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Medial artery calcification in ESRD patients is associated with deposition of bone matrix proteins

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Medial artery calcification in ESRD patients is associated with deposition of bone matrix proteins.

Background. In non-ESRD patients, recent studies have demonstrated that the process of vascular calcification resembles developmental osteogenesis. Patients with ESRD are known to have excessive vascular calcification, but this has previously been attributed to the non-cell-mediated process of metastatic calcification.

Methods. To determine if the calcification observed in patients with ESRD is related to a cell-mediated process, we removed a piece of inferior epigastric artery at the time of renal transplant. Calcium content of the entire vessel was quantified with spiral computed tomography (CT). The vessel was then examined histologically for calcification and the presence of bone matrix proteins by immunohistochemistry, and medial and intimal thickness quantified by histomorphometry. These findings were correlated with demographic, clinical and laboratory values.

Results. The proximal inferior epigastric artery was obtained from 41 patients undergoing renal transplantation, but two were inadequate for histologic examination. Twenty-seven of the remaining vessels had no evidence of calcification by MacNeal's or Alizarin red pH 4.2 staining, five vessels had mild/moderate calcification, and seven had severe calcification, all in the medial layer. Calcification assessed histologically was closely correlated with calcification score as assessed by spiral CT, normalized for vessel weight ($P = 0.027$). Positive immunostaining for the bone matrix proteins osteopontin, type I collagen, bone sialoprotein, and alkaline phosphatase was strongly correlated with calcification (all $P \leq 0.001$), as was a history of coronary artery disease ($P < 0.001$), and diabetes ($P = 0.034$). The calcification score by spiral CT correlated with these same factors and the serum phosphorus and calcium $\times$ phosphorus product ($P = 0.032$ and 0.037). The location of immunostaining for the bone proteins was strongly associated with the presence of calcification. However, positive immunostaining also was observed in association with disorganization of the vascular smooth muscle cells in the medial layer due to deposition of a matrix-like substance, prior to overt calcification.

Conclusions. In patients with ESRD undergoing renal transplantation, vascular calcification of the medial layer of the inferior epigastric artery is common (44%), can be detected by spiral CT, and is associated with deposition of bone matrix proteins. This implies an active cell-mediated process, raising hope that directed intervention can arrest this process.

Key words: calcium, dialysis, phosphorus, vascular calcification, metastatic calcification, atherosclerotic disease, extra-skeletal calcification.

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Atherosclerotic disease remains a major cause of morbidity and mortality in the general population. The assessment of coronary arteries by new non-invasive imaging techniques such as electron beam computed tomography (EBCT) scan has heightened the awareness that over 90% of atherosclerotic plaques observed in the aging population are calcified [1]. Furthermore, the magnitude of calcification by EBCT correlates with the severity of obstructive coronary artery disease by angiography [2, 3] and with clinical cardiac events [3]. Cardiovascular disease and stroke are also the leading causes of death in patients with end-stage renal disease that require dialysis (ESRD), at a risk that is 10- to 20-fold the age and sex matched general population [4, 5]. 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. Furthermore, in a follow-up of these 57 hemodialysis patients, every patient had an increase in their calcification score when followed up just one to two years later [6]. Goodman et al recently demonstrated this process also affects young adults on dialysis, with a sharp increase in the magnitude of coronary artery calcification by EBCT after age 20 [7].

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, several recent studies have not found an association between elevated parathyroid hormone (PTH) and coronary artery calcification [7, 8] or calcification of the arterioles of the skin in calcific uremic arteriolopathy (CUA calciphylaxis) [9]. Instead, recent studies evaluating vascular calcification using new techniques in dialysis patients demonstrate that an elevated serum phosphorus, an elevated serum calcium × phosphorus product, or increased calcium load are risk factors [7, 9–11]. However, the mechanism by which the elevated concentration of these ions leads to calcification in dialysis patients is unknown. Recent studies in non-dialysis patients have demonstrated that vascular calcification in coronary arteries of patients with atherosclerosis resembles developmental bone formation, with the production of "bone" proteins by vascular smooth muscle cells, including osteopontin, bone sialoprotein, alkaline phosphatase, and type I collagen [12–17]. Recently, we have found evidence of osteopontin expression at the site of calcification in small arterioles in the skin of patients with calcific uremic arteriolopathy [9]. This would imply that the deposition of mineral into vascular tissue in dialysis patients is not simply metastatic, but rather an active process, perhaps paralleling that observed in atherosclerotic calcification in non-dialysis patients. To test the hypothesis that generalized vascular calcification in dialysis patients is also cell mediated, and at least in part regulated by the synthesis of these same "bone" matrix proteins by vascular smooth muscle cells, we examined arteries obtained from patients undergoing renal transplantation and compared our histologic findings with clinical parameters and laboratory tests.

METHODS

All patients over the age of 18 undergoing a renal transplant at Indiana University Hospital were eligible for the study. At the time of transplant, patients had sera drawn from their central line prior to the administration of any immunosuppressive medications. During the transplant procedure, the Transplant Surgeon normally ligates the inferior epigastric artery. For this study, the proximal portion of that artery was removed after ligation and placed immediately in saline. The vessel was dissected free of fat and subcutaneous tissue, weighed, and then fixed by placing in 4% paraformaldehyde for 48 hours, followed by 0.25% paraformaldehyde. The vessel was taken to the Radiology Department and placed on a dish and moved through a spiral CT scanner (see below) to quantify the calcium content. Once analyzed for calcium content, the vessel was cut into two pieces and embedded in paraffin for sectioning. The patient's medical record was reviewed for demographic, historical, and medication information, supplemented with questions directly to the transplant recipient. The study was approved by the Indiana University Purdue University at Indianapolis/Clarian Institutional Review Board.

