Kidney International, Vol. 67 (2005), pp. 2295–2304

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.

Received for publication August 18, 2004
And in revised form November 30, 2004, and December 16, 2004
Accepted for publication January 6, 2005

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

results with several controls and thus we were only able to do immunostaining for fetuin-A and MGP.

Determination of expression of inhibitors in BVSMCs incubated in the presence of pooled normal human or pooled uremic human serum

We used cultures of BVSMCs, isolated as primary cultures by the explant method as previously described [19]. The BVSMCs were incubated in Dulbecco's modified Eagle's medium (DMEM) media (Sigma Chemical Co.), with 10% serum for 24 to 72 hours, and then protein isolated or mRNA isolated for assessment of fetuin-A or OPG by Western blot, or MGP by reverse transcription-polymerase chain reaction (RT-PCR). As previously described [19, 20], the serum was either fetal bovine serum (FBS) (Sigma Chemical Co.), pooled uremic serum from patients undergoing hemodialysis without residual renal function (uremic serum), or pooled serum from age matched healthy controls (normal serum). In addition we used a mineralization model where BVSMC were incubated in 10 mmol/L β-glycerophosphate, 10 U/mL insulin, and ascorbic acid in the presence of 15% serum. In this model, the calcification over time was quantified by extraction with HCl at various time points, as previously described [19]. In some experiments, conditioned media were collected preincubation and postincubation to determine secretion of OPG into culture media by ELISA (Alpco Diagnostics) by net change (post minus pre). In the OPG experiments, the osteoblast cell lines Saos (ATCC, Manassas, VA, USA) were used as control cells. In additional experiments designed to determine the effect of inhibitors in mineralizing cultures of BVSMC, we added fetuin-A (Sigma Chemical Co.) at doses of 100, 500, and 1000 μg/mL, or 200 ng/mL of OPG (R&D Laboratories, Minneapolis, MN, USA) and assessed calcium deposition at 7 and 14 days.

For the Western blot analyses, BVSMCs were grown in a 6-well culture plate and incubated with the various reagents for indicated times. Cells were then washed with cold phosphate-buffered saline (PBS) and incubated with ice-cold lysis buffer containing 5 mmol/L Hepes (pH 7.9), 150 mmol/L NaCl, 26% glycerol (vol/vol), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mmol/L dithiothreitol (DTT), and 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF). Whole cell lysates (20 μg) were mixed with equal volume of 4× Laemmli sample buffer. The mixture and prestained molecular weight markers were boiled for 5 minutes and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to a polyvinylidene diflouride (PVDF) membrane (Millipore Corp., Bedford, MA, USA). The membrane was blocked in Tris-buffered saline containing 5% nonfat dry milk and 0.05% Tween-20 (TBST) for 1 hour, then incubated overnight at 4°C with rabbit antibodies against bovine fetuin-A (IgG fraction, 3 μg/mL) or OPG and RANK-L (1:100) (both from R&D Laboratories). The membrane was washed with TBST buffer, then incubated with goat antirabbit IgG peroxidase conjugate (1:5000 dilution). Immunodetection was done with the enhanced chemiluminescence (ECL) kit (NEN Life Science Products, Boston, MA, USA). The band intensity was analyzed by scanning densitometry (Molecular Analysis; Bio-Rad, Hercules, CA, USA).

The RT-PCR was performed using Titan one tube RT-PCR kit (Roche Diagnostics, Indianapolis, IN, USA). Briefly, total RNA was RT at 50°C for 30 minutes followed by PCR reaction with Titan enzyme mix (AMV reverse transcriptase and Taq DNA polymerase) in a 50 μL mixture containing 0.2 mmol/L deoxynucleoside triphosphate (dNTP), 5 U RNAse inhibitor, 5 mmol/L DTT, 20 pmol of sense and antisense primers. The sense primer for MGP was 5′-TACCAGACCGAGACCAACAGAG-3′ and the antisense primer was 5′-CACCACCGGGTCACGTCGC-3′. The reaction went for 35 cycles consisting of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 68°C, followed by a final extension step at 68°C for 10 minutes before cooling at 4°C. The RT-PCR products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. The housekeeping gene β-actin was used for internal control. The band density was analyzed by scanning densitometry (Molecular Analysis; Bio-Rad).

Statistical analyses

The relationship of demographic, laboratory, and calcification scores with serum levels of inhibitors was determined by Pearson correlation coefficients or Spearman rank-sum analysis. Differences in levels of inhibitors in patients with varying degrees of calcification were analyzed by Mann-Whitney U test or analysis of variance (ANOVA) with Tukey post hoc testing. Serum levels pre- and posttransplant were analyzed by Mann-Whitney U test. Immunostaining of arteries, or mineralization between groups of BVSMCs, were analyzed by ANOVA with Tukey post hoc testing. All studies were approved by the local Institutional Review Board.

