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Endochondral bone formation is involved in media calcification in rats and in men

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Arterial media calcification is often considered a cell-regulated process resembling intramembranous bone formation, implying a conversion of vascular tissue into a bone-like structure without a cartilage intermediate. In this study, we examined the association of chondrocyte-specific marker expression with media calcification in arterial samples derived from rats with chronic renal failure (CRF) and from human transplant donors. CRF was induced in rats with a diet supplemented with adenine. Vascular calcification was evaluated histomorphometrically on Von Kossa-stained sections and the expression of the chondrocyte markers sox9 and collagen II with the osteogenic marker core-binding factor alpha1 (cbfa1) was determined immunohistochemically. Media calcification was detected in more than half of the rats with CRF. In over half of the rats with severe media calcification, a typical cartilage matrix was found by morphology. All of the animals with severe calcification showed the presence of chondrocyte-like cells expressing the markers sox9, collagen II, and cbfa1. Human aorta specimens showing mild to moderate media calcification also showed sox9, collagen II, and cbfa1 expression. The presence of chondrocytes in association with calcification of the media in aortas of rats with CRF mimics endochondral bone formation. The relevance of this association is further demonstrated by the chondrogenic conversion of medial smooth muscle cells in the human aorta.

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KEYWORDS: media calcification; endochondral bone formation; chondrocytes

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Vascular calcification is a major risk factor for cardiovascular mortality, particularly in patients with end-stage renal disease[1,2] and diabetes.[3,4] Calcification may develop in two distinct layers of the artery, namely the tunica intima and tunica media. Intima calcification occurs in advanced atherosclerotic lesions and is associated with lipid accumulation and infiltration of inflammatory cells, such as macrophages and T cells. The presence of osteoblasts and chondrocyte-like cells within calcified atherosclerotic plaques, indicating the formation of structures resembling bone and cartilage, has been reported in humans[5,6] and apoE knockout mice.[7] The observation of cartilage metaplasia in these lesions provides evidence that intima calcification may occur at least partially by a process resembling endochondral bone formation, in which a cartilage template is replaced by bone.

Media calcification develops independently of atherosclerosis and is mainly associated with aging, diabetes, and end-stage renal disease. In contrast with intima calcification, calcification in the tunica media is generally believed to develop analogous to intramembranous osteogenesis, that is, the direct conversion from condensations of mesenchymal cells into bone without a cartilage intermediate. Indeed, several studies have shown that cultured vascular smooth muscle cells can transdifferentiate into osteoblast-like cells producing a mineralized matrix and expressing osteogenic proteins.[8–10] In addition, Moe *et al.*[11,12] have demonstrated the presence of osteoblast-like cells, expressing the osteogenic transcription factor core-binding factor alpha1 (cbfa1) and matrix protein collagen I, in the calcified media of the inferior epigastric artery of uremic patients.

In this study, the process of uremia-related media calcifications was investigated in rats with adenine-induced chronic renal failure (CRF), which was demonstrated to be a useful model for the study of vascular calcification in this setting.[13–15] Cells with the morphological appearance of chondrocytes in the vicinity of media calcifications were identified immunohistochemically as chondrocytes, demonstrating that a process analogous to endochondral bone formation is an additional mechanism by which media calcification may develop. In addition, the presence of chondrocyte-like cells was also demonstrated in the calcified media of human arteries. To our knowledge, this is the first

study reporting the localization of chondrocytes in the calcified tunica media of human arteries in the absence of atherosclerotic plaque formation.

RESULTS

Experimental study

In this study, rats were fed a diet containing 0.75% adenine for 4 weeks to induce CRF. Both uremic and control rats were allocated to two groups receiving a different high phosphate diet (1.03 or 1.2% phosphate) before and after the adenine diet (or cellulose diet in the control rats) to achieve optimal uremia-related vascular calcification (Figure 1). As neither biochemical parameters nor the degree of vascular calcification differed significantly between both high phosphate groups, biochemical and microscopic results of both groups were pooled.

Biochemical analysis

Serum and urine parameters are shown in Table 1.