Spiral CT scan

The arterial specimen was placed in air on a plastic cup in the center of the CT gantry with the long axis of the vessel aligned with the long axis of the CT table. Scanning was performed on the model MX-Twin, (Marconi Medical Systems, Inc., Cleveland, OH, USA). Data were acquired with the following parameters: kVP = 120; mAs = 320; focal spot size = small; gantry rotation time = 1 second; slices/gantry rotation = 2; beam collimation = 2 mm; nominal slice width = 1 mm; table speed = 1.5 mm/sec; pitch = 0.75 (× 2 slices/rotation). Data were reconstructed with the following parameters: reconstruction field of view = 18 cm; matrix = 512 × 512 pixels; reconstruction algorithm = 360° linear; effective slice width=1.1 mm; longitudinal reconstruction increment = 0.5 mm; edge enhancement = 0%; filter = standard body (C). Calcium scoring was performed with software installed on the scanner console (Marconi Medical Systems). All pixels with density greater than or equal to 90 Hounsfield Units (HU) were highlighted in (pink) color on the black and white images [18]. A region of interest was placed by the operator around all colored areas of the artery on every image. Calcified lesions were defined as those lesions with density equal to or greater than 90 HU with an area of 0.1 square mm or greater [18]. Smaller lesions were not scored. A weighting factor was applied to each lesion. The weighting factor (F) = (D/100) − 0.5, where D is the average density (HU) of all pixels in each lesion on each image. The score for each lesion was calculated by multiplying the area of each lesion in square mm by the weighting factor. The calcium score for the entire specimen was equal to the sum of the scores for each lesion. In addition, the area was also determined. Small microcentrifuge tubes containing various concentrations of calcium chloride were scanned with the same technique described above, establishing the linear function of detection at low calcium concentration in a sample of comparable size. The spiral CT calcification score and area results were divided by vessel weight, to normalize for the variation in the size of the vessels.

Laboratory values

The serum collected at the time of transplant was analyzed for calcium, phosphorus and total alkaline phosphatase by colorimetric methods using a Roche Autoanalzyer (Boehringer Manheim, Indianapolis, IN, USA). The results for calcium and phosphorus were averaged with other values obtained during the hospitalization (prior to transplant, 1 to 3 different measurements), and the Ca × P product determined by multiplying these

two values. Serum obtained at the time of transplant also was analyzed by enzyme-linked immunosorbent assay (ELISA) for intact PTH (IRMA Nichols Institute, San Juan Capistrano, CA, USA) and Bone Specific Alkaline Phosphatase (Metra Biosystems, Mountain View, CA, USA). Values for total cholesterol, if available, were obtained from the patients' medical record from the previous six months.

Clinical history

The medical records were reviewed to determine the duration of dialysis, type of dialysis (defined as modality in use at the time of transplant), cause of ESRD, presence or absence of diabetes, history of peripheral vascular disease, past history of smoking, and known coronary artery disease. The latter was defined as stenotic lesions on coronary artery angiogram >30% in any vessel, history of angioplasty or bypass surgery, or previous myocardial infarction. Medication lists were reviewed for past or present use of vitamin D, and current use of hormone replacement therapy (women), lipid lowering agents, anti-hypertensives, and phosphate binders. The phosphate binder calcium intake was calculated by taking the number of pills per day × the known elemental calcium content as defined in the package insert for each type of calcium containing phosphate binder.

Histologic analysis

Vessels were stained for the presence of calcification by MacNeal's tetrachrome and Alizarin red staining. The presence or absence of bone matrix proteins was assessed by immunohistochemistry on paraffin embedded sections after fixation as described above.

MacNeal's stain. Specimens were deparaffinized in xylene and rehydrated in graded alcohol followed by distilled water. Sections were then placed in 0.2% silver nitrate for 10 minutes in the dark, rinsed, and placed in sodium carbonate-formaldehyde for two minutes. After rinsing, the sections were placed in Farmer's diminisher for 30 seconds and rinsed until clear. The sections were counterstained with tetrachrome solution, rinsed, dehydrated and mounted. This stains calcification black, and non-mineralized collagen and non-collagenous proteins (such as unmineralized bone/osteoid) a light blue/purple color.

Alizarin red stain. Alizarin red is a pH dependent stain, which can differentiate calcium oxalate from calcium carbonate and phosphate. 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 three minutes, rinsed with distilled water, and then dehydrated through graded alcohol [9].

Immunostaining for "bone proteins." Unstained slides of tissue sections were deparaffinized in xylene and rehydrated in descending 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, USA) for 15 minutes, and then incubated in the primary antibody at appropriate dilutions for one hour. The secondary antibody peroxide conjugated goat-anti-rabbit IgG or goat-anti-mouse at 1:400 dilution (Southern Biotechnology, Birmingham, AL, USA) was applied for 30 minutes, developed with DAB (Vector Biotechnology, Burlingame, CA, USA), and the section counterstained with Harris Hematoxylin (Sigma). The antibodies utilized were: anti-osteopontin (1:100), bone sialoprotein (1:100) and type I collagen (1:50; all gifts of Larry Fisher, Ph.D., National Institutes of Health). CD68, type IV collagen and factor VIII were done with a DAKO autostainer (with primary antibodies 1:100; all from DAKO Corp, Carpinteria, CA, USA). Sections of kidney and/or bone served as positive controls. Staining without the primary antibody served as a negative control and was done for each section.

