Nephrol Dial Transplant (2015) 0: 1–9
doi: 10.1093/ndt/gfv321

ndt
Nephrology Dialysis Transplantation

Original Article

The matrix metalloproteinases 2 and 9 initiate uraemic vascular calcifications

Eva Hecht[1,2,*], Christian Freise[1,2,*], Karoline v. Websky[3], Hamoud Nasser[1,2], Nadja Kretzschmar[1,2], Philipp Stawowy[1,4], Berthold Hocher[3] and Uwe Querfeld[1,2]

[1]Center for Cardiovascular Research, Charité–University Medicine, Campus Mitte, Berlin, Germany, [2]Department of Pediatric Nephrology, Charité–University Medicine, Campus Virchow Clinic, Berlin, Germany, [3]Institute of Nutritional Science, University of Potsdam, Potsdam-Rehbrücke, Germany and [4]Department of Medicine and Cardiology, German Heart Institute, Berlin, Germany

Correspondence and offprint requests to: Uwe Querfeld; E-mail: uwe.querfeld@charite.de
[*]These authors contributed equally to this work.

ABSTRACT

Background. The matrix metalloproteinases (MMP) MMP-2 and MMP-9 are physiological regulators of vascular remodelling. Their dysregulation could contribute to vascular calcification. We examined the role of the MMP-2 and MMP-9 in uraemic vascular calcification *in vivo* and *in vitro*.
Methods. The impact of pharmacological MMP inhibition on the development of media calcifications was explored in an aggressive animal model of uraemic calcification. In addition, the selective effects of addition and inhibition, respectively, of MMP-2 and MMP-9 on calcium-/phosphate-induced calcifications were studied in a murine cell line of vascular smooth muscle cells (VSMCs).
Results. High-dose calcitriol treatment of uraemic rats given a high phosphate diet induced massive calcifications, apoptosis and increased gene expressions of MMP-2, MMP-9 and of osteogenic transcription factors and proteins in aortic VSMC. The MMP inhibitor doxycycline prevented the VSMC transdifferentiation to osteoblastic cells, suppressed transcription of mediators of matrix remodelling and almost completely blocked aortic calcifications while further increasing apoptosis. Similarly, specific inhibitors of either MMP-2 or -9, or of both gelatinases (Ro28-2653) and a selective knockdown of MMP-2/-9 mRNA expression blocked calcification of murine VSMC induced by calcification medium (CM). In contrast to MMP inhibition, recombinant MMP-2 or MMP-9 enhanced CM-induced calcifications and the secretion of gelatinases.
Conclusions. These data indicate that both gelatinases provide essential signals for phenotypic VSMC conversion, matrix remodelling and the initiation of vascular calcification. Their inhibition seems a promising strategy in the prevention of vascular calcifications.

Keywords: chronic kidney disease, matrix metalloproteinases, vascular calcification, vascular smooth muscle cells

INTRODUCTION

Matrix metalloproteinases (MMPs) are proteolytic enzymes with important physiological roles in tissue remodelling, including bone and vasculature [1]. The MMPs comprise a broad spectrum of enzymes with diverse physiological functions, but MMP-2 and MMP-9 (gelatinase A and B, respectively) have emerged as important contributors in the pathophysiology of various cardiovascular diseases, including atherosclerosis, aortic aneurysm formation and others [2]. Matrix degradation by MMPs is a universal feature of vascular remodelling regulated by haemodynamic forces, vascular injury, inflammation and oxidative stress [3]. Recent studies suggest that enhanced vascular MMP activity is associated with ageing and subclinical arterial disease and that chronic MMP inhibition retards age-associated arterial proinflammatory signalling and blood pressure increase [4]. Elastin, the most abundant protein in the arterial wall and main constituent of elastic fibres, can be degraded by MMP-2 and MMP-9, which may also catabolize other matrix proteins such as laminin, type IV collagen and fibronectin. Of note, degradation of elastic fibres may be an important initial step in uraemic media calcification [5, 6].

Thus, dysregulation of MMP-2 and MMP-9 could significantly contribute to vascular calcification, a hallmark of advanced cardiovascular disease in the general population and especially, in patients with chronic kidney disease (CKD). Recent clinical studies have indeed shown upregulation of MMP-2 in arterial biopsies of patients with CKD, which was associated with the extent of media calcification and arterial stiffness [7]. Similarly, MMP-2 and MMP-9 were found upregulated in arterial biopsies of diabetic CKD patients and associated with endothelial dysfunction and impaired angiogenesis [8].

Taken together, recent studies have provided evidence that increased matrix degradation by MMPs could provide an early signal in the pathophysiology of calcifications indicating that inhibition of MMPs could be of therapeutic value. However, it is unknown whether MMP inhibition *in vivo* could impede the development of vascular calcifications in CKD, which is known to provide a pro-calcifying milieu by a multitude of pathogenetic mechanisms. Therefore, we have performed studies *in vivo* and *in vitro* to examine the role of MMP-2 and MMP-9 in uraemic vascular calcification.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Charles River, France) with a mean body weight of 100 g were used for the experiments. They had free access to water and food. After an acclimatization period of 1 week, the animals were randomly allocated to experimental groups. All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals. Uraemia was created by two-stage 5/6 nephrectomy (5/6-Nx) as previously described [9]. Rats received a high phosphate (HP) diet containing 1.2% phosphorus, 0.9% calcium and 20% crude protein (Altromin, Altromin, Co., Lage, Germany) beginning after the removal of the right kidney.

Arterial calcifications were induced by oral treatment with calcitriol (250 ng/kg/day) beginning 1 week after the 5/6-Nx. Inhibition of MMPs was achieved by oral application of the MMP inhibitor doxycycline (100 mg/kg/day), administered in drinking water. Five groups of rats were studied: sham-operated rats (sham-op + HP diet; $n = 10$), uraemic rats (5/6-Nx + HP diet; $n = 10$), uraemic rats treated with doxycycline (5/6-Nx + HP diet + dox; $n = 6$), uraemic rats treated with calcitriol (5/6-Nx + HP diet + calc; $n = 14$) and uraemic rats treated with calcitriol and doxycycline (5/6-Nx + HP diet + calc + dox; $n = 14$). After 33 days of calcitriol treatment, animals were sacrificed by removal of the heart under isoflurane anaesthesia and the extent of media calcifications was quantified in aortic sections by von Kossa staining. Osteoblastic transition of vascular smooth muscle cells (VSMCs) was quantified by real-time PCR (RT–PCR) for proteins controlling bone mineralization. Aortic content of MMP-2 and MMP-9 was quantified by immunohistological staining and RT–PCR.

