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Phosphorus and uremic serum up-regulate osteopontin expression in vascular smooth muscle cells

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Phosphorus and uremic serum up-regulate osteopontin expression in vascular smooth muscle cells.

Background. Dialysis patients have accelerated atherosclerosis, with extensive calcification of both the intima and media. Cross-sectional studies have implicated hyperphosphatemia in this process, but the mechanism is unclear.

Methods. To test the hypothesis that hyperphosphatemia and/or uremia induces vascular calcification, bovine vascular smooth muscle cells (BVSMC) were treated with increasing concentrations of β-glycerophosphate, a phosphate donor, in the presence or absence of inhibitors for sodium/phosphate (Na/Pi) co-transport (foscarnet) or alkaline phosphatase (levamisole) for 48 hours. BVSMC also were incubated for various times with DMEM plus 15% pooled uremic sera from patients with low (LP) or high serum phosphorus (HP), or from pooled healthy control serum. Calcification in BVSMC was examined by quantitation of calcium deposition. Osteopontin expression and alkaline phosphatase activity were assessed by Western blotting and a colorimetric assay.

Results. β-glycerophosphate increased osteopontin expression and alkaline phosphatase activity in BVSMC. Inhibition of either alkaline phosphatase activity or Na/Pi co-transport abolished this effect. Compared to incubation with control human serum, BVSMC cultured with uremic sera had increased mineral deposition. Uremic sera also increased alkaline phosphatase activity and osteopontin expression in BVSMC. The addition of β-glycerophosphate to uremic HP or LP sera did not further augment osteopontin expression. Blocking Na/Pi co-transport or alkaline phosphatase activity only partially inhibited uremic sera-induced osteopontin expression, indicating that other non-Na/Pi co-transport dependent mechanisms also are involved.

Conclusion. β-glycerophosphate and uremic sera induce calcification and osteopontin expression in BVSMC. The uremic sera-induced osteopontin expression in BVSMC is partially mediated through alkaline phosphatase activity and a Na/Pi co-transporter dependent mechanism. However, other non-Na/Pi dependent mechanisms also contribute to accelerated vascular calcification in patients with ESRD.

Key words: calcification, hyperphosphatemia, uremia, intima and media, end-stage renal disease, atherosclerotic placques.

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Cardiovascular disease and stroke are the leading causes of death in patients with end-stage renal disease (ESRD), at a risk that is 10- to 20-fold the age- and sex-matched general population [1]. The assessment of coronary arteries by new non-invasive imaging techniques such as electron beam CT scan (EBCT) has heightened the awareness that over 90% of atherosclerotic plaques observed in the aging population are calcified [2]. Studies evaluating coronary calcification by EBCT in patients with ESRD have demonstrated two- to fivefold more coronary artery calcification than age and sex matched individuals with angiographically-proven coronary artery disease [3]. Furthermore, in a follow-up of these hemodialysis patients, every patient had an increase in their calcification score just one to two years later [4]. Recent studies evaluating vascular calcification using EBCT and other techniques in dialysis patients have demonstrated an elevated serum phosphorus value, an elevated serum calcium X phosphorus product, or increased calcium load as risk factors [3, 5–7]. However, the mechanism by which the elevated concentration of these ions leads to calcification in dialysis patients is unknown.

Until recently, vascular calcification was considered to be a passive process. However, considerable evidence now suggests that vascular calcification in non-dialysis patients is regulated in a manner very similar to that of developing bone, with apatite crystal nucleation, growth, and possible degradation in association with an extracellular matrix that regulates tissue mineralization (reviewed by Giachelli [8] and Farzaneh-Far et al [9]). In dialysis patients, the presence of extra-skeletal calcification has been previously attributed to secondary hyperparathyroidism and metastatic calcification, whereby the elevated concentrations of calcium and phosphorus in the serum of dialysis patients leads to supersaturation and subsequent deposition in the form of hydroxyapatite. However, we have recently demonstrated the expression of "bone" matrix proteins by vascular smooth muscle cells, including osteopontin, bone sialoprotein, alkaline phosphatase, and type I collagen, in arterioles of skin

and in the inferior epigastric artery of patients undergoing renal transplant [7, 10]. This implies that vascular calcification in dialysis patients is not simply metastatic, but rather an active, cell-mediated process.

We hypothesize that the uremic state leads to the deposition of these bone proteins, with subsequent, perhaps accelerated calcification, in the presence of the positive calcium and phosphorus balance so common in ESRD patients. To test this hypothesis and further clarify the molecular mechanisms regulating vascular calcification, we characterized the response of bovine vascular smooth muscle cells (BVSMC) to β-glycerophosphate, a phosphate donor, or pooled uremic sera from patients with persistently low versus high serum phosphorus and from pooled healthy control serum. Our results demonstrate that uremic sera potentiate calcification and enhance osteopontin expression in BVSMC. In addition to hyperphosphatemia, other uremic factors may contribute to the accelerated vascular calcification in patients with ESRD.

