Uremia induces the osteoblast differentiation factor Cbfa1 in human blood vessels

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Background. Bone matrix proteins are expressed in calcified arteries from dialysis patients, suggesting that vascular smooth muscle cells (VSMCs) may transform to osteoblast-like cells. One of the key transcriptional regulators of osteoblast differentiation is Cbfa1. Thus, we hypothesized that this may be a key factor in arterial calcification.

Methods. To test this hypothesis, we examined sections of the inferior epigastric artery from uremic patients for the presence of Cbfa1 and type I collagen and osteopontin by in situ hybridization and immunostaining. We also examined the effect of pooled uremic sera from dialysis patients on the expression of Cbfa1 by reverse transcription-polymerase chain reaction (RT-PCR) in bovine VSMCs in vitro.

Results. Cbfa1 and osteopontin were expressed in both the media and the intima in vessels that were calcified, but there was only minimal staining in non-calcified vessels. In vitro studies demonstrated that pooled uremic serum, compared to pooled control human serum induced the expression of Cbfa1 by RT-PCR in bovine VSMCs in a time-dependent, nonphosphorus-mediated mechanism.

Conclusion. These results support that Cbfa1 is a key regulatory factor in the vascular calcification observed in dialysis patients and is up-regulated in response to many uremic toxins.

The assessment of coronary arteries by new noninvasive imaging techniques such as electron beam computed tomography scan (EBCT) and intracardiac ultrasound has heightened the awareness that over 70% of atherosclerotic plaques observed in the aging population are calcified [1], and the magnitude of calcification correlates with the severity of obstructive coronary artery disease by angiography and clinical events [2, 3]. Cardiovascular disease and stroke are the leading causes of death in patients with end-stage renal disease (ESRD) that re-

quire dialysis, at a risk that is 10- to 20-fold greater than the general population [4, 5]. Studies evaluating coronary calcification by EBCT in patients with ESRD have demonstrated excessive coronary artery calcification, even in young adults [6, 7]. Pathologic analysis of arteries from nondialysis patients demonstrates that vascular calcification resembles developmental bone mineralization, with the production of "bone" proteins by vascular smooth muscle cells (VSMCs), such as osteopontin, bone sialoprotein, alkaline phosphatase, and type I collagen [8–11]. We have confirmed these findings in arteries from dialysis patients [12, 13]. This would imply that the calcification of vascular tissue is an active process, with VSMCs transforming to osteoblast-like cells. However, the mechanism by which this occurs is not yet clear.

In vitro experiments in both human and bovine VSMCs have demonstrated that phosphorus, in the form of β-glycerophosphate (which is cleaved by alkaline phosphatase to form free phosphate), can induce calcification similar to that observed in osteoblast cultures [14, 15]. The mechanism by which phosphorus induced calcification was dependent on the sodium-phosphate (Na/Pi) co-transporter [16]. Furthermore, in a recent study by Jono et al [17], exogenous phosphate added to human VSMCs culture up-regulated Cbfa1 expression. Cbfa1 is a transcription factor critical for osteoblast differentiation and the expression of the bone matrix proteins, osteopontin, osteocalcin, and type I collagen [18]. In addition, Cbfa1 knockout mice fail to form mineralized bone, proving that Cbfa1 is critical for the terminal differentiation of osteoblasts [19]. Thus, the in vitro data in VSMCs support that phosphorus can lead to calcification, and that phosphorus can induce Cbfa1 and the expression of bone matrix proteins. However, in vivo evidence of this relationship to vascular calcification is lacking. We have previously demonstrated that pooled serum collected from hemodialysis patients can also induce mineralization of bovine VSMCs to a greater extent and at an earlier time point than bovine VSMCs incubated with pooled serum from healthy controls [20]. In addition,

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uremic serum can induce osteopontin protein expression in bovine VSMCs at levels twofold that induced by control serum, despite similar final media concentrations of phosphorus of 0.5 to 0.6 mmol/L [20], which is markedly less than concentrations of phosphorus that have been previously shown to upregulate Cbfa1 expression in cultured VSMCs [17]. Furthermore, the addition of exogenous phosphorus in the form of β-glycerophosphate doubled osteopontin expression in bovine VSMCs incubated with control serum, but had no additive effect in bovine VSMC incubated with uremic serum [20]. These data suggest that uremic serum, regardless of the phosphorus concentration, can induce bovine VSMC to become osteoblast-like cells, capable of mineralization and production of bone matrix proteins. We, therefore, hypothesized that nonphosphorus uremic toxins may also lead to the up-regulation of Cbfa1 with subsequent expression of bone matrix proteins in vascular tissue, resulting in calcification.

