Calciphylaxis Is Associated With Hyperphosphatemia and Increased Osteopontin Expression by Vascular Smooth Muscle Cells

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• **Calciphylaxis or calcific uremic arteriolopathy (CUA) is a fatal disease in dialysis patients due to calcification of cutaneous blood vessels. The pathogenesis has been attributed to elevated parathyroid hormone (PTH). However, recent studies evaluating vascular calcification in nondialysis patients have found that the smooth muscle cells play an active role, including production of the bone matrix protein osteopontin. To examine the involvement of various clinical parameters and smooth muscle cells of CUA, we performed a case-control analysis comparing 10 CUA patients with our current dialysis patients. Available histologic sections were immunostained for osteopontin, markers of smooth muscle cells, endothelial cells, and macrophages. Compared with our current dialysis population, patients with CUA were more likely to be obese, white, and female ($P < 0.02$). Comparison of laboratory values found CUA patients with lower serum albumin, greater serum phosphorus, and greater calcium X phosphorus product ($P < 0.01$). In contrast, there was no difference in the concentration of PTH or calcium between the 2 groups. Immunostaining of calcified blood vessels showed that all calcified vessels stained positive for osteopontin, whereas all the noncalcifed vessels showed no osteopontin localization. Staining for smooth muscle α-actin decreased in the medial layer with calcification, with cells appearing to be sloughed off, leading to near occlusion of the vessel lumen. Our case-control study demonstrates that hyperphosphatemia and an elevated calcium X phosphorus product is associated with CUA. Histologic examination suggests that the calcification is associated with increased expression of osteopontin by smooth muscle cells.**

INDEX WORDS: Calciphylaxis; osteopontin; α-smooth muscle actin; parathyroid hormone (PTH); phosphorus; vascular smooth muscle cells; matrix vesicles.

HANS SELYE COINED the term calciphylaxis in 1962 to describe his experimental observations of diffuse soft tissue calcification in the skin and internal organs of rats. The calcification was precipitated by "sensitizing" the animals with high-dose vitamin D, high calcium, and phosphorous diets or parathyroid hormone (PTH) and then "challenging" the animals with trauma or injection of iron salts or egg albumin.[1] Although the term has been used to define a syndrome in patients with renal failure characterized by indurated, painful, violaceous, subcutaneous nodules, which progress to ulceration and necrosis of the skin,[2-8] the pathology differs from that observed by Seyle.[1] As a result, recent authors have suggested the term calcific uremic arteriolopathy (CUA) replace calciphylaxis as it more accurately reflects the histologic finding observed in patients with renal failure.[7] The clinical manifestations of CUA have been the subject of several recent reviews.[6,9,10] CUA affects 1% to 4% of dialysis patients and can present in a proximal (abdomen, thigh, or buttock) or distal distribution (below the knee or elbow), with the former carrying a worse prognosis.[3,6] The prevalence may be increasing as there is a recent resurgence of publications on the disease.

Diagnosis is confirmed by histologic evidence of calcification of the tunica media of the small arterioles of the skin.[4,11] Various other findings have been noted in the literature, including intimal hyperplasia,[2] thrombosis,[7] calcification of adipocytes,[7] and infiltration of inflammatory cells.[5] The pathogenesis of the disorder is largely speculative, and no uniform consensus has emerged. Over the years, there have been only small series and sporadic case reports with contradictory findings describing potential patho-

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Received July 27, 2000; accepted in revised form December 8, 2000.

Supported by the National Kidney Foundation of Indiana and National Institutes of Health Grant No. DK02775-01.

Presented in part at the 25th Annual National Kidney Foundation Clinical Meetings, April 13-18, 2000, Atlanta GA.

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0272-6386/01/3706-0021$35.00/0

doi:10.1053/ajkd.2001.24533

genic mechanisms of CUA (reviewed in Chan et al[3]). The hypothesized pathogenesis for a number of years has been high parathyroid hormone levels, leading to parathyroidectomy as the treatment of choice.[2,3,12,13] However, this assumption was based on retrospective, uncontrolled trials. Recently, Bleyer et al[8] performed a case control study and found no relationship of CUA with elevated PTH, bringing the role of altered PTH into question. The literature to date on CUA suggests there is no single clear-cut pathophysiologic mechanism that can explain the phenomenon of CUA in the context of renal failure.