METHODS

Cell cultures

Bovine vascular smooth muscle cells were obtained by a modification of the explant method originally described by Ross [11]. Briefly, medial tissue was separated from segments of bovine aorta after removal of endothelial cells from aorta segments. Small pieces of tissue (1 mm^2) were placed in a 6-well culture dish and cultured for two weeks in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose supplemented with 10% fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO, USA) in a 95%/5% air/CO_2 humidified environment at 37°C. Cells that migrated from the explants were collected and maintained in DMEM containing 10% FBS supplemented with 100 U/mL of penicillin G, 0.25 μg/mL of amphotericin B and 100 μg/mL of streptomycin. The media were replaced every two to three days. Only cells between passages 2 and 8 were used for experiments [12]. To examine the role of phosphorus in osteopontin expression, BVSMC were treated with various concentrations of β-glycerophosphate, a phosphate donor, in the presence or absence of an inhibitor for sodium/phosphate (Na/Pi) co-transport (foscarnet, 1 mmol/L; Sigma) or alkaline phosphatase (ALP; levamisole, 100 μmol/L; Sigma) for 48 hours. In order to determine whether uremic factors further contribute to accelerated vascular calcification in dialysis patients, BVSMC were incubated for 2, 3, 7, 10 and 14 days with DMEM plus 10% sera from pooled uremic sera from patients with persistently low or high serum phosphorus and from pooled healthy control serum as described below.

Collection of pooled uremic and control sera

Uremic and control sera were pooled from patients who were on dialysis for at least two years. Utilizing our dialysis database, patients were identified with the following: those with a mean serum phosphorus ≤5.5 mg/dL over the preceding two years, and patients with a mean serum phosphorus ≥6.5 mg/dL. Thirteen patients in the high (>6.5 mg/dL) phosphorus groups consented to have sera collected (HP). These patients were then matched for age and presence or absence of diabetes with 14 patients who had phosphorus persistently <5.5 mg/dL (LP). Sera from normal healthy individuals of similar ages served as controls. The sera were pooled and frozen at –20°C in aliquots for use in tissue culture. The pooled sera were analyzed for pH by Corning pH meter 240; total alkaline phosphatase, electrolytes, calcium, and phosphorus by chemistry autoanalyzer (Boeringer Roche Diagnostics, Indianapolis, IN, USA); parathyroid hormone (PTH) by intact assay (Nichols Laboratory, San Juan Capistrano, CA, USA); bone alkaline phosphatase by enzyme-linked immunosorbent assay (ELISA; Metra Biosystems, Mountain View, CA, USA); and C-reactive protein (Alpha Diagnostics, San Antonio, TX, USA).

Induction of calcification

Bovine VSMC were cultured in DMEM + 10% FBS until confluent and then switched to calcification medium (DMEM containing 10 mmol/L sodium pyruvate, 10^{-7} mol/L insulin, 50 μg/mL ascorbic acid, 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mmol/L β-glycerophosphate), in the presence of either 15% normal human serum or 15% uremic (HP) serum for up to 21 days. The medium was replaced with fresh medium every two to three days and the first day of culture in calcification media was defined as day 0.

Quantification of calcium deposition

Bovine VSMC were decalcified with 0.6 N HCl for 24 hours. The calcium content of HCl supernatants was determined colorimetrically by the *o*-cresolphthalein complexone method (Calcium kit; Sigma) as previously described by Jono et al [13]. After decalcification, the cells were washed three times with PBS and solubilized with 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS). The protein content was measured by a DC protein assay kit (Bio-Rad, Richmond, CA, USA). The calcium content of the cell layer was normalized to protein content.

Western blot analysis

Bovine VSMC were grown in a 6-well culture plate and incubated with various reagents for 48 hours. 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 pre-stained molecular weight markers were boiled for five minutes and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrophoretically transferred to a polyvinylidene difluoride (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 one hour, then incubated with rabbit antibodies against osteopontin (1:1000; gift of Dr. Larry Fisher, NIH, Bethesda, MD, USA) overnight at 4°C. The membrane was washed with TBST buffer and then incubated with goat anti-rabbit IgG peroxidase conjugate (1:5000 dilution). The immunodetection was accomplished using the Enhanced Chemiluminescence Kit (NEN Life Science Products, Boston, MA, USA). The band intensity was analyzed by scanning densitometry (Molecular Analysis; Bio-Rad).

Alkaline phosphatase and phosphorus ion assays

For cellular alkaline phosphatase activity measurements, cells were washed three times with PBS and cellular proteins solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged. Supernatants were assayed for alkaline phosphatase activity as described previously by Bessey and Brock [14]. One unit was defined as the activity producing 1 nmol of p-nitrophenol for 30 minutes. Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad), and alkaline phosphatase activity normalized to cellular protein content.

