Exposure to Uremic Serum Induces a Procalcific Phenotype in Human Mesenchymal Stem Cells

Rafael Kramann, Simone K. Couson, Sabine Neuss, Uta Kunter, Manfred Bovi, Jörg Bornemann, Ruth Knüchel, Willi Jahnen-Dechent, Jürgen Floege, Rebekka K. Schneider

Objective—Medial artery calcification in patients with chronic kidney disease proceeds through intramembranous ossification resulting from osteoblast-induced calcification of the collagen extracellular matrix. The current study is based on the hypothesis that mesenchymal stem cells (MSC) constitute critical cells for procalcific extracellular matrix remodeling in patients with chronic kidney disease.

Methods and Results—Human MSC were cultured in media supplemented with pooled sera from either healthy or uremic patients (20%). Exposure to uremic serum enhanced the proliferation of MSC (cell counting, BrdU incorporation) whereas apoptosis and necrosis were not affected (annexin V and 7-amino-actinomycin staining). Uremic serum–exposed MSC recapitulated osteogenesis by matrix calcification and expression of bone-related genes (bone morphogenetic protein [BMP]-2 receptor, alkaline phosphatase, osteopontin, and Runx2) in 35 days. The uremic serum–induced osteogenesis was completely blocked by a BMP-2/4 neutralizing antibody or the natural antagonist NOGGIN. Calcification and matrix remodeling were further analyzed in a collagen-embedded osteogenesis model recapitulating the vascular collagen I/III environment. The uremic serum–induced calcification was shown to occur along collagen fibers as shown by scanning electron microscopy, energy-dispersive X-ray spectroscopy, and von Kossa staining and was accompanied by extensive matrix remodeling.

Conclusion—Uremic serum induced in a BMP-2/4-dependent manner an osteoblast-like phenotype in MSC accompanied by matrix remodeling and calcification. (Arterioscler Thromb Vasc Biol. 2011;31:e45-e54.)

Key Words: apoptosis ■ calcification ■ matrix ■ vascular biology

With advanced age, hypertension, metabolic disorders (eg, diabetes, obesity, hyperphosphatemia, dyslipidemia), and chronic kidney disease, calcium accumulates in the arterial vasculature. Once considered a passive process, vascular calcification has emerged as a tightly regulated, coordinated, and osteoblastic process resembling bone morphogenesis.1 Executive cell types familiar to bone biology are seen in calcified vasculature. One major hypothesis proposes that the presence of osteoblast-type cells in the vessel wall has a role in how vascular calcification proceeds.2,3 Nevertheless, it remains controversial whether these cells are derived from resident cells as vascular smooth muscle cells and pericytes or from circulating progenitor cells.4 Osteoblasts, smooth muscle cells, adipocytes, fibroblasts, and chondrocytes all share a common mesenchymal progenitor stem cell, and recent studies suggest that mesenchymal stem cells (MSC) contribute to the ectopic osteogenic program of vascular calcification in their role as vascular pericytes.5-7 The current study is based on the hypothesis that in the case of endothelial dysfunction/damage, MSC are recruited from the vascular tube or the bone marrow as the stem cell niche to support regenerative processes by their paracrine and biosynthetic activity (Supplementary Figure I).8,9 In response to metabolic, inflammatory, or mechanical stress, they (mal)differentiate into osteoblasts, adipocytes, or chondrocytes.10,11

Differentiation along the osteogenic lineage requires crucial factors, including bone morphogenetic proteins (BMP). In response to the stressors described above, large vessels produce BMP, especially BMP-2, which exerts a paracrine influence on regional mesenchymal progenitors.1 BMP–induced osteogenesis involves lineage-specific transcription factors such as Runx2 and Osx, which increase target genes such as alkaline phosphatase, osteocalcin, osteopontin, bone sialoprotein, and collagen I.12 In patients with chronic kidney disease (CKD), calcification can be found in atherosclerotic plaques and in the vascular media, smooth muscle cells, and elastic laminae of large elastic and medium muscular arteries as well as in cardiac valves.4,13,14