Adenine feeding induced a stable, moderate to severe CRF as indicated by an increase in the serum creatinine concentration from 0.45 ± 0.06 mg/dl before the start of the study to 2.61 ± 0.52 mg/dl 4 weeks after the start of adenine treatment. Creatinine levels remained high throughout the remainder of the study. Renal function impairment resulted in a significantly decreased urinary phosphate excretion, and uremic rats also developed hyperphosphatemia. After 2 weeks of adenine feeding, serum phosphate increased significantly to reach maximum values around 16.35 ± 8.30 mg/dl at the end of the study. Serum calcium levels were significantly decreased in CRF rats at week 4 and remained low until week 10. During the whole study period, urinary calcium excretion was lower in the CRF group compared to control rats, reaching significant decreases at 2, 8, and 10 weeks after CRF induction. Adenine-induced CRF caused a major increase in parathyroid hormone levels achieving values up to 2040 ± 636 pg/ml at week 10, indicating the development of severe hyperparathyroidism.

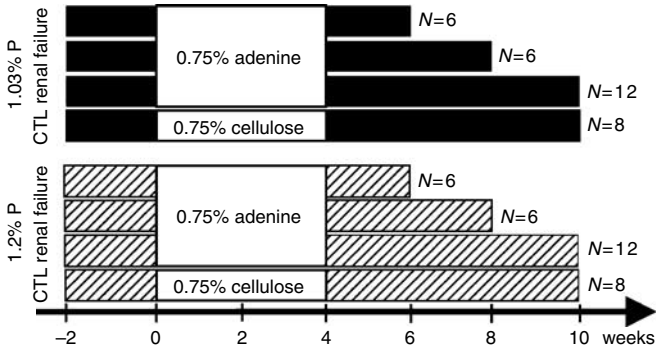

Figure 1 | Study setup. Animals were fed a 0.75% adenine diet for 4 weeks to induce CRF. Before and after the adenine treatment period (or cellulose in controls), rats were loaded with a high phosphate diet, either 1.03% P (black bars) or 1.2% P (hatched bars). CRF rats were killed at week 6, 8, or 10. Control animals (CTL) were killed at week 10.

Aortic calcification

Evaluation of media calcifications in the aorta showed a large biological variability, going from no calcification to focal, partial, or circumferential mineralization in aortic cross-sections. Overall, 56% of uremic rats developed calcifications in the tunica media. A good correlation was found between microscopic (Von Kossa staining) and biochemical (total calcium content) measurements of vascular calcification ($r_s = 0.83$, $P < 0.01$). The area % vessel calcification and the calcium content in the aorta did not differ between uremic rats killed at week 6, 8, or 10 (data not shown), indicating that vascular calcification developed mainly before week 6 and that the degree of calcification did no longer increase significantly between weeks 6 and 10.

To correlate the amount of mineral deposition in the media with the expression of the chondrocyte markers, all adenine-treated rats were divided into four groups according to the degree of media calcification: (1) severe calcification, (2) moderate calcification, (3) mild calcification, and (4) no calcification. The area % media calcification as well as the calcium content in the aorta is presented in Table 2. Twelve out of 39 uremic rats had severe media calcification with an area Von Kossa positivity of on average 22.08 ± 8.36% and calcium content of 35.77 ± 20.63 mg/g wet tissue. Animals with a few partially calcified aortic sections (moderate calcification) showed a calcified area of 2.07 ± 1.07% and calcium concentrations of 1.15 ± 0.64 mg/g. Five animals showed mild calcification as indicated by some focal calcification spots in the media (calcified area: 0.28 ± 0.14%;

Table 1 | Serum and urine biochemistry

		Pre-CRF	2-week CRF	4-week CRF	6-week CRF	8-week CRF	10-week CRF	10-week CTL
Serum	Creatinine (mg/dl)	0.45 ± 0.06	1.93 ± 0.50^b	2.61 ± 0.52^b	2.27 ± 0.46^b	2.00 ± 0.47^b	2.60 ± 0.71^b	0.53 ± 0.06
	Phosphate (mg/dl)	6.47 ± 0.60	8.52 ± 1.86^b	9.20 ± 1.83^b	7.88 ± 2.01^b	8.17 ± 1.06^b	16.35 ± 8.30^b	6.26 ± 0.76
	Calcium (mg/dl)	6.82 ± 0.98	5.93 ± 1.26	5.27 ± 1.24^b	4.52 ± 0.98^b	4.05 ± 1.22^b	4.86 ± 1.89^b	8.25 ± 0.50
	PTH (pg/ml)	ND	ND	ND	1820 ± 438^c	2018 ± 426^c	2040 ± 636^c	228 ± 89
Urine	Phosphate (mg/24 h)	173.9 ± 26.4^a	23.8 ± 13.7^b	18.9 ± 8.7^b	26.7 ± 8.2^b	38.9 ± 16.5^b	36.2 ± 16.1^b	102.5 ± 33.8
	Calcium (mg/24 h)	1.08 ± 0.45^a	0.97 ± 0.44^a	0.85 ± 0.42	1.52 ± 0.56	1.04 ± 0.46^a	0.68 ± 0.37^a	1.25 ± 0.50