Phosphorus concentration in the culture media was measured prior to addition to BVSMC and after 48 hours of incubation by the phosphomolybdate complex method (phosphorus kit; Sigma).

Statistical analysis

The difference of alkaline phosphatase activity levels in response to various reagents was compared by analysis of variance (ANOVA) with Fisher's post-hoc analysis. Statistical significance of difference in expression of osteopontin and calcium deposition was performed by one-way ANOVA. Comparisons were made between densitometric analyses of Western blots of treated and control cells. The results are expressed as mean ± SEM, with $P < 0.05$ considered significant (StatView; SAS Institute, Inc., Cary, NC, USA).

RESULTS

Phosphorus-induced osteopontin expression and the induction is dependent on Na/Pi co-transport and alkaline phosphatase activity

We first examined the effect of phosphorus on osteopontin expression by incubating BVSMC with increasing concentrations of β-glycerophosphate for 48 hours. Cell lysate was extracted and Western blotting was performed. As shown in Figure 1, β-glycerophosphate induced osteopontin expression in a dose dependent manner. While treatment of cells with 4 mmol/L β-glycerophosphate had little effect on osteopontin expression, 8 mmol/L β-glycerophosphate induced a detectable increase in osteopontin expression. The transport of phosphorus into cells is mediated through the Na/Pi co-transporter; thus, we next examined the role of Na/Pi co-transporter on osteopontin expression. BVSMC were incubated with phosphonoformic acid (foscarnet), a competitive inhibitor of Na/Pi co-transporter, in the presence or absence of 12 mmol/L β-glycerophosphate for 48 hours. The results show that foscarnet completely inhibits the induction of osteopontin in response to β-glycerophosphate as demonstrated by Western blotting (Fig. 2). This suggests that Na/Pi co-transport is necessary for phosphorus-induced osteopontin expression in BVSMC. To confirm the importance of alkaline phosphatase, which can cleave β-glycerophosphate into free phosphorus, in the phosphorus-induced osteopontin expression in BVSMC, cells were treated with levamisole, a specific inhibitor of alkaline phosphatase in the presence of β-glycerophosphate. Western blotting showed that inhibition of alkaline phosphatase activity by levamisole abolished β-glycerophosphate-induced osteopontin expression in BVSMC (Fig. 2). This indicated that hydrolysis of β-glycerophosphate by alkaline phosphatase is required for its induction of osteopontin expression in BVSMC. To further clarify the role of alkaline phosphatase in osteopontin expression in response to β-glycerophosphate, we measured alkaline phosphatase activity in BVSMC. β-glycerophosphate increased alkaline phosphatase activity by threefold and

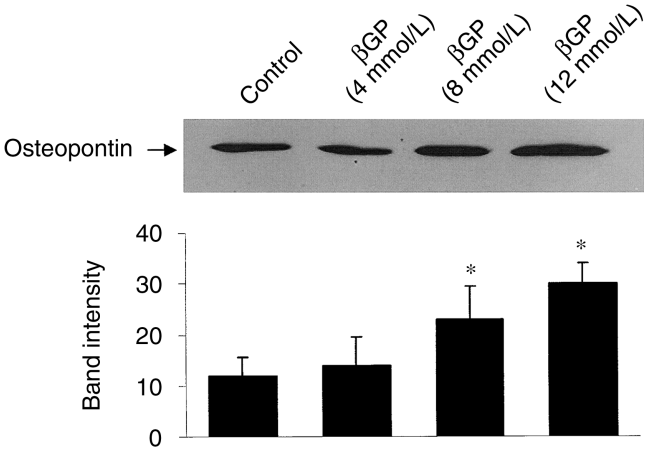

Fig. 1. Western blot analysis of β-glycerophosphate (βGP)-induced osteopontin expression in bovine vascular smooth muscle cells (BVSMC). Cells were treated with 4 to 12 mmol/L of β-glycerophosphate (βGP) for 48 hours. Cells were lysed and Western blotting was performed using osteopontin antibody. The band intensity was analyzed by scanning densitometry. Data are shown as mean ± SD from three experiments. Groups with * are different from controls ($P < 0.05$).