RESULTS

In vivo studies

The serum level of fetuin-A was negatively correlated with the coronary artery calcification score ($r = -0.30$, $P = 0.034$) ($N = 51$), although the fetuin-A levels in subjects with no calcification (0.51 ± 0.04) ($N = 14$) did not differ from those with mild calcification (score 1 to 200) (0.47 ± 0.02) ($N = 15$) or with severe calcification (score > 200) (0.44 ± 0.2) ($N = 20$) although a trend was

present. There was no correlation with serum fetuin-A levels and aorta calcification score, age, time on dialysis, or serum levels of calcium, albumin, phosphorus, parathyroid hormone (PTH), and MGP. However the fetuin-A levels were lower in males (0.45 ± 0.11) ($N = 40$) than females (0.53 ± 0.16) ($N = 19$) ($P = 0.019$). The serum levels of OPG were positively correlated with the magnitude of coronary artery calcification ($r = 0.29$, $P = 0.045$) ($N = 48$). The serum levels of OPG were different with different levels of calcification (no calcification 6.23 ± 0.68) ($N = 18$); coronary artery calcification scores of 1 to 200, 9.63 ± 0.96 ($N = 19$); and coronary artery calcification >200, 11.16 ± 0.02 ($N = 29$) ($P < 0.002$). Furthermore, the serum OPG levels were greater in subjects with coronary artery calcification (10.56 ± 0.69, range 2.86 to 29.48) ($N = 48$) than with no coronary artery calcification (6.23 ± 0.68, range 1.47 to 12.06) ($N = 18$) ($P = 0.001$). Similarly, the serum OPG levels were greater in subjects with aorta calcification (10.88 ± 0.76, range 4.14–29.48) ($N = 35$) compared to no aorta calcification (7.67 ± 0.81, range 1.47–22.21) ($N = 31$) ($P = 0.005$). Serum OPG levels correlated with duration of dialysis in months ($r = 0.32$, $P = 0.008$) ($N = 68$), and increasing age ($r = 0.46$, $P < 0.001$) ($N = 68$), but did not correlate with serum levels of PTH, calcium, phosphorus, or calcium × phosphorus product. In contrast to fetuin-A and OPG levels, there was no correlation of serum MGP levels ($N = 54$) and calcification of the coronary arteries or aorta, no difference in MGP in those with and without calcification, nor any relationship with demographic or laboratory variables. Further analyses were done to determine if a ratio of one inhibitor to another, or a ratio of the calcium × phosphorus product to an inhibitor altered results. These analyses demonstrated no change to the relationships stated above.

To determine if the serum levels of these inhibitors changed with a functioning renal allograft, we measured serum levels in a subset of patients 15 to 20 months posttransplant. Serum fetuin-A levels ($N = 11$) ($P = 0.049$) and MGP levels ($N = 11$) ($P = 0.042$) increased after renal transplant, whereas serum OPG levels declined ($N = 19$) ($P < 0.001$) after renal transplant in patients with a well functioning allograft (Fig. 1).