CRF, chronic renal failure; CTL, values of control; ND, not done.
Serum and urine parameters were determined before the start of the study and every 2 weeks after CRF induction. CTL rats at week 10 are shown in the last column.
[a] $P < 0.05$ vs control rats at the same time point.
[b] $P < 0.001$ vs control rats at the same time point.
[c] $P < 0.002$ vs control rats at week 10.

calcium content: 0.39±0.15 mg/g). The calcium concentration in the aorta of uremic rats without development of media calcifications was comparable with control rats.

Presence of chondrocyte-like cells in severely calcified aortas

Adjacent to media calcifications, cells with the morphological appearance of chondrocytes were observed in 7/12 animals with severe media calcifications (Figure 2a). At higher magnification (Figure 2b), these cells were surrounded by a typical lacuna and cartilage capsule. At some sites, the matrix, in which these chondrocyte-like cells were located, was mineralized.

To identify these cells, we performed immunohistochemical stainings for two specific chondrocyte markers, the transcription factor sox9 and the chondrocyte-specific matrix protein collagen II. As shown in Figure 3, both sox9 (Figures 3b and f) and collagen II (Figure 3c and g) were highly expressed in these chondrocyte-like cells. In addition, the osteogenic transcription factor cbfa1 also stained positive (Figure 3d and h).

The relationship between the expression of the chondro-/osteogenic markers and the degree of media calcification was further investigated. An animal was considered positive for a particular marker when at least two cross-sections stained positive. Immunohistochemical investigation revealed that all animals with severe calcification in the aorta showed expression of sox9 and cbfa1 and 83% of this group also expressed collagen II. Only one out of five animals with moderate media calcification was positive for the expression of sox9 or cbfa1. No expression of these markers was found in aortic sections of uremic rats with mild calcification and rats with normal renal function. However, the transcription factor sox9 was also highly expressed in one noncalcifying uremic animal.

To study the role of apoptosis in the process of media calcification, apoptotic cells were examined in the rat aorta. In 9/12 animals with a severely calcified aorta, a few apoptotic cells were present in the vicinity of calcium depositions. In four out of these, a number of cells resembling hypertrophic chondrocytes, which are located in areas with the morphological appearance of cartilage tissue, were apoptotic (data not shown). No apoptotic cells were found in CRF animals with moderate, mild, or no media calcification and control animals.

Table 2 | Evaluation of media calcifications in the aorta

Degree of calcification	Number of animals	Area % calcification	mg Ca/g wet tissue
Severe	12	22.08±8.36	35.77±20.63
Moderate	5	2.07±1.07	1.15±0.64
Mild	5	0.28±0.14	0.39±0.15
None – CRF rats	17	0.06±0.01	0.20±0.06
None – control rats	16	0.00±0.00	0.17±0.03

CRF, chronic renal failure.
Since the severity of vascular calcification did not correlate with the time of killing, uremic rats were divided into four groups according to the degree of vascular calcification. Data are thus from weeks 6, 8, and 10 after onset of chronic renal failure. For each group, the number of animals, the mean area % vessel calcification, and the mean calcium content were calculated.

Presence of chondrocyte-like cells in human arteries with media calcification

To examine the presence of chondrocyte-like cells in the calcified tunica media of human arteries, immunohistochemistry for the expression of sox9, collagen II, and cbfa1 was performed on aortic sections of seven transplant donors.

In three vessels, no intima or media calcification was detectable on Von Kossa-stained sections (Figure 4a). These noncalcified vessels showed no or very weak, scattered expression of sox9 (Figure 4b), and no expression of collagen II (Figure 4c) and cbfa1 (Figure 4d) could be detected.