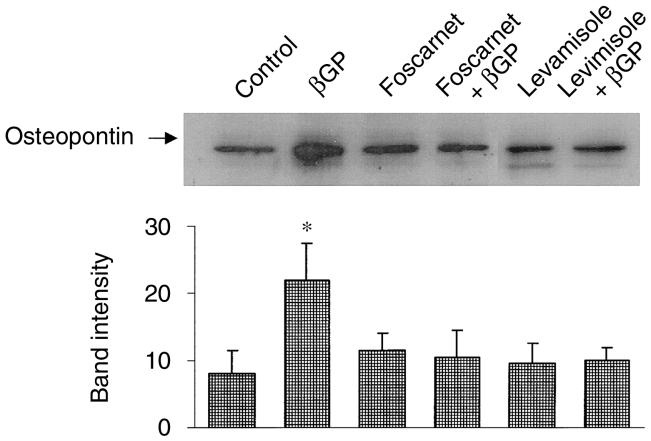

Fig. 2. Western blotting of osteopontin expression in BVSMC treated with β-glycerophosphate (βGP) in the presence or absence of various inhibitors. Cells were treated with β-glycerophosphate (βGP, 12 mmol/L) in the presence or absence of inhibitors for Na/Pi co-transporter (foscarnet) or alkaline phosphatase activity (levamisole) for 48 hours. Cells were lysed and Western blotting performed using osteopontin antibody. The band intensity was analyzed by scanning densitometry. Data are shown as mean ± SD from three experiments. Groups with * are different from controls ($P < 0.05$).

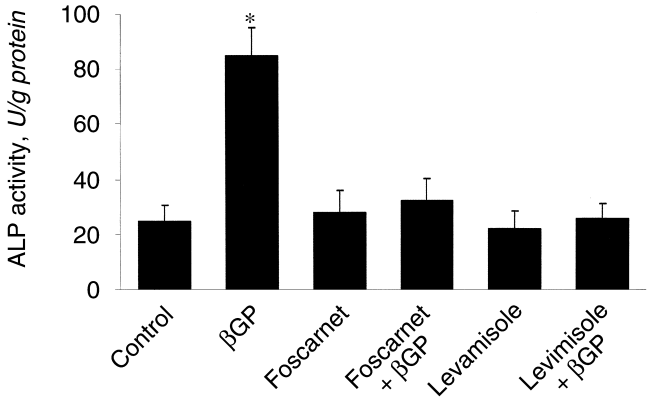

Fig. 3. Alkaline phosphatase (ALP) activity in BVSMC treated with β-glycerophosphate (βGP) in the presence or absence of various inhibitors. Cells were treated with β-glycerophosphate (βGP, 12 mmol/L) in the presence or absence of inhibitors for Na/Pi co-transporter (foscarnet) or alkaline phosphatase activity (levamisole) for 48 hours. Cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and supernatants were assayed for alkaline phosphatase activity. One unit was defined as the activity producing 1 nmol of p-nitrophenol for 30 minutes. Alkaline phosphatase activity was normalized to cellular protein content. Data are shown as mean ± SD from three to four experiments. Groups with * are different from controls ($P < 0.05$).

the induction of alkaline phosphatase activity by β-glycerophosphate was inhibited when cells were treated with an inhibitor of Na/Pi co-transport (foscarnet; Fig. 3). As expected, levamisole, a specific inhibitor for alkaline phosphatase also abolished the increase in alkaline phosphatase activity in response to β-glycerophosphate in BVSMC (Fig. 3). Thus, β-glycerophosphate-induced osteopontin expression in BVSMC is indeed dependent on Na/Pi co-transport and alkaline phosphatase activity.

Uremic serum increased calcium deposition and osteopontin expression in BVSMC and inhibition of Na/Pi co-transport partially blocked the effect of uremia

To determine if uremia contributes to accelerate vascular calcification, the effect of uremic sera on calcification of BVSMC cultures was examined. The analysis of the sera is given in Table 1. As expected, compared to control serum, the uremic sera had elevated levels of phosphorus, parathyroid hormone, total and bone alkaline phosphatase, and C-reactive protein. In the presence of uremic sera plus calcification medium, calcium deposition significantly increased in a time-dependent manner in BVSMC, as compared to cells treated with normal human serum plus calcification medium (Fig. 4). This indicates that uremic serum potentiates calcification in BVSMC.

To examine the phosphorus levels in the cultured media of BVSMC as a result of addition of β-glycerophosphate, the inorganic phosphorus concentration was measured in media before its addition to BVSMC, and after 48 hours of incubation (Pre-Post). Results showed that pre-post values of phosphorus were 0.30 to 0.25 mmol/L for DMEM with 10% FBS; 0.22 to 8.55 mmol/L for DMEM with 10% FBS plus 10 mmol/L β-glycerophosphate; 0.58 to 0.46 mmol/L for DMEM with 10% normal human serum; 0.69 to 9.67 mmol/L for DMEM with 10% FBS plus 12 mmol/L β-glycerophosphate; 0.67 to 0.60 mmol/L for DMEM with 10% uremic serum; and 0.87 to 10.45 mmol/L for DMEM with 10% uremic serum plus 12 mmol/L β-glycerophosphate. Thus, β-glycerophosphate was converted by cells to inorganic phosphorus in