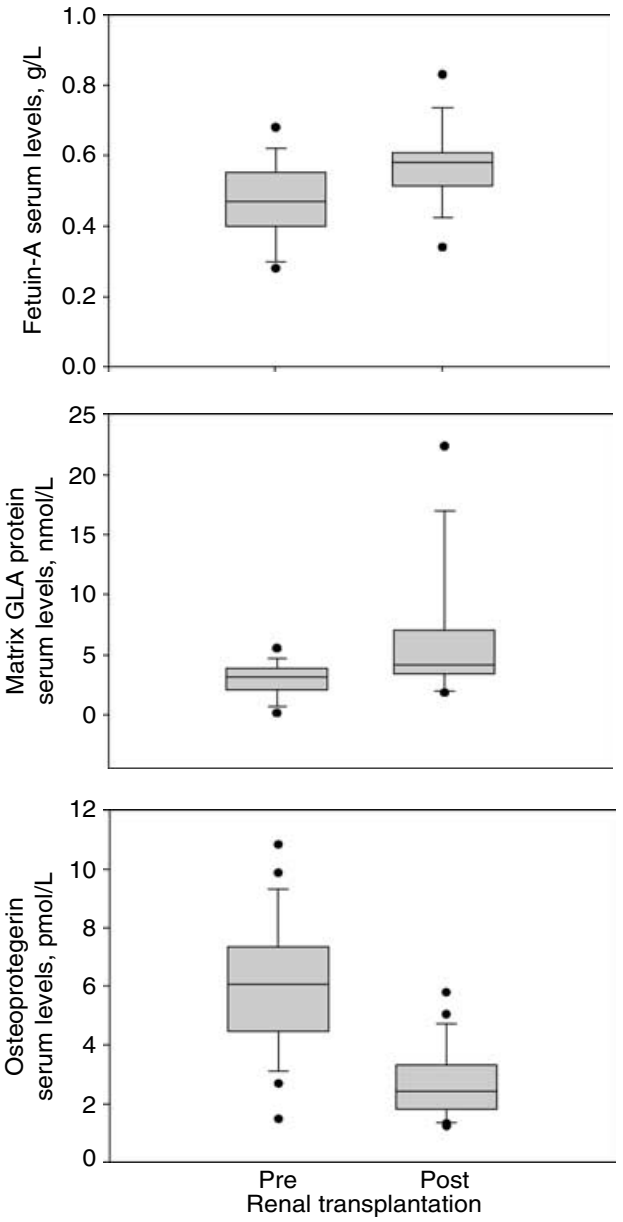

Fig. 1. Change in serum levels after renal transplantation. Patients had serum level for fetuin-A (top panel), matrix gla protein (MGP) (middle panel), and osteoprotegerin (OPG) (lower panel) at the time of renal transplant, and approximately 15 months later with a well-functioning allograft. The results show that serum levels for fetuin-A ($P = 0.049$) ($N = 11$) and MGP ($P = 0.042$) ($N = 11$) increased posttransplant, whereas OPG levels decrease ($P < 0.001$) ($N = 19$).

Ex vivo studies

We analyzed the inferior epigastric artery obtained at the time of renal transplant. Thirty-three arteries were of sufficient quantity and quality to analyze for the presence of mineralization by silver stain (MacNeal's stain), and for the relationship of the mineralization to immunostaining for fetuin-A and MGP, graded semiquantitatively (0 to 4+). The results of the scoring are presented in Figure 2, and demonstrate that as mineralization progresses histologically, there is increased immunostaining of both fetuin-A ($P = 0.003$) and MGP ($P < 0.001$). Representative staining are shown in Figure 3 and although there was a similar pattern of staining for fetuin-A and MGP, there was not complete overlap and double staining was not able to be performed.

In vitro studies

We have previously demonstrated that pooled human serum collected from dialysis patients can induce

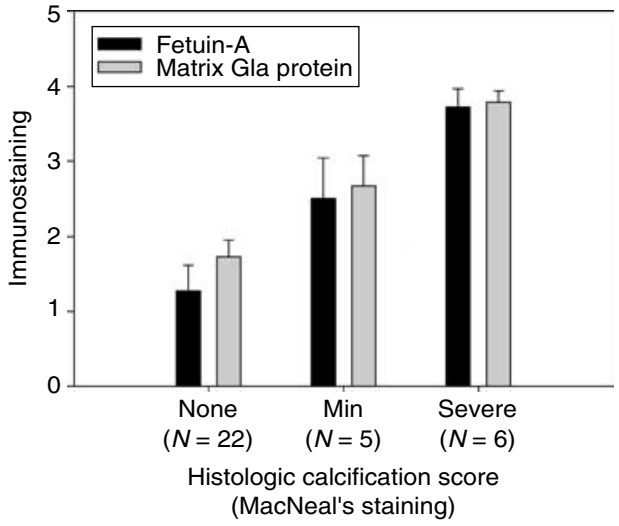

Fig. 2. Immunostaining for fetuin-A and matrix gla protein (MGP) in inferior epigastric arteries. Arteries obtained at the time of renal transplant were fixed and embedded in paraffin. The sections were stained by MacNeal's stain (black, calcification), and by immunostaining with anti-fetuin-A (■) and MGP (□) and graded semiquantitatively (0 to 4+). The result show a progressive increase in immunostaining with progressive calcification ($P = 0.003$ for fetuin-A and $P < 0.001$ for MGP).

mineralization in BVSMCs earlier and to a greater extent than pooled normal human serum [19, 20]. Therefore, we tested whether pooled uremic human serum also had a differential effect compared to pooled normal human serum in the local expression of calcification inhibitors.