This report describes the novel finding of ex vivo expression of Cbfa1 in both medial and intimal calcification of arteries from dialysis patients. Furthermore, pooled serum from dialysis patients can induce expression of Cbfa1 in bovine VSMCs in vitro through a nonphosphorus-dependent mechanism. These data implicate Cbfa1 as a potential regulatory factor important in vascular calcification in dialysis patients.

METHODS

To test the hypothesis that uremia up-regulates the expression of Cbfa1, we performed two sets of experiments: (*1*) ex vivo examination of sections of the inferior epigastric artery (obtained from patients undergoing renal transplantation) by in situ hybridization for Cbfa1, and immunostaining for Cbfa1, type I collagen, and osteopontin; and (*2*) in vitro determination of Cbfa1 expression in bovine VSMC by reverse transcription-polymerase chain reaction (RT-PCR). The cells were incubated with pooled sera collected from control (nonuremic) individuals or dialysis patients on hemodialysis for at least 2 years to eliminate any residual renal function. Both studies were approved by the local Institutional Review Board and all patients gave written informed consent.

Ex vivo studies

The inferior epigastric artery was obtained from patients undergoing renal transplantation as previously described [13]. Briefly, during the surgery, the proximal inferior epigastric artery is normally ligated. For the present study, a 2 to 3 cm piece of vessel was removed and placed in 4% paraformaldehyde, then snap frozen in 22-oxacalcitriol (OCT) embedding compound (Triangle Biomedical Science, Durham, NC, USA). Cryosections

(7μ) were utilized for both immunohistochemistry and in situ hybridization.

Immunostaining was performed on frozen cryostat sections that were mounted onto treated glass slides (Superfrost/plus, Fisher Scientific, Pittsburgh, PA, USA), post-fixed in 4% paraformaldehyde for 10 minutes at 4°C, and washed in phosphate-buffered saline (PBS) followed by deionized H_2O. The sections were incubated for 10 minutes in hydrogen peroxide, followed by blocking with 1.5% blocking serum in PBS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour. The sections were incubated with the primary antibody against Cbfa1 (a gift from Dr. Gerald Karsenty, Baylor College of Medicine, Houston, TX; 1:300 dilution). To confirm our findings, two additional Cbfa1 antibodies were used (Santa Cruz Biotechnology and Oncogene, Boston, MA, USA), both at 1:10 dilution for 1 hour. The sections were developed by the aviding-biotin-complex (ABC) staining system (Santa Cruz Biotechnology), followed by color development with 3,3′ diaminobenzaidine tetrahydrochloride (DAB). Sections were counterstained with hematoxylin. Negative controls were obtained by substituting the primary antibody with PBS. To determine if the downstream proteins of Cbfa1 transcription were also up-regulated, immunostaining for type I collagen and osteopontin (gifts of Larry Fisher, Ph.D; National Institutes of Health; 1:100 dilution) was similarly performed. The presence of macrophages was determined utilizing anti-CD68 antibody (Dako, Carpenteria, CA). Images were recorded on a digital camera (Nikon Coolpix 950) using a Nikon Eclipse E400 microscope through 10× or 40× objective (1.4 numerical aperture). Sections were also examined by MacNeal's stain (toluidine blue with silver stain) to assess calcification as previously described [13].

In situ hybridization was performed on the same frozen sections of the inferior epigastric artery prepared as above. Sections were treated with 1 N HCl for 10 minutes followed by 0.1 mol/L Triethanolamine (TEA) buffer for 20 minutes. The specimens were prehybridized for 2 hours at 42°C and hybridized with the labeled riboprobe (5 ng/uL) for 16 hours. Riboprobes were made by in vitro transcription of linearized plasmids (pBluescript SK) containing cDNA for Cbfa1 [American Type Culture Collection (ATCC), Manassas, VA, USA]. In vitro transcription was performed in the presence of digoxygenin-uridine triphosphate (dUTP) and T3 RNA polymerase (antisense probe) or T7 RNA polymerase (sense probe), according to the manufacturer's instruction (Roche Diagnostics, Indianapolis, IN, USA). Specimens were washed under stringent conditions. After hybridization, sections were washed for 30 minutes in 2× SSC at room temperature and then 1 hour each in 1× standard sodium citrate (SSC), 0.5× SSC, and 0.1× SSC in the presence of 50% formamide at 48°C. Probe binding was localized by a colorimetric reaction with an alkaline phosphatase-

conjugated antidigoxygenin antibody (Roche Diagnostics, Indianapolis, IN, USA). Methylgreen was used for counterstaining. For negative controls serial sections were processed using sense probe.