Four aorta specimens showed mild to moderate calcification in the tunica media (Figure 4e and i), but none of these vessels had atherosclerotic plaques or intima calcification. Both the chondrocyte-specific markers sox9 (Figure 4f and j) and collagen II (Figure 4g and k) and the osteogenic transcription factor cbfa1 (Figure 4h and l) were highly expressed in the tunica media of these vessels, both in direct proximity of mineral deposition (Figure 4i) and in areas without calcification (Figure 4e).

DISCUSSION

This study demonstrates the localization of chondrocyte-like cells adjacent to media calcifications in rats with

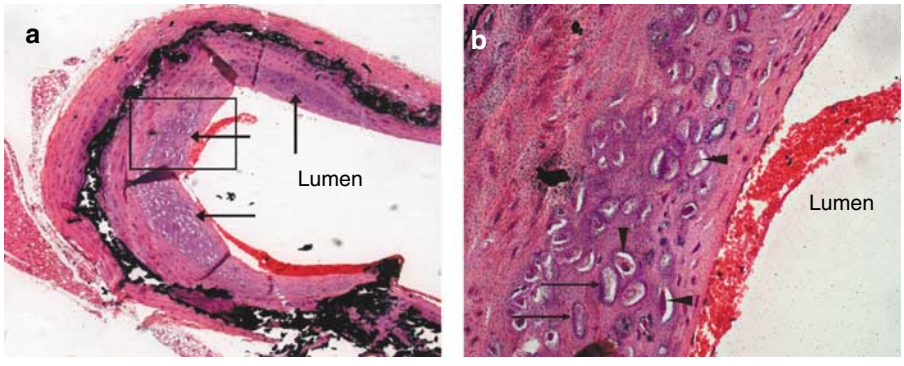

Figure 2 | Presence of chondrocyte-like cells in a Von Kossa-stained cross-section of a rat aorta with severe, circumferential media calcification. Sections were counterstained with hematoxylin and eosin. (**a**) A band of tissue with the morphological appearance of cartilage (arrows) is present at the luminal side of the calcified area. Original magnification ×50. (**b**) Higher magnification of the boxed area in (**a**) shows chondrocyte-like cells surrounded by a lacuna (arrowheads) and cartilage capsule (arrows). Original magnification ×320.

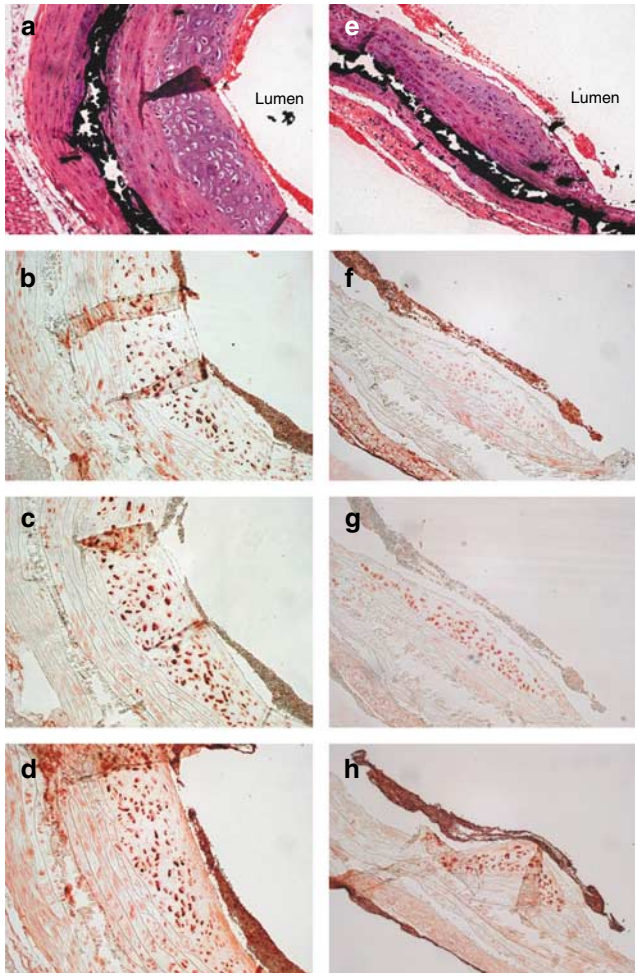

Figure 3 | Immunohistochemical evidence for the presence of chondrocyte-like cells in the calcified tunica media. Aortic cross-sections of the same area from a CRF rat killed at week 10 (**a**–**d**) and week 8 (**e**–**h**). Original magnification × 160. (**a**) and (**e**) Von Kossa staining shows severe calcification in the media. (**b** and **f**) Sox9, (**c** and **g**) collagen II, and (**d** and **h**) cbfa1 are highly expressed in cells with the morphological appearance of chondrocytes.