Cell culture

The murine VSMC cell line (MOVAS-1) was purchased from ATCC (ATCC® CRL-2797™), and cells were cultured in a humidified atmosphere at 37°C and 5% CO_2. Standard culture medium consisted of DMEM with 862 mg/L L-alanyl-L-glutamine, 6 mmol/L glucose, 50 µg/mL streptomycin, 50 U/mL penicillin, 0.2 mg/mL G418, supplemented with 10% heat-inactivated foetal bovine serum (Biochrom, Berlin, Germany). Vehicles of the different drugs used served as respective controls in the cell culture experiments.

Induction of VSMC calcification

VSMCs were grown in 6- and 12-well plates or Petri dishes (Becton Dickinson, Heidelberg, Germany) to 80–90% confluency (Day 0). Calcification was induced by a calcification medium (CM) consisting of standard culture medium supplemented with NaH_2PO_4 and $CaCl_2$ to final concentrations of PO_4^{3-} (2.0 versus 1.0 mmol/L) and Ca^{2+} (2.7 versus 1.8 mmol/L), respectively. Cells were cultured for up to 9 days as indicated. Media were replaced every 2–3 days.

To determine the effects of recombinant MMPs or MMP inhibitors on VSMC calcification, they were added to the standard culture medium or the CM. The respective concentrations used are given in Supplementary data, Table S1.

Quantification of calcification in aortas and VSMCs

Arterial calcification was determined by homogenization of parts of the abdominal rat aortas in 0.1 mmol/L HCl and subsequent photometric measurement of calcium and phosphate concentrations as described [10] and normalized to the respective protein contents, which were determined by the BCA method.

VSMCs were decalcified with 0.1 mmol/L HCl for 30 min. Calcium content in the supernatants was determined by the *o*-cresolphthalein complexone method and normalized to respective protein contents determined by the BCA method.

Enzymatic activities of alkaline phosphatase (ALP) as a marker of VSMC calcification were assessed in VSMC supernatants using the Quanti Blue reagent (InvivoGen, San Diego, CA, USA). Measured ALP activities were normalized to total protein contents.

In addition, mineralized VSMCs were stained with alizarin red. VSMCs were fixed for 10 min with 4% formalin followed by incubation with 2% alizarin red solution. Photographs were taken with a digital Biorevo BZ-9000 microscope (Keyence, Mechelen, Belgium). Staining was quantified by resolving bound red dye with cetylpyridinium chloride and measurement of absorbance using an ELISA reader (Biorad, Munich, Germany).

Quantitative evaluation of aortic calcification and lumen area of the aorta was performed using specific software (BZ II Analyzer, Keyence): from each animal, the area of stained regions of five different sectors with three sections each was analysed, and the respective means were calculated and displayed as percentage of total aortic wall area.

Tissue processing

Aortas were dissected and eight equally sized segments (each ~0.3 cm) from the descending thoracic aorta to the iliac branch were removed per rat. Five segments were placed into Carnoy's fixative (60% absolute alcohol, 30% chloroform and 10% glacial acetic acid) for paraffin embedding and histological analysis;

three segments were immediately placed in liquid nitrogen for protein and RNA extraction.

Immunohistochemistry

Preparation and immunhistochemical analysis of 8-µm-thin arterial paraffin sections were performed as described previously [11]. Apoptosis-positive aortic areas were stained with primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, USA, 1 : 300). After counterstaining with haematoxylin, caspase-3-positive stained areas were quantified by digital microscopy using the BZ II Analyzer software (Keyence). The area of stained regions was measured and displayed as percentage of total aortic wall area. Six arterial sections of each experimental animal were quantified.

Determination of MMP activities in supernatants of VSMCs and aortic sections

Activities of MMP-2 and MMP-9 were measured by cleavage of 0.01 mg/mL dye-quenched (DQ)-gelatine (Molecular Probes, Life Technologies GmbH, Darmstadt, Germany) as described [12]. Cells were treated as indicated. Afterwards, supernatants were discarded and replaced by fresh serum-free growth medium for 20 h. Fifty microliters of the supernatants were transferred to black 96-well plates (Greiner Bio-One) and mixed with 150 µL DQ-gelatine. Fluorescence signals were monitored for 100 min at 30°C using a Victor^3 microplate reader (Perkin Elmer, Rodgau, Germany).

Quantitative RT–PCR

Total RNA was isolated as described previously [13]. The relative expressions of runx2, osterix, osteocalcin, osteopontin, MMP-2 and MMP-9 were analysed by RT–PCR using specific primers (BioTez Berlin-Buch GmbH, Berlin, Germany). The relative amount of RNA was calculated using the $2-\Delta\Delta Ct$ method and normalized to mRNA expressions of housekeeping genes.

The gene expressions of bone morphogenetic protein 2 (BMP-2), transforming growth factor-beta1 (TGF-β1), elastin, collagen1A2 (col1A2) and collagen6A2 (col6A2) were determined by a Custom RT^2 Profiler™ PCR Array (Qiagen, Hilden, Germany).

MMP gelatine zymography

The activity of MMP-2 and MMP-9 was checked by SDS–PAGE zymography as described [14] using the same supernatants, which were used for the DQ-gelatine measurements.

Analysis of VSMC proliferation

VSMC proliferation was studied using the cell proliferation kit II (XTT) (Roche Diagnostics GmbH, Mannheim, Germany). Cell cycle-synchronized VSMCs were treated as indicated for 24 h in 96-well plates. Proliferation was assessed by adding specific substrates during the last 4 h of treatment and measurement of the absorbance at 475/650 nm using an ELISA reader (Biorad).

Transient transfection of VSMCs

VSMCs were transiently transfected with siRNAs specific for MMP-2 or MMP-9 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; see Supplementary data, Figure S7) using the FuGENE® HD Transfection Reagent (Promega) and white 96-well plates (NUNC, Roskilde, Denmark). Twenty-four hours after transfection, the cells were treated as indicated.

Statistical analyses

Data sets from animal studies and *ex vivo* experiments were analysed by the non-parametric Kruskall–Wallis test followed by Dunn's Multiple Comparison test. Results from cell culture studies were analysed by one-way ANOVA followed by the Tukey *post hoc* test using GraphPad PRISM, version 5.01 (GraphPad Software, USA), and differences with $P \leq 0.05$ (*) were considered to be statistically significant.

