Original Article

Renal Handling of Matrix Gla-Protein in Humans with Moderate to Severe Hypertension

Roger J.M.W. RENNENBERG[1)], Leon J. SCHURGERS[2)], Cees VERMEER[2)], Jan B.J. SCHOLTE[1)], Alphons J.H.M. HOUBEN[1)], Peter W. de LEEUW[1)], and Abraham A. KROON[1)]

Vascular calcifications are common among patients with hypertension. The vitamin K–dependent protein matrix Gla-protein plays an important role in preventing arterial calcification. Since a decrease in renal clearance is a prevalent clinical problem in patients with hypertension, we aimed to study the renal clearance of matrix Gla-protein from the circulation in these patients having a wide range of creatinine clearances. Ninety moderate to severe hypertensive patients who were scheduled for renal angiography were enrolled in the study. In these patients, renal arterial and renal venous blood was sampled prior to the administration of contrast material in order to determine the total renal and single kidney clearance of matrix Gla-protein. The average renal fractional extraction of matrix Gla-protein was 12.8%. There was no significant correlation between creatinine clearance (range 26–154) and renal fractional extraction of matrix Gla-protein in this population. The extraction of matrix Gla-protein was not influenced by the presence of a renal artery stenosis. In conclusion, we demonstrate that the kidney is able to extract matrix Gla-protein from the plasma at a constant level of 12.8%, independent of renal function in hypertensive subjects. (*Hypertens Res* 2008; 31: 1745–1751)

***Key Words*: calcification, renal clearance, blood flow, matrix Gla-protein, hypertension**

Introduction

Small peptides are usually able to freely pass the glomerular barrier, and their extraction by the kidney does not drop until the glomerular filtration rate has fallen substantially. Previously, we assessed the renal extraction of small hormonal peptides such as leptin and growth hormone (*1*, *2*). Another peptide whose renal extraction is relevant to measure is matrix Gla-protein (MGP), a 10 kDa vitamin K–dependent protein and a potent inhibitor of vascular calcifications. Almost all tissues, including the kidney, contain carboxylase and vitamin K, which are necessary to convert uncarboxylated MGP into active carboxylated MGP. Although first discovered in bone, MGP is mainly produced by vascular smooth muscle cells and chondrocytes and appears to accumulate at sites of calcifications (*3*–*8*). Part of the MGP synthesized in the tissues spills over into plasma, where it can be measured in concentrations between 5 and 19 nmol/L. Because of the insolubility of MGP, this protein likely circulates complexed with "chaperone" (lipo)proteins or other structures. An example is the MGP-mineral complex in which MGP is bound to fetuin to form a soluble complex that can be transported by the plasma (*9*).

Circulating levels of MGP correlate inversely with arterial calcifications and cardiovascular disease (*10*–*12*). Low serum levels of MGP are associated with a greater degree of coronary calcification and angina pectoris (*11*, *12*). The carboxylation status of MGP is also correlated with markers of active calcification in dialysis patients (*13*). Moreover, hypertensive

From the [1)]Department of Internal Medicine, University Hospital Maastricht and Cardiovascular Research Institute Maastricht (CARIM), Maastricht, The Netherlands; and [2)]VitaK, University of Maastricht and Cardiovascular Research Institute Maastricht (CARIM), Maastricht, The Netherlands.

Address for Reprints: Roger J.M.W. Rennenberg, M.D., Department of Internal Medicine, University Hospital Maastricht and Cardiovascular Research Institute Maastricht (CARIM), P. Debyelaan 25, 6229 HX, Maastricht, The Netherlands. E-mail: rre@sint.azm.nl

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patients have more vascular calcifications than normotensive subjects, which may contribute to their increased vascular risk (*14*). Lower levels of circulating MGP may be due either to less spillover into or to enhanced clearance from the circulation. So far, the mechanisms by which MGP is removed from the circulation are still elusive and, in particular, no information is available on the role of the kidney in MGP clearance. This prompted us to design the present study to explore whether the kidneys extract MGP and, if so, to what extent. Due to the invasive nature of this study, measurements were confined to hypertensive patients for whom renal angiography was indicated. Furthermore, this protocol allowed us to distinguish the potential effect of a renal artery stenosis on MGP handling. This is the first publication concerning MGP extraction in the human kidney.