Laser capture microdissection. Tissue samples were selectively dissected using the PixCell II® Laser Capture System (Arcturus, Mountain View, CA, USA). Briefly, cryosections were sliced at a 7 μm thickness and mounted on HistoGene™ laser capture microdissection (LCM) Slides (Arcturus). Slides were then stained using the HistoGene™ LCM Frozen Section Staining Kit (Arcturus) in order to maintain RNA integrity. Once completely dry, the stained sections were brought to focus on the microscope and the section of interest was covered with the LCM cap. The laser was focused and intensity varied based on slide conditions. The 30 μm diameter laser pulse setting was used to melt areas of the LCM cap film, adhering those cells directly beneath to the cap surface. The harvested cells were then digested from the cap and the total RNA was purified using the PicoPure™ RNA Isolation Kit (Arcturus). RNA was eluted twice from the provided column. RNA was harvested from 4 to 6 consecutive sections per patient and pooled for RT-PCR. A similar caliber vessel (superior epigastric artery) was obtained from a patient undergoing a Whipple's procedure and similarly processed. The subject did not have renal failure or diabetes, but was elderly and had calcification of the aorta present on CT scan, suggesting the presence of atherosclerosis. Unfortunately, we were not able to obtain a truly healthy young vessel of similar caliber.

RT-PCR was performed using Titan One Tube RT-PCR Kit (Roche Diagnostics, Indianapolis, IN, USA). Briefly, total RNA was isolated by LCM, and reverse-transcribed at 50°C for 30 minutes followed by PCR reaction with Titan enzyme mix [avian myeloblastosis virus (AMV) reverse transcriptase and Taq DNA polymerase] in a 50 μL mixture containing 0.2 mmol/L desoxynucleoside triphosphate (dNTPs), 5 U RNAse inhibitor, 5 mmol/L dithiothreitol (DTT), 20 pmol sense and antisense primers. The sense primer for Cbfa1 was 5′-CCG CACGACAACCGCACC-3′; the Cbfa1 antisense primer was 5′-CGCTCCGGCCCACAAATC-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 7 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, Richmond, CA, USA).

In vitro studies

Bovine VSMCs were obtained by a modification of the explant method originally described by Ross [21]

as previously described [20]. Briefly, medial tissue was separated from segments of bovine aorta after removal of endothelial cells. Small pieces of tissue (1 mm²) were placed in a 6-well culture dish and cultured for 2 weeks in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical Company, St. Louis, MO, USA) containing 4.5 g/L glucose supplemented with 10% fetal bovine serum (FBS; Sigma) 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 with 100 U/mL penicillin and 100 μg/mL streptomycin. The media was replaced every 2 to 3 days. Only cells between passages 2 to 8 were used for experiments [14]. In order to determine whether accelerated vascular calcification in dialysis patients is due to the induction of Cbfa1 expression, bovine VSMCs were incubated for 24 or 48 hours with DMEM plus 10% uremic serum from dialysis patients (see below) or healthy control patients and analyzed by RT-PCR. β-actin was used as an internal control.

Uremic sera were pooled from patients who were on dialysis for at least 2 years to eliminate residual renal function. 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 (Roche Diagnostics); parathyroid hormone (PTH) by intact assay (Nichols Laboratory, San Juan Capistrano, CA); bone alkaline phosphatase by enzyme-linked immunosorbent assay (ELISA) (Metra Biosystems, Mountain View, CA, USA); and C-reactive protein by ELISA (Alpha Diagnostics, San Antonio, TX, USA). Ten percent of the control or uremic serum was added to media for use with cell culture. The phosphorus concentration of the final media plus 10% serum was measured using a colorimetric method (Sigma Chemical Company).

Statistical analysis

The immunohistochemistry and in situ hybridization tissue sections were read semiquantitatively by a single reader (0 = no staining; 1+ = scattered positive cells; 2+ = small patches positive cells; 3+ = diffuse patches positive staining; 4+ = entire medial layer positive). The RT-PCR was quantified by densitometry, and the difference in the Cbfa1/β-actin ratio between the groups compared by analysis of variance (ANOVA) with Fisher's post-hoc analysis (StatView, SAS Institute, Inc., Cary, NC, USA); $P < 0.05$ was considered significant.

RESULTS

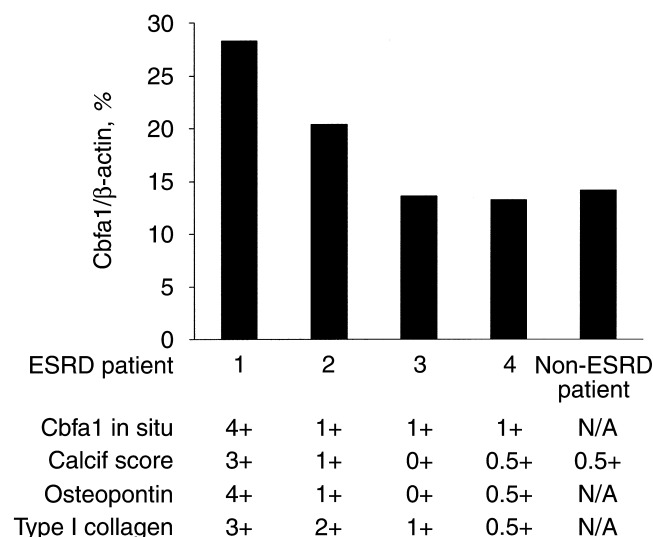### Ex vivo examination of the inferior epigastric artery