adenine-induced CRF. Immunohistochemical analysis showed the expression of two specific chondrocyte markers, that is the transcription factor sox9 and the cartilage matrix protein collagen II, in severely calcified rat aortas. Sox9 is a crucial regulator in the early steps of the chondrocyte differentiation pathway.[16,17] It regulates the conversion of mesenchymal cells to chondroprogenitors and the establishment of the chondrogenic mesenchymal condensations during endochondral bone formation, and promotes the expression of downstream proteins, such as collagen II. In severely calcified aortas, a large subset of cells positive for sox9 and/or collagen II also expressed the osteogenic transcription factor cbfa1. The latter protein is required for osteoblast differentiation,[18,19] but is also expressed in hypertrophic chondrocytes in the growth plate.[20–22] The expression of cbfa1 in arterial smooth muscle cells is mostly considered a transdifferentiation towards osteoblast-like cells, but in this study, the co-expression of both specific chondrocyte markers together with cbfa1 in the same cell, as shown in Figure 3, indicates that cells with the phenotype of hypertrophic chondrocytes are present in the tunica media of calcifying vessels.

This is the first study reporting the involvement of cartilage metaplasia in the process of media calcification in the setting of CRF. These findings are in line with previous gene knockout studies showing the expression of collagen II in association with media calcification in mice lacking matrix Gla protein,[23] an inhibitor of vascular calcification, and mice lacking PC-1/nucleotide pyrophosphatase phosphodiesterase 1,[24] an enzyme that generates the calcification inhibitor pyrophosphate. In addition, chondrocyte-like cells expressing S100 protein were found in the calcified media of aortic rat allografts.[25] Our observations together with these experimental studies imply that media calcification may proceed at least partially through a process similar to endochondral bone formation. Strikingly, this is in contrast to what has been reported in recent reviews,[26–29] stating that endochondral ossification is involved in intimal plaque calcification, whereas media calcification is described as a process in which vascular tissue develops directly towards a bone structure, without a cartilage intermediate.

Chondrocyte-like cells, highly expressing sox9 and collagen II, were further localized in human arteries with mild to moderate media calcifications, strengthening the clinical relevance of the above experimental findings and suggesting that chondrogenic conversion of arterial smooth muscle cells during media calcification is more important than believed previously. Shanahan *et al.*[30] used reverse transcription-polymerase chain reaction to demonstrate a significant increase in collagen II expression in human peripheral arteries of diabetic patients with Mönckeberg's sclerosis. As RNA was extracted from sections of the whole vessel and intimal plaque formation/calcification was also present in these arteries, no conclusions could be drawn concerning the localization of the chondrocyte-like cells. A few years ago, a case report of two diabetic patients with renal failure described cartilaginous metaplasia in intimal plaque tissue and the tunica media of lower extremity arteries with extensive intima and media calcifications.[6] In extension to these findings, this study reports the localization of an early (sox9) and late (collagen II) chondrocyte marker associated with mild to moderate media calcifications in arteries with no accompanying atherosclerosis, obtained from transplant donors representing relatively healthy humans.

In adenine-induced CRF rats, chondrocyte-like cells were mainly present in severely calcified aortas, which may suggest that endochondral ossification plays a role in a later stage of the calcification process, when massive hydroxyapatite depositions are already present in the media. However, sox9 was also highly expressed in one noncalcifying uremic rat and, more importantly, both chondrocyte-specific markers were abundantly expressed in human arteries with only mild media calcifications and in noncalcified areas of human