Recent evidence in the cardiovascular literature examining vascular calcification has found that the process is not passive, but cell-mediated. Experimental studies have demonstrated that isolated smooth muscle cells of vascular tissue can dedifferentiate into mesenchymal precursors, or osteoblast-like cells, capable of calcifying in vitro (reviewed in Campbell and Campbell[14]). This is accompanied by the production of several matrix proteins, traditionally felt to be produced by osteoblasts in bone, including osteopontin, alkaline phosphatase, matrix gla protein, osteocalcin, bone morphogenic protein, and bone sialoprotein.[15-18] Osteopontin contains an RGD-domain suggesting its role in cell adhesion and migration.[19] Although originally isolated from bone, it is localized in many tissues and appears to have increased expression in tissues at risk or known to calcify.[19] We, therefore, hypothesized that CUA may also be due to an active cellular process in which the vascular smooth muscle cells transform to an "osteoblast"-like phenotype, with an ability to actively mineralize. To test this hypothesis, we performed immunohistochemistry on CUA skin biopsies to examine the expression of osteopontin and smooth muscle α-actin, a marker of smooth muscle cell differentiation.[20] In addition, we performed a case-control analysis to identify clinical and biochemical risk factors for the development of CUA, comparing the patients with CUA with our present dialysis patient population. The study was approved by the Indiana University-Purdue University at Indianapolis Institutional Review Board.

METHODS

A search of the Indiana University Department of Dermatopathology computer database from 1995 to 2000 showed 12 skin biopsies with a diagnosis of calciphylaxis. The medical records of the 12 patients were reviewed to confirm a clinical picture consistent with CUA. Two patients did not have renal failure. The remaining 10 patients were all undergoing dialysis. Their medical records were reviewed to obtain demographic data, body mass index (BMI), cause of end-stage renal disease (ESRD), duration and mode of dialysis, presence of any precipitating factors, such as skin trauma prior to presentation, use of anticoagulants, subcutaneous injections, and hormone replacement therapy. The clinical course including presentation and treatment was also reviewed. All laboratory values from the previous 6 months for calcium (corrected for albumin), phosphorous, intact PTH, and albumin were averaged. Similar demographic and biochemical data from our current dialysis patient population was obtained for comparative purposes.

The histologic blocks were retrieved, and new sections were cut and stained by hematoxylin and eosin (H&E) to determine the presence of calcified vessels in the remaining tissue block. Blocks from 5 of the 10 patients had sufficient tissue to analyze with special stains. These sections were then examined by (1) H&E; (2) alizarin red staining to examine calcium content; (3) immunostaining with antibodies directed against the bone matrix protein osteopontin (LF 123; a gift of Larry Fisher, PhD at the National Institutes of Health[21]), the smooth muscle cell marker, α-actin (monoclonal mouse antihuman, N 1584; DAKO Corp, Carpinteria, CA), markers of endothelial cells von Willebrand factor (polyclonal rabbit antihuman, N1505 DAKO), CD34 (mouse monoclonal antibody, M7165 DAKO), and marker of macrophages CD68 (mouse monoclonal antihuman, N1577 DAKO); (4) stain for iron deposition; (5) examination for apoptosis by DNA fragmentation; and (6) electron microscopy by the following methods.

Alizarin Red Staining

Alizarin red is a pH-dependent stain, which can differentiate calcium oxalate from calcium carbonate and phosphate.[22] Calcium oxalate stains reddish-orange at pH 7, but not at pH 4.2. Calcium carbonate and calcium phosphate stain reddish-brown at both pHs. A 2% aqueous solution of alizarin red was prepared and the pH adjusted to 7 or 4.2 with NaOH. Tissue sections were deparaffinized, incubated with alizarin red for 3 minutes, rinsed with distilled water, and then dehydrated through graded alcohol.[22]

Immunostaining for Osteopontin

Unstained slides of tissue sections were deparaffinized in xylene and rehydrated in ascending alcohol. Slides were placed in 3% hydrogen peroxide to inhibit endogenous peroxidase. After washing in Tris saline, sections were blocked by 3% bovine serum albumin (Sigma Chemical Co, St Louis, MO) for 15 minutes, and then incubated in the primary antibody at 1:100 for 1 hour. The secondary antibody (peroxide conjugated goat antirabbit immunoglobulin G [IgG] of 1:400 dilution, Southern Biotechnology, Birmingham, AL) was applied for 30 minutes, developed with 3,3′-diaminobenzidine (DAB; Vector Biotechnology, Burlingame, CA), and the section counterstained with hematoxy-