RESULTS

MMP inhibition by doxycycline prevents vascular calcification in uraemic rats

First, we studied the effect of MMP inhibition by doxycycline in a model of uraemic calcification in 5/6-nephrectomized rats treated with calcitriol and a HP diet. The creatinine, calcium and phosphorus levels were higher in the two groups receiving calcitriol, without significant differences within these groups, i.e. if treated with or without doxycycline (Table 1). The systolic blood pressure (tail-cuff method) was similar in all groups (Table 1).

Rats treated with calcitriol (5/6-Nx + calc; 5/6-Nx + calc + dox) showed a decrease in weight gain and substantial mortality (~30%), which was not influenced by treatment with doxycycline (Supplementary data, Figure S1).

Massive calcifications were found in 5/6-Nx rats treated with calcitriol and an HP diet whereas uraemic rats treated

Table 1. Serum chemistry and blood pressure in uraemic rats (means ± SD)

	sham-op	5/6-Nx	5/6-Nx + dox	5/6-Nx + cal	5/6-Nx + cal + dox
Creatinine (mg/dL)	0.37 ± 0.24	0.49 ± 0.13	0.75 ± 0.35	1.52 ± 0.82*,**	0.94 ± 0.76***
Calcium (mmol/L)	2.9 ± 0.25	2.8 ± 0.37	2.6 ± 0.38	3.3 ± 0.43	3.3 ± 0.16**,****
Phosphorus (mmol/L)	0.98 ± 0.15	1.14 ± 0.43	1.00 ± 0.23	2.59 ± 1.35**,***,****	2.08 ± 1.74**
Blood pressure (mmHg)	152 ± 14	143 ± 10	160 ± 10	146 ± 21	147 ± 28
PTH (pg/mL)	273 ± 131	563 ± 157	500 ± 215	98 ± 110**	56 ± 57**,****

*P < 0.001 versus sham-op.
**P < 0.05 versus 5/6-Nx.
***P < 0.05 versus sham-op.
****P < 0.05 versus 5/6-Nx + dox.

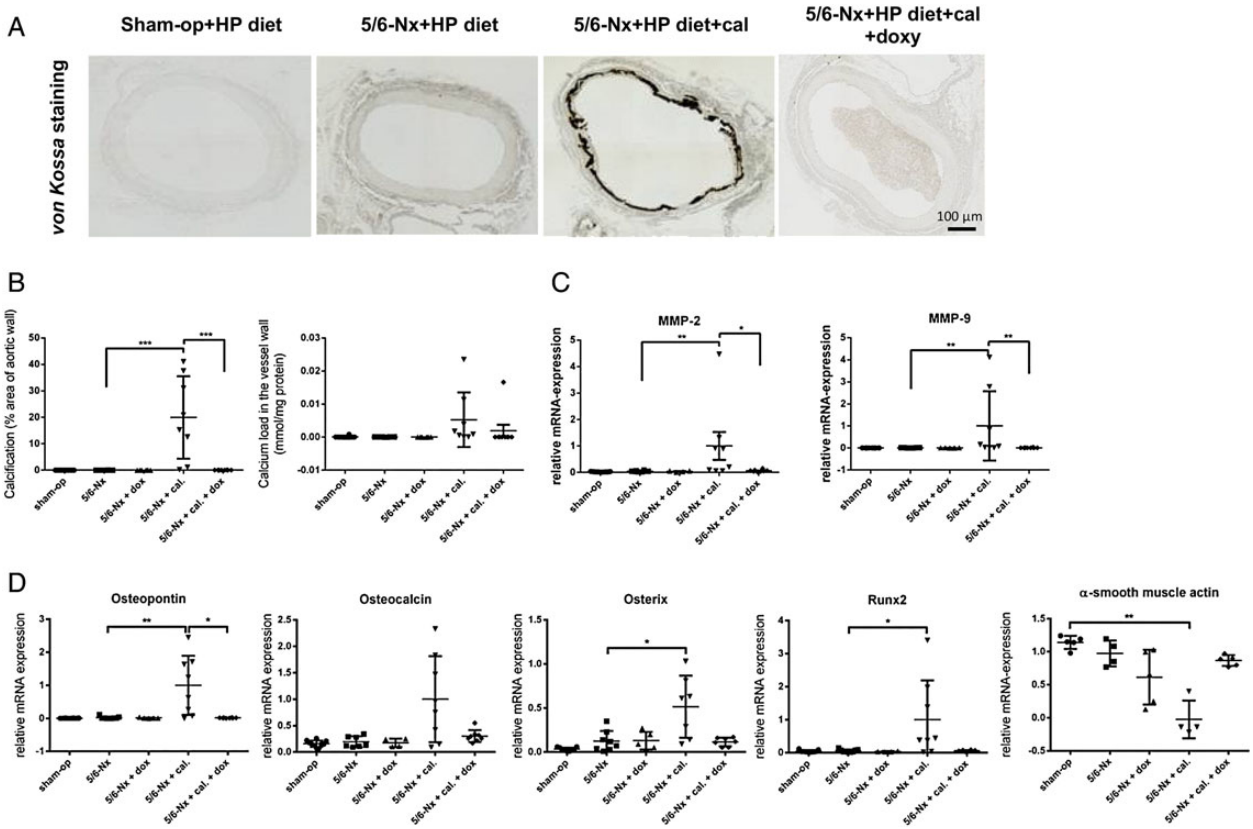

FIGURE 1: Calcifications, calcium load and gene expression in rat aortas. (**A**) Representative images of aortic sections (von Kossa staining) from four groups of rats treated as indicated (4-fold magnification). Of note, 5/6-Nx + dox groups were not different from the 5/6-NX group (not shown). All animals received an HP diet. (**B**) Quantification of calcified area in the arterial media. (**C**) Aortic mRNA expression of MMP-2 and MMP-9. (**D**) mRNA expression levels of specific proteins/transcription factors involved in the transdifferentiation/calcification of VSMCs. Shown are means ± SD. *P < 0.05, **P ≤ 0.01.

with calcitriol, an HP diet and doxycycline showed almost complete absence of media calcifications as also indicated by a decreased vessel load of calcium (Figure 1A and B) and phosphorus (data not shown). Treatment with doxycycline resulted in strong inhibition of aortic MMP-2 and MMP-9, as shown by a significant decrease of mRNA expression (Figure 1C) and decreased protein expression (Supplementary data, Figure S2).

MMP inhibition by doxycycline prevents phenotypic conversion of VSMCs in uraemic rats and blocks calcitriol-induced expression of MMP-2

Uraemic rats treated with calcitriol and an HP diet showed significantly increased gene expression levels of runx2, osterix and osteopontin, whereas treatment with doxycycline reduced gene expression levels of these proteins to those of uraemic controls (Figure 1D). In addition, expression of alpha-smooth muscle actin (αSMA) indicating loss of the contractile phenotype of VSMCs was significantly reduced in calcitriol-treated rats, but not in the doxycycline-treated animals (Figure 1D). Thus, inhibition of MMPs by doxycycline abrogated the chondro-osteogenic conversion of VSMCs in uraemic rats resulting in the prevention of calcification in spite of calcitriol treatment and an HP diet.