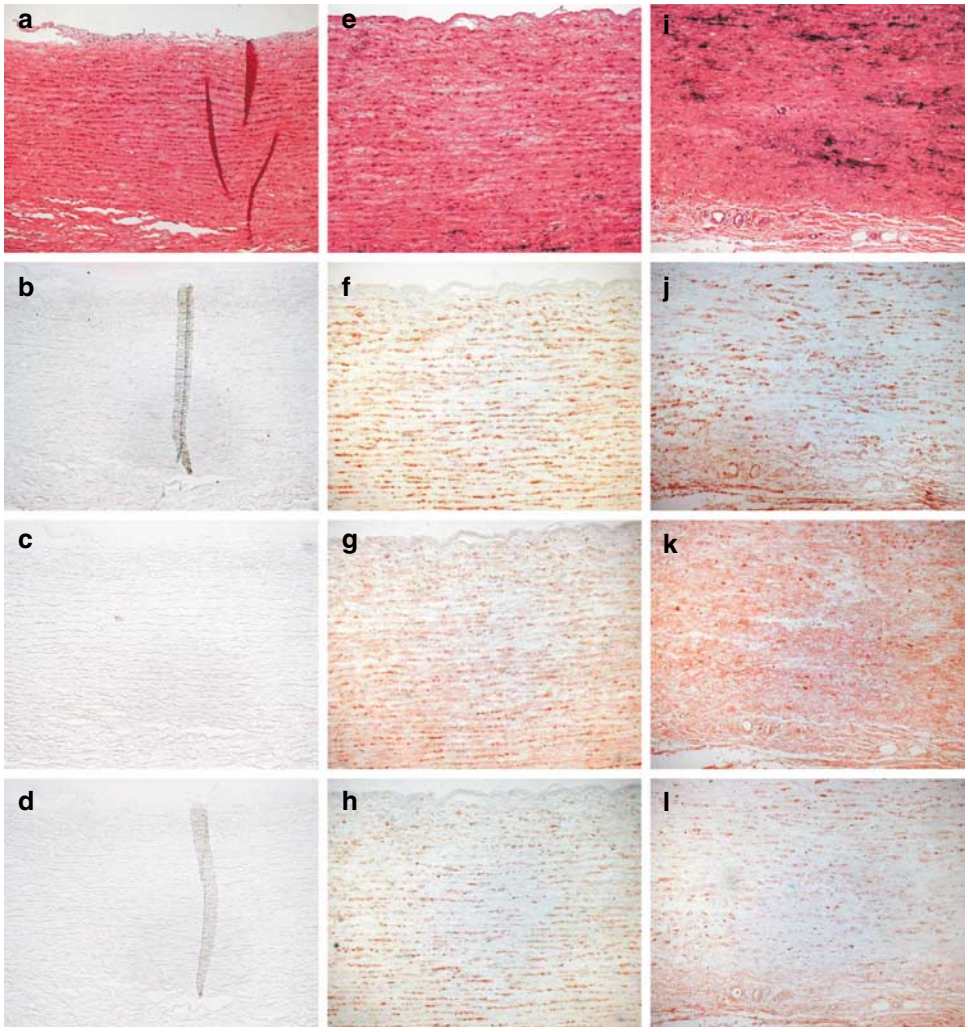

Figure 4 | Presence of chondrocyte-like cells in a human aorta specimen with moderate media calcification. (**a**–**d**) Cross-sections of a human aorta without calcification, (**e**–**h**) a noncalcified area at some distance of mineral deposition, and (**i**–**l**) a calcified area in a human vessel with moderate media calcifications. Original magnification $\times$ 100. (**a**, **e**, **i**) Von Kossa staining; (**b**, **f**, **j**) sox9 immunostaining; (**c**, **g**, **k**) collagen II immunostaining; (**d**, **h**, **l**) cbfa1 immunostaining. Expression of the three chondro-/osteogenic markers was absent in the noncalcified aorta specimen (**b**–**d**). A noncalcified area (**f**–**h**) and a calcified area (**j**–**l**) in a vessel with moderate media calcification show high expression of the chondrocyte-specific markers sox9, collagen II, and the osteogenic transcription factor cbfa1.

vessels with moderate media calcification. This refutes the above assumption and indicates that chondrocytes and a process analogous to endochondral bone formation also contribute to the initial phase of media calcification. A possible explanation for the absence of chondrocyte-like cells in rats with mild media calcifications may be that uremia-induced media calcifications in the rat seems to be an 'all or none' phenomenon in which the calcifications develop within a very short-time period, by which initial steps in this process may be missed. Experimental studies with the rat model of adenine-induced CRF performed in our laboratory revealed that most of the animals either are resistant to vessel calcification or develop massive-media calcifications over a 2-week time period, that is between weeks 4 and 6 after CRF induction. It is indeed remarkable that, despite a comparable, stable, moderate to severe CRF in all animals fed an adenine-rich diet, only 56% of them developed media calcifications. In order to explain this high biological variability, which is also reported in humans,[11,31,32] various serum, urine, and bone parameters relevant to the mineral metabolism were statistically correlated with the development of vascular calcification. However, no singular parameter significantly differed between calcifying and noncalcifying uremic animals, suggesting that a combination of different factors must be responsible for the induction of vessel calcification. It is indeed well known that various inducers, such as phosphate[9,10] and calcium,[33,34] and inhibitors, such as matrix Gla protein,[23] fetuin A,[35,36] and pyrophosphate,[37] and the balance between both, play an important role in the calcification process.