Fetuin-A. As detailed above, the human arteries demonstrated positive immunostaining for the presence of fetuin-A when calcification was present. However, in the adult, fetuin-A is only synthesized by the liver, and is primarily deposited in bone [12]. To confirm this, we examined BVSMCs incubated in the presence of albumin (no fetuin-A), FBS (large amounts bovine fetuin-A), and human normal and uremic serum (contains human fetuin-A), and examined cell lysate for fetuin-A content by Western blot. The results demonstrated the presence of fetuin-A in BVSMCs cultured with FBS and human serum, but not albumin (data not shown) suggesting that the cells take up fetuin-A rather than synthesize it.

To determine if fetuin-A could specifically inhibit mineralization, we incubated BVSMCs in the presence of calcification media (β-glycerophosphate, insulin, and ascorbic acid) with 15% pooled human normal or uremic serum. We then added increasing amounts of fetuin-A to

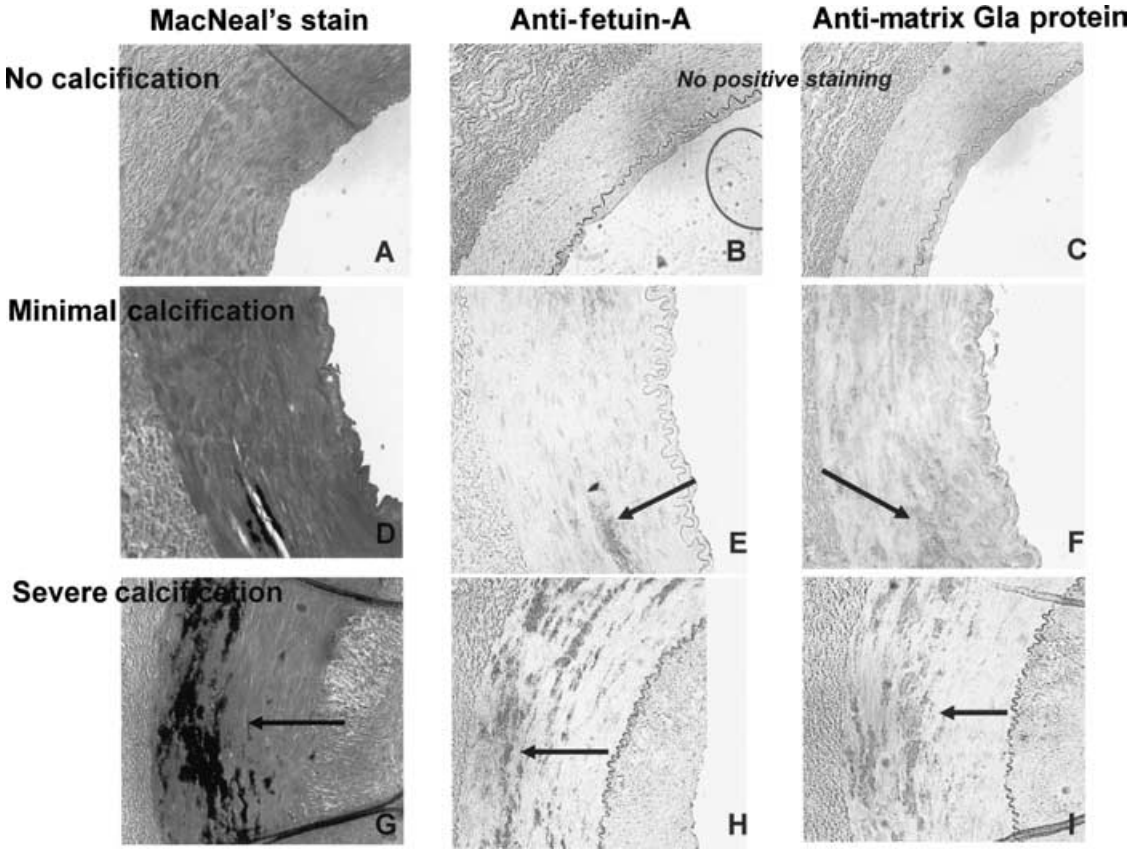

Fig. 3. Immunostaining for fetuin-A and matrix gla protein (MGP) in inferior epigastric artery. Arteries obtained at the time of renal transplant were fixed and embedded in paraffin. The sections were stained by MacNeal's stain (left column; black = calcification), and by immunostaining with anti-fetuin-A (middle column) and MGP antibodies (right column). This panel demonstrated representative sections from a noncalcified artery (upper row), 2+ calcified artery (middle row) and 4+ calcified artery (lower row). There was positive immunostaining for both fetuin-A and MGP in areas of calcification (arrows in *E*, *F*, *H*, *I*).