Although mouse cardiovascular transplantation models implicate an osteogenic differentiation of MSC in the patho-

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genesis of atherosclerotic lesions, the effect of uremia on
MSC has not yet been identified.\textsuperscript{5,7,11} In vitro assays allow the
characterization of a single cell type subjected to a specific, 
well-defined pathological stimulus.\textsuperscript{16,17} In the present study, 
we have analyzed the effect of established uremic culture
conditions on proliferation,\textsuperscript{18} apoptosis,\textsuperscript{19,20} extracellular ma-
trix (ECM) remodeling,\textsuperscript{21} and osteogenic differentiation of
MSC,\textsuperscript{22} representing key events in CKD vascular calcification.
The gold standard for cell biological imaging has been
2D tissue culture. However, this environment is rather arti-
ficial, and cells within a 3D matrix mimic the complex cellular
organization much better and allow the study of extracellular
matrix remodeling. Among the 26 different collagen types
described to date, types I and III collagen are the major
fibrilar collagens in the vasculature.\textsuperscript{23–25} Previously, we
described a collagen I/III-embedded osteogenesis model that
allows the study of the calcification processes beyond osteo-
genesis by the accompanying matrix remodeling.\textsuperscript{26} This
organotypic model representing the major collagens of the
arterial wall was applied for the first time to study uremic
seum-induced extracellular matrix remodeling.

\section*{Methods}

\subsection*{Isolation of MSC}
Human MSCs were isolated from the bone marrow of femoral heads, 
as previously described.\textsuperscript{26–29} MSCs were expanded in MSC medium 
(PAN Biotech, Aidenbach, Germany) consisting of 60% Dulbecco
minimum essential medium low glucose and 40% MCDB-201
supplemented with 2% FCS, 1× ITS-plus (insulin-transferrin-selenic
acid + BSA-linoleic acid), 1 mmol/L dexamethasone, 100 μmol/L
ascorbic-acid-2-phosphate, and 10 ng/mL epidermal growth factor.

\subsection*{Experimental Culture Conditions}
To assess the effect of uremic medium, the MSC medium was
modified to contain 20% serum content with either serum of healthy
patients or serum of patients undergoing dialysis. The pool of sera
from patients was obtained from 40 patients with end-stage renal
disease on maintenance hemodialysis after informed consent was
given (22 men and 18 women; mean age, 60 ± 17 years). Patients with
diabetes mellitus or former kidney transplantation were ex-
cluded. Blood samples were always collected immediately before
initiating the hemodialysis session. Sera were heat-inactivated for 40
minutes at 56°C and sterile-filtered through 0.22-
m filters (Millex,
Millipore Corp, Bedford, MA) before supplementing the stem cell
medium. The pool of control sera was obtained from healthy subjects
(n = 10) after informed consent was given (6 men, 4 women; mean
age, 59.76 ± 22.88 years). Basic biochemical data (creatinine, urea,
phosphate, and calcium) of the media are shown in Supplementary
Table I. The study was approved by the ethics committee of the
RWTH Aachen University Hospital and carried out according to the
principles of the Declaration of Helsinki.

To demonstrate the osteogenic differentiation of isolated MSC,
cells were induced to differentiate into osteoblasts according to
standard protocols.\textsuperscript{26} A neutralizing monoclonal mouse anti-human BMP-2/4 antibody
(1 μg/mL; R&D systems, Minneapolis, MN; MBA3552) and human
recombinant NOGGIN (10 μg/mL, R&D systems) were used to
evaluate the influence of BMP-2/4 signaling on osteogenic differen-
tiation. Monoclonal mouse IgG1 antibody (1 μg/mL, R&D systems)
was used as an isotype control.