Table 1. Results of analysis of pooled sera

	Control sera	Uremic sera	
		Low phosphate	High phosphate
Number of patients	14	14	13
Age *years*	48.7	50.2	46.2
Gender *% female*	36	36	46
Years on HD		6.5	6.9
% Diabetic		21	23
% on Vitamin D		64	23
Kt/V		1.55	1.45
Na *meQ/L*	142	141	140
K *meQ/L*	4.3	4.8	5.1
Albumin *g/L*	4.8	4.2	4.0
pH	8.05	8.37	8.15
Calcium *mg/dL*	9.4	9.4	8.3
Phosphate *mg/dL*	3.5	5.5	7.4
PTH *pg/mL*	28	266	496
Total alkaline phosphatase *IU*	62	126	155
Bone alkaline phosphatase *IU*	11.3	24.3	40
C-reactive protein *μg/mL*	0.004	0.086	32.4

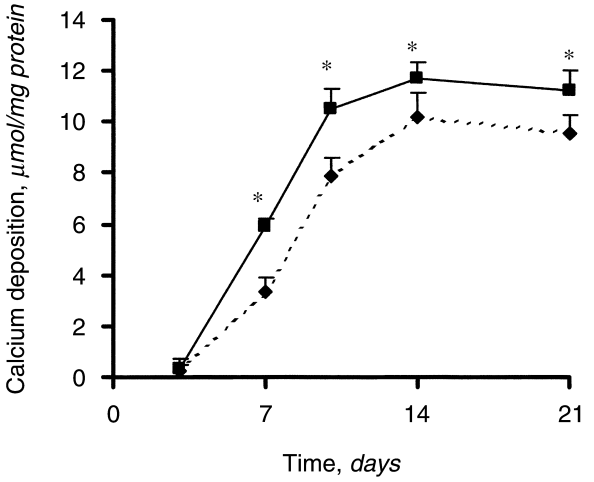

Fig. 4. Effects of uremic serum on calcium deposition in BVSMC. After confluence, BVSMC were incubated with calcification media in the presence of either 15% normal human serum (◆) or 15% uremic serum (■) for up to 21 days. The medium was changed every 2 to 3 days. The calcium content was measured at the indicated times by *o*-cresolphthalein complexone method, normalized by cellular protein content, and are presented as mean ± SD. Groups with * are significantly different between normal and uremic treated cells.

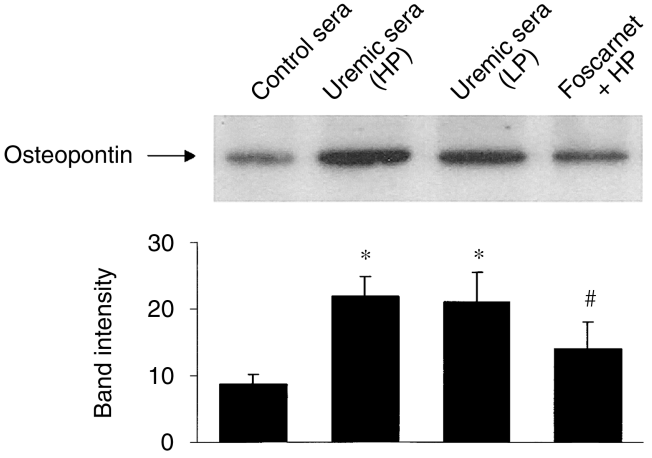

Fig. 5. Western blotting of osteopontin expression in BVSMC treated with uremic sera. Cells were incubated in the presence of pooled sera from controls, uremic high phosphorus (HP) or uremic low phosphorus (LP) for 48 hours. To determine the role of Na/Pi co-transport in the uremic response, cells were treated with HP in the presence of inhibitor of Na/Pi co-transport (foscarnet) for 48 hours. Cells were lysed and Western blotting was performed using osteopontin antibody. The band intensity was analyzed by scanning densitometry. Data are shown as mean ± SD from three experiments. Groups with * and # are different from controls and different between groups ($P < 0.05$).

DMEM with FBS, normal human serum and uremic serum. In addition, when diluted to 10% sera, the final phosphorus concentration in uremic sera was not different from that in the control serum groups.

To test whether up-regulation of osteopontin is due to elevated phosphorus and/or other uremic factors, we incubated BVSMC in the presence of pooled sera from healthy volunteers or dialysis patients with low or high phosphorus. As shown in Figure 5, BVSMC treated with uremic sera had increased expression of osteopontin compared to cells treated with normal serum, suggesting an important role of uremic serum in vascular calcification. However, there was no significant difference in osteopontin expression between cells treated with uremic low phosphorus serum or uremic high phosphorus serum. Blocking Na/Pi co-transporter with foscarnet only partially inhibited uremic serum-induced osteopontin expression, indicating that in addition to Na/Pi co-transport dependent mechanisms, there are other uremic factor(s) that may modulate osteopontin expression in BVSMC.

