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Role of calcification inhibitors in the pathogenesis of vascular calcification in chronic kidney disease (CKD)

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Background. The majority of patients with chronic kidney disease (CKD) have excessive vascular calcification; however, most studies demonstrate that a subset of CKD patients do not have, nor develop, vascular calcification despite similar exposure to the uremic environment. This suggests protective mechanisms, or naturally occurring inhibitors, of calcification may be important.

Methods. In order to determine the role of three inhibitors, fetuin-A, matrix gla protein (MGP), and osteoprotegerin (OPG) in the vascular calcification observed in patients with CKD-5, we (*1*) measured serum levels of these inhibitors and compared the levels to calcification assessed by computed tomography (CT); (*2*) examined arteries from CKD-5 patients by immunostaining for these inhibitors; and (*3*) examined the expression and effect of these inhibitors in cultured bovine vascular smooth muscle cells (BVSMCs) incubated in serum pooled from uremic patients compared to healthy controls.

Results. There was a negative correlation of coronary artery calcification scores with serum fetuin-A levels ($r = -0.30$, $P = 0.034$) and a positive association with OPG levels ($r = 0.29$, $P = 0.045$). There was increasing immunostaining for both fetuin-A and MGP in arteries with increasing calcification graded semiquantitatively ($P < 0.003$). In vitro, fetuin-A added to mineralizing BVSMCs inhibited mineralization ($P < 0.001$). Compared to normal serum, BVSMCs incubated with uremic serum had a progressive increase in MGP expression with mineralization ($P < 0.001$) and increased expression of OPG in BVSMCs ($P < 0.04$).

Conclusion. These data demonstrate that fetuin-A, OPG, and MGP play an important role in the pathogenesis of uremic vascular calcification.

Key words: vascular calcification, dialysis, fetuin-A, osteoprotegerin, matrix gla protein, CKD.

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Cardiovascular disease and stroke is the leading cause of death in patients with end-stage renal disease (ESRD) that require dialysis [chronic kidney disease, stage 5 (CKD-5)], at a risk that is 10- to 20 -fold the age- and gender-matched general population[1]. However, traditional Framingham risk factors can not completely account for the excessive prevalence [2], suggesting dialysis specific risk factors may contribute. Recent data have linked hyperphosphatemia and elevations in the calcium × phosphorus product in CKD-5 patients with increased mortality [3]. In addition, studies have linked these disorders of mineral metabolism with vascular calcification in vitro, in vivo animal models, and in human studies (reviewed in [4]).

A study evaluating coronary calcification by electron-beam computed tomography scan (EBCT) in patients with ESRD has demonstrated two- to fivefold more coronary artery calcification than age- and gender-matched individuals with angiographically proven coronary artery disease [5]. Histologic analyses confirm increased calcification of arterial lesions from dialysis patients compared to the general population with known coronary artery disease [6]. Others have demonstrated that this process is rampant, even in children and young adults [7]. Furthermore, the presence of vascular calcification may have prognostic implications for dialysis patients. Indeed, the greater the degree of peripheral vascular calcification by ultrasound [8] and plain radiographs [9], and coronary artery disease by helical CT [10], the greater risk of mortality. Despite this high prevalence of vascular calcification, in most series 0% to 50% (mean 17%) of CKD subjects studied do not have detectable vascular calcification, despite similar risk factors (reviewed in [11]). Furthermore, patients who do not have vascular calcification rarely develop calcification on follow-up [10]. While this is in part due to younger age, these data also imply a potential role of naturally occurring inhibitors of vascular calcification. Even in non-CKD patients, serum is supersaturated with respect to calcium phosphate, and thus

inhibitors must exist to prevent widespread extraskeletal ossification [12].