For the alkaline phosphatase immunostaining, slides were deparaffinized in xylene and hydrated in graded alcohols. Endogenous peroxidase activity was quenched by incubating slides in 1.5% H_2O_2 in ddH_2O, rinsed, and incubated in Pepsin Solution (Sigma), rinsed, and blocked, (ABC Staining System, Santa Cruz, CA, USA). Primary antibody (1:500 mouse anti-human placental alkaline phosphatase; Chemicon, Temecula, CA, USA) was added for one hour followed by secondary antibody-biotin and AB enzyme (ABC Staining System). Slides were counterstained with Harris Hematoxylin.

Histomorphometry

Histomorphometric measurements were collected on a Nikon Optiphot II fluorescence microscope (Nikon Inc., Garden City, NY, USA) equipped with an Optronics NTSC-CE camera head. Bioquant 98 image analysis system (R&M Biometrics, Inc., Nashville, TN, USA) was used to measure section profiles captured using the camera head. The following parameters were measured on sections stained with MacNeal's stain: vessel and lumen area and diameter, and intimal and medial width (thickness). The results represent the average of measurements on 2 to 4 sections per specimen. Sections where the complete circumference of the vessel was not intact, or were tangentially cut, were not quantified.

Electron microscopy

One block from one patient was examined by electron microscopy as previously described [9]. Sections from the specimen were deparaffinized in xylene overnight. They were then immersed in 1% osmium tetraoxide/xylene

for two 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 four 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 Phillips CM-10 transmission electron microscope (FEI Co., Hillsboro, OR, USA) operated at 60 kV.

Data analysis

Semiquantitative analysis was done for all sections by a single reader, who was blinded to patient characteristics. Scores for 2 to 6 sections per stain per subject were determined and averaged. For the histologic calcium content, sections were read for MacNeal's and were graded from 0 to 4+: 0 = no calcification (black/silver stain); 1 = spots; 2 = single segments of black staining; 3 = multiple segments; and 4 = diffuse, circumferential staining. A similar grading system was utilized for Alizarin red (pH 4.2) staining, and the purple-blue staining by MacNeal's stain within the medial layer of the vessel wall.

Histologic calcification score. The histologic calcification score was obtained by averaging all of the scores from all sections for both MacNeal's and Alizarin red stains, where a score of <1 was labeled "no calcification"; score of 1 to 2.5 = mild/moderate calcification; and >2.5 to 4 = severe calcification. The immunohistochemistry was read in a similar blinded, semiquantitative manner with grading of 0 to 4+.

Statistical analysis. Data are expressed as mean ± SD. Since many of the variables were not normally distributed, non-parametric statistical methods have been used. Spearman Rank Correlation was used to assess the relationship between two continuous variables. Relationships between continuous and discrete variables were examined with either the Mann-Whitney U test or Kruskal-Wallis non-parametric analysis of variance depending on the number of discrete values. Fisher's Exact Test was used when both variables were discrete.

RESULTS

Forty-one patients entered the study over an eight-month period. Seventeen patients underwent cadaveric transplants, 19 living related transplants, and three living non-related transplants. The patients were 45 ± 13 years old (range 18 to 64) and had been on dialysis for 25 ± 20 months (0 to 72). Twenty-five patients were undergoing hemodialysis at the time of transplantation, 14 were undergoing peritoneal dialysis, and two were not yet on dialysis. Thirty-one patients were male and ten were female; 35 patients were Caucasian, five were African American and one was an American Indian. The cause of ESRD was glomerulonephritis in 15, diabetes mellitus in ten, polycystic kidney disease in six, genitourinary problems in five, hypertension in three, and unknown in two patients. These demographics reflect the large percentage of living related transplants and young age of these patients. Other clinical and laboratory indices for the subjects are listed in Table 1.

Inferior epigastric arteries were assessed for the presence of calcification by staining with MacNeal's stain and Alizarin red as described in the methods. Twenty-seven vessels had no evidence of calcification (Fig. 1A), five vessels had mild/moderate calcification (Fig. 1B), seven vessels had severe calcification (Fig. 1C), and two could not be quantified due to inadequate sections. In all but one patient, the calcification was located exclusively in the medial layer with segmented spots of calcification observed in mild/moderate disease (Fig. 1 B and D, higher magnification of the box in 1B). In severe calcification, the calcification was circumferentially located in the media, effectively dissecting the vessel into two pieces (Fig. 1C). There was calcified intimal plaque in only one patient, who was diabetic, in addition to severe medial calcification. The histologic calcification content was positively correlated with the presence of diabetes mellitus ($P = 0.034$) and a history of CAD ($P < 0.001$). No other demographic variable was associated with the histologic calcification content, although those patients being treated for hyperlipidemia approached significance ($P = 0.068$). No laboratory values correlated with the histologic calcification content.