As apoptosis has been proposed to initiate the calcification process *in vitro*,[33,38] we investigated the involvement of apoptotic cells in media calcifications *in vivo*. Only a few

apoptotic cells could be detected in the vicinity of severely calcified areas in arteries of uremic rats. Interestingly, some morphologically distinguishable hypertrophic chondrocytes in the media were apoptotic. This is in agreement with the fate of hypertrophic chondrocytes in the epiphyseal growth plate during endochondral bone formation, where they either undergo apoptosis[39–42] or transdifferentiate to osteoblasts,[43,44] which replace the cartilage matrix by bone (reviewed by Archer and Francis-West[45]). Analogous to endochondral bone formation, it is possible that hypertrophic chondrocyte-like cells in the vessel wall also undergo either of these processes and in this way contribute to the mechanism of media calcification. However, further investigation is necessary to confirm this hypothesis.

In conclusion, the presence of chondrocyte-like cells in association with media calcification in both rat and human arteries indicates that, in addition to intramembranous ossification, a process resembling endochondral bone formation is another mechanism by which media calcification may occur. As previously reported, osteoblast-like cells are also involved in media calcification. In our study, the osteoblast markers collagen I, osteopontin, and bone sialoprotein were found to be associated with calcified areas (Neven *et al.*, *J Am Soc Nephrol* 2006; **17**: 506A), suggesting that intramembranous bone formation can also play an important role in the process of media calcification in this animal model. However, it remains unclear to what extent each of the processes contributes to the development of media calcification. Finally, this study contributes to a better understanding of the cellular mechanisms involved in vessel calcification, which is important for future therapeutic intervention studies.

MATERIALS AND METHODS

Experimental study

Study design. Male Wistar rats (Iffa Credo, Brussels, Belgium) were purchased at 10 weeks of age and housed in plastic cages at constant room temperature with a 12-h light/dark cycle and with tap water and food available *ad libitum*. All experimental procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals 85-23 (1996) and approved by the Antwerp University Ethics Committee. The study set-up is presented in Figure 1. In order to assess optimal conditions for uremia-related media calcifications, two different high phosphate diets (SSNIFF Spezialdiäten, Soest, Germany) were used. Before the start of the study, animals were conditioned on the respective high phosphate diets (1.06% calcium and 1.03 or 1.2% phosphate) for 2 weeks. Moderate to severe CRF was induced by feeding rats a diet containing 0.75% adenine (0.92% phosphate and 1.0% calcium) for 4 weeks, after which the animals were again administered the high phosphate diet until killing after 6, 8, or 10 weeks of CRF. Control animals received 0.75% cellulose instead of the adenine-enriched diet and were killed at week 10. Before induction of CRF and every 2 weeks until killing, animals were placed in metabolic cages for 24 h urine collection, followed by blood sampling from the tail vein. Serum and urine samples were stored at -80°C until further analysis.

At killing, animals were exsanguinated through the retro-orbital plexus after anesthesia with sodium pentobarbital 60 mg/kg via intraperitoneal injection.

Serum and urine biochemistry. Serum creatinine, calcium, and phosphorus were measured using the Vitros 750 XRC autoanalyzer system. Urinary creatinine was measured with a colorimetric method (Creatinine Merckotest, Diagnostica Merck, Germany). Urinary calcium was determined with flame atomic absorption spectrometry (Perkin-Elmer, Wellesley, MA, USA) and urinary phosphorus was measured with the Ecoline®S Phosphate kit (DiaSys, Holzheim, Germany). Serum parathyroid hormone was measured with a rat PTH-IRMA kit (Immutopics Inc., San Clemente, CA, USA) according to the manufacturer's instructions.