As previously described, 41 inferior epigastric arteries were obtained and examined for immunohistochemistry
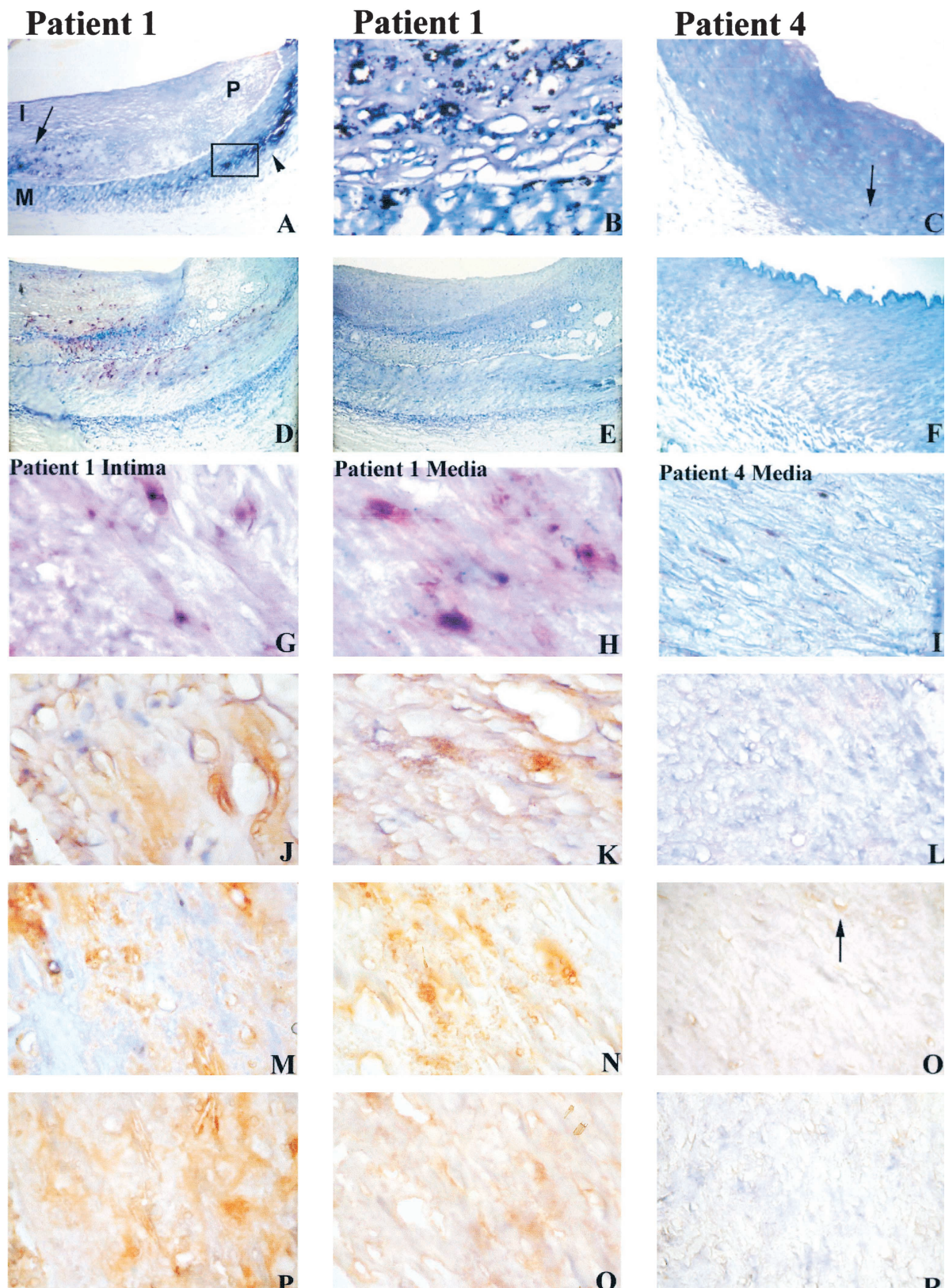
Fig. 1. Histopathologic analysis of vascular calcification in the inferior epigastric artery from dialysis patients. The left two columns are sections from an artery from Patient 1, a 40-year-old diabetic on hemodialysis for 24 months. This vessel demonstrates calcification adjacent to an intimal plaque (arrow) and in the media (arrowhead; ×10) (*A*). Abbreviations are: I, intima; M, media; P, plaque. (*B*) shows the boxed area from (*A*) at a higher magnification (×40) demonstrating the calcification surrounds the cells and is located in the area between cells. The results from this 3+ calcified vessel in (*A*) are compared to the results from Patient 4, a 55-year-old on dialysis for 14 months with only 0.5 + calcification (*C*). The