Table 1. Clinical Characteristics of 10 CUA Patients

Patient No.	Type	Age/Gender	Race	BMI (kg/m²)	DM	ESRD Cause	RRT Mode	RRT Duration (mo)	PTH Gland Removed	FU (mo)	Outcomes
1	Proximal	55/F	W	46	N	?	HD	7	N	6	Expired
2	Proximal	46/F	W	28.5	N	SLE	HD	60	N	9	Alive
3	Proximal	70/F	W	42	N	Sclero	HD	8	N	2	Expired
4	Proximal	50/F	W	39	Y	?	HD	24	N	4	Expired
5	Proximal	67/F	W	39	Y	DM	HD	24	Y	7	Alive
6	Proximal	34/F	W	34	Y	DM	HD	18	Y	4	Expired
7	Proximal	52/F	W	28.5	Y	DM	PD	2	N	3	Expired
8	Proximal	43/F	W	NA	N	GN	PD	2	N	1	Expired
9	Distal	72/M	W	32	Y	?	HD	24	N	4	Alive
10	Distal	46/F	B	25	N	HTN	HD	72	N	22	Alive
Cases		55.9 ± 10.9/F:90%	W:90%	34.9 ± 7.1							
Control		56.1 ± 15.8/F:49.7%	W:32.6%	26.2 ± 6.5							
P value		NS/<0.020	<0.001	<0.01							

Abbreviations: F, female; M, male; W, white race; B, black race; BMI, body mass index; DM, diabetes mellitus; SLE, sytemic lupus erythromatosus; Sclero, scleroma; GN, glomerulonephritis; HTN, hypertension; RRT, mode of renal replacement therapy; HD, hemodialysis; PD, peritoneal dialysis; PTH, parathyroid hormone.

lin. Sections of kidney served as positive controls. Staining without the primary antibody served as a negative control.

Immunostaining for α-Actin, von Willebrand Factor, CD34 and CD68

Immunostaining with these primary antibodies was performed with the above protocol using a LSAB2 kit with a DAKO autostainer (DAKO). Sections were incubated with the primary anti–α-actin antibody (prediluted) for 20 minutes and the anti-von Willebrand factor (1:200), anti-CD34 (1:50), and anti-CD68 (prediluted) antibodies for 10 minutes. The positive controls for α-actin, von Willebrand factor, CD34, and CD68 were muscle, prostate, prostate and spleen, respectively. The negative controls were tissue sections stained without primary antibodies.

Iron Stain

The location of iron was examined with Perl's iron stain.[23] Sections were incubated with a 10% potassium ferrocyanide and 20% HCL solution for 30 minutes followed by nuclear fast red counterstain.

Apoptosis

Cellular apoptosis was measured using a Klenow-FragEL DNA Fragmentation Detection Kit (Oncogene, Cambridge, MA). Specimens were deparaffinized, gradually rehydrated in alcohol, and permeabilized with proteinase. Endogenous peroxidases were inactivated with 3% H_2O_2. DNA fragment ends were labeled with an optimized ratio of Klenow enzyme; biotin-labeled deoxynuclear triphosphates (dNTPs) and unlabeled dNTPs. Biotin-labeled dNTPs were detected using a streptavidin-horseradish peroxidase conjugate that

Table 2. Laboratory Values of 10 CUA Patients Compared With Control Patients

Patient No.	Albumin (mg/dL)	Calcium (Ca)* (mg/dL)	Phosphorus (P) (mg/dL)	Ca × P (mg²/dL²)	PTH (pg/dL)
1	2.6	9	8.6	77	148
2	2.7	9.3	8.3	78	333
3	2.6	10	7.3	73	81
4	2.8	9	7.1	64	52
5	3.7	9.2	6.6	61	504
6	2.6	8.5	7.5	64	307
7	3.1	9.5	7.1	67	298
8	2.2	9.5	5	48	97
9	3.6	10.3	5.9	61	588
10	3.5	9.1	5.8	53	592
Cases	2.94 ± 0.51	9.34 ± 0.52	6.80 ± 1.22	63.4 ± 10.9	300 ± 207
Control	3.52 ± 0.37	9.19 ± 0.93	5.45 ± 1.75	50.0 ± 16.9	341 ± 342
P value	<0.001	NS	<0.01	<0.01	NS

*Corrected for serum albumin.