Recent animal "knockout" models have shown that key gene products may be protective of vascular calcification including matrix gla protein (MGP), osteoprotegerin (OPG), and fetuin-A (α2-Heremans-Schmid glycoprotein) (Ahsg). Animals deficient in OPG [13] and MGP [14] develop spontaneous medial calcification of arteries. Animals deficient in fetuin-A, when bred on a DBA/2 background or fed a high calcium diet also develop extraskeletal calcification, including soft tissue and perivertebral arterial calcification [15]. We therefore hypothesized that naturally occurring inhibitors may be deficient or aberrantly regulated in CKD patients with vascular calcification. Specifically, we hypothesized that fetuin-A, OPG, and MGP are important inhibitors of vascular calcification in CKD-5 patients. To test this hypothesis, we performed a series of experiments: (*1*) we measured these inhibitors in vivo in the serum of CKD-5 subjects and determined the association of serum levels with the magnitude of coronary artery and aorta calcification quantified by spiral CT; (*2*) we assessed the presence of these inhibitors by immunostaining in arteries obtained from CKD-5 patients with and without calcification; (*3*) in vitro experiments were done to determine expression of these inhibitors in bovine vascular smooth muscle cells (BVSMCs) incubated with pooled serum from dialysis patients compared to pooled normal healthy control human serum; and (*4*) we determined the ability of fetuin-A and OPG to inhibit mineralization in BVSMCs. The results demonstrate a role for all three inhibitors in the vascular calcification observed in CKD.

METHODS

Association of vascular calcification and serum levels of inhibitors

We measured fetuin-A, OPG, and MGP levels in serum from patients with ESRD who were undergoing assessment of coronary artery and aorta calcification by spiral CT to determine the relationship of calcification and serum inhibitor levels. The patient characteristics and calcification scores have been previously published [10, 16]. Briefly, the patients consisted of two cohorts, a group of 30 hemodialysis patients and a group of 38 patients undergoing a renal transplant. All but two of this latter group were undergoing dialysis. CT scans were performed with quad-slice technique on the model MX 8000 scanner using a retrospective gating protocol with a threshold of 130 Hounsfield units (Philips Medical Systems, Cleveland, OH, USA) as previously described in detail [17]. A single reader scored each coronary artery, the summation of which was the total coronary artery calcification score.

As previously published, in both cohorts, only advancing age and duration of dialysis were associated with the coronary artery calcification score, and only advanced age was associated with aorta calcification score [17]. For the present study, these two groups are combined for a cross-sectional analysis of the association of the coronary artery calcification scores and aorta scores with serum levels of inhibitors. Because this is a secondary analysis using stored serum, not all patients had enough serum for all analyses, thus the final number (*N*) for each serum test differs. The fetuin-A was measured by nephelometry using antihuman fetuin-A antibody from Chiron Behring, Marburg, Germany [18], MGP was measured by enzyme-linked immunosorbent assay (ELISA) (Biomedica, Vienna, Austria), and OPG was measured by ELISA (Alpco Diagnostics, Windham, NH, USA). In the cohort who received a renal transplant, additional serum levels were measured 15 to 20 months later in some patients to determine the net change in serum levels of inhibitors with a functional allograft.

Determination of expression of inhibitors in arteries with and without calcification

As previously described, we have performed immunostaining using standard methodology on paraffin sections of inferior epigastric arteries obtained from patients undergoing renal transplantation, demonstrating the presence of medial calcification in association with the expression of "bone" matrix proteins and transcription factors [16]. During the surgery, the proximal inferior epigastric artery was ligated and a 2 to 3 cm piece of vessel was removed and placed into 4% paraformaldehyde, followed by paraffin embedding and sectioning [16]. MacNeal's stain was also done, which uses a combination of tetrachrome solution for blue nuclear staining, together with silver stain to highlight calcification. For the present study, unstained slides from these tissue sections were deparaffinized in xylene and rehydrated in descending alcohol, followed by 3% hydrogen peroxide and washing in Tris saline. Sections were blocked by 3% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA) for 15 minutes, and then incubated for 1 hour in the primary antibody, human fetuin-A (antiserum diluted 1:1000 in blocking buffer) or human matrix gla protein (1:200 from Dr. Reider Wallin, Wake Forest University, NC, USA). The sections were then incubated with ABC staining system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by color development with diaminobenzidine (DAB), and counterstained with Harris Hematoxylin (Sigma Chemical Co.). Staining without the primary antibody served as a negative control. Unfortunately, despite trying several commercially available and other antibodies to OPG, none demonstrated consistent