The calcium score as determined by spiral CT scan, normalized for the weight of the vessel, was significantly related to the histologic presence of calcification (Table 2). The calcification by spiral CT, when assessed by area, reached borderline significance. The calcium score and area values were tightly correlated ($r = 0.92$, $P < 0.001$). The serum phosphorus correlated with the calcium score by area/weight ($r = 0.33$, $P = 0.032$) and was borderline significant for score/weight ($r = 0.37$, $P = 0.058$). Similarly, the Ca × P product was significantly correlated with both the score/weight ($r = 0.36$, $P = 0.037$) and the area/weight ($r = 0.37$, $P = 0.031$). Patients on peritoneal dialysis had greater score/weight ($P = 0.012$) and area/weight ($P = 0.014$) spiral CT calcium content than hemodialysis patients.

On histologic examination, the specimens without calcification showed a nice linear arrangement of vascular smooth muscle cells (Fig. 1F). In contrast, the medial layer of vessels with calcification showed a haphazard arrangement of cells, with deposition of a purple staining material (Fig. 1G). The immunostaining for each of the four "bone" proteins examined ranged from 0 to 4. The positive immunostaining for the bone proteins was almost exclusively in the media, and generally most prominent in areas adjacent to or surrounding overt calcifica-

Table 1. Clinical and laboratory variables of subjects

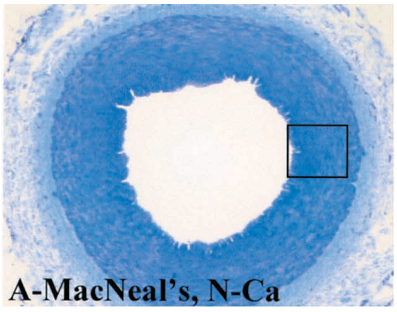
Variable	With/without	Mean ± SD	Range
Diabetes mellitus	14/28		
History of CAD	8/33		
History of PVD[a]	2/39		
History of hypertension[a]	37/3		
Ever smoked	12/29		
History of hyperlipidemia	10/31		
On medication for hyperlipidemia	9/32		
On hormone replacement[a]	3/5 (33 n/a)		
On vitamin D therapy	16/25		
Body mass index		25.5 ± 4.5	16.1–33.9
Elemental calcium content of phosphate binder *mg/day*		2247 ± 2095	0–7500
Serum calcium *mg/dL*		8.9 ± 0.8	6.1–10.2
Serum phosphorus *mg/dL*		5.2 ± 1.3	2.8–8.2
Serum Ca × P product mg^2/dL^2		46.5 ± 13.1	24.9–71.7
Serum intact PTH *pg/mL*		452 ± 398	30–1632
Serum total alkaline phosphatase *IU*		77.6 ± 52.4	19–295
Serum bone specific alkaline phosphatase *IU*		19.7 ± 21	3.3–117.8
Serum albumin *g/L*		3.9 ± 0.5	2.5–4.8
Serum total cholesterol[b] *IU*		174 ± 56	85–287

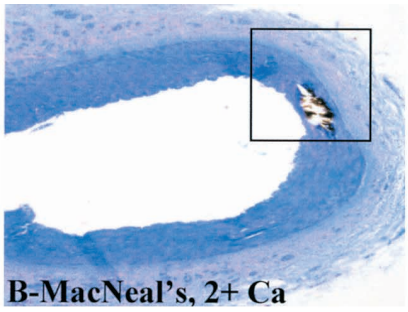
Abbreviations are: CAD, coronary artery disease; PVD, peripheral vascular disease; PTH, parathyroid hormone; n/a, not applicable.
[a] Distribution not suitable for further analysis
[b] Only available for 29 patients

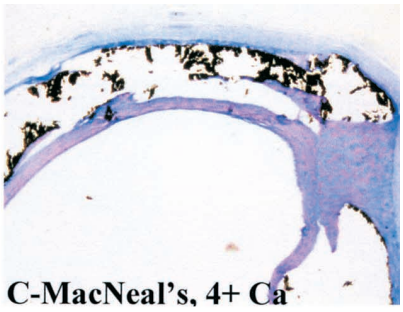
tion (Fig. 1 H–O), corresponding to areas with the haphazard arrangement of cells and purple staining material. The mean ± SD and (median) immunostaining score was 1.38 ± 1.44 (1.00) for osteopontin, 1.36 ± 1.00 (1.00) for bone sialoprotein, 1.46 ± 1.25 (1.50) for alkaline phosphatase, and 1.66 ± 1.65 (1.00) for type I collagen. The degree of immunostaining for the bone proteins was strongly associated with the severity of the histologic calcification score (Table 2). In vessels with more advanced calcification, there was an apparent loss of type IV collagen (Fig. 1N) and gain of type I collagen (Fig. 1O) in the same area, although quantification of this was not possible. Of the 27 vessels without calcification, 16 had evidence of deposition of the purple staining material in the medial layer by MacNeal's stain (Fig. 1G). Of these 16 vessels with positive purple staining by MacNeal's stain, 12 (75%) had positive immunostaining (≥1+) for at least one bone protein. In contrast, of the 11 vessels without positive purple staining, only one (9%) had positive immunostaining for any bone protein ($P = 0.001$). Thus, it appeared that positive immunostaining was associated with deposition of the purple staining substance by MacNeal's stain and could be found in the absence of calcification as assessed by silver stain (MacNeal's) or Alizarin red.