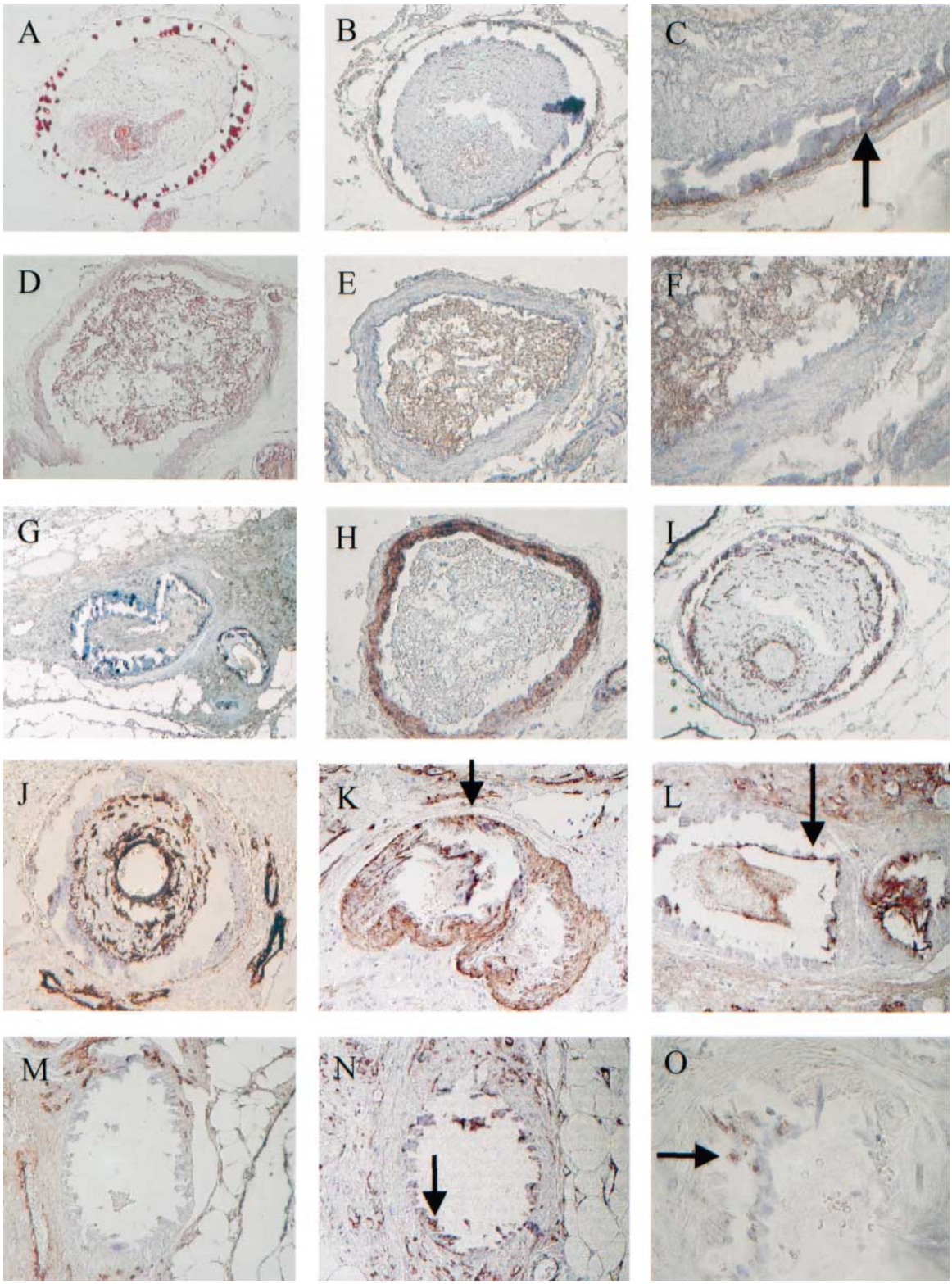

Fig 1.

reacted with DAB to produce an insoluble colored substrate. Specimens were counterstained with methyl green.

Electron Microscopy

One block from patient 5 was examined by electron microscopy. Sections from the specimen were deparaffinized in xylene overnight. They were then immersed in 1% osmium tetraoxide/xylene for 2 hours at room temperature. The sections were rinsed in xylene followed by propylene oxide and then placed in 1:1 mixture of propyleneoxide and polybed 812 resin for 4 hours. Specimens were embedded in fresh resin and polymerized in a 60°C oven for 48 hours. Thin sections of the specimens were stained with uranyl acetate and lead citrate and examined with a Philips CM-10 transmission electron microscope (FEI Co, Hillsboro, OR) operated at 60 kV.