Methods

Patients

During a 19-month period, consecutive patients were recruited from the Internal Medicine outpatient hypertension clinic. All hypertensive patients between 18 and 65 years of age were routinely screened for the presence of clinical clues indicating renal artery stenosis, as defined by the Working Group on Renovascular Hypertension and others (*15–17*). A total of 133 patients with at least one clinical clue in addition to hypertension were scheduled for renal angiography on the suspicion of having renal artery stenosis. In 90 of the patients, antihypertensive drugs and other types of medication that could influence the flow through the kidney could safely be withheld for a period of 3 weeks preceding angiography. These patients were enrolled in the present study after they had given written informed consent. The study was approved by our local ethics committee. The 43 remaining patients who were excluded did not differ from the study group with respect to relevant clinical variables (blood pressure levels, sex, age, body mass index [BMI], smoking habits, blood glucose, cholesterol levels and estimated creatinine clearance).

Angiography and Sampling

At the angiosuite, catheters were placed into the renal artery and vein without the use of contrast material. Subsequently, blood samples were drawn simultaneously from the artery and both the left and right renal vein for determination of MGP levels. In a subgroup of the patients, we also measured arterial and venous osteocalcin. Blood sampling was followed by selective measurements of renal blood flow by means of the ^{133}xenon washout technique (*18–20*). Angiography was performed only after all measurements had been completed in order to avoid disturbing renal hemodynamics or tubular function by the contrast material. Measurements were performed in both the left and the right renal veins because previous studies have shown a side difference in renal blood flow irrespective of the presence of a renal artery stenosis (*21*).

To determine the possible excretion of MGP into the urine, we also measured MGP concentrations in the overnight morning urine of five healthy volunteers.

Measurements

Blood samples were centrifuged immediately at 3,000 × *g* for 15 min at 4°C. Serum was transferred into separate tubes and stored at −80°C until use. Test kits for MGP detection in serum were obtained from Biomedica (Vienna, Austria). The principle of the assay is that of a competitive enzyme-linked immuno-sorbent assay (ELISA) in which microwell plates are coated with a mouse monoclonal antibody against human MGP. Synthetic MGP peptide in human serum, which was affinity-stripped to remove native human MGP, was used as calibrator. Biotinylated MGP that was added simultaneously to the wells with standards, samples or controls was used as tracer. Detection of bound tracer was accomplished with horseradish peroxidase (HRP)–labeled streptavidin (*12*). The test detects total MGP in any of the bound, unbound, carboxylated or uncarboxylated forms. The intra- and inter-assay coefficients of variation of this test were both approximately 10%. Osteocalcin (OC) was measured using a commercially available ELISA kit (Takara Bio Inc., Otsu, Japan) (*22*).

Biochemical variables, including total cholesterol, creatinine and glucose, were measured using an automated chemistry analyzer (Beckmann Synchron CX 7-2; Fullerton, USA). Endogenous creatinine clearance (ECC) was calculated from peripheral venous creatinine concentrations with the formula of Cockcroft and Gault (*23*). Human kidney tissue was obtained from our department of pathology. After deparaffinization and rehydration, tissues were stained with hematoxylin and eosin to demonstrate tissue integrity. For immunostaining with MGP monoclonal antibody, sections were heated in 0.2% (w/v) citric acid at pH 6.0 for 15 min before washing with phosphate-buffered saline and incubation with anti-MGP3-15 (5 μg/mL). All antibodies were diluted in blocking reagent (Roche Diagnostics, Mannheim, Germany). Substitution with an irrelevant antibody served as a negative control. Biotinylated sheep anti-mouse IgG (Amersham Biosciences, Little Chalfont, UK) was used as a secondary antibody, followed by incubation with an avidin-linked alkaline phosphatase complex (Dako, Golstrup, Denmark). Staining was carried out with the alkaline phosphatase kit I (Vector Laboratories, Burlingame, USA). All specimens were counterstained with hematoxylin, and the sections were mounted using imsol-mount.

Calculations

Mean renal blood flow (MRBF) was determined by means of the ^{133}xenon-washout technique, as described previously (*20*, *24*). Flow data were analyzed for both the right and the left kidney separately and expressed in mL/min/100 g of renal tis-