Evaluation of vascular calcification in the aorta. At killing, the aorta was dissected out. The aortic arch and the proximal thoracic aorta were fixed in neutral buffered formalin and cut into 2–3 mm rings before embedding. All rings from the same aorta were embedded upright in the same paraffin block, so that every section comprises on average eight to nine cross-sections (minimum six) at different sites along the vessel. Four micrometer sections were stained for calcification with Von Kossa's method and counterstained with hematoxylin and eosin. Vascular calcifications were evaluated histomorphometrically on Von Kossa-stained sections with the Kontron 400 2.0 image analysis software (Kontron, Nashville, TN, USA). Sections were analyzed at magnification $\times$ 100 with two different color separation thresholds, one to measure the area % of the slide covered with tissue, and another to measure the area % of Von Kossa positivity. The possibility for manual user intervention was built into the program to eliminate artefacts outside the aorta, which falsely increase the tissue area, and tissue folds, which can give rise to overestimation of the calcified tissue area. The absolute areas of tissue and calcified tissue were summed for each animal and the ratio is expressed as area % aortic calcification. For noncalcifying animals, two microscopic fields were measured per cross-section, whereas in calcifying animals all cross sections were analyzed completely. The distal part of the thoracic aorta was used to determinate the total calcium content. Therefore, tissue samples were weighed shortly after removal, subsequently digested in 65% nitric acid at 65°C overnight and diluted in 0.1% lanthanum nitrate to eliminate chemical interference during total calcium content measurement with flame atomic absorption spectrometry (Perkin-Elmer, Wellesley, MA, USA). Results are expressed as mg calcium/g wet tissue. Calcifying animals were allocated to three groups according to the degree of calcification. Animals with an area % Von Kossa positivity $\geqslant 5$ were considered to have severe media calcification. Those with a calcified area between 1 and 5% were classified as moderate media calcification and those with values between 0.1 and 1% to have mild media calcification.

Apoptosis

Apototic cells were examined by the TUNEL assay (ApopTag® Peroxidase *In situ* Apoptosis Detection Kit, Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions, omitting the pre-treatment step with proteinase K. Instead, the sections were decalcified in 3% citric acid for 1 h at room temperature before starting the protocol. A cross-section of a calcified rat aorta, pre-treated with DNAse I in a concentration of 1000 U/ml for 30 min at 37°C, was used as a positive control. Cross-sections were counterstained with hematoxylin and analyzed by light microscopy at original magnification $\times$ 1000.

Human tissue

Aorta specimens were obtained from seven transplant donors with normal kidney function, after approval by the Antwerp University Ethics Committee. The tissue was fixed in neutral buffered formalin or formol calcium and embedded in paraffin. Calcification in the vessel wall was stained with Von Kossa's method. Four micrometer serial sections were cut for immunohistochemical analysis of sox9, collagen II, and cbfa1.

Immunohistochemistry

Immunohistochemical stainings for sox9, collagen II, and cbfa1 were performed on aortic sections of both rats and humans with goat polyclonal antibodies against the human antigen, showing a good crossreactivity in rat tissue. Chondrocytes were identified by the expression of the transcription factor sox9 (sc-17340) and chondrocyte-specific matrix protein collagen II (sc-7764) (Santa Cruz Biotechnology, CA, USA). Sections were also incubated with a primary antibody against the osteogenic transcription factor cbfa1 (sc-8566, Santa Cruz Biotechnology). For all immunohistochemical stainings, a biotinylated horse anti-goat immunoglobulin G (Vector Laboratories, Burlingame, CA, USA) was used as secondary antibody. Subsequently avidin and biotinylated horseradish peroxidase (VECTASTAIN® ABC KIT, Vector Laboratories) were added as signal amplifiers. 3-Amino-9-ethylcarbazole (AEC, Sigma-Aldrich, Bornem, Belgium) was used as chromogen. Sections of human cartilage were included as positive controls for the chondrocyte markers. Sections in which the primary antibody was omitted served as negative controls.

Statistics

Data are expressed as mean $\pm$ s.d. Statistical analysis was performed with SPSS 12.0 for Windows software. Differences between CRF and control animals were assessed for each time point with a Mann–Witney *U*-test, in combination with Bonferroni correction when multiple comparisons were made. A Kruskal–Wallis test was used when more than two groups were compared. A value of $P<0.05$ was considered significant. The Spearman Correlation Coefficient (r_s) was used to evaluate the correlation between the biochemical and histomorphometric results of media calcification. The correlation is significant when $P<0.01$.

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