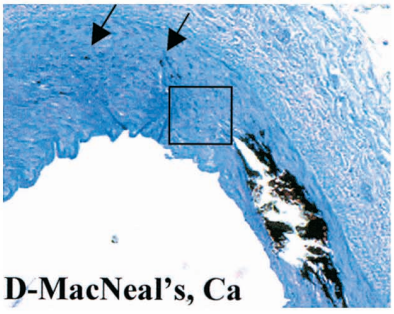
The endothelial layer was intact and positive for Factor VIII in all sections and occasionally thickened. In all sections, the internal elastic lamina was intact, indicating that the medial calcification did not result from encroachment of the intimal layer into the media. There was CD68 + positive cells (macrophage) in the intima in only 12 sections. CD68 + cells were not observed in the me-

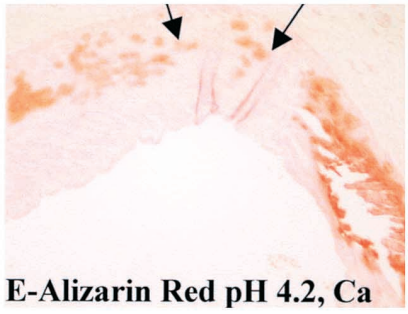
Fig. 1. Histologic evaluation of calcified (Ca) and non-calcified (N-Ca) vessels from patients undergoing renal transplantation: Examination of the arteries revealed a spectrum of calcification changes by MacNeal's stain from normal appearing vessels (*A*, non-calcified = N-Ca; ×10 objective), mild/moderate (2+) calcification (*B*, calcified = Ca; ×10) and severe (4+) medial calcification (*C*, calcified = Ca; ×10). When the area in the black box in vessel B is examined at higher magnification (×20), there is some punctate calcification by MacNeal's stain (*D*, arrows) and by Alizarin red, pH 4.2 (*E*, arrows). To assess the histologic changes further, we compared a part of the normal vessel (A, black box = N-Ca) to this area in the 2 + Ca vessel (D, black box = Ca) with various stains at higher magnification (×40, panels *F–O*, corresponding to non-calcified (N-Ca) in A, and the calcified vessel (Ca in D). By MacNeal's stain, the normal vessel (F, which corresponds to the black box in A) revealed a nice linear pattern of vascular smooth muscle cells. In contrast, in the minimally calcified area of the moderately calcified vessel (G, corresponding to the black box in D) revealed a haphazard arrangement of cells, in part, due to the deposition of a purple staining material (arrow in G). Immunostaining for alkaline phosphatase (α-Alk Phosph) revealed minimally positive staining in the uncalcified vessel (H), but positive staining in the calcified vessel (I, arrow) in an area corresponding to the purple substance in G. Immunostaining for osteopontin (α-Osteopontin) revealed a similar pattern: in the non-calcified vessel (J) there was only scattered positive staining, whereas the staining for osteopontin in the calcified vessel (K, arrow) was very strong, and appeared to be in the area surrounding cells. Similarly, immunostaining for bone sialoprotein (α-BSP) was negative in the non-calcified vessel (L), but positive in the calcified vessel (M, arrow). It is important to point out that the positive immunostaining for these bone proteins was in the area with only mild specs of calcium by MacNeal's stain (D square) and Alizarin red pH 4.2 (E). Thus, the immunostaining was positive even in the absence of overt calcification. Staining for type IV collagen, a major vascular wall collagen was strong in all vessels, but there were areas of absent staining for type IV collagen (N, arrow) in calcified vessels. In contrast, staining for type I collagen, the predominant collagen in bone, was positive in these same areas (O, arrow), suggesting a replacement of type IV collagen with type I collagen.