Table 1. Clinical Characteristics of the Study Population

Variables	All subjects (n=90)	Subjects with RAS (n=23)	Subjects without RAS (n=67)	p value
Age (years)	52±10	58±6	50±10	0.0001†
Body mass index (kg/m^2)	27±5	26±5	28±4	0.11
Male/female (n)	51/39	16/7	35/32	0.15
(ever) Smoking (%)	62	83	55	0.02
Systolic blood pressure (mmHg)	164±24	171±23	162±24	0.15
Diastolic blood pressure (mmHg)	97±14	97±14	98±14	0.73
Creatinine (μmol/L)	87±26	93±34	85±23	0.29†
Creatinine clearance (mL/min)	97±26	86±29	101±24	0.02
(median, range)	98, 26–154	80, 29–153	99, 26–154	0.02
Fasting serum glucose (mmol/L)	6.1±2.0	6.7±2.4	5.9±1.8	0.14†
Total cholesterol (mmol/L)	5.5±1.1	5.5±1.0	5.6±1.2	0.72

Data are shown as mean±SD. RAS, renal artery stenosis; MGP, matrix Gla-protein. There was no significant correlation between body mass index or age and MGP levels. †Unpaired t-test with Welch's correction, comparing subjects with and without RAS.

Table 2. Renal Clearance Assessments Analyzed per Kidney

Variables	All subjects (n=90)	Left kidney (n=90)	Right kidney (n=90)	Kidneys with RAS (n=31)	Kidneys without RAS (n=149)	p value*
Left renal vein MGP (nmol/L)	5.5±1.4	5.5±1.4	—	5.7±1.2	5.4±1.4	0.47
Right renal vein MGP (nmol/L)	5.7±0.7	—	5.7±0.7	6.3±1.9	5.6±1.2	0.20†
Arterial MGP (nmol/L)	6.4±1.5	—	—	6.8±1.7	6.3±1.4	0.16
Mean renal plasma flow (mL/min/100 g of renal tissue)	—	109±36.8	131±40.2	87.3±35.8	127.9±35.8	<0.0001
Total venous efflux (nmol/L)	1,307±409.3	—	—	—	—	
Total arterial MGP delivery (nmol/L)	1,508±467.9	—	—	1,187±454.4‡	1,555±468.3‡	0.002
Total renal uptake (pmol/min/100 g of renal tissue)	198±186.4	—	—	—	—	
Total renal uptake left kidney (pmol/min/100 g of renal tissue)	94.9±99.8	—	—	105.0±84.3	92.7±103.4	0.67
Total renal uptake right kidney (pmol/min/100 g of renal tissue)	95.6±160.2	—	—	10.2±84.9	111.1±166.1	0.004†
SKFE (%)	—	14±12.8	10±14.5	—	—	
Renal fractional extraction (%)	12.8±11.1			11.5±13.2	12.6±10.5	0.98
MGP clearance (mL/min/100 g of renal tissue)	28	15	13	10	16	0.07

Data are shown as mean±SD. *Comparison between stenosis present and absent. RAS, renal artery stenosis; MGP, matrix Gla-protein; SKFE, single kidney fractional extraction. †Unpaired t-test with Welch's correction because of significantly different variances. ‡Results applicable to patient, not to single kidney.

sue. Mean renal plasma flow (MRPF) was calculated with the formula: $MRPF = MRBF \times (1 - Ht)$, where Ht is the arterial hematocrit. Total arterial MGP delivery to the kidneys amounts to: arterial $MGP \times (MRPF_L + MRPF_R)$, where $MRPF_L$ and $MRPF_R$ represent mean renal plasma flow through the left and right kidney, respectively. Single kidney fractional extraction (SKFE) of MGP was derived from the formula: $((A - V)/A) \times 100$ (%), where A and V represent the MGP concentrations in the renal artery and vein, respectively. Renal MGP uptake by each kidney was computed as mean renal plasma flow×(arterial MGP concentration – renal venous MGP concentration) and expressed in pmol/min/100 g. Total renal uptake was taken as the sum of the uptake by the right and the left kidney. Total venous efflux of MGP was calculated as: $(V_L \times MRPF_L) + (V_R \times MRPF_R)$, where V_L and V_R represent the concentrations of MGP in the left and right renal vein, respectively. Renal fractional extraction (RFE) of MGP was derived from the formula: (total arterial delivery – total venous efflux)×(total arterial delivery)$^{-1}$ ×100 (%). Total renal clearance of MGP was calculated as: $(SKFE_L \times MRPF_L)$