\subsection*{Generation of 3D Collagen Gels}
For the generation of 3D collagen gels, 8 volumes of acidic collagen
G (3 mg/mL collagen III in 12 mmol/L HCl) was mixed with 1
volume of 10-fold Dulbecco minimum essential medium (with 4.5
g/L D-glucose, both Biochrom, Berlin, Germany) followed by
neutralization with 1 mol/L sodium hydroxide.\textsuperscript{27,30} One volume of
culture medium containing 1×10^6 MSC was added. Then, 300 μL of
the collagen/cell suspension was poured into 24-well culture plates
(Corning Life Sciences, Schiphol-Rijk, The Netherlands). Gelation
was performed by incubating the collagen solution for 2 hours at
37°C in a humidified incubator. The study is based on 6 independent
experiments with primary cultures of human MSC from bone
marrow.

\subsection*{Apoptosis Assay}
APC-labeled annexin V was used to quantitatively determine the
percentage of cells within the cell populations treated with 4 different
cultivation media that were undergoing apoptosis. Cells were stained
according to manufacturer’s instructions (APC Apoptosis Detection
Kit, BD Pharmingen, San Jose, CA). By costing with 7-amino-
actinomycin (7-AAD), viable cells were distinguished from nonvia-
able cells. Cells were analyzed by flow cytometry (FACS Canto II,
BD Biosciences) within 1 hour, and data were analyzed by using
Flow Jo software (Version 7.5, Tree Star Inc, Ashland, OR).

\subsection*{Determination of Proliferation}
The colorimetric BrdU ELISA assay (Roche Applied Science,
Mannheim, Germany) is based on the detection of BrdU incorpo-
rated into the genomic DNA of proliferating cells. Cells grown under
the 4 culture conditions in 96-well tissue-culture microplates were
labeled according to manufacturer’s instructions. The reaction pro-
duct was quantified after adding the stop solution (25 μL/well, 1
mol/L H_2SO_4) by measuring the absorbance using a scanning
multwell spectrophotometer (Infinite M 200, Tecan Group AG,
Maennedorf, Switzerland).

\subsection*{Histomorphological Analysis}
For histological and immunohistochemical analyses, 3D cell/colla-
gen constructs were fixed in 3.7% formaldehyde for 24 hours. The
constructs were paraffin-embedded, cut with a rotating microtome at
3-μm thickness (Leica), and stained according to routine histology
protocols. Immunohistochemical analysis was performed with the
use of the primary antibody specific for vimentin (mouse monoclo-
nal, 1:4000; DAKO, Hamburg, Germany), α-smooth muscle actin
(SMA) (mouse monoclonal, 1:3000; Sigma, Hamburg, Germany),
collagen I (mouse monoclonal, 1:2000; Sigma), laminin (mouse
monoclonal, 1:1000; Sigma), fibronectin (mouse monoclonal, 1:500;
Sigma), and osteopontin (mouse monoclonal, 1:3000; Santa Cruz,
Biotecnology, Heidelberg, Germany). Antigen retrieval was
achieved by microwave pretreatment in citrate buffer (pH 6.0). Slide
preparations were stained by using an autostainer for immunohisto-
chemistry (DAKO cytometry). Primary antibodies were diluted in a
commercial antibody diluent (ready-to-use, DAKO) and applied
for 25 minutes. After incubation of the biotinylated secondary antibody
(rabbit/mouse, ready-to-use; DAKO) for 25 minutes, color develop-
ment was performed with StreptABC complex/HRP (DAKO) and
with 3,3′-diaminobenzidine (DAB +; DAKO). Finally, slide prepara-
tions were counterstained with hematoxylin, dehydrated, and
mounted in Vitro-Clud (Langenbrinck, Emmendingen, Germany).
For transmission and scanning electron microscopy, collagen/cell
constructs were fixed in 3% glutaraldehyde for at least 24 hours. Pre-
paration for further analysis proceeded as previously
described.\textsuperscript{26,31}

\subsection*{Real-Time Reverse Transcriptase–Polymerase 
Chain Reaction}
Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad,
CA). One microgram of RNA was reverse-transcribed, using the
high-capacity cDNA Reverse Transcription Kit (Applied Biosys-
tems, 7300 Real-Time PCR System, Foster City, CA). cDNA was
diluted 1:2 with DEPC water. Quantitative polymerase chain reac-
tions were carried out with Power SYBR Green PCR Master Mix
(Applied Biosystems, 7300 Real-Time PCR System). The cDNA
subjected to control medium 1 of each MSC donor was used as a
relative standard for the genes of interest. For each sample, 1.2 μL of cDNA was added as a template in polymerase chain reactions. Amplification was monitored with the ABI Prism 7300 (Applied Biosystems). The expression of the genes of interest was normalized against the housekeeping gene GAPDH in all samples, and relative gene expression was analyzed with the $2^{-\Delta\Delta Ct}$ method. Primers are shown in Supplementary Table II.

**Calcium Determination**

Calcium was extracted overnight with HCl (0.6 mol/L) and quantified with the use of cresolphthalein complexone chemistry for a colorimetric assay at 578 nm (Randox Laboratories Ltd, Crumlin, England). The calcium content was normalized to the protein content to allow for comparisons. The protein content was measured colorimetrically at 562 nm (BC-Assay, Uptima, Montlucon, France). The protein content of the collagen gels without cells was subtracted of the total protein amount.

**Statistical Analysis**

Data are presented as mean±SD. Data analyses were performed with the use of 1-way ANOVA or Mann-Whitney U test where appropriate. For multiple group comparison, ANOVA with post hoc Bonferroni correction was applied. Statistical significance was defined as $P<0.05$. Analyses were performed using PASW Statistic 18.0 (SPSS Inc, Chicago, IL).

**Results**

**Uremic Culture Conditions Enhance the Proliferative Activity and Do Not Induce Apoptosis in MSC**

A proposed mechanism for vascular calcification is an increased rate of intramural apoptosis. To evaluate the effect of uremic serum on MSC in culture, we analyzed cell proliferation and apoptosis. Exposure of MSC to growth media containing pooled uremic serum did not alter the cell morphology. As confirmed by cell counting (Figure 1A, i) and BrdU incorporation (Figure 1A, ii), MSC exposed to uremic culture conditions exhibited an enhanced proliferative activity as compared with cells maintained in healthy pooled
serum after 7 and 14 days. As the result of contact inhibition of cells in overgrown culture wells, BrdU incorporation decreased after 5 days of culture (Figure 1A, ii). Cells subjected to osteogenic differentiation revealed the lowest proliferative activity. Uremic medium did not exert an apoptotic effect on cultured MSC (Figure 1B). By co-staining with 7-amino-actinomycin (7-AAD) and an antibody against annexin V, we determined the number of viable, apoptotic (positive for annexin V, negative for 7-AAD), and necrotic cells (positive for both annexin V and 7-AAD). The rate of apoptotic and necrotic cells increased slightly after 15 days under all culture conditions as the result of contact inhibition and aging of cells, without significant differences between uremic and control conditions. The highest levels of apoptotic cells were seen in osteogenic differentiated MSC (Figure 1B).

Uremic Serum Induces an Osteogenic Phenotype in MSC Resulting in Matrix Calcification

Induction of osteogenesis in MSC under conditions of osteogenic differentiation usually takes 15 to 21 days. BMP-2 and BMP-4 have been implicated in mineralization by BMP receptor 2 (BMPR2) phosphorylation and subsequent phosphorylation of regulatory Smads that modulate the target genes, for example, Runx2, alkaline phosphatase (ALP), and osteopontin.32 MSC exposed to uremic serum and osteogenic differentiation medium significantly upregulated BMP2-receptor expression compared with cells maintained in MSC medium and healthy serum starting at day 7 (Figure 2). The BMP2-receptor upregulation was followed by a significantly increased expression of the downstream target gene ALP after 14 days, which is known as an early indicator of osteogenic conversion. The consecutive biosynthetic activity was reflected in significant upregulation of collagen I after 21 days of culture, which was more pronounced than that observed in MSC-derived osteoblasts (Figure 2). Osteopontin and Runx2 expression are known to increase during osteoblast differentiation and maturation.33,34 After 35 days, Runx2 and osteopontin were significantly upregulated compared with MSC in MSC medium and healthy serum indicating the osteogenic phenotype.

Uremic Serum–Induced Osteogenesis Is Mediated by BMP-2/4 Signaling

The osteogenic differentiation of MSC in uremic medium was associated by intense matrix calcification. MSC exposed to uremic serum and osteogenic differentiation medium formed von Kossa–positive, partly nodular, calcium/phosphate crystals (Figure 3). The determination of the calcium load revealed that uremic serum–exposed MSC accumulated significantly more calcium than MSC in MSC medium and in healthy serum (Figure 3C). Because BMP-2/4 appears to induce an osteoblastic phenotype in cells in the vasculature, we explored the influence of BMP-2/4 signaling on uremic serum–induced calcification. MSC were incubated with an anti-human inhibitory BMP-2/4 antibody, isotype mouse IgG1, or the natural BMP-2/4 antagonist NOGGIN for up to 35 days. Blockade of BMP-2/4 in uremic and osteogenic medium completely abolished the calcification, as shown by negative von Kossa staining (Figure 3). Additionally, the calcium content in the BMP2/4-antagonized cultures was not significantly enhanced compared with control conditions (Figure 3C).

Uremic Serum Induces Matrix Calcification in the Collagen-Embedded Osteogenesis Model

The vessel wall mainly consists of collagen I/III and elastic lamellae, and the vascular calcification was described to occur along the collagen fibers.35 Besides calcification,
excessive extracellular matrix remodeling occurs in the vessel wall. In previous work, we established a collagen-embedded osteogenesis model that allows the study of osteogenic differentiation and, in parallel, extracellular matrix synthesis and degradation. To evaluate both matrix calcification and remodeling, we cultured MSC in the established collagen scaffolds under the various conditions for up to 35 days (Figure 4). After 35 days, we observed dense crystalline calcium/phosphate deposits in MSC exposed to uremic serum and after osteogenic differentiation (Figure 4). Von Kossa staining and scanning electron microscopy demonstrated that these partly crys-
talline, partly nodular calcium accumulations are lined along the collagen fibers and aggregate to form nodules. Analysis of the mineral content in collagen-cultured MSC by energy-dispersive X-ray analysis (EDX analysis) confirmed the presence of calcium and phosphate in a calcium-phosphate molar ratio of \( \frac{1}{1.6} \), matching that of hydroxyapatite mineral. Finally, the determination of the calcium load verified that uremic serum–exposed MSC and osteogenic-stimulated MSC accumulated significantly more calcium than MSC cultivated under control conditions and nearly the same amount of calcium as under osteogenic cultivation conditions in the 3D cultivation system. Scale bars, 20 \( \mu \text{m} \) (A) and 50 \( \mu \text{m} \) (B). *P<0.05 versus healthy serum. #P<0.05 versus MSC medium.

Uremic Serum Induces a Myofibroblastic Phenotype in MSC

MSC in the collagen-embedded osteogenesis model significantly contracted and densified the collagenous matrix when exposed to uremic serum compared with control conditions with healthy serum and MSC medium (Figure 5A). Immunohistochemistry demonstrated that MSC under uremic culture conditions acquire a myofibroblastic phenotype because they coexpressed the mesenchymal marker vimentin and \( \alpha \)-SMA (Figure 5B). In contrast, MSC in MSC medium and healthy serum expressed vimentin but were negative for \( \alpha \)-SMA. Osteogenic-stimulated MSC, which were also able to contract the collagenous matrix, coexpressed \( \alpha \)-SMA and vimentin, underlining their contractile phenotype.

Uremic Serum Induces Extensive Extracellular Matrix Remodeling in the Collagen-Embedded Osteogenesis Model

The most abundant protein in the osteocytic environment is collagen I. It can also be found in high levels in calcified vessels. After 21 days, MSC exposed to uremic serum overexpressed collagen I and significantly outperformed osteogenic-differentiated cells (Figure 6). Immunohistochem-
istry revealed the evenly porous reticular structure of the collagen I fibers of the collagen gels containing MSC in MSC medium and healthy serum after 35 days. In MSC under uremic conditions, the collagen gel stained strongly positive for collagen I at the cell/collagen interface indicating collagen I synthesis and remodeling. The collagen fibers were not evenly distributed but showed a nodular aspect. Under osteogenic differentiation conditions, MSC remodeled the collagenous matrix and formed lamellar, strongly collagen I–positive structures. Laminin, a key regulator of cell adhesion and migration, was significantly upregulated in uremic serum–exposed MSC after 35 days. The immunohistochemical staining showed a slight staining at the MSC/collagen interface under uremic culture conditions, whereas laminin was not expressed in cells under control and osteogenic differentiation culture conditions. Fibronectin, as a provisional matrix component during the wound-healing processes, was also termed a proangiogenic factor. Although MSC under all culture conditions expressed this matrix component at the mRNA level, immunohistochemistry showed a pronounced expression surrounding MSC in uremic serum, underlining the densified, nodular aspect of the collagen matrix. Osteopontin is not specifically expressed by osteoblasts but was shown to play a crucial role for physiological and pathological processes including wound healing, bone turnover, tumorigenesis, inflammation, ischemia, and immune responses. We detected a significant osteopontin upregulation in osteogenic-stimulated MSC after 21 days and in uremic serum–exposed MSC after 35 days. Immunohistochemistry showed a faint osteopontin expression in the collagen gel under all culture conditions that was enhanced after exposure to uremic serum and after osteogenic differentiation, indicating the osteogenic maturation step.

Scanning as well as transmission electron microscopy of MSC exposed to uremic serum further confirmed the matrix maturation and remodeling evidenced by an increase in matrix density with numerous collagen fibers (Supplementary Figure IIIA and IIIB). A sign of a high biosynthetic activity, MSC exhibited a dense rough endoplasmatic reticulum and secreting vesicle/lipid droplets (Supplementary Figure IIIB). In contrast, MSC in MSC medium and serum from healthy donors were embedded in a reticular and amorphous matrix with only a few collagen fibers.

Given that we observed matrix production without significant signs of matrix degradation, we assessed the expression of matrix metalloproteinases as MMP1, MMP2, MMP7, and MMP13. All MMPs were only expressed in the first days of the collagen culture, that is, during early remodeling and cell migration, but downregulated over of the subsequent 35 days (Supplementary Figure IV).

Thus, our data suggest a potential role of MSC in uremic vascular remodeling and calcification.

**Discussion**

In the present study, we analyzed the effect of uremic serum on MSC as potential procalcifying cells regarding key events of vascular calcification in CKD patients: (1) cell proliferation, (2) apoptosis and necrosis, (3) osteogenic gene regulatory programs, and (4) procalcific ECM remodeling. MSC from 6 independent donors were characterized according to the minimal criteria published by the International Society for Cellular Therapy and subjected to identical isolation and cultivation conditions. MSC exposed to uremic medium were shown to be robust and able to survive the pathological stimulus. They maintained their typical spindle-shaped morphology and increased their proliferation activity, as determined by cell counting and BrdU assay. In fact, mediators in...
uremia appear to stimulate proliferation and serve as mitogenic signals. We did not observe apoptotic bodies in our cultured cells nor did we determine enhanced apoptosis or necrosis caused by uremic culture media. This high proliferative activity and adoptability to pathological, inflammatory stimuli supports our hypothesis of MSC as one of the critical cell types for initiating the process of procalcific ECM remodeling in the vessel wall.

Our first major finding was that uremic serum induces an osteogenic phenotype in MSC resulting in matrix calcification. The uremic serum–induced osteogenesis recapitulated the distinct phases of bone formation by expression of cell growth and bone-related genes in 35 days. First, BMP2-receptor upregulation initiated the differentiation of the proliferating mesenchymal stem cells to an osteoprogenitor cell. Second, the enhanced collagen I expression and alkaline phosphatase upregulation induced the maturation into preosteoblasts resembling to the so-called ECM maturation phase. Finally, the uremic serum induced mature osteoblasts in MSC that were characterized by mineralization of the ECM and

Figure 6. Semiquantitative real-time reverse transcriptase–polymerase chain reaction (days 7, 21, and 35) and immunohistochemistry (day 35) of mesenchymal stem cells cultivated under the 4 different conditions in a 3D collagen matrix demonstrated the immense production of extracellular matrix proteins under uremic cultivation conditions. Scale bars, 20 μm.
expression of Runx2 and osteopontin. Previous studies demonstrated that uremic serum induces mineralization of bovine vascular smooth muscle cells independent of phosphorus. About 90 uremic toxins have been identified to date (www.uremictoxins.org). Some of these toxins have been individually examined in vascular smooth muscle cell cultures and have been described to induce calcification including elevated phosphorus, PTH and PTH-related peptide, calcitriol, alterations of lipoproteins, and homocysteine. Combinations of these factors probably are more potent and cause the stepwise calcification in patients with CKD. Thus, we exposed MSC to pooled uremic serum as a surrogate for the uremic milieu. This pool of uremic toxins induced osteogenesis and matrix calcification in uremic serum–exposed MSC by upregulating typical mediators of osteogenesis as BMP-2 receptor and Runx2. This calcification process was never observed under our control conditions.

Our second major finding was that antagonism of BMP-2 inhibited uremic serum–induced osteogenesis over 35 days. Previous studies demonstrated the importance of BMP-2 in uremic serum–induced calcification of vascular smooth muscle cells. On the basis of these studies, BMP-2 was also proposed to be a potential “uremic toxin,” as it is found in increased levels in uremic compared with normal human serum. To date, BMP-2 expression in vascular walls, first identified by Demer in 1993, is considered to promote ectopic osteogenic gene regulatory programs. Pathophysiological stimuli for BMP-2 expression are inflammatory and redox-dependent. Examples of this are an increase in cytokine levels, bacterial products, advanced glycation end products, hyperhomocysteinemia, hypercholesterinemia, and oxidized lipoproteins. Most of these factors have also been described in patients with CKD. Further, BMP-2 is a potent osteoinductive factor in MSC. Our results indicate BMP-2–dependent calcification and osteogenic differentiation of MSC exposed to uremic serum.

Our third major finding was that uremic serum–induced osteogenesis was accompanied by the acquisition of a myofibroblastic phenotype as well as extensive matrix remodeling. In previous studies, we demonstrated that 2D culture systems can induce osteogenesis but that its value is limited in simulating osteogenesis-related matrix remodeling processes. Because collagen I and III are the major collagens in the vessel wall, our established model is highly suitable for recapitulating pathophysiological processes in the vessel wall in an in vitro system. Thus, we cultured MSC in the established collagen–embedded osteogenesis model and subjected them to the different culture conditions. We observed intensive matrix remodeling through upregulation of extra-cellular matrix proteins as well as matrix modification by contraction in uremic serum–exposed MSC. Interestingly, the matrix deposition was found to be predominantly at the cell/collagen interface, indicating the MSC-driven remodeling. Further, scanning electron microscopy, von Kossa staining, and EDX analysis confirmed that the dense calcium/phosphate crystals were positioned along the collagen fibers and displayed the quality of hydroxyapatite crystals in bone. This observation is in accordance with a study of Karsenty et al., who demonstrated that premature expression stages of osteogenesis as ectopic expression of ALP combined with collagen I deposition are sufficient to drive heterotopic mineralization. Watson et al. reported that a permissive matrix is needed for calcification and that exposure to collagen I stimulates robust calcification by otherwise slowly mineralizing cells. Once thought to be biologically inert, collagens are now known to be bioactive components of the ECM that exert profound effects on many cell types. Strikingly, the osteoblast-induced, intramembranous calcification of a collagenous ECM is the main pathophysiological mechanism of media artery calcification (also known as Mönckeberg sclerosis) in CKD patients. In accordance with our hypothesis that recruited MSC cause vascular matrix remodeling and calcification in CKD, Vattikuti and Towler argued that media artery calcification is a consequence of a migratory population that acquires an osteoblastic phenotype.

In conclusion, our study shows the BMP-2/4–dependent calcification of MSC in an in vitro uremic microenvironment. By far, the most extensive vascular calcification occurs in patients with CKD. MSC exposed to uremic serum recapitulate osteogenesis, mainly characterized by extensive ECM remodeling and collagen synthesis with adjacent expression of the bone transcription factor Runx2 and the matrix proteins osteopontin, collagen, and fibronectin. Our data support the hypothesis that uremic serum induces a procalcific phenotype in human MSC.

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Disclosures
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Supplementary Figure I: Hypothesis for the origin of osteoblast-type cells in the vessel wall: Mesenchymal stem cells as circulating osteoprogenitor cells derived from the bone marrow migrate into the vessel wall, act like pericytes and differentiate into osteoblast-type cells and calcifying vascular cells. Also, resident pericytes may differentiate into osteoblast-type cells and promote calcification of the media through ECM remodeling.
Supplementary Figure II: Semi-quantitative real-time RT-PCR (n = 3) in the collagen-embedded osteogenesis model showed, similarly to MSC in the monolayer (Figure 2), an up-regulation of BMP2 receptor under uremic serum after 7 days followed by increased ALP expression after 21 days and Runx 2 upregulation after 35 days.
**Supplementary Figure III:** (A) Scanning electron microscopy showed a densification of the reticular collagen matrix when exposed to uremic serum. (B) Transmission electron microscopy confirmed the enhanced ECM remodeling and collagen synthesis in uremic serum-exposed MSC. Sfa= substantia fundamentalis amorpha, N = nucleus, Cf = collagen fibres, Ncl = nucleolus, M = mitochondria , Vs = vesiculae secretae, RER = roug endoplasmatic reticulum. Scale bar 50 µm (B), 2 µm (C).
Supplementary Figure IV: Semi-quantitative realtime RT-PCR (n = 3) in the collagen-embedded osteogenesis model showed expression of MMP1, MMP2, MMP7 and MMP13 mainly after 7 days of culture and their downregulation after 21 days.
Supplementary Table I:
Basic biochemical data of the four cultivation media

<table>
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<th>MSC-medium</th>
<th>healthy serum</th>
<th>uremic serum</th>
<th>osteogenic</th>
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<td>0,8</td>
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<tr>
<td>phosphate [mmol/l]</td>
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<td>calcium [mmol/l]</td>
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### Supplementary Table II:

**Primer:**

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<th>Gene</th>
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<td>Rv 5´-TGGACTCCACGACGTACTCA-3´</td>
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<td>Osteopontin</td>
<td>Fw5´-AATTGCAGTGATTTGCTTTTGC-3´</td>
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<td>Rv5´-CAGAACTTCCAGAATCACGCTTGT-3´</td>
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<td>Runx2</td>
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<td>Rv5´-AAGACGGTTATGGTCAAGGTGAAAA-3´</td>
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<td>ALP</td>
<td>Fw5´-CCGTGGCAACTCTATCTTTTG-3´</td>
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<td></td>
<td>Rv5´-CAGGCCCATTGCCATACAG-3´</td>
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<td>BMPR2</td>
<td>Fw 5´-AGTGCCAACCTCGTTATGG-3´</td>
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MMP 13  Fw5'-AAGGAGCATGGCGACTTCT-3'
Rv5'-TGGTTCAGGAAAAGC-3'