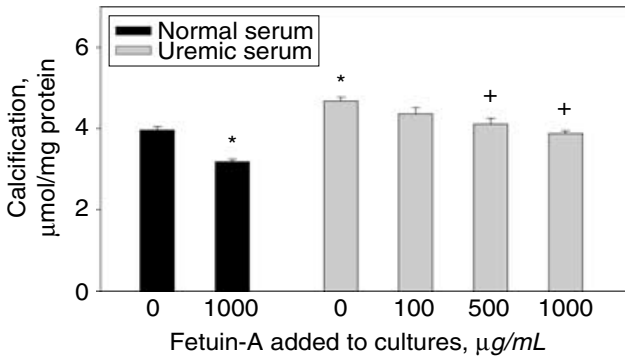

Fig. 4. Fetuin-A inhibits mineralization of bovine vascular smooth muscle cells (BVSMCs). BVSMCs were incubated in mineralizing conditions (β-glycerophosphate, insulin, and ascorbic acid) in the presence of normal control serum (■) or uremic serum (□) and fetuin-A added at 0, 100, 500, and 1000 μg/mL. Mineralization was assessed at 14 days with HCl extraction. The results demonstrate that fetuin-A inhibits mineralization in both control and uremic treated BVSMCs. $^*P < 0.001$ compared to normal serum-treated BVSMCs without fetuin-A; $+P < 0.01$ compared to same serum-treated BVSMCs without fetuin-A.

the mineralizing cultures and determined net calcium deposition by HCl extraction. The results show that 500 and 1000 μg/mL of added fetuin-A inhibited uremic serum mineralization down to baseline levels of mineralization observed in cells incubated with normal human serum without additional fetuin-A. In addition, similar findings were observed in BVSMCs incubated in the presence of normal serum when 1000 μg/mL fetuin-A was added to normal serum, indicating the inhibitory effects of fetuin-A were observed in both normal and uremic serum, suggesting that the fetuin-A inhibitory effects is not specific (Fig. 4) ($P < 0.01$). Of importance, the levels of fetuin-A in the pooled normal and uremic serum were not different (0.6 and 0.5 g/L) and this difference would be further minimized when the sera is diluted to 15% for the experiments.

OPG. To determine if the effect of OPG is via the vasculature, bone or both, we incubated BVSMCs and Saos osteoblasts in the presence of pooled human normal or uremic serum and determined the expression of OPG and RANK-L in cell lysate by Western blot. The results demonstrate that BVSMCs incubated with uremic serum had increased expression of OPG ($P = 0.008$) and RANK-L ($P = 0.03$) by Western Blot (Fig. 5, left panel) compared to BVSMCs incubated with normal serum. In contrast, uremic serum only increased RANK-L ($P = 0.01$), but not OPG in osteoblasts (Fig. 5, right panel). In addition, BVSMCs incubated with uremic serum had a net increase in secretion of OPG into the media (0.79 ± 0.12 pmol/L) (difference between media postincubation and media preincubation) versus no detectable OPG secretion in BVSMCs incubated in normal serum. When BVSMCs were induced to mineralize in the presence of uremic or normal serum with β-glycerophosphate, the addition of 200 ng/mL of OPG [21] did not affect mineralization in either normal or uremic serum at day 7 or day 14 [day 7 normal, 100%; normal + OPG, $106 \pm 6.8\%$; normal uremic, $124 \pm 19.1\%$; uremic + OPG $132 \pm 12.4\%$; day 14 normal, $133 \pm 6.7\%$ normal day 7; normal + OPG, $139 \pm 11.2\%$; uremic, $164 \pm 19.1\%$; uremic + OPG, $166 \pm 12.4\%$ ($N = 8$ or 9 per group from three separate experiments)].

MGP. To determine the role of MGP in uremic serum induced vascular calcification, we examined BVSMCs incubated in normal or uremic serum for 24, 48, or 72 hours for the expression of MGP by RT-PCR. However, only very faint expression was seen despite appropriate positive controls (data not shown). We then examined the expression of MGP by RT-PCR during BVSMC incubated in calcification media (β-glycerophosphate, insulin, and ascorbic acid) and found that MGP expression progressively increased with time and mineralization ($P < 0.001$), and was greater in the BVSMCs incubated with uremic serum compared to control human serum at 10 and 14 days (Fig. 6) ($P < 0.001$). Even if the expression of MGP was divided by the magnitude of calcification at each time point (as calcification was greater in uremic cultures), the expression was still greater in the BVSMCs treated with uremic serum, indicating that at a given level of calcification, MGP expression was still greater in BVSMCs incubated with pooled uremic serum compared to pooled normal serum. Unfortunately, MGP is insoluble [22], and thus in vitro experiments could not be done.