for the expression of various bone matrix proteins, including type I collagen and osteopontin, downstream protein products induced by Cbfa1 activation. This protein expression was present in vessels without calcification, suggesting that deposition of bone proteins preceded overt calcification [10]. Eight of these vessels were also prepared as frozen sections and were utilized for the present study. These vessels showed various levels of calcification by MacNeal's stain, (three vessels with no calcification; four vessels with 0.5 to 2+ calcification; one vessel with 3+ calcification).

Examination of the vessels by in situ hybridization demonstrated two vessels with positive Cbfa1 staining in the intima; both of these had thickened intima, with other cells positive for the macrophage marker CD68. One of these vessels had an intimal plaque. The other six vessels had nonthickened intima and no expression of Cbfa1 or CD68 staining in the intima. In contrast, in the medial layer, there was no expression of CD68 by immunostaining but positive expression for Cbfa1 by in situ hybridization in all eight vessels. Four vessels had only scattered Cbfa1 positive cells by in situ hybridization (1+); the remaining four vessels had more patchy expression (2 to 3+). Qualitative results of immunostaining for Cbfa1 paralleled the RNA expression of Cbfa1 in both the intima and the medial layers. The staining pattern was similar with all three Cbfa1 antibodies, although expression was best visualized with the antibody that corresponds to the 17 amino acids of the N-terminals portion of Cbfa1 (a gift from Dr. Gerard Karsenty). The location and scoring for the immunostaining for the Cbfa1-induced downstream proteins osteopontin and type I collagen parallel the Cbfa1 expression by in situ hybridization and immunostaining. Of note, this expression of Cbfa1 and its downstream proteins was found even in areas of sections without overt calcification. This suggests that this transformation of VSMCs to osteoblast-like cells with deposition of matrix proteins occurs before overt calcification by up-regulation of Cbfa1. These results are further demonstrated in Figure 1 where expression in a vessel with both intimal and medial calcification and strong Cbfa1 expression (Patient 1; left two

	ESRD patient 1	2	3	4	Non-ESRD patient
Cbfa1 in situ	4+	1+	1+	1+	N/A
Calcif score	3+	1+	0+	0.5+	0.5+
Osteopontin	4+	1+	0+	0.5+	N/A
Type I collagen	3+	2+	1+	0.5+	N/A

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) of total RNA isolated from vessel section by laser capture microdissection (LCM). Sections from four vessels from end-stage renal disease (ESRD) patients, and one vessel from a non-ESRD patient were utilized for total RNA isolation by laser capture microdissection (see **Methods** section). The results of the ratio of Cbfa1 to β-actin, assessed by densitometry, are shown graphically. RT-PCR was performed from total RNA isolated from 4 to 6 consecutive sections of vessel per patient. Patient 1, due to the extensive calcification, had many fewer viable cells for the same amount of tissue collected. These studies parallel the results of the in situ hybridization (Cbfa1 in situ), calcification score by MacNeal's stain (Calcif score), and immunostaining for osteopontin and type I collagen (Type I collagen).

panels) is compared to a minimally calcified vessel with only scattered Cbfa1 positive cells in the medial layer (Patient 4; right panel).

To confirm the in situ hybridization results, total RNA was isolated from available sections of vessels of Patients 1 through 4 by laser capture microdissection. RT-PCR was performed and the Cbfa1 expression was normalized to β-actin. These results and corresponding results for calcification, in situ hybridization, and immunostaining for osteopontin and type I collagen are shown in Figure 2. The results demonstrate that in Patients 1 and 2, where calcification was present, there was greater Cbfa1, osteopontin, and type I collagen expression than in Patients