To further characterize the mechanism by which uremic serum induced osteopontin expression, BVSMC were treated with normal serum or uremic sera with high or low phosphorus and alkaline phosphatase activity was measured. Uremic serum doubled alkaline phosphatase activity in BVSMC ($P < 0.05$; Fig. 6A), although the degree of increase in alkaline phosphatase activity was similar between cells treated with high phosphorus uremic serum and low phosphorus uremic serum. Inhibition of alkaline phosphatase with levamisole also partially blocked uremic sera-induced osteopontin expression in BVSMC (Fig. 6B). These results again suggest that increased alkaline phosphatase activity and Na/Pi co-transporter are only partially responsible for uremic sera-induced osteopontin expression in BVSMC. To determine if the effects of phosphorus are additive to uremic serum, we incubated BVSMC with control, uremic low phosphorus or high phosphorus sera plus 12 mmol/L β-glycerophosphate and osteopontin expression analyzed by Western blotting. There was no additive effect of β-glycerophosphate above that induced by uremic serum for osteopontin expression in BVSMC, whereas the addition of β-glycerophosphate nearly doubled osteopontin expression in BVSMC incubated in control serum (Fig. 7).

DISCUSSION

Dialysis patients are known to have many risk factors for vascular disease such as a history of hypertension, diabetes, oxidized lipids, elevated homocysteine and C-reactive protein [1, 15]. In addition to these traditional vascular risk factors, there is increasing evidence that elevated serum phosphorus, serum calcium X phosphorus product, and/or calcium load in the form of calcium-containing phosphate binders are associated with various vascular end-points including coronary artery calcification by electron beam computed tomography [3], calcific uremic arteriolopathy (calciphylaxis) [7], carotid and aortic calcification [6, 16], hemodynamic abnormalities [5], and mortality (abstract; Levin et al, *J Am Soc Nephrol* 10:A1109, 1999) [17]. One potential mechanism by which

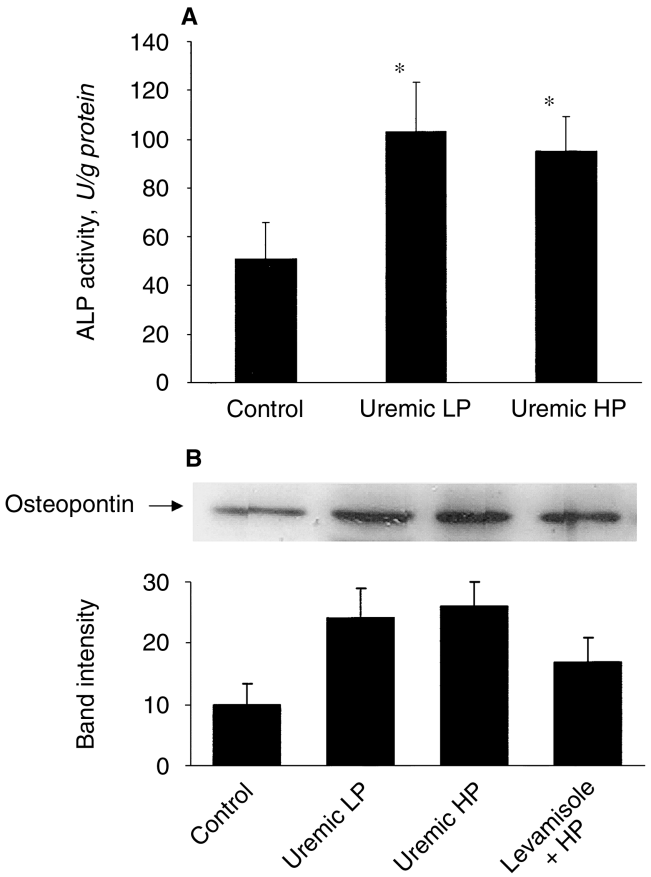

Fig. 6. Alkaline phosphatase (ALP) activity and osteopontin expression in BVSMC treated with uremic sera. (*A*) Cells were incubated in the presence of pooled sera from controls, uremic high phosphorus (HP) or uremic low phosphorus (LP) for 48 hours. Cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and supernatants were assayed for alkaline phosphatase activity. One unit was defined as the activity producing 1 nmol of p-nitrophenol for 30 minutes. Alkaline phosphatase activity was normalized to cellular protein content. Data are shown as mean ± SD from three to four experiments. Groups with * are different from controls ($P < 0.05$). (*B*) Cells were incubated in the presence of pooled sera from controls or uremic high phosphorus (HP) for 48 hours. To determine the role of alkaline phosphatase activity in the uremic response, cells were treated with HP in the presence of an inhibitor of alkaline phosphatase (levamisole) for 48 hours. Cells were lysed and Western blotting was performed using an anti-osteopontin antibody. The band intensity was analyzed by scanning densitometry.

