephrectomy (NX) with killing at 22 weeks; CKD-2–28, same as CKD-2 but killing at 28 weeks (6 additional weeks of slowly progressive CKD); CKD-3, *ldlr−/−* high fat–fed mice undergoing moderate renal injury (EC*) and NX with killing at 22 weeks; CKD-3–28, same as CKD-3 but killing at 28 weeks.

FGF23 levels in the presence of normal plasma Ca, Pi, PTH, and calcitriol, and a decrease in bone formation rates. These results confirmed earlier reports before FGF23 studies from our laboratory that when Ca, Pi, calcitriol, and PTH were maintained normal in CKD, decreased bone formation rates and the adynamic bone disorder were observed.[26] However, it is unknown whether vascular calcification and renal osteodystrophy produced by kidney disease[27,28] are present in the early stages of the CKD–MBD.

These studies were conducted to test the hypothesis that the CKD–MBD begins early in kidney disease, including the onset of heightened cardiovascular risk related to vascular calcification. We developed an animal model of early CKD using the atherosclerosis-bearing low-density lipoprotein–deficient mouse (*ldlr−/−*) fed high-fat, Western-type diets (40% of calories from fat). The phenotype of these mice is further characterized by insulin resistance progressing to type 2 diabetes over time. The mice respond as humans to atherosclerosis with neointimal plaque calcification that is stimulated by advanced CKD.[29] Using inulin clearances to determine glomerular filtration rate (GFR), we staged CKD in mice and developed a model of CKD equivalent in GFR to stage 2 human CKD using unilateral renal injury and contralateral nephrectomy. This model mimics acute kidney injury (AKI) and incomplete recovery in humans with atherosclerosis and insulin resistance/diabetes. The peak GFR (representing a 25–40% decrease from normal GFR) established after recovery from AKI slowly diminished over weeks related to interstitial inflammation and fibrosis, allowing the development of the CKD–MBD. By using this model, we characterized the early CKD–MBD discovering stimulated vascular calcification in early CKD before hyperphosphatemia. The discovery of vascular calcification early in CKD produced a requisite search for mechanisms, and we discovered mesenchymal transition in vascular cells and newly recognized abnormalities in the arterial tree that cause vascular calcification, reduction of vascular klotho,[30–32] expression of FGF23, and increased circulating klotho (c-klotho). The elevations in c-klotho explain the early stimulation of skeletal osteocytes and FGF23 secretion independent of changes in the serum phosphorus.

RESULTS

Studies in early CKD

The overall experimental design is shown in Figure 1. The serum/plasma chemistries (blood urea nitrogen (BUN), Ca, Pi, and PTH) determined in the experimental groups are shown in Table 1. The BUN of wild-type and sham-operated mice ranged from 20 to 23 mg/dl (Table 1). In mice with mild renal ablation, referred to in this paper as CKD-2, the mean BUN of the group was not elevated at 22 weeks and only increased to 24–30 mg/dl at 28 weeks (CKD-2–28). Inulin clearances confirmed a 33% reduction in GFR in the 22-week *ldlr−/−* CKD-2 group (Figure 2). A 40% reduction from normal GFR is the low end of the GFR range in human stage 2 CKD. The CKD-2 animals were normocalcemic and normophosphatemic at 22 weeks (Table 1). In the 28-week CKD-2–28 animals, compatible with the slight progression of the kidney insufficiency, hyperphosphatemia had developed (12.7 ± 3.9 mg/dl). These findings are compatible with our previous findings of hyperphosphatemia in mice with more severe ablation and GFR in the human stage 3 CKD range.[14,33] PTH levels were elevated to 120 ± 48 pg/ml in the CKD-2 animals at 22 weeks, but were only 90.8 ± 20 pg/ml at 28 weeks (not significantly different from the normal levels of the sham-operated animals). The elevation of PTH at 22 weeks in the CKD-2/3 groups following mild renal injury suggests that the elevation may have been related to changes associated with the AKI phase of the model. As shown in Figure 3a, longitudinal analysis of PTH levels