first row is the vessels stained by MacNeal's tetrachrome stain, where calcification is indicated by a black color. In contrast, the vessel from Patient 4 in (C) shows only scattered spots of faint calcification (arrow; ×10). In situ hybridization for Cbfa1 expression demonstrates significant expression in both the intima and media of the vessel from Patient 1 (D) (×10). The negative control (sense probe) is shown in (E) (×10). Higher magnification of Cbfa1 expression by in situ hybridization is shown for the intima (G) (×40) and media (H) (×40). In contrast, in the vessel from Patient 4, there is very little expression of Cbfa1 by in situ hybridization with only scattered positive cells (F) (×10) and (I) (×40). Immunostaining using an anti-Cbfa1 antibody paralleled the findings of in situ hybridization in the calcified vessel in both the intima (J) (×40) and media (K) (×40) of vessel from Patient 1. In contrast, there was no detectable immunostaining for Cbfa1 in the vessel from Patient 4 (L) (×40). Up-regulation of Cbfa1 leads to the expression of the downstream bone matrix proteins osteopontin and type I collagen. Immunostaining for osteopontin demonstrated expression in areas also positive for Cbfa1 in both the intima (M) (×40) and media (N) (×40) of vessel from Patient 1. In contrast, there was minimal expression of osteopontin in the vessel with minimal Cbfa1 expression (O) (arrow) (×40). Similar results were demonstrated with type I collagen where immunostaining was similarly localized in both the intima (P) (×40) and media (Q) (×40) in the vessel from Patient 1, whereas there was essentially no expression of type I collagen in the area without Cbfa1 expression in the minimally calcified vessel (R) (×40).

Table 1. Results of analysis of pooled serum

	Control sera	Uremic serum
Number of patients	14	13
Age *years*	48.7	46.2
Gender *%F*	36	46
Hemodialysis *years*	—	6.9
Diabetic *%*	—	23
Vitamin D usage *%*	—	23
Kt/V	—	1.45
Sodium *meq/L*	142	140
Potassium *meq/L*	4.3	5.1
Albumin *g/L*	48	40
pH	8.05	8.15
Calcium *mmol/L*	2.3	2.1
Phosphorus *mmol/L*	1.13	2.39
Parathyroid hormone *pmol/L*	2.8	49.6
Total alkaline phosphatase *IU*	62	155
Bone alkaline phosphatase *IU*	11.3	40
C-reactive protein *μg/mL*	0.004	32.4

3 and 4 who had minimal or no calcification (0 to 1+ = no or scattered rare cells positive by Van Kossa staining). The sample from the non-ESRD patient also had rare cells positive by Von Kossa, with similar Cbfa1/β-actin ratio by LCM as ESRD Patients 3 and 4. These findings of basal expression are consistent with basal expression of Cbfa1 observed by Jono et al [17] in human vascular smooth muscle cells.

In vitro expression of Cbfa1

Bovine VSMCs were incubated in the presence of normal control human sera or uremic sera from patients on hemodialysis for at least 2 years as previously described [20]. As expected, the analysis of these pooled sera revealed that the uremic serum had elevated levels of phosphorus, total and bone alkaline phosphatase, PTH, and C-reactive protein compared to the control serum (Table 1). However, it is important to emphasize that the serum was subsequently diluted to 10% for use in vitro cell cultures.