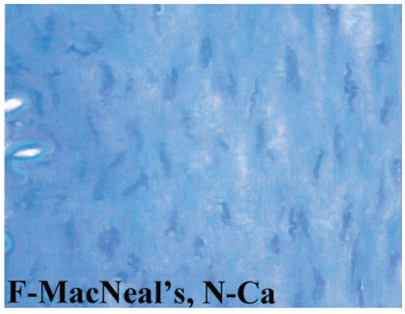

A-MacNeal's, N-Ca

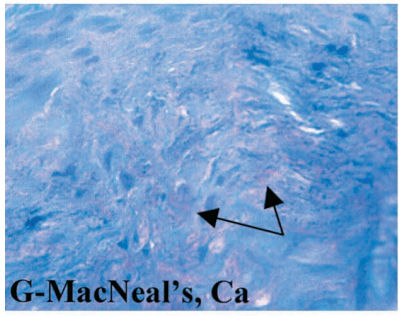

B-MacNeal's, 2+ Ca

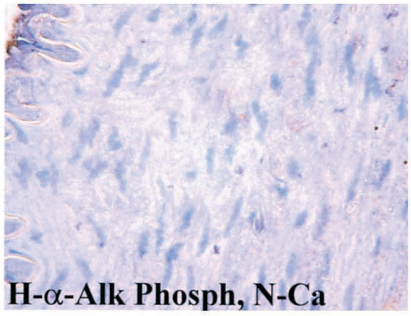

C-MacNeal's, 4+ Ca

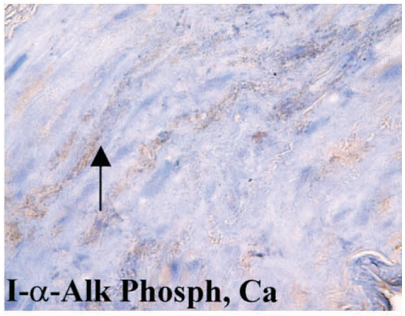

D-MacNeal's, Ca


E-Alizarin Red pH 4.2, Ca


F-MacNeal's, N-Ca


G-MacNeal's, Ca


H-α-Alk Phosph, N-Ca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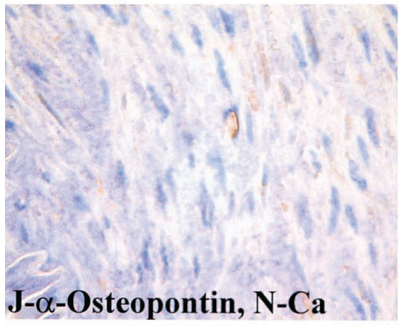
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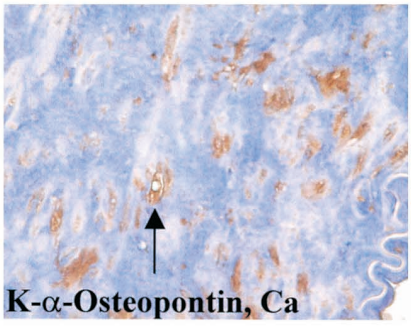
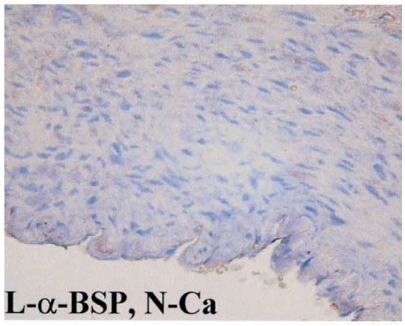
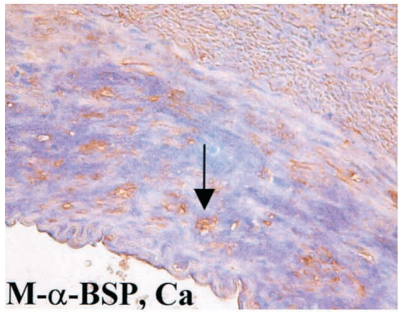
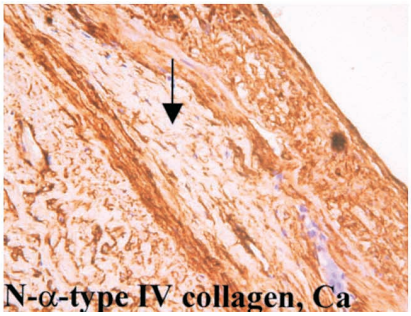

K-α-Osteopontin, Ca


L-α-BSP, N-Ca


M-α-BSP, Ca


N-α-type IV collagen, Ca

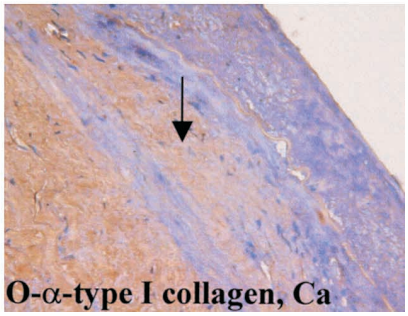

O-α-type I collagen, Ca

Table 2. Relationship of bone protein staining and spiral CT calcification score to histologic calcification content

Bone protein	Histologic calcification content			*P* value
	None	Mild/mod	Severe	
Number vessels	27	5	7	
Osteopontin[a]	0.5	2.5	4.0	<0.001
Alkaline phosphatase[a]	0.5	2.0	3.0	<0.001
Bone sialoprotein[a]	0.5	1.5	3.5	=0.001
Type I collagen[a]	0.5	2.0	4.0	<0.001
Spiral CT score/weight[b] *mg*	0.067	0.020	1.061	=0.027
Calcification area/weight[b] *mg*	0.073	0.040	0.288	=0.078

[a] Sections were scored semiquantitatively from 0 (no staining) to 4+ (staining diffusely in nearly all of vessel). Results are the median staining score for vessels in each of the three calcification score categories.

[b] Vessels, prior to sectioning were weighed and then analyzed by spiral CT scanner for presence of calcification (see **Methods**)

dial layer even with advanced medial calcinosis. A history of coronary artery disease was associated with positive immunostaining of osteopontin ($P = 0.001$), type I collagen ($P = 0.015$), alkaline phosphatase ($P = 0.008$) and bone sialoprotein ($P = 0.092$). Similarly, vessels from patients with diabetes, compared to non-diabetics, was associated with positive immunostaining for osteopontin ($P = 0.001$), type I collagen ($P = 0.012$), and alkaline phosphatase ($P = 0.015$). Increasing age correlated with alkaline phosphatase immunostaining ($r = 0.40$; $P = 0.015$). Increasing body mass index correlated with immunostaining for both alkaline phosphatase ($r = 0.43$, $P = 0.008$) and bone sialoprotein ($r = 0.34$; $P = 0.039$). The relationship of laboratory values and immunostaining was not consistent for the different proteins. However, there was a significant negative correlation of type I collagen immunostaining and serum calcium $\times$ phosphorus product ($r = -0.34$, $P = 0.043$) and serum calcium level ($r = -0.42$, $P = 0.010$); alkaline phosphatase immunostaining with serum phosphorus ($r = -0.41$, $P = 0.012$); and bone sialoprotein immunostaining with serum bone alkaline phosphatase ($r = -0.41$, $P = 0.012$).