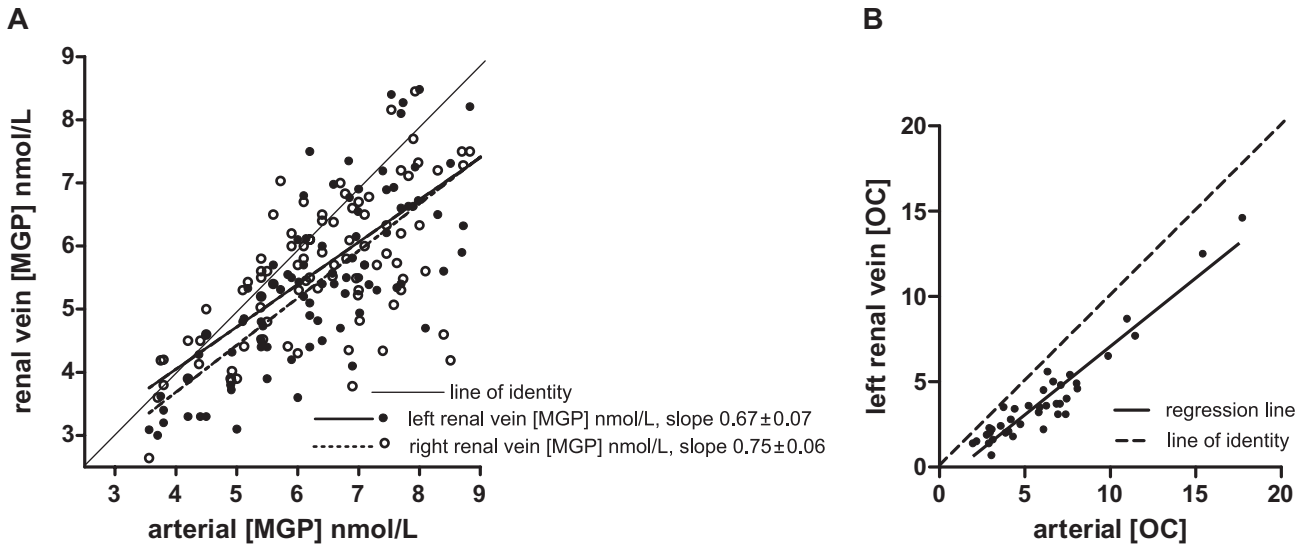

Fig. 1. *Correlation between arterial and venous concentrations of matrix Gla-protein and osteocalcin. A: Matrix Gla-protein (MGP). B: Osteocalcin (OC). Extraction of MGP and OC vs. arterial concentration of MGP and OC, showing increased extraction with increasing arterial concentration. The line of identity represents no effect on MGP and OC concentration when blood passes through the kidney.*

$+(SKFE_R \times MRPF_R)$. For OC, we calculated the single kidney fractional extraction and renal uptake for the left kidney, since only samples from the left side were taken for comparison.

Statistical Analysis

Normally distributed variables were expressed as means with standard deviations. Data that showed a non-normal distribution were expressed as medians and ranges. Renal MGP and OC extraction were correlated with clearance parameters by use of the Spearman correlation. To assess differences between means, the unpaired *t*-test was used. Welch's correction was applied when the variances were significantly different. Statistical calculations and data management were performed using SPSS for Windows V11.0 (SPSS, Chicago, USA). A p-value of <0.05 was considered to denote statistical significance.

Results

Of the 133 hypertensive patients who underwent angiography, 90 patients were not receiving medication at the time of the procedure. Table 1 shows the anthropometric characteristics of these patients. In a total of 18 kidneys in 12 patients, ^{133}xenon washout measurements were impossible or incomplete due to the misplacement of the catheter or because the renal artery was too narrow to allow the catheter to be advanced far enough. In 23 of the 90 patients, renal angiography showed renal artery stenosis of varying degrees, with a minimum of 20%. Bilateral renal artery stenosis was present in eight patients. Patients with renal artery stenosis were significantly older, (had) smoked more often and had a significantly lower ECC (Table 1). Concentrations of MGP were lower in renal venous blood than in arterial blood. Arterial

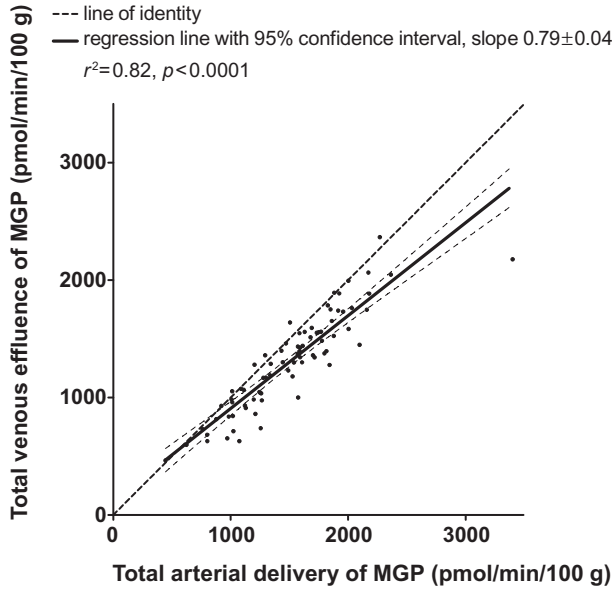

Fig. 2. *Total venous effluence vs. total arterial delivery of matrix Gla-protein (MGP). Total venous efflux of MGP plotted against total arterial delivery. The line of identity represents no effect on MGP concentration when blood passes through the kidney. The majority of the patients are depicted below the line of identity, indicating extraction of MGP from the circulation.*

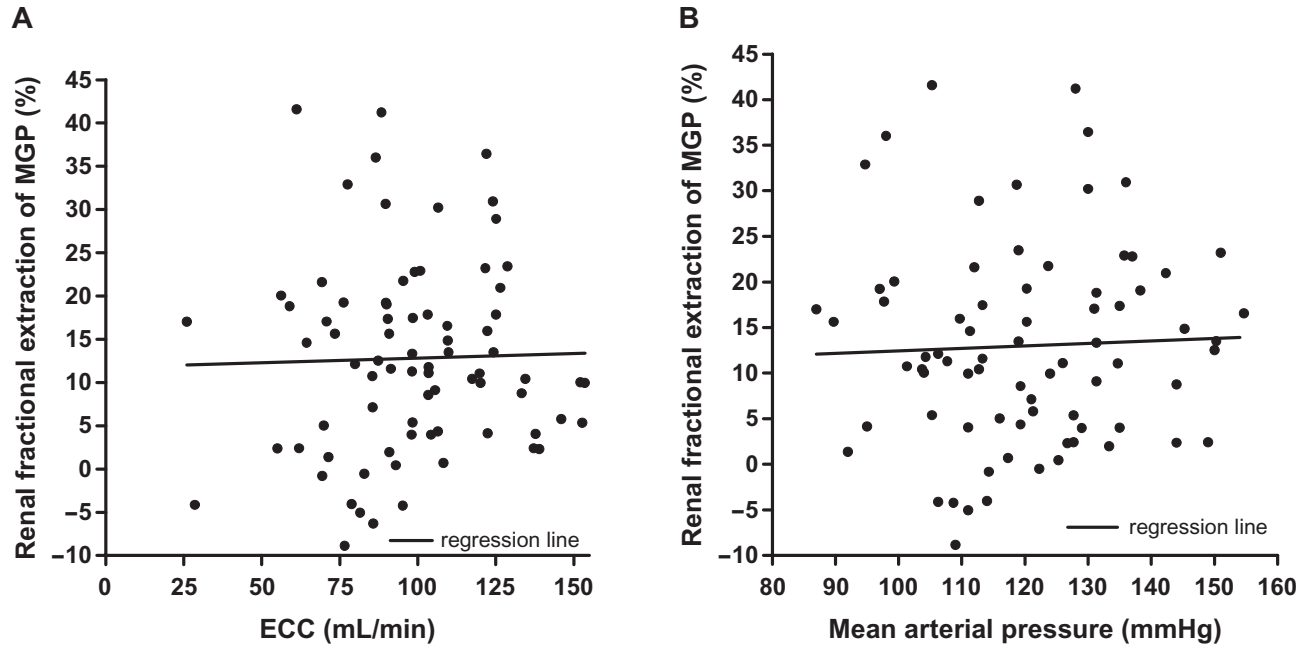

Fig. 3. *A: Correlation between renal function and renal fractional extraction of MGP. Renal fractional extraction of MGP showing no correlation with endogenous creatinine clearance (ECC), as estimated by the Cockcroft and Gault formula. B: Correlation between mean arterial pressure and renal fractional extraction of MGP. Renal fractional extraction of MGP showing no correlation with mean arterial pressure (calculated from 24 h ambulatory blood pressure measurement).*

MGP averaged 6.4±1.5 nmol/L (mean±SD) whereas the average MGP concentrations in the left and the right renal vein were 5.5±1.4 and 5.7±0.7 nmol/L, respectively (Table 2). A significant positive relationship was found between arterial MGP concentrations and both the left and right venous MGP concentrations ($r=0.78$, $p<0.0001$ and $r=0.63$, $p<0.0001$, respectively), as well as between the arterial and renal vein concentrations of OC ($r=0.89$, $p<0.0001$, Fig. 1). Plotting the total renal venous outflow against arterial delivery (Fig. 2) demonstrates that MGP is extracted by the kidney and that the renal extraction efficiency is constant over a wide range of arterial delivery. Overall fractional renal extraction of MGP was 12.8%. Overall fractional renal extraction of OC was 31.7%.

Thirty-five of the patients showed normal renal function, while 22, 26, 5, 2 and 0 patients were in chronic kidney disease stages 1, 2, 3, 4 and 5, respectively. There was no correlation between either serum creatinine or ECC and the renal fractional extraction of MGP ($r=0.15$, $p=0.2$ and $r=0.04$, $p>0.7$, respectively) (Fig. 3A). However, renal fractional extraction of OC, which was only measured on the left side, showed a significant but weak correlation with ECC ($r=0.35$ $p=0.03$). Renal extraction of MGP and OC did not correlate with age, blood pressure (Fig. 3B), the presence of stenosis or 24 h albumin excretion.

The fact that the kidneys are involved in the extraction of MGP from the circulation may imply that MGP-related peptides are found in urine. However, in the overnight morning urine of five healthy volunteers, urine-MGP concentrations averaged only 1.14 nmol/L (range 0 to 1.8). This is close to the lower detection limit and suggests that virtually no detectable forms of MGP are excreted in the urine. Unfortunately, proteolytic decay cannot be excluded completely because no protease inhibitors were added to the collected urine.

Discussion

In the present study, we demonstrated that the kidney is able to extract MGP from the circulation. This extraction of MGP is independent of renal function as well as the presence of renal artery stenosis or albuminuria. However, patients with more advanced stages of chronic kidney disease were either excluded (stage V) or relatively underrepresented (stage III and IV). As there is virtually no excretion of MGP into the urine of healthy volunteers, our data are compatible with the view that the extracted MGP is metabolized on its way through the kidney. However, we cannot fully exclude the possibility that normal tubular cell and brush border function may be impaired in subjects with renal artery stenosis or renal insufficiency, thus resulting in incomplete reabsorption and breakdown of MGP that may then be detectable in urine. Given its small size, MGP is likely to be filtered by the glomerulus and then reabsorbed and broken down in the proximal tubuli. This mechanism has been demonstrated for many

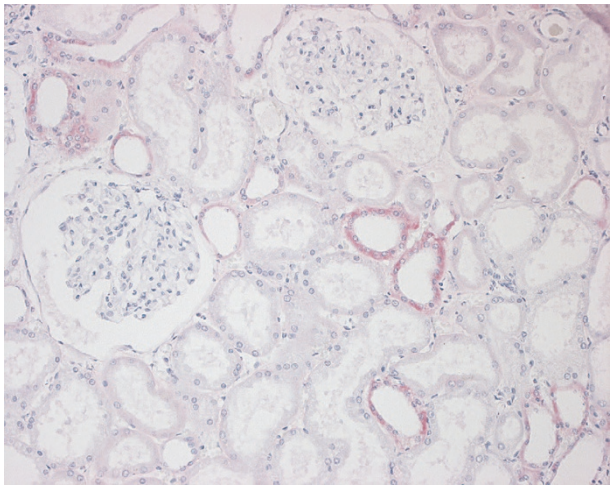

Fig. 4. *MGP in a human renal biopsy specimen. The immunohistochemical localization of MGP in a human kidney specimen. A section displaying distal tubules was stained with anti-MGP antibodies (red) and counterstained with haematoxylin. The control sample, which was stained with an irrelevant antibody, was negative (data not shown).*

other small proteins, like leptin and growth hormone (*1*, *2*). Moreover, on the basis of its small size, Price *et al*. suggested that MGP is rapidly cleared by the kidney (*9*). Although clearance may be impaired because MGP may be complexed with larger structures, experimental data on this issue are lacking. So far, attempts to determine the exact molecular size of the fetuin mineral complex have been unsuccessful, but it is likely to be greater than 250 kDa (*9*). Thus, it is unlikely that the complexed form of MGP is filtered by the glomerulus. The fractional extraction of total MGP (bound or unbound to carriers) passing through the kidney was 12.8%. It should be emphasized, however, that our data on renal extraction of MGP are valid only when there is no renal production of this protein; we do not know whether this is truly the case. Indeed, high levels of MGP mRNA have been reported in the kidney where it was found in discrete tissue-specific cell types (*6*, *10*, *25*, *26*). Using an MGP antibody, we stained apparently healthy kidney tissue