DISCUSSION

Vascular calcification in patients with CKD-5 is associated with increased cardiovascular morbidity and mortality [9, 10, 23]. However, studies evaluating the prevalence of vascular calcification indicate that from 0% to 50% (mean 17%) of patients on dialysis do not develop vascular calcification (reviewed in [11]). Animal "knockout" studies provide further support that calcification is normally actively inhibited in extraskeletal tissues, and that a lack of activity or absence of these inhibitors must be a factor when pathologic calcification occurs in extraskeletal locations [12]. Given that most, but not all CKD-5 patients, have vascular calcification there is likely an important role for naturally occurring inhibitors in the pathogenesis of vascular calcification in dialysis patients [24]. Each of the three inhibitors tested in this study, fetuin-A, OPG, and MGP, appear to be involved in vascular calcification, but differ in the magnitude and mechanism by which these proteins regulate calcification in uremia.

In the present study, we demonstrated a weak association with lower fetuin-A serum levels in CKD-5 patients with greater levels of coronary artery calcification,

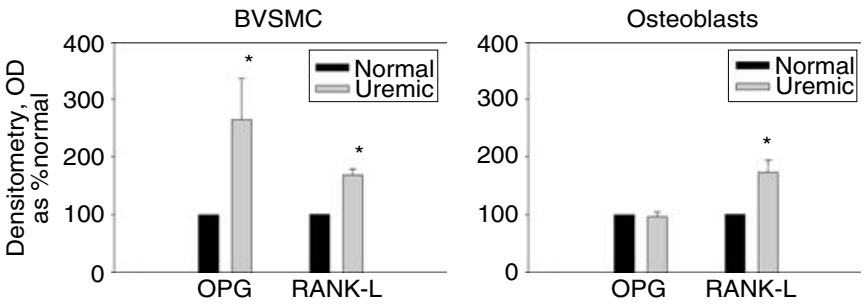

Fig. 5. Osteoprotegerin (OPG) and RANK-L expression in bovine vascular smooth muscle cells (BVSMCs) and Saos osteoblasts. BVSMCs (left panel) and osteoblasts (right panel) were incubated in normal control human serum (■) or pooled uremic serum (□) and OPG and RANK-L expression assessed in cell lysate by Western blot. In BVSMCs (left panel), uremic serum increased the OPG ($P = 0.008$) and RANK-L ($P = 0.03$) expression in BVSMCs compared to normal serum. In contrast, in osteoblasts (right panel) uremic serum only increased RANK-L ($P = 0.01$), but not OPG compared to normal serum.

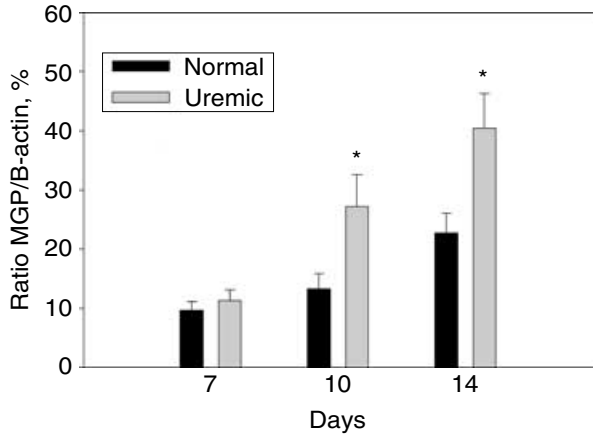

Fig. 6. Matrix gla protein (MGP) expression in mineralizing bovine vascular smooth muscle cells (BVSMCs). BVSMCs were incubated in mineralizing conditions (β-glycerophosphate and insulin) in the presence of healthy normal serum (■) or uremic serum (□) and MGP expression assessed at 7, 10, and 14 days by reverse transcription-polymerase chain reaction (RT-PCR). The results demonstrate that MGP expression is increased in uremic serum treated BVSMCs during mineralization ($P < 0.001$) and that at 10 and 14 days, uremic serum further increases MGP compared to normal serum-treated cells. $^*P < 0.001$.