Total RNA was isolated from bovine VSMCs treated with control serum or uremic serum, for 24 or 48 hours. RT-PCR demonstrated an increase in Cbfa1 expression in the uremic groups compared to control serum treated cells by 48 hours. The up-regulation of Cbfa1 by 48 hours was similar to that observed with control human serum plus dexamethasone (control human = 26 ± 7.26; uremic, 24 hours = 32.3 ± 7.57; uremic, 48 hours = 45.3 ± 5.78; dexamethasone = 52 ± 5.1; $P < 0.05$) for uremic 48 hours and dexamethasone-treated bovine VSMCs compared to control results pooled from three experiments. To determine the role of phosphorus in uremic serum induced Cbfa1 expression, bovine VSMCs were incubated in control or uremic serum with or without 12 mmol/L β-glycerophosphate, which is cleaved to form free phosphorus. β-glycerophosphate increased the expression of Cbfa1 by twofold in the control human serum,
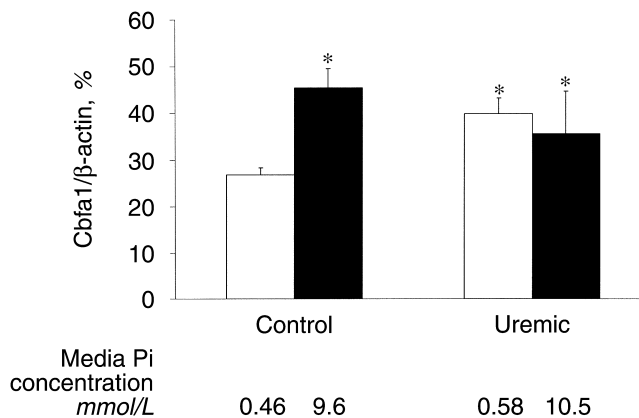
Fig. 3. Effect of phosphorus on uremic serum induced Cbfa1 expression. Reverse transcription-polymerase chain reaction (RT-PCR) for Cbfa1 expression in bovine vascular smooth muscle cells (VSMCs) incubated in the presence of pooled sera from control or uremic subjects, without (□) or with (■) the addition of 12 mmol/L β-glycerophosphate for 48 hours. The media [Dulbecco's modified Eagle's medium (DMEM) plus serum] concentration of phosphorus (Pi) was measured at the end of the incubation period and confirmed an increase in the Pi concentration in the presence of β-glycerophosphate. The results demonstrate that bovine VSMC incubated with uremic serum had increased expression of Cbfa1 compared to control serum, despite similar final concentrations of phosphorus. Furthermore, the addition of β-glycerophosphate doubled the expression of Cbfa1 in bovine VSMCs incubated in control serum, but had no additive effect on bovine VSMCs incubated in uremic serum. *$P < 0.05$ compared to control.
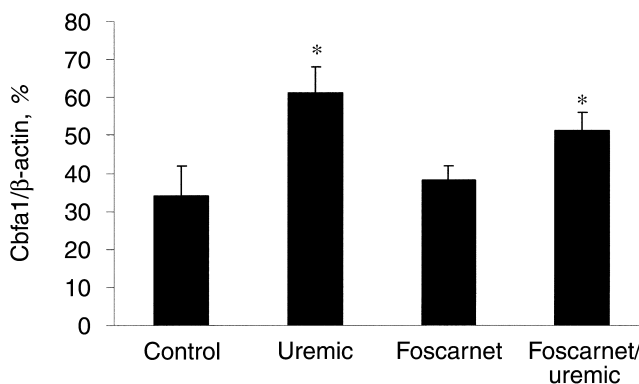
Fig. 4. Effect of sodium/phosphate co-transport on uremic serum–induced Cbfa1 expression. Reverse transcription-polymerase chain reaction (RT-PCR) for Cbfa1 expression in bovine vascular smooth muscle cells (VSMCs) incubated in the presence of pooled sera from control or uremic subjects, without or with foscarnet (an inhibitor of sodium-phosphate co-transport). The results demonstrate that foscarnet had no effect on basal Cbfa1 expression and did not inhibit uremic serum induced Cbfa1. These results indicate that the effects of uremic serum are not dependent on sodium-phosphate co-transport. *$P < 0.05$ compared to control.

but had no additive effect in the uremic serum, despite a large increase in the media concentration of phosphorus with the addition of the β-glycerophosphate (Figure 3). Furthermore, inhibition of sodium/phosphate co-transport with foscarnet had no effect on Cbfa1 expression in bovine VSMCs incubated in uremic serum (Figure 4).

These data support that uremic serum induces Cbfa1 expression through a nonphosphorus mediated mechanism.

DISCUSSION

In the present study, we have demonstrated the novel finding that the RNA and protein expression of the osteoblast differentiating factor Cbfa1 is increased in both the intima and media of the inferior epigastric artery of dialysis patients with calcified vessels. This expression corresponds to the expression of type I collagen and osteopontin proteins known to be regulated by Cbfa1. The expression of Cbfa1, osteopontin, and type I collagen was present even without overt calcification, suggesting that Cbfa1 transformation of bovine VSMC to osteoblast-like cells occurs prior to overt calcification. To our knowledge, this is the first demonstration of Cbfa1 expression in intimal and medial human arterial calcification, supporting the hypothesis that VSMCs transform or dedifferentiate to osteoblast-like cells. This also lends support to the hypothesis that vascular calcification is a regulated process. Furthermore, we have demonstrated that uremic serum can up-regulate Cbfa1 through a non-phosphorus-mediated mechanism, suggesting that the etiology of vascular calcification in dialysis patients is multifactorial.

Osteoblasts differentiate from a pluripotent mesenchymal stem cell that is the progenitor for osteoblasts, chondrocytes, adipocytes, myocytes, and fibroblasts. The switch that controlled the differentiation to osteoblasts had remained elusive until the experimental findings of the Cbfa1 knockout mice were reported [18, 19, 22]. These mice lack mineralized bone and the normal ossification areas in the shafts of long bones demonstrate an arrest of the normal progression from cartilage to mineralized bone [19]. Subsequent studies have demonstrated that Cbfa1 controls the expression of the bone matrix proteins, osteopontin, type I collagen, and osteocalcin in osteoblasts, by binding to osteoblast-specific elements (OSE2) in the promoter region [18]. The importance of Cbfa1 in controlling normal bone mineralization is further demonstrated by the finding that osteoprotegerin, the protein product of osteoblasts that controls the osteoclast, is also induced by Cbfa1 [23]. Thus, Cbfa1 is an important factor in the control of normal bone turnover by regulating the deposition of type I collagen and noncollagenous proteins such as osteopontin that guide and control subsequent mineralization.