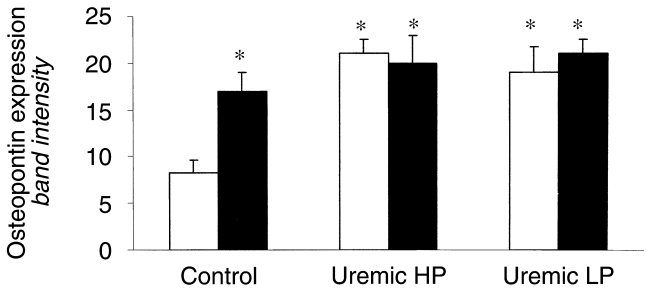

Fig. 7. Densitometry analysis of Western blotting of osteopontin expression in BVSMC treated with uremic sera. Cells were incubated with pooled sera from controls, uremic high phosphorus (HP) or uremic low phosphorus (LP) in the presence (■) or absence (□) of β-glycerophosphate (BGP, 12 mmol/L) for 48 hours. Cells were lysed and Western blotting was performed using an anti-osteopontin antibody. The band intensity was analyzed by scanning densitometry. Data are shown as mean ± SD from three experiments. Groups with * are different from controls ($P < 0.05$). There is no significant difference between uremic groups treated with or without β-glycerophosphate.

disordered mineral metabolism may contribute to vascular calcification is through the regulation of the deposition of bone matrix proteins, such as osteopontin. The present study found that the up-regulation of osteopontin expression by β-glycerophosphate, a phosphate donor, is dependent on the activity of Na/Pi co-transporter and alkaline phosphatase in BVSMC. We also demonstrated, to our knowledge for the first time, that uremic serum potentiated calcification and osteopontin expression in BVSMC. The ability of uremic serum to induce osteopontin expression is associated, at least partly, with the Na/Pi co-transport and the activity of alkaline phosphatase in BVSMC.

The identification of bone matrix proteins in vascular calcification in non-uremic [18–22] and uremic individuals [7, 10] suggests an active process that may parallel mineralization in bone. In bone, osteoblasts differentiate from pluripotent cells with specific gene products at different stages of differentiation and mineralization. In vitro, a model of this process, bone nodules, can be induced in osteoblasts in the presence of β-glycerophosphate and ascorbic acid. In these bone nodules, a discrete zone of hydroxyapatite-containing mineral is formed within the collagen fibrils and along the non-collagenous proteins such as osteopontin [23–25]. The β-glycerophosphate is cleaved to free phosphate by the enzyme alkaline phosphatase for which ascorbic acid is an essential co-factor. Alkaline phosphatase is commonly considered a phenotypic marker of osteoblasts that is critical for bone mineralization [26]. A recent study in osteoblast MC3T3 cells has further demonstrated the importance of this enzyme in the induction of osteopontin expression [25].

Several groups have recently demonstrated that both human and bovine VSMC also can calcify their extracellular matrix in vitro in the presence of β-glycerophosphate and ascorbic acid, in a manner similar to osteoblasts [12, 27]. In vitro, these vascular smooth muscle cells produce alkaline phosphatase, type I collagen, and non-collagenous proteins such as osteopontin and osteocalcin [12, 13], suggesting that vascular smooth muscle cells retain their pluripotential and transform into osteoblast-like cells. We have recently demonstrated the expression of osteopontin, alkaline phosphatase, type I collagen and bone sialoprotein by VSMC in arteries of dialysis patients undergoing a renal transplant [10]. Furthermore, the deposition of these "bone" proteins precedes overt calcification, suggesting that the transformation of VSMC to osteoblast-like cells with expression of these proteins is an essential first step. These proteins may then regulate subsequent calcification.

The mechanism by which these bone matrix proteins may regulate calcification is not completely clear. For example, the addition of osteopontin to mineralizing BVSMC cultures inhibited calcification [28]. However, Jono et al recently demonstrated that the phosphorylation of osteopontin was critical for this activity: non-phosphorylated osteopontin (converted by the addition of alkaline phosphatase) did not inhibit mineralization, whereas phosphorylated osteopontin did inhibit mineralization [29]. Therefore, the net effect of osteopontin is dependent on its state of phosphorylation, suggesting a potential control mechanism by which this non-collagenous protein may be involved in the calcification of VSMC. In the present study, uremic serum induced mineralization, alkaline phosphatase activity, and osteopontin expression. However, we did not determine if the osteopontin was phosphorylated, nor did we perform serial studies in vitro. Thus, it is unclear whether the increased osteopontin production is to try to enhance or inhibit mineralization. Clearly, more work is needed to understand this complex process, but these data support that the process is at least partly regulated, even in uremia.

In the present study in BVSMC incubated with FBS and phosphorus, in the form of β-glycerophosphate, can induce the expression of osteopontin. This process is dependent on alkaline phosphatase activity and Na/Pi co-transport, as both levamisole (an inhibitor of alkaline phosphatase) and foscarnet (an inhibitor of Na/Pi co-transport) completely abolished β-glycerophosphate-induced alkaline phosphatase activity and osteopontin expression in BVSMC. Although the inhibitors used in the current study are specific for alkaline phosphatase and Na/Pi co-transport, both levamisole and foscarnet may have other mechanisms of action. Levamisole can inhibit Na^+,K^+-ATPase [30] and foscarnet can inhibit DNA polymerase [31].