although levels could not discriminate between those with and without calcification. However, our observations are consistent with previous studies demonstrating that low levels of fetuin-A are associated with increased cardiovascular mortality in dialysis patients [18]. Low levels of fetuin-A are also observed in dialysis patients with calcific uremic arteriolopathy [15]. Furthermore, adding fetuin-A back to serum of patients with calcific uremic artriolopathy can reduce mineral precipitation in vitro. Fetuin-A has diverse biologic activity, is constitutively secreted by liver parenchyma cells, is an abundant circulating protein in serum, and is primarily located in bone (reviewed in [12]). Fetuin-A avidly binds calcium phosphate [25], and thus acts as a buffer of serum calcium-phosphate to avoid extraskeletal calcification in states of serum supersaturation. Indeed, the fetuin-A knockout mice develop extraskeletal calcification, but only in situations of excess calcium by either feeding the animals calcium and vitamin D, or breeding them on a strain of mice predisposed to extraskeletal calcification [15]. This role of fetuin-A as a serum "buffer" for calcium phosphate is also consistent with our in vitro findings that fetuin-A dose-dependently inhibited mineralization in BVSMCs, similar to previous data in osteoblasts [26]. Thus, our findings of low serum levels of fetuin-A associated with vascular calcification in dialysis patients may be due to decreased (or overwhelmed) buffering capacity of serum in the presence of low serum fetuin-A levels, leading to extraskeletal calcification.

We also demonstrated positive fetuin-A immunostaining of arteries of CKD-5 patients in areas of calcification. However, we were unable to determine why fetuin-A was localized to areas of calcification, although there are several possible explanations. First, the fetuin-A may bind excess calcium phosphate in the serum, then precipitate to allow efficient removal by vascular smooth muscle cells of excess calcium phosphate from the circulation. This is supported by data demonstrating that fetuin-A facilitates the uptake of cationic macrophage deactivating molecules in macrophages [27]. Similarly, a second alternative is that if the normal removal system (presumably reticuloendothelial system) is overwhelmed, this serum fetuin-mineral complex deposits into arteries leading to local cell injury and vascular abnormalities. Our findings that fetuin-A appeared to be taken up, as opposed to synthesized, by the vascular smooth muscle cells is consistent with either of these possibilities. A third possibility is that the fetuin-A-calcium-phosphorus complex may also deposit in the vasculature in states of abnormal bone remodeling. Price et al [28–31] in a series of experiments has demonstrated that fetuin-A complexed to calcium and phosphorus is released from bone in a rat model of high turnover bone and vascular calcification induced by high dose vitamin D. Furthermore, this vascular calcification could be inhibited in the presence of bisphosphonates, and the serum levels of this fetuin-A complex correlated with arterial calcification in the rat. A last, less likely, possibility is that the serum fetuin-A binds to calcium that is already deposited in the arteries, acting as a "homing pigeon" to go to the deposits, presumably as an attempt to stop extension of the deposit, or enable phagocytosis of the deposit. Clearly, more work is needed to fully understand the role of fetuin-A in the regulation of vascular calcification.

The interpretation of our OPG results is also complex. OPG knockout mice develop arterial calcification, implying that OPG is an important inhibitor of vascular calcification. However, in the present study, we demonstrated that increased OPG serum levels were associated with increased coronary artery and aorta calcification. This apparent paradoxic association has also been observed in the general population [32], and in hemodialysis patients [33, 34]. There are several potential explanations for these findings. One potential explanation is that the serum assays detect nonfunctional OPG fragments that are normally cleared in CKD. Supporting this is our finding that serum levels decreased posttransplant, whereas both fetuin-A and MGP levels increased. In addition, serum OPG was positively correlated with decreasing creatinine clearance and increased duration of dialysis, and vascular calcification is more common with increased duration of dialysis. Against this possibility is that multivariate analysis in the study by Nitta et al [33, 34] demonstrated OPG levels were independent predictors of vascular calcification, and the study Kazama et al [35] demonstrating that serum from CKD patients was able to appropriately bind RANK-L in vitro, suggesting intact biologic activity. A second possibility is that the elevated serum OPG is released by BVSMCs that are calcifying, and therefore behaving like osteoblasts. Our in vitro data support this, in that uremic serum increased OPG and RANK-L production by vascular smooth muscle cells. The role of OPG in bone is to serve as a decoy receptor, binding to the RANK-L receptor on osteoblasts, thereby preventing osteoclast binding and activation. Thus, it is possible that elevated OPG levels reflect an ongoing attempt of arteries to remodel, supported by the observation of an occasional osteoclast like cell in arterial calcification [36]. The lack of effect of exogenously added OPG in vitro in our studies may be due to the absence of osteoclast like cells in this culture system. A third explanation for elevations in OPG associated with arterial calcification is that the circulating OPG is released from bone, and reflects the state of bone turnover, which in turn determines the degree of vascular calcification. Data supporting this view include that OPG administration to OPG−/− mice reverses the bone phenotype but can only prevent, not reverse, vascular calcification [37]. In postmenopausal osteoporosis women, and in rats, the administration of OPG reduces bone resorption and therefore bone turnover [38, 39]. London et al [40] recently demonstrated in CKD-5 patients that low bone turnover by biopsy was associated with increased vascular calcification and in a study of CKD-5 patients by Coen et al [41], elevations in serum OPG were associated with low turnover bone disease. Thus, the patients with the higher OPG levels may have had lower bone turnover, which does not allow bone to take up a mineral load [42], thereby leading to extraskeletal calcification. However, others have found that elevated OPG in CKD-5 patients was associated with high turnover bone disease [43]. This conflicting data are likely due to the complex interaction of PTH and OPG, and more research is required to fully understand the physiologic function of OPG in vascular calcification.