Statistical Analysis

Calcium (corrected for albumin), phosphorus, PTH, calcium X phosphorus product, albumin, race, gender, BMI, and age of CUA patients were compared to a cross-sectional sample of our general "control" dialysis patient population (n = 180) by group *t* test or Fisher's exact test (for sex and race) using SAS software (Cary, NC). Because the samples were so disparate in size, separate variance estimates for the 2 groups were used in the *t* tests. All results are expressed as mean ± SD. A P value of < 0.05 was considered significant.

RESULTS

Clinical Results

In all patients, the presenting symptoms were classic: indurated, painful, violaceous, subcutaneous lesions, which progressed to ulceration and skin necrosis. The clinical characteristics of the 10 CUA patients are described in Table 1. Two patients presented with the distal/acral location, and the remaining 8 had proximal distribution of skin lesions. In comparing the 10 CUA patients with our "control" general dialysis patient population, the CUA patients were more likely to be of white race ($P < 0.001$) and female ($P < 0.02$). The BMI of the CUA patients was greater than the control group ($P < 0.01$). Thirty percent of the CUA patients had diabetic nephropathy, and 4 patients were on subcutaneous insulin. Two of the 10 patients were receiving peritoneal dialysis. The duration of renal replacement therapy (RRT) ranged between 2 to 72 months in CUA patients. Six of 10 patients were receiving anticoagulation with coumadin, and 1 patient had documented protein C deficiency. Six of 10 patients were receiving intravenous or oral vitamin D3 therapy, and 8 patients were receiving subcutaneous injections of erythropoietin. No CUA patient was receiving hormone replacement therapy. There was no history of trauma or injections at the site of the CUA lesions. Treatments included local wound care (10 of 10), surgical debridement (6 of 10), and systemic antibiotics (10 of 10). Six patients died as a result of complications related to CUA. Only 2 patients underwent parathyroidectomy for the treatment of CUA; 1 of these died.

Table 2 shows the serum levels of albumin, calcium, phosphorus, calcium X phosphorus product (CaXP), and intact PTH in the CUA patients. The CUA patients had lower serum albumin concentrations compared with controls

Fig 1. Paraffin blocks from skin biopsies were retrieved from archived samples and stained as described in the Methods section. (A) Patient (Pt) 8, stained with alizarin red pH 4.2 demonstrating multiple deposition in aggregates of calcium, 10 X objective. (B) Pt 8, same vessel stained with antiosteopontin antibody (Ab) demonstrating positive staining at the base of the calcium deposits, 10 X objective. (C) Pt 8, antiosteopontin Ab staining of same vessel as (A) and (B) at higher magnification, again demonstrating positive staining at the base of the calcium deposits (arrow), 40 X objective. (D) Pt 8, different vessel, which was negative for calcification by alizarin red pH 4.2, 20 X objective. (E) Pt 8, same noncalcified vessel as (D) stained with antiosteopontin Ab, demonstrating no reactivity, 20 X objective. (F) Pt 8, same vessel as (D), (E) at 40 X objective. (G) Pt 5, stained with antiosteopontin Ab, again demonstrating deposition of osteopontin at base of calcium deposition in aggregates in side by side vessels, 20 X objective. (H) Pt 8, noncalcified vessel as in (D through F) above, stained with anti-alpha smooth muscle actin Ab, demonstrating intact smooth muscle layer, 20 X objective. (I) Pt 8, calcified vessel as in (A through C) above, stained with anti-alpha smooth muscle actin Ab, demonstrating shearing of smooth muscle layer, 10 X objective. (J) Pt 7, stained with anti-alpha smooth muscle actin demonstrating the medial layer being rolled into a mass of tissue with recannulation, which may have been mistaken for a thrombus or intimal hyperplasia by H&E stain, 20 X objective. (K) Pt 5, stained with antismooth muscle actin Ab, demonstrating lifting of actin positive tissue with calcification, 20 X objective. (L) Pt 5, same vessels as in (K), stained with anti-von Willebrand factor Ab, demonstrating sloughing of endothelial layer as calcium is deposited, 20 X objective. (M) Pt 5, different vessel with advanced calcification and complete absence of endothelial layer by anti-von Willebrand factor Ab staining, 20 X objective. (N) Pt 5, same vessel as (M), stained with anti–α-actin Ab, demonstrating virtually no staining in remaining media or surrounding adventitia, 20 X objective. (O) Pt 5, same vessels stained with anti-CD68 Ab, demonstrating only a few CD68$^+$ cells, 20 X objective.