MMP inhibition suppresses inducers of bone formation and aortic remodelling

MMP inhibition by doxycycline also suppressed gene expressions of BMP-2 and TGF-β1, both of which are important inducers of bone formation (Figure 2). In addition, MMP inhibition resulted in suppression of matrix proteins associated with matrix remodelling, aortic dilatation and aneurysm formation, i.e. elastin, col1A2 and col6A2 (Figure 2).

MMP inhibition by doxycycline is not associated with suppression of apoptosis

Since apoptosis is considered to be an important mechanism in the pathogenesis of arterial calcification [15], we quantified apoptotic cells in aortic sections of experimental groups. As expected, we found that calcitriol-induced aortic calcifications (5/6-Nx + calc, Figure 1A) were accompanied by elevated levels of apoptotic cells compared with controls (sham-op) (Figure 3). Interestingly, doxycycline, which prevented calcitriol-induced aortic calcifications, was not associated with suppression of apoptosis, but instead, further increased aortic apoptosis (Figure 3).

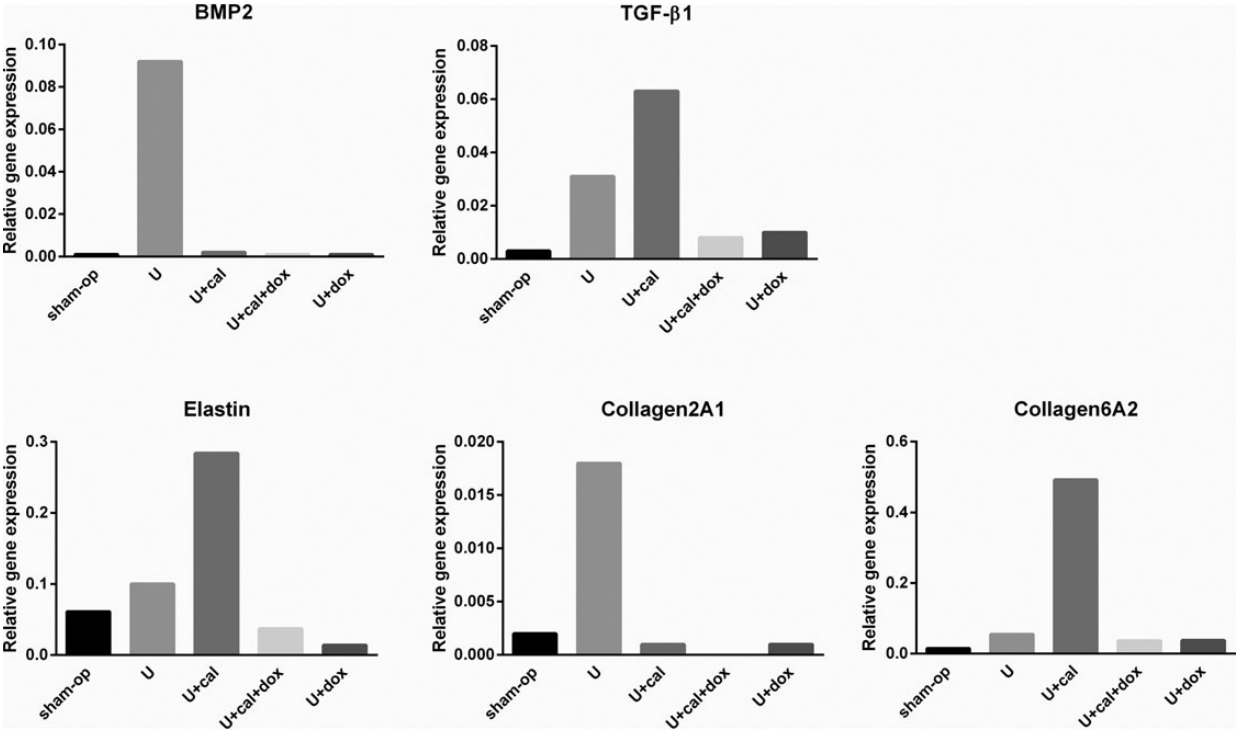

FIGURE 2: Transcription levels of inducers of osteogenesis and matrix remodelling. Aortic mRNA expressions of BMP-2, TGF-β1, elastin, collagen1A2 and collagen6A2 in experimental groups. Shown are data from measurements using pooled probes consisting of isolated RNA from five individual animals per treatment group. sham-op, sham-operated animals; U, uraemic animals; U + cal, uraemic animals treated with calcitriol; U + cal + dox, uraemic animals treated with calcitriol and doxycycline; U + dox, uraemic animals treated with doxycycline only.

Selective effects of MMP-2 and MMP-9 and their respective selective inhibitors in murine VSMCs

To better understand the individual contribution of each gelatinase to the calcification process, we studied the selective effects of recombinant MMP-2 and MMP-9 in murine VSMCs. After 9 days of incubation with CM, cultured cells showed distinct calcifications indicated by marked alizarin red staining (Supplementary data, Figure S3) and a significantly increased cellular calcium content (Figure 4A). Addition of both, recombinant MMP-2 or MMP-9, resulted in significantly enhanced VSMC calcifications (Figure 4A, Supplementary data, Figure S3).

Moreover, addition of either recombinant MMP-2 or MMP-9 also increased total gelatinolytic activity in supernatants of CM-treated cells (cultured in serum-free medium without MMPs for 24 h) but not untreated cells (without CM) (Figure 4B), indicating a positive feedback loop for the activity of each gelatinase. This was confirmed by gelatine zymographies showing an increased secretion of gelatinases under these conditions (Supplementary data, Figure S4).

The effect of a selective inhibition of each gelatinase was studied by using specific inhibitors of MMP-2 or of MMP-9 or the selective inhibitor for both gelatinases Ro28-2653. The latter served as a substitute for doxycycline, which (corresponding to the *in vivo* experiments) could not be used *in vitro* due to complex formation with divalent cations. Incubation with these inhibitors significantly decreased the CM-induced elevation of cellular calcium content to a similar degree (Figure 5A). In line with decreased calcium content, VSMC cultures treated with a combination of CM and MMP inhibitors showed reduced alizarin red staining, and respective VSMC supernatants showed reduced enzymatic activities of the calcification marker ALP compared with control cultures treated with CM alone (Supplementary data, Figure S5A and S5B). To further confirm these findings, we studied the effects of selective inhibition of MMP-2 and MMP-9 gene transcription on VSMC calcification. A selective knock down of MMP-2 or MMP-9 by specific siRNA (Supplementary data, Figure S6) attenuated the CM-induced calcifications of VSMCs (Figure 5B).