The vessels that were histologically intact ($N = 32$) were analyzed by histomorphometry. The maximum intimal width/thickness was 0.059 ± 0.072 mm (range 0 to 0.246 mm). The maximum medial width was 0.372 ± 0.117 mm (0.201 to 0.658 mm). The maximum vessel diameter was 2.21 ± 0.71 mm (1.07 to 3.98 mm). The maximum lumen diameter was 1.48 ± 0.57 mm (0.45 to 3.16 mm). In the vessels with severe (4+) calcification, the vessel wall was so disrupted that morphometric analysis could not be done. The maximum intimal thickness correlated with the presence of diabetes ($P = 0.005$), African American race ($P = 0.016$), the duration of dialysis ($r = 0.38$, $P = 0.030$) and the calcium load ($r = 0.39$, $P = 0.030$). The maximum medial thickness was greatest in males compared to females ($P = 0.004$), but did not correlate with any other laboratory or demographic characteristic. The maximum vessel diameter was greatest in males compared to females ($P = 0.049$), peritoneal dialysis versus hemodialysis patients ($P = 0.039$), and older age ($r = 0.36$, $P = 0.041$). The maximum lumen diameter was also greater in peritoneal dialysis compared to hemodialysis patients ($P = 0.001$), and negatively correlated with the calcium load from phosphate binders ($r = -0.041$, $P = 0.023$). The maximum vessel and lumen diameter were strongly correlated with the maximum medial thickness ($r = 0.68$, $P < 0.001$ and $r = 0.54$, $P = 0.001$, respectively). Not surprisingly, the maximum vessel diameter correlated with the maximum lumen diameter ($r = 0.84$, $P < 0.001$).

Electron micrographs revealed the presence of matrix vesicles adjacent to the collagen fibrils, similar to those we observed with calcific uremic arteriolopathy [9].

DISCUSSION

The results of this study in patients with ESRD demonstrate that calcification of the inferior epigastric artery is in the form of medial calcinosis. In addition, for the first time in dialysis patients to our knowledge, we have demonstrated that this calcification is associated with expression of "bone" matrix proteins, similar to findings in vessels of non-dialysis patients with both atherosclerotic coronary arteries [12–16] and small distal vessels in medial calcinosis [17]. The presence of positive immunostaining for these bone proteins was found more frequently than was overt calcification, which suggests that the deposition of these proteins precedes calcification. Furthermore, in vessels without histologic evidence of calcification but positive immunostaining for these bone matrix proteins, there was a deposition of purple-stain material in the same location in the medial layer of the arteries. In bone biopsies using a MacNeal's stain, this purple staining correlates with deposition of osteoid, or unmineralized bone, composed of collagen and noncollagenous proteins. Thus, our findings suggest that the initial changes that occur in the vessels of dialysis patients are the deposition of these bone matrix proteins, followed by calcification. However, a single time point study such as this cannot truly assess the order in which this process occurs. Nevertheless, these histologic changes observed in the arteries of ESRD patients clearly parallel observations in bone.

Calcification in bone proceeds as a tightly regulated process. Osteoblasts produce matrix, most predominantly type I collagen, which is subsequently mineralized by the ordered deposition of hydroxyapatite on to the collagen and matrix lattice [19]. The hydroxyapatite is believed to form in the osteoblast and is concentrated in cell membrane vesicles. These vesicles then pinch off from the cell membrane to form matrix vesicles, which attach to the type I collagen fibrils and other non-collage-

nous matrix proteins [20]. Numerous cytokines and hormones are known to regulate this process in bone. In the present study, we were able to demonstrate a replacement of type IV collagen, found in blood vessels, with type I collagen, predominately found in bone (Fig. 1 N, O). Watson et al have previously demonstrated that bovine vascular smooth muscle cell isolates that rapidly mineralized in vitro produced three times the type I collagen, and markedly less type IV collagen than cells that did not mineralize [21]. In addition, we observed the presence of matrix vesicles by electron micrographs. These findings imply that an active cell-mediated process of mineralization exists in vascular tissue, similar to that in bone.

In atherosclerotic disease, the calcification is felt to be a late phenomenon after initial plaque formation in response to atherosclerotic risk factors such as lipid oxidation, macrophage infiltration and inflammation. In the present study, we were unable to find any evidence of macrophages or other cellular infiltrates in the medial calcification, which implies that an inflammatory process is not a requirement for calcification of the vascular smooth muscle cells. Despite past thoughts that medial calcification and atherosclerotic/plaque calcification are mechanistically distinct, thus far, there is no evidence to support this hypothesis. However, it is possible that different factors lead to similar cellular events in these two forms of calcification, up-regulating specific genes and transcription factors important in the calcification process. In vitro studies have demonstrated in vascular smooth muscle cells that the expression of these genes and transcription factors proceed in a sequence similar to that in bone [14, 16, 17, 22]. Indeed, cultures of vascular smooth muscle cells can be induced to form bone-like nodules in the presence of calcification media, consisting primarily of β-glycerophosphate, as a phosphorus donor [12, 23, 24] similar to that observed in osteoblasts in culture [25].

What makes this process so prominent in dialysis patients? Dialysis patients are known to have many vascular risk factors such as a history of cardiac disease, diabetes, oxidized lipids, and hypertension. In the present study a history of coronary artery disease and diabetes were correlated with calcification by both histology and spiral CT, and correlated with bone protein immunostaining, albeit weakly. Advancing age and increased body mass index also appear to be contributing factors, as has been found in vascular disease in non-dialysis patients.