In contrast to fetuin-A and OPG, MGP expression appears to be predominantly a local regulator of vascular calcification. MGP knockout mice have excessive cartilage and growth plate mineralization and excessive arterial medial calcification [14]. In patients undergoing cardiac valve replacement warfarin is associated with increased valve calcification [44], and in dialysis patients, warfarin is associated with calcific uremic arteriolopathy [45]. The mechanism by which MGP inhibits extraskeletal calcification appears to be via modulation of bone morphogenic protein-2 (BMP-2) activity, and BMP-2 is known to induce vascular calcification [22, 46]. In the present study we demonstrated expression of MGP in the inferior epigastric artery, with increased expression associated with increased calcification. The antibody used in the present study was against total MGP, and thus we cannot confirm whether the MGP was active or not. Nonetheless, these results confirm that MGP is up-regulated in the presence of arterial calcification in humans as has been previously demonstrated in arteries from non-CKD patients [47, 48]. In addition, we demonstrated that MGP expression was minimal in BVSMCs that were not mineralizing, similar to recent data by Zebboudj, Shin, and Bostrom [49]. However, it increased with progressive mineralization in BVSMCs and further increased in the presence of uremic serum compared to normal serum, even when normalized for the increased calcification in the presence of uremic serum. This data confirms that MGP is increased in vitro in response to mineralization as has been previously shown [49, 50]. The data also imply that MGP is not defective in the presence of pooled uremic serum. In addition, we measured serum levels of MGP, but found no association with the presence of arterial calcification in the CKD patients. This is in contrast to the study by Jono et al [51], demonstrating an association of low MGP levels in non-CKD patients with coronary artery calcification. These differences may be due to a small sample size in our study, or different assays. Alternatively, circulating MGP levels in CKD-5 patients may not reflect the increased local activity. Thus, at the present time, there is no data supporting abnormal function of MGP in uremia to account for the increased vascular calcification in CKD patients, although additional studies are required.

CONCLUSION

We have presented in vivo, ex vivo, and in vitro studies supporting that naturally occurring calcification inhibitors are important in the excess vascular calcification

observed in CKD-5. Although more work is required to fully elucidate the precise role of these inhibitors, we can hypothesize the following. Fetuin-A appears to function as a circulating inhibitor, as a decrease in the serum level in CKD is associated with increased calcification. This is likely due to a deficiency of this important naturally occurring inhibitor that binds calcium phosphate as a soluble complex for efficient clearance, and/or that the available fetuin-A is depleted in dealing with the elevated serum calcium phosphate product so commonly found in CKD. What remains unclear is the process by which fetuin-A is deposited into the arterial wall, and the fate of these deposits. We believe that the predominant reason that elevated serum levels of OPG in CKD patients and the general population are associated with increased vascular calcification, is due to a primary effect of OPG on bone turnover, the natural reservoir for mineral. Thus, when OPG is elevated, there is less bone turnover, impairing the ability of bone to take up a calcium load. In contrast, MGP functions locally and our data suggest that this inhibitor is appropriately functional in CKD. It should be emphasized that our in vivo and ex vivo data are associations, and not direct cause-effect because of the cross-sectional nature of the studies, and that further work using in vitro models and animal models is necessary. Despite this limitation, the data are intriguing and suggest that not only is calcification an active cell-mediated process, but that there are natural defenses to calcification. This implies that augmenting these defenses may have an important therapeutic potential in preventing the devastating effects of vascular calcification observed in CKD patients.

ACKNOWLEDGMENTS

This work was presented in part at the 35th and 36th Annual Meeting of the American Society of Nephrology in 2002 and 2003, and was supported by grants from the Veterans Affairs Department (S.M.M.), National Institutes of Health (S.M.M.), National Kidney Foundation of Indiana (S.M.M.), and Genzyme Corporation (N.X.C.). The authors wish to thank Michelle Murray for her excellent secretarial assistance.

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