Contrary to the effects of recombinant MMP-2 and MMP-9 (Figure 4B), addition of either the specific inhibitors of MMP-2 and MMP-9 or of the specific inhibitor of both gelatinases Ro28-2653 decreased total gelatinolytic activity in the medium to the levels of control-treated cells (Figure 5C). These data indicate a negative feedback loop for the activity of each gelatinase when inhibited.

We then investigated whether MMP inhibition affects proliferation of VSMCs, an integral part of vascular remodelling. As shown in Figure 5D, VSMC treatment with CM induced the proliferation of VSMCs, whereas pharmacological inhibition of MMP-2 and/or MMP-9 markedly decreased basal and CM-induced VSMC proliferation.

DISCUSSION

We here show that inhibition of MMP-2 and MMP-9 by doxycycline, an unspecific MMP inhibitor, prevented arterial

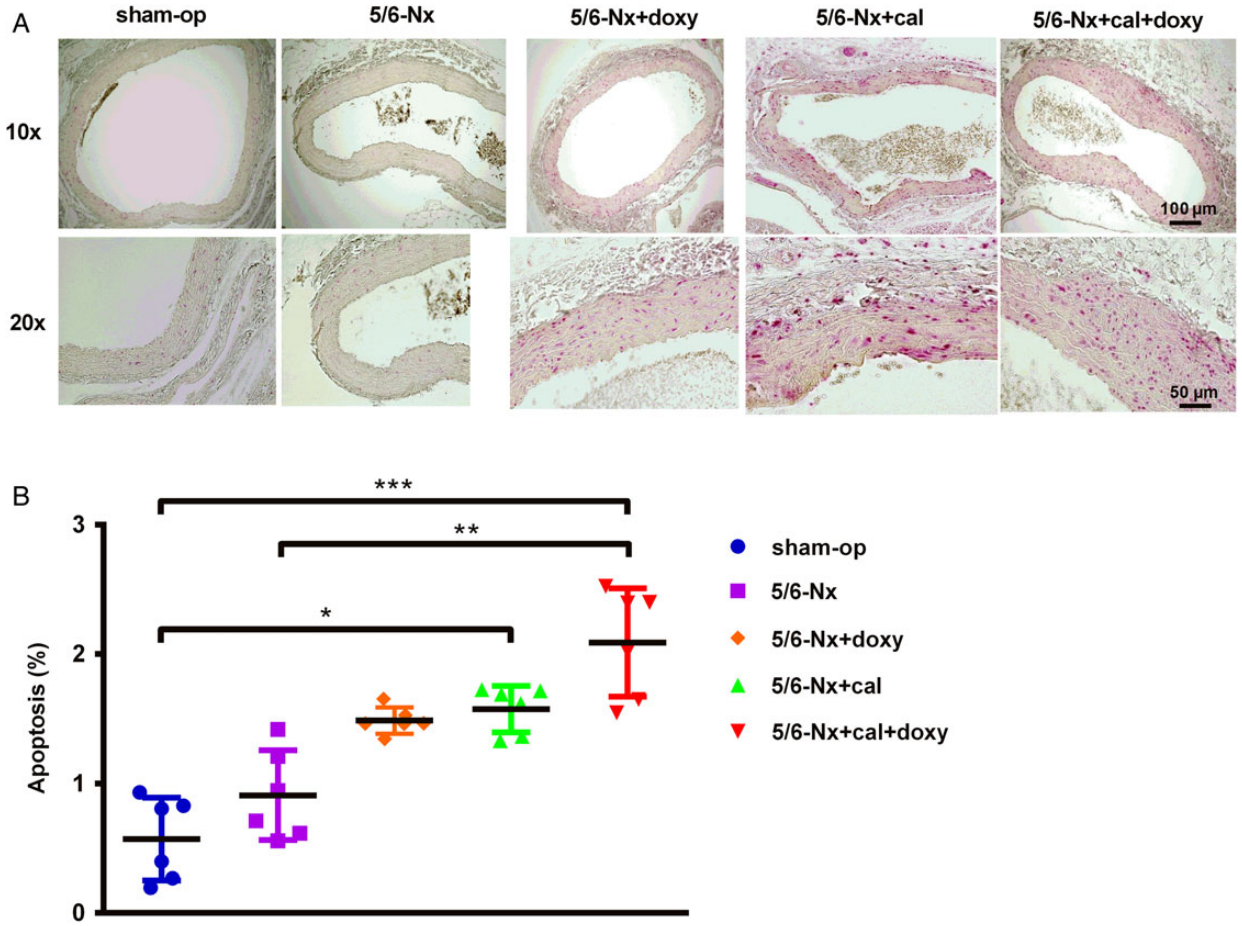

FIGURE 3: Apoptosis in the arterial media of uraemic rats. (**A**) Representative images of aortic sections stained for apoptotic areas with antibodies against cleaved caspase-3 (shown in red) from five groups of rats treated as indicated (10-fold and 20-fold magnification). (**B**) Quantification of apoptotic areas in relation to the total area of the arterial media. Shown are means ± SD ($n = 6$). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 (only significant differences are indicated).

calcifications in 5/6 nephrectomized rats on an HP diet treated with high doses of calcitriol. As previously demonstrated [11], this aggressive model resulted in severe media calcifications and a further increase in creatinine levels in the calcitriol-treated groups. The calcification-preventing effect of MMP inhibition by doxycycline treatment was evident in spite of higher calcium and phosphorus serum levels, and independent of blood pressure and renal function. However, MMP inhibition had no effect on mortality, which was most likely explained by a similar severity of CKD (resulting in anaemia, poor feeding and weight loss) in both calcitriol-treated experimental groups. These results are in line with previous studies in non-uraemic rats showing attenuation of calcification by MMP inhibition with doxycycline [16–18].

It is currently assumed that elastin degradation in the arterial media is a key event initiating vascular calcification [6, 19, 20]. In our study, transcription levels of not only elastin but also of the collagens col1A2, col6A2 and the multifunctional cytokine TGF-β1 were elevated in calcitriol-treated uraemic rats and suppressed by doxycycline. While upregulation of elastin transcription probably reflects elastin remodelling [5], overexpression of each of these matrix proteins and of TGF-β1 is associated with aortic dilatation and aortic dissection [21–23]. Of note, aortic aneurysmatic dilatation is a distinct feature of calcitriol-induced aortic calcification in uremic rats [24].

BMP-2 is a mediator of osteoblastic transdifferentiation of VSMCs and the induction of matrix mineralization [25]. In our study, BMP-2 was upregulated by 5/6 nephrectomy, but significantly suppressed by both, calcitriol and doxycycline treatment. This is in line with previous studies showing a downregulation by calcitriol [26, 27] and doxycycline [28], respectively. Therefore, calcitriol-induced vascular calcifications and their prevention could not be attributed to effects on BMP-2.

Increased apoptosis of VSMCs is a significant contributing factor in the initiation of vascular calcification [10, 29, 30]. In the present study, doxycycline inhibited calcification, but stimulated apoptosis. It is known that doxycycline induces apoptosis *in vitro* in various cell lines by caspase-dependent and -independent mechanisms [31, 32], and our study confirms these effects in arterial tissue *in vivo*. While it is interesting to speculate whether an increase in apoptosis (of calcifying cells) might be an additional mechanism by which doxycycline treatment prevented media calcification, these data indicate that

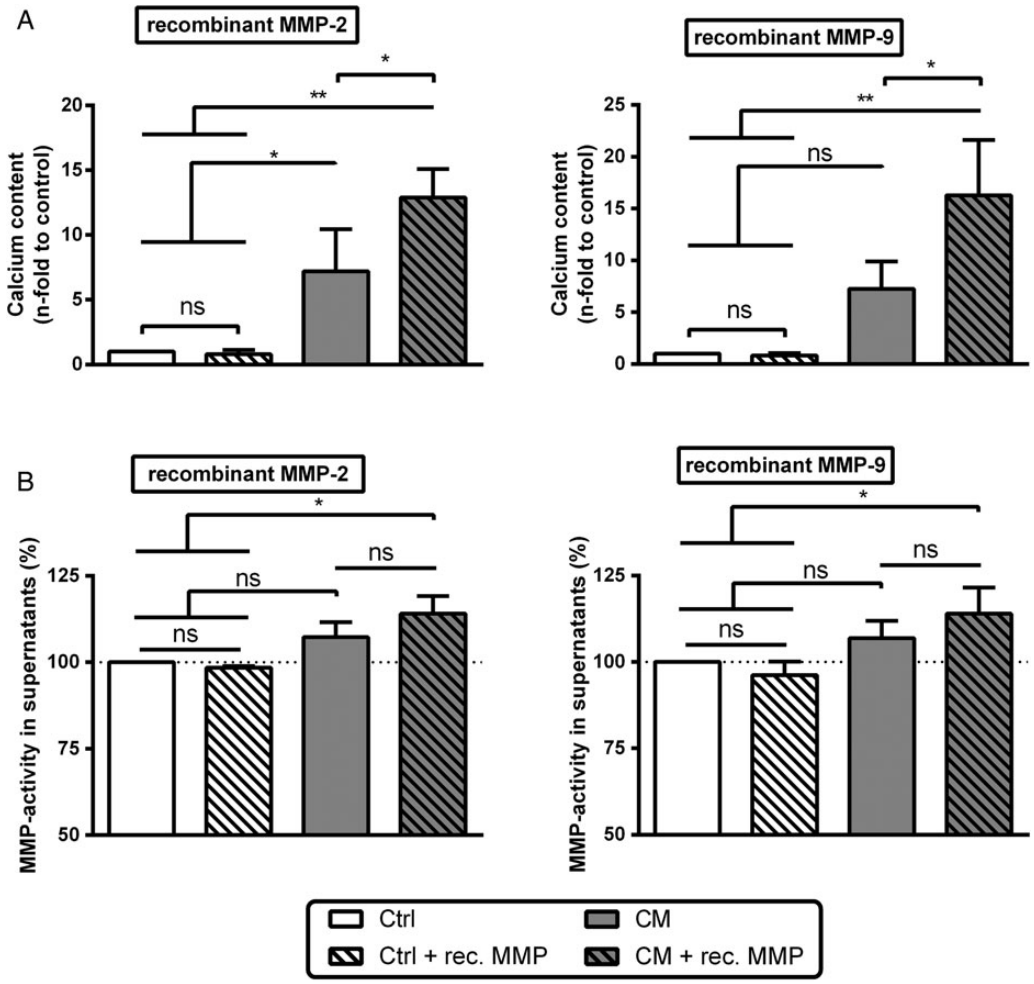

FIGURE 4: Effects of recombinant (rec.) MMP-2 and MMP-9 on VSMC calcification and gelatinase secretion. (**A**) VSMCs were treated for 9 days as indicated with or without addition of recombinant MMP-2 or MMP-9. Calcium contents in the VSMC cultures were determined by the *o*-cresolphthalein complexone method. (**B**) Gelatinolytic activities in supernatants of VSMCs as an indicator for MMP secretion were determined by conversion of DQ-gelatine substrate after 9 days of treatment as indicated. Shown are means ± SD ($n = 4$). ns, not significant, *$P < 0.05$, **$P \leq 0.01$.

under the conditions of MMP inhibition, a significant increase in apoptotic VSMCs was not associated with calcifications or an increased aortic calcium content.

It has been shown that osteogenic conversion of VSMCs and elastin degradation precede the development of vascular calcifications [5], and in our study, MMP inhibition prevented osteogenic conversion of VSMCs and calcifications. It is conceivable that MMP-mediated matrix degradation provides an early signal for osteogenic conversion. It has indeed been shown that activated proteinases may generate extracellular matrix-derived peptides, which regulate intracellular signalling pathways, functioning as 'matrikines' [33, 34]. Future studies are needed to identify putative peptides inducing phenotypic conversion and the intracellular pathways involved.

Our *in vitro* data show that exogenous MMP-2/-9 promoted enhanced mineralization of VSMCs in the presence of elevated concentrations of calcium and phosphorus. Vice versa, pharmacological blockade and transcriptional inhibition by siRNAs reduced calcification of VSMCs. Thus, both, MMP-2 and MMP-9 provided extracellular calcification signals, and their effect was mainly due to their enzymatic activity, as indicated by the effect of inhibitors, which reduced calcium uptake almost to the level of untreated controls. Interestingly, both MMP-2 and MMP-9 enhanced their own secretion by VSMCs as indicated by increased gelatinolytic activity and zymography measurements, and vice versa, MMP inhibitors decreased MMP secretion, suggesting a feedback loop in both directions. Additionally, we found that MMP inhibition blocked the calcium/phosphorus-induced proliferation of VSMCs, a key element of vascular remodelling.

It is a limitation of our study that other MMPs participating in bone formation and remodelling have not been investigated. In this regard, it cannot be excluded that additional mechanisms such as suppressive effects on other MMPs than MMP-2 and MMP-9 may have contributed to the prevention of calcification in rats given the unspecific MMP inhibitor doxycycline.

In summary, these data indicate that both gelatinases provide essential signals for phenotypic VSMC conversion and

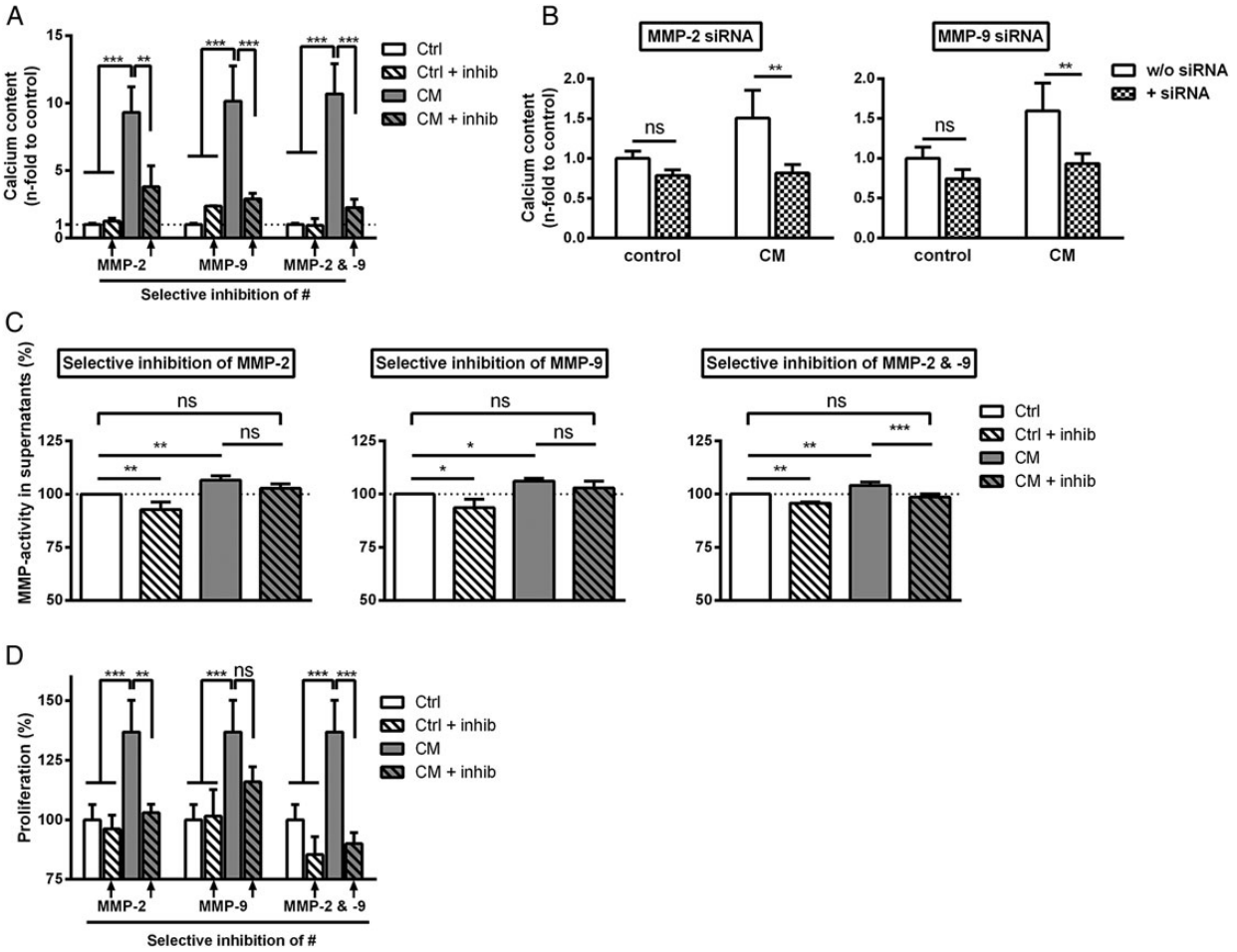

FIGURE 5: Effects of specific MMP inhibitors and selective knock down of mRNA expressions of MMP-2 or MMP-9 on the calcification, gelatinase secretion and proliferation of VSMCs. VSMC calcification was induced by 9 days of treatment with CM ± inhibitors specific for MMP-2, MMP-9 or both MMPs. (**A**) Calcium content in the culture was determined by the *o*-cresolphthalein complexone method. (**B**) MMP-2 and MMP-9 mRNA expressions were transiently knocked down by specific siRNAs. VSMCs were treated with CM for 5 days to induce VSMC calcification. VSMC calcifications were detected by alizarin red staining and quantified spectrophotometrically. (**C**) Gelatinolytic activities in supernatants of VSMCs as an indicator for MMP secretion were determined by conversion of DQ-gelatine substrate after 9 days of treatment as indicated. (**D**) Effects of specific MMP inhibitors on the proliferation of VSMCs were determined by proliferation measurements using an XTT-based colorimetric cell proliferation kit. Shown are means ± SD ($n = 4$). ns, not significant, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

the initiation of vascular calcification. Inhibition of MMP-2 and MMP-9 seems a promising strategy in the prevention of vascular calcifications.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

ACKNOWLEDGEMENTS

We gratefully acknowledge the help of Heike Meyborg, Thore Dietrich, Christoph Reichetzeder, Jan Rahnenführer, Kerstin Sommer, Ursula Schulz, Caroline Paeschke and Dr Elena Kaschina.

FUNDING

This study was supported by the Peter Stiftung für die Naturwissenschaften, Berliner Sparkassenstiftung Medizin, Charité Funds for Research and Else Kröner-Fresenius Stiftung (Grant No 2011_A19).

CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES

1. Chow AK, Cena J, Schulz R. Acute actions and novel targets of matrix metalloproteinases in the heart and vasculature. Br J Pharmacol 2007; 152: 189–205

2. Benjamin MM, Khalil RA. Matrix metalloproteinase inhibitors as investigative tools in the pathogenesis and management of vascular disease. EXS 2012; 103: 209–279
3. Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res 2002; 90: 251–262
4. Wang M, Zhang J, Telljohann R *et al.* Chronic matrix metalloproteinase inhibition retards age-associated arterial proinflammation and increase in blood pressure. Hypertension 2012; 60: 459–466
5. Pai A, Leaf EM, El-Abbadi M *et al.* Elastin degradation and vascular smooth muscle cell phenotype change precede cell loss and arterial medial calcification in a uremic mouse model of chronic kidney disease. Am J Pathol 2011; 178: 764–773
6. Basalyga DM, Simionescu DT, Xiong W *et al.* Elastin degradation and calcification in an abdominal aorta injury model: role of matrix metalloproteinases. Circulation 2004; 110: 3480–3487
7. Chung AW, Yang HH, Kim JM *et al.* Upregulation of matrix metalloproteinase-2 in the arterial vasculature contributes to stiffening and vasomotor dysfunction in patients with chronic kidney disease. Circulation 2009; 120: 792–801
8. Chung AW, Yang HH, Sigrist MK *et al.* Matrix metalloproteinase-2 and -9 exacerbate arterial stiffening and angiogenesis in diabetes and chronic kidney disease. Cardiovasc Res 2009; 84: 494–504
9. Zebger-Gong H, Muller D, Diercke M *et al.* 1,25-Dihydroxyvitamin D3-induced aortic calcifications in experimental uremia: up-regulation of osteoblast markers, calcium-transporting proteins and osterix. J Hypertens 2011; 29: 339–348
10. Shroff RC, McNair R, Figg N *et al.* Dialysis accelerates medial vascular calcification in part by triggering smooth muscle cell apoptosis. Circulation 2008; 118: 1748–1757
11. Jung S, Querfeld U, Muller D *et al.* Submaximal suppression of parathyroid hormone ameliorates calcitriol-induced aortic calcification and remodeling and myocardial fibrosis in uremic rats. J Hypertens 2012; 30: 2182–2191
12. Freise C, Ruehl M, Erben U *et al.* The synthetic hydroxyproline-containing collagen analogue $(Gly\text{-}Pro\text{-}Hyp)_{10}$ promotes enzymatic activity of matrixmetalloproteinase-2 in *vitro*. Eur J Microbiol Immunol 2012; 2: 186–191
13. Freise C, Ruehl M, Seehofer D *et al.* The inhibitor of Ca(2+)-dependent K+ channels TRAM-34 blocks growth of hepatocellular carcinoma cells via downregulation of estrogen receptor alpha mRNA and nuclear factor-kappaB. Invest New Drugs 2013; 31: 452–457
14. Freise C, Erben U, Muche M *et al.* The alpha 2 chain of collagen type VI sequesters latent proforms of matrix-metalloproteinases and modulates their activation and activity. Matrix Biol 2009; 28: 480–489
15. Shanahan CM, Crouthamel MH, Kapustin A *et al.* Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. Circ Res 2011; 109: 697–711
16. Qin X, Corriere MA, Matrisian LM *et al.* Matrix metalloproteinase inhibition attenuates aortic calcification. Arterioscler Thromb Vasc Biol 2006; 26: 1510–1516
17. Chen NX, O'Neill KD, Chen X *et al.* Activation of arterial matrix metalloproteinases leads to vascular calcification in chronic kidney disease. Am J Nephrol 2011; 34: 211–219
18. Sasaki T, Nakamura K, Sasada K *et al.* Matrix metalloproteinase-2 deficiency impairs aortic atherosclerotic calcification in ApoE-deficient mice. Atherosclerosis 2013; 227: 43–50
19. Hosaka N, Mizobuchi M, Ogata H *et al.* Elastin degradation accelerates phosphate-induced mineralization of vascular smooth muscle cells. Calcif Tissue Int 2009; 85: 523–529
20. Bouvet C, Moreau S, Blanchette J *et al.* Sequential activation of matrix metalloproteinase 9 and transforming growth factor beta in arterial elastocalcinosis. Arterioscler Thromb Vasc Biol 2008; 28: 856–862
21. Suh JH, Yoon JS, Kwon JB *et al.* Identification of genomic aberrations by array comparative genomic hybridization in patients with aortic dissections. Korean J Thorac Cardiovasc Surg 2011; 44: 123–130
22. Jones JA, Zavadzkas JA, Chang EI *et al.* Cellular phenotype transformation occurs during thoracic aortic aneurysm development. J Thorac Cardiovasc Surg 2010; 140: 653–659
23. Xu S, Liu AC, Gotlieb AI. Common pathogenic features of atherosclerosis and calcific aortic stenosis: role of transforming growth factor-beta. Cardiovasc Pathol 2010; 19: 236–247
24. Haffner D, Hocher B, Muller D *et al.* Systemic cardiovascular disease in uremic rats induced by 1,25(OH)2D3. J Hypertens 2005; 23: 1067–1075
25. Hruska KA, Mathew S, Saab G. Bone morphogenetic proteins in vascular calcification. Circ Res 2005; 97: 105–114
26. Guerrero F, Montes de Oca A, Aguilera-Tejero E *et al.* The effect of vitamin D derivatives on vascular calcification associated with inflammation. Nephrol Dial Transplant 2012; 27: 2206–2212
27. Castro MM, Tanus-Santos JE. Inhibition of matrix metalloproteinases (MMPs) as a potential strategy to ameliorate hypertension-induced cardiovascular alterations. Curr Drug Targets 2013; 14: 335–343
28. Muthukuru M, Sun J. Doxycycline counteracts bone morphogenic protein 2-induced osteogenic mediators. J Periodontol 2013; 84: 656–665
29. Proudfoot D, Skepper JN, Hegyi L *et al.* Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies. Circ Res 2000; 87: 1055–1062
30. Clarke MC, Littlewood TD, Figg N *et al.* Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. Circ Res 2008; 102: 1529–1538
31. Iwasaki H, Inoue H, Mitsuke Y *et al.* Doxycycline induces apoptosis by way of caspase-3 activation with inhibition of matrix metalloproteinase in human T-lymphoblastic leukemia CCRF-CEM cells. J Lab Clin Med 2002; 140: 382–386
32. Onoda T, Ono T, Dhar DK *et al.* Tetracycline analogues (doxycycline and COL-3) induce caspase-dependent and -independent apoptosis in human colon cancer cells. Int J Cancer 2006; 118: 1309–1315
33. Maquart FX, Pasco S, Ramont L *et al.* An introduction to matrikines: extracellular matrix-derived peptides which regulate cell activity. Implication in tumor invasion. Crit Rev Oncol Hematol 2004; 49: 199–202
34. Duca L, Floquet N, Alix AJ *et al.* Elastin as a matrikine. Crit Rev Oncol Hematol 2004; 49: 235–244

Received for publication: 13.5.2015; Accepted in revised form: 30.7.2015