To date, three types of Na/Pi co-transporters have been identified [32]. Recently, type III Na/Pi co-transporter was found to be expressed constitutively in human VSMC [13]. Although no specific type III Na/Pi inhibitor is available, foscarnet significantly inhibited osteopontin expression in our study, and inhibited the expression of osteocalcin and a transcription factor Cbfa1 in the study of Jono et al [13]. Indeed, a recent study demonstrated that phosphorus induced the expression of Cbfa1, a transcription factor critical for osteoblast differentiation and the regulation of osteopontin and other bone matrix proteins [33]. Taken together, these results indicate that high extracellular phosphorus generated from β-glycerophosphate by alkaline phosphatase enters the cell through the action of Na/Pi co-transport. The elevated intracellular phosphorus may then regulate the vascular calcification process by inducing the transformation of VSMC into osteoblast-like cells with production of bone matrix proteins via a Cbfa1 dependent mechanism. Further supporting this mechanism is our recent result identifying the expression of Cbfa1 in both the intima and media of calcified arteries in areas with strong expression of type I collagen and osteopontin (abstract; Moe, *J Am Soc Nephrol* 12:756A, 2001). These results are exciting and offer a potential pathophysiologic link between the mineral metabolism abnormalities observed clinically, and vascular calcification observed pathologically in dialysis patients.

To examine further the role of hyperphosphatemia and vascular calcification observed in dialysis patients, we analyzed the effects of pooled uremic sera from two groups of dialysis patients: Those with persistently elevated serum phosphorus levels (≥6.5 mg/dL; pooled sera level = 7.4 mg/dL), and those with serum phosphorus in the "normal" target range for dialysis patients (≤5.5 mg/dL [34]; pooled sera phosphorus level = 5.5 mg/dL). Using these pooled sera in vitro, we demonstrated that uremic sera potentiate calcium deposition in BVSMC. We further demonstrated that both groups of uremic sera led to increased expression of osteopontin in BVSMC. Similar to the exogenous addition of phosphorus in the form of β-glycerophosphate, the uremic serum-induced osteopontin expression is dependent on both alkaline phosphatase and Na/Pi co-transport. However, in contrast to the effect observed with β-glycerophosphate, the induction of uremic serum-induced osteopontin expression was only partially blocked by foscarnet, indicating that the mechanism was not completely dependent on Na/Pi co-transport.

We were unable to demonstrate a difference in osteopontin expression between the two uremic groups: low phosphorus (LP) or high phosphorus (HP). One potential explanation is that cells were treated with 10% uremic sera in which the serum, and thus the phosphorus concentration was diluted, bringing the final media concentration well below the lower limits of phosphorus (2 mol/L = 6.2 mg/dL) previously shown to induce vascular cell calcification in human VSMC in vitro [13], and well below levels generated with 12 mmol/L β-glycerophosphate. However, dilution does reveal a potent effect of uremic sera in general, as even with this low final concentration of phosphorus there was induction of osteopontin expression and uremic serum also augmented calcification. This supports that other uremic factors are critical, and/or that the effect of phosphorus in vivo may be to increase the production of one or more circulating proteins present in our pooled sera. We also were unable to demonstrate an additive effect of β-glycerophosphate on uremic serum induced osteopontin expression, despite a clear increase in the media inorganic phosphorus concentration. Taken together with the finding that foscarnet and lavamisole were only partially inhibitory, these data suggest that uremic sera induces osteopontin expression via additional, non-Na/Pi co-transporter dependent mecha-

nisms. Clearly, further studies are warranted to understand this process. This will almost certainly be multifactorial, as there are numerous abnormal, altered, and absent proteins in uremic serum that may be involved in this process, and clear differences in measured proteins between our uremic and non-uremic sera (Table 1). Nevertheless, our results provide direct evidence that uremic sera play an important role in vascular calcification in BVSMC.

In conclusion, β-glycerophosphate induces osteopontin expression in BVSMC through an alkaline phosphatase and Na/Pi co-transporter dependent mechanism. We also have demonstrated that uremic serum leads to calcification in vitro in BVSMC, and induces osteopontin expression in BVSMC in both Na/Pi co-transporter dependent and independent mechanisms. These results suggest that in addition to hyperphosphatemia, other factors in uremia may be involved in the accelerated vascular calcification in ESRD. Further studies to determine what factor(s) in uremia contribute to the deposition of matrix proteins and subsequent calcification are needed. Hopefully, this will lead to the development of therapeutic interventions to stop, or reverse, the vascular calcification that plagues patients with ESRD.

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