In addition to these traditional vascular risk factors, there is increasing evidence that elevated serum phosphorus, serum calcium × 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 [7], calcific uremic arteriolopathy (calciphylaxis) [9], carotid and aortic calcification [11, 26], and hemodynamic abnormalities [10]. In addition, Block et al have recently found elevated phosphorus and calcium × phosphorus product were associated with increased mortality [27], principally due to cardiovascular death (abstract; Hafeez et al, *J Am Soc Nephrol* 10:282A, 1999). These studies suggest a relationship of these laboratory values and positive calcium and phosphorus balance with vascular disease. The present study was able to demonstrate that the serum phosphorus and calcium × phosphorus product correlated with the calcification score of inferior epigastric artery as assessed by spiral CT. However, we were unable to demonstrate a clear relationship with serum values and immunostaining of these bone proteins, and in fact, the only significant relationships were actually inverse. The reasons for these inconsistent findings is unclear, but may be due to the fact that we were only able to assess values of calcium and phosphorus at the time of transplantation due to the large referral base for our transplant program. These single values may not reflect previous calcium and phosphorus balance, may be falsely low due to an altered dialysis schedule in the immediate pre-transplant period, and/or reflect increased attention to dietary factors and changes in binder therapy in response to known vascular disease. Clearly, longitudinal clinical studies and more in vitro work is needed to fully understand this process and demonstrate a true cause effect of hyperphosphatemia, calcium × phosphorus product, and/or calcium load on the induction of vascular calcification.

In dialysis patients, calcification of the coronary arteries and aorta have been assessed by new imaging methods such as EBCT [6, 7] and spiral CT (abstract; Ahmed et al, *J Am Soc Nephrol* 11:552A, 2000). Results in dialysis patients have revealed markedly greater calcification scores of the coronary arteries than non-dialysis patients with documented severe three-vessel atherosclerotic coronary artery disease [6]. We found in the ex vivo tissue that medial calcification observed by histology was strongly correlated with that detected by spiral CT. Thus, one potential explanation for these astronomical scores observed clinically in the coronary arteries of dialysis patients is the presence of significant calcification in the medial layer, in addition to that observed in atherosclerotic plaques. However, the present study evaluated the inferior epigastric artery, and it is possible that the location of calcification in coronary arteries is different. In the vessels taken from transplant recipients, the medial thickness was increased, whereas the intimal thickness was decreased, when compared to a published morphometric evaluation of the same vessels in non-dialysis patients undergoing cardiac revascularization [28]. Furthermore, calcification was uncommon in the inferior epigastric artery of these non-dialysis patients, even though they had coronary artery disease of a magnitude

requiring surgery [28]. Schwartz et al, in examining coronary arteries from autopsy sections, did not find a difference in the number and size of atherosclerotic plaques between non-ESRD patients and ESRD patients, but did find that ESRD patients had a thickened medial layer. Calcification was more common in the ESRD patients in the atherosclerotic plaques compared to non-dialysis patients, and medial calcification was only found in the medial layer of ESRD patients [29]. The presence of predominately medial calcification in the present study parallels earlier reports [30, 31]. However, none of the above studies performed immunostaining for bone proteins. Thus, although we suggest that some of the higher calcification scores observed in coronary arteries of dialysis patients may be due to an additional component of medial calcification secondary to the deposition of bone matrix proteins and hydroxyapatite, confirmation of this hypothesis is required.

Interestingly, the presence of medial calcification, while detectable by imaging techniques such as EBCT and spiral CT, may not always be detected by coronary angiography. The latter only detects lumenal obstruction, which is observed with intimal thickening/plaque formation that extends into the lumen, and not with medial calcification. In contrast, EBCT or spiral CT detects both intimal, plaque and medial calcification and cannot differentiate the location. This difference potentially may explain a recent report from Canada that demonstrated that in patients referred for coronary arteriography, dialysis patients were less likely to have obstructive lesions than were patients without renal failure. However, mortality of the dialysis patients was nearly threefold greater compared to non-renal patients with obstructive disease by angiography [32]. Similarly, Braun et al found the calcification scores of dialysis patients were two- to fivefold higher than non-ESRD patients with obstructive coronary artery disease by angiography. It is not hard to imagine that heavily calcified vessels in the medial layer would lose their vascular reactivity, and thus patients with high calcification scores due to medial calcification would be at high risk for sudden death, even without obstructive disease. Clearly, further investigation is warranted.

In conclusion, this study demonstrates that even in fairly young patients undergoing renal transplantation, there is evidence for vascular calcification of the inferior epigastric artery in many patients. Microscopically, this calcification is in the form of medial calcinosis, and is associated with, or likely preceded by, the production of bone like proteins from vascular smooth muscle cells. Although the single time point analysis of this study precludes confirmation of direct cause and effect relationships, risk factors for this appear to be a history of coronary artery disease, diabetes, older age, and possibly an elevated phosphorus and Ca $\times$ P product. We hypothesize that the uremic state leads to the deposition of these bone proteins, with subsequent calcification, perhaps accelerated, in the presence of the positive calcium and phosphorus balance so common in ESRD patients. Confirming this hypothesis and determining what factor(s) in uremia contribute to the initial changes will require further study. However, the findings of an active process lend hope to the possibility of interventions to stop, or even reverse, the calcification process in patients with end-stage renal disease.

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