($P < 0.001$). Calcium, corrected for albumin, and intact PTH levels were similar. However, serum phosphorus and the CaXP were significantly higher in CUA subjects compared with the controls ($P < 0.01$).

Histologic Results

Examination of H&E-stained sections showed calcium deposition in aggregates in the blood vessels of remaining blocks from 5 of 10 patients. Sections from these 5 blocks were then stained as described in the Methods. Alizarin red staining of the calcified vessels was positive at both pH 7.0 and 4.2, indicating the presence of calcium phosphate and/or carbonate (Fig 1A), but no staining in noncalcified vessels (Fig 1D). These observations are consistent with earlier spectral analysis indicating calcium-phosphate as the mineral.[7] In addition, calcification was observed in the small vessels, in the septae of the subcutaneous fat, and some venules. All calcified vessels showed positive immunostaining for osteopontin, which was localized uniformly at the base of the calcium aggregates in each of the vessels (Fig 1B, C, and G). In contrast, none of the noncalcified vessels were positive for osteopontin (Fig 1E and F). Staining for iron was negative in all vessels examined.

Immunostaining performed to characterize the surrounding cells showed a thickened medial layer that was strongly positive for smooth muscle α-actin in noncalcified vessels (Fig 1H). However, in the vessels that were calcified, the α-actin staining pattern was decreased (Fig 1I), absent (Fig 1J), or localized in the debris or "thrombus" within the lumen (Fig 1I and J). The staining for α-actin was inversely related to the degree of calcification. In Fig 1, section H and I represent the α-actin staining from a calcified and noncalcified vessel from the same patient. In advanced lesions, there was no staining for α-actin (Fig 1N). In Fig 1K, as part of the vessel has begun to calcify, the α-actin is decreased, staining the sloughed tissue (arrow). This staining pattern suggests the media may be lifted off by the calcification. In some sections, there was marked staining in the debris found in the vessel lumen (Fig 1J). No sections showed positive staining for α-actin in the adventitia.

In sections in which there was no calcification, there was an intact endothelial layer as shown by staining with anti-von Willebrand factor antibody (Fig 1L, right vessel). Staining with CD34 antibody confirmed these observations. In another part of the adjacent vessel, it appeared that the endothelium was being "lifted" off as the calcium became deposited (Fig 1L, arrow). However, most of the sections were advanced lesions, and there was no endothelial layer remaining (Fig 1M). There was a marked paucity of macrophage infiltration by CD68 with the exception of 1 vessel (Fig 1O). In both the early and advanced lesions, there was no evidence for apoptosis of the smooth muscle cell layer surrounding the calcification. There were, however, scattered areas of apoptosis in the adipocytes in a few sections. Taken together, our analysis suggests that the calcification occurs simultaneously with the presence of osteopontin. The presence of osteopontin was a uniform finding in calcified vessels, but never observed in noncalcified vessels. The medial layer is then severed by the calcium deposits with either loss of the smooth muscle cells into the lumen or dedifferentiation of the smooth muscle cells, as the α-actin staining was more pronounced in the debris inside the lumen than the remaining vessel wall. In contrast to data collected from an animal model of injury to coronary vessels, the adventitia did not gain α-actin staining.[24]

Electron microscopy was performed on 1 section in which a calcified vessel was easily identified at the dermis-subcutaneous layer (Fig 2). Ultrastructural examination of this vessel demonstrated electron dense regions of calcification in the form of large masses and in small 50 to 150 nm round particles consistent with matrix vesicles. The sites of calcification were intertwined among collagen fibrils. The pattern of calcification observed was identical to that seen in in vitro calcification of smooth muscle cells[18] and in normal calcification in bone.[25] The architecture of intracellular organelles was unable to be clearly identified due to paraffin embedding in this retrospective analysis.