Jono et al [17] first demonstrated that this osteoblast transcription factor Cbfa1 is expressed in vitro in cultured human VSMCs and up-regulated in the presence of phosphorus at concentrations of 2 mmol/L or greater. Levels of phosphorus in this range have also been associated with clinical evidence of vascular calcification by EBCT [7], and ultrasound [24], as well as cardiovascular

and all-cause mortality [25, 26]. This association of elevated phosphorus levels with cardiovascular disease in vivo and Cbfa1 expression in vitro suggested that the elevated phosphorus levels observed in uremic patients may be the primary mechanism for increased vascular calcification. However, the results of the present in vitro study demonstrate that uremic serum has a potent ability to induce the expression of Cbfa1, over and above that due to hyperphosphatemia. The other uremic toxin(s) that may be responsible is (are) unknown. Indeed, uremic serum contains numerous proteins and altered forms of proteins due to decreased renal clearance and incomplete removal with dialysis. We performed a proteonomic analysis on the normal and uremic serum and identified 285 proteins that were increased, decreased, or different in the normal versus pooled uremic serum samples. Indeed, potential candidate toxins include calcitriol [27], oxidized low-density lipoprotein cholesterol [28] and alterations of cytokines [29, 30], all of which are abnormal in the serum of dialysis patients and have been associated with vascular calcification in vitro or ex vivo. PTH was increased in the pooled uremic serum compared to normal serum, however, in studies utilizing the in vitro model used for the present study, both PTH and PTHrp have been found to inhibit, not enhance, vascular calcification [31]. Of note, our in vitro evidence demonstrating that phosphorus is not additive to the effect of other uremic factors may not reflect the in vivo state where it is likely that phosphorus and other uremic toxins are additive. Supporting such a complexity, our previous work demonstrated that blocking sodium-phosphate cotransporter with foscarnet only partially inhibited uremic serum-induced osteopontin expression in bovine VSMCs [20]. Thus, the precise interaction of phosphorus with other uremic toxins, and determining which uremic toxin, or more likely the interaction of multiple toxins, that are responsible for the induction of Cbfa1 and its protein products will require further extensive study.

Interestingly, it has been previously assumed that calcification of the media and the intima are different processes, with macrophage infiltration of critical importance in plaque formation in the intima, but not the media. We observed CD68-positive (macrophage) cells in the intima of two of eight vessels from dialysis patients along with Cbfa1 staining. However, we found Cbfa1 staining without CD68-positive cells in the medial layer. This would imply that although there may be different initiation factors, calcification may ultimately proceed via a Cbfa1-dependent mechanism in both the intima and media. Interestingly, the expression of Cbfa1 in the intima of one vessel was adjacent to the calcified plaque, suggesting that plaque formation may induce Cbfa1 expression in adjacent smooth muscle cells. As plaque formation in smaller arteries is unusual, this finding will require confirmation. In addition, it is unknown if these

results in dialysis patients can be extrapolated to the general population. However, the expression of bone proteins in both atheromatous and medial calcification of non-dialysis [8–11, 32, 33] and dialysis patients [12, 13] suggests similar pathogenesis of calcification in these two patient populations. In addition, the presence of basal expression of Cbfa1 in human VSMCs in vitro [17] and in the non-ESRD patient artery examined by LCM in the present study suggests that Cbfa1 expression may be up-regulated by many factors as is true in osteoblasts.

CONCLUSION

In conclusion, the novel results of the present study confirm and extend the hypothesis that vascular calcification in uremia is an active process, and provides a mechanism by which pooled uremic serum upregulates osteopontin expression [20]. In addition, we have demonstrated, for the first time, the in vivo expression of the osteoblast differentiating factor Cbfa1 in intimal and medial VSMCs in calcified arteries of dialysis patients, correlating with calcification and the expression of the downstream protein products, type I collagen and osteopontin. Furthermore, uremic serum can induce Cbfa1 expression in bovine VSMC in vitro. We, therefore, hypothesize that vascular calcification in dialysis may be a three-step process. First, VSMCs are stimulated by uremic toxins (of which there are likely many) to transform into osteoblast-like cells. These cells then lay down a bone matrix of type I collagen and noncollagenous proteins. The final step may be mineralization of this matrix, in part through a physiochemical process and in part through an active process "guided" by the matrix proteins and osteoblast-like cells. This latter step is likely to be accelerated in the presence of elevated calcium x phosphorus [7, 34, 35] product in the serum as well as excessive positive calcium balance due to calcium containing phosphate binders [7, 34, 36]. Should our hypothesis prove true, the presence of Cbfa1 as a key regulatory factor in the pathogenesis of early stages of vascular calcification offers the potential hope of developing specific therapeutic agents to arrest this process. In addition, continued efforts to optimize calcium and phosphorus balance are of paramount importance.

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