DISCUSSION

In the present study, we compared the clinical, demographic, and laboratory results of our 10 patients in a case-control analysis and evaluated the available sections by immunohistochemistry with various cell markers to try to understand the

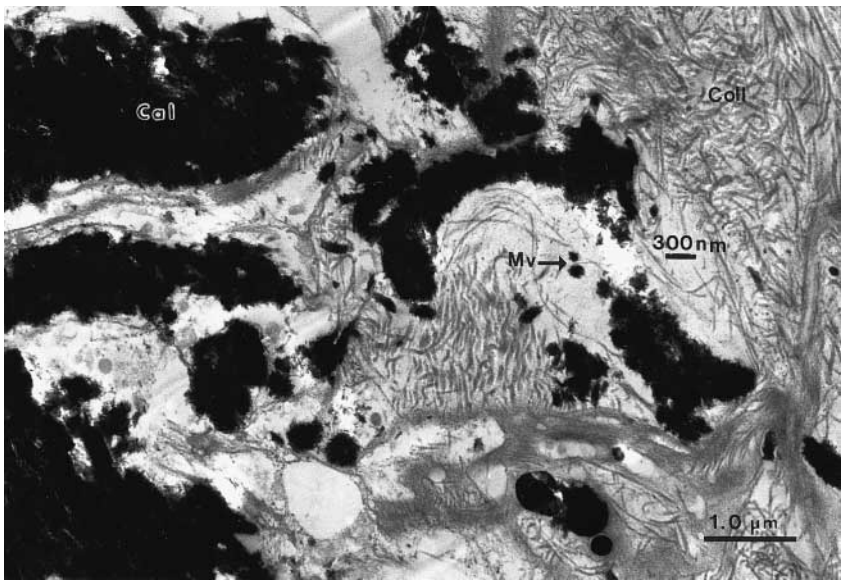

Fig 2. Electron microscopic analysis of a calcified vessel from Pt 5 (1,800 X). Calcium deposits are dense black areas in the form of large masses (cal) or as small 50 to 100 nm round dense particles, consistent with matrix vesicles (Mv). The calcification is interspersed among collagen fibrils (Coll).

pathophysiology of CUA. Our study is unique in its extensive histologic analysis of CUA specimens in association with the case-control analysis of the demographic, clinical, and metabolic profiles of the patients. We found a significant association of CUA with white race, female gender, obesity, and hypoalbuminemia. Presumably, the latter is a result of the disease process rather than a cause. Bleyer et al,[8] in a similar case-control analysis of their CUA cases, also found white race, obesity, female gender, and hypoalbuminemia associated with CUA. In addition, we found an elevated serum CaXP and hyperphosphatemia associated with CUA. The level of CaXP in the cases was less than the commonly accepted 70 mg^2/dL^2.[26] Interestingly, the CaXP in the CUA patients and control patients (63.4 ± 10.9 compared with 50.0 ± 16.9 mg^2/dL^2) are remarkably similar to that of Goodman et al[27] in children and adolescents with and without cardiac calcification by electron beam computed tomography (CT) (65 ± 10.6 compared with 56.4 ± 12.7 mg^2/dL^2). However, in both studies, the elevated phosphorus appeared to be the critical ion that elevated the product. The serum phosphorus levels in Bleyer's study were more elevated in CUA patients, but did not reach statistical significance. In contrast to previous uncontrolled studies,[2,3,12,13] neither our study nor that of Bleyer et al[8] found an association with elevated PTH levels. Further supporting this is the recent study by Mawad et al[28] in which they describe their center's experience with CUA and found 5 of 7 patients had biopsy proven adynamic bone disease with low PTH. It is possible that the earlier "cures" with parathyroidectomy were a result of lowering the serum phosphorus or CaXP as a result of hungry bone syndrome rather than an effect of changes in PTH.

Other studies have also implicated hyperphosphatemia in vascular disease. Block et al[29] and Levin et al[30] recently found elevated serum phosphorus has been shown to increase mortality in dialysis patients in association with increased cardiac death, and Marchais et al[31] found hyperphosphatemia was associated with increased tensile stress of vessels. One potential mechanism to explain these findings is the demonstration that smooth muscle cells from vascular tissue may dedifferentiate into "osteoblast"-like cells capable of mineralizing in vitro and producing bone matrix proteins, such as osteopontin (reviewed in Campbell and Campbell[14]). These cells can form mineralized nodules in vitro, but only in the presence of beta-glycerophosphate, a source of phosphorus.[16-18] Further supporting the importance of phosphorus is the recent study by Jono et al,[32] demonstrating that incubating human vascular smooth muscle cells in vitro with concentrations of phosphorus greater than 1.4 mmol/L (4.5 mg/dL) leads to the induction of the transcription factor Cbfa-1, which is an impor-

tant factor in osteoblast differentiation. The mechanism by which phosphorus increases calcification in vascular smooth muscle cells is believed to be due to elevated concentration of calcium and phosphorus within matrix vesicles, which form as out pockets from the cell membrane.[25,33] This parallels calcification in osteoblasts and chondrocytes.[25] We also observed matrix vesicles in CUA, supporting a similar mechanism of calcification.

We performed extensive histologic examination of the CUA vessels to determine if the cells surrounding the calcification had dedifferentiated to a more "osteoblast"-like phenotype. Our interpretation of the data is limited due to the single time point nature of this study. However, based on some sections, which had vessels at various stages of calcification, we interpret our histologic findings as follows. The initial event may be calcification of the tunica media with production of osteopontin, although which comes first is unknown, as all calcified sections had osteopontin. The distribution of the calcification was clearly in the media, and there was no evidence of lipid-laden macrophages, intimal hyperplasia, or other evidence of a classic atherogenic process. The endothelium was absent in all advanced cases. The "thrombus" that is commonly described in CUA was mostly composed of sloughed smooth muscle cells, as staining for α-actin was strong. We did not find evidence of apoptosis of the vascular smooth muscle cells, but the cells did lose staining for smooth muscle α-actin. This may be due to a loss of the number of cells as they were sloughed off into the lumen or some component of dedifferentiation. Clearly, full understanding of the pathogenesis will require an animal model to follow sections serially.

According to our histologic findings, CUA is a type of Mönckeberg's calcification, characterized by calcification in the medial layer.[34,35] This is in contrast to classic atherogenesis in large vessel disease and coronary arteries, which is associated with lipid-laden macrophages and intimal hyperplasia, with late calcification.[36] Shanahan et al[37] recently demonstrated that isolated arterial vascular smooth muscle cells from both atherogenic and Mönckenberg's pattern of calcification become osteoblast-like, producing bone matrix proteins, such as osteopontin.[17] We also found a similar expression of osteopontin at the base of calcium aggregates in calcified vessels from CUA patients, whereas no osteopontin expression was observed in noncalcifed vessels from the same patient (Fig 1B, C, E, and F). In fact, the pattern of osteopontin staining observed in the present study is identical to the findings by Shanahan et al[17] in Mönckeberg's sclerosis. Perhaps some cellular injury, such as ischemia, leads to changes that allow calcification to occur. Obesity, which was a risk factor in both our study and that of Bleyer et al, may lead to cellular ischemia due to decreased blood flow to the truncal regions (reviewed in Janigan et al[10]). Perhaps all of the various "risk factors" for CUA that have been reported in the literature[2-10] are simply causative agents leading to cellular injury of vascular smooth muscle cells, with dedifferentiation and calcification as a response. It is possible that the injury must occur in an environment conducive to calcification, such as hyperphosphatemia. In that regard, there is some similarity between CUA and the original Seyle calciphylaxis model[1] of sensitizer (ie, elevated phosphorous) and challenging agents (ie, ischemia). Clearly, much work remains to be done to fully understand the pathogenesis of CUA, including the reasons for increased risk in Caucasians.

In summary, we demonstrate for the first time in a controlled study that elevated serum phosphorus and CaXP is associated with CUA. In addition, our histologic analysis demonstrates that the pattern of calcification is that of a Mönckeberg's, or medial calcinosis, indicating the principal cell involved is the vascular smooth muscle cell of the medial layer. It appears that these cells dedifferentiate into osteoblast-like cells, capable of producing the bone matrix protein osteopontin and forming matrix vesicles. The relationship between hyperphosphatemia and these findings deserve further study. However, it is alarming that the elevated product found in both the present study of CUA and that of Goodman et al,[27] which examined coronary artery calcification in dialysis patients, was near 50 mg^2/dL^2, well below the commonly "accepted" value of 70 mg^2/dL^2. This data, together with the increased mortality found in dialysis patients with CaXP of 73 mg^2/dL^2 or greater,[29] point to an increasing need for much

tighter control of serum phosphorus and CaXP than previously recognized.

ACKNOWLEDGMENT

The authors would like to thank Dr Naomi Fineberg for help with the statistical analysis and Dr Carrie Phillips for her support of the project. We also thank Lee Ann Baldridge, Sharon Bledsoe, Michael Goheen, Taffy Hooser, and Farrukh Azmi, MD, PhD, for their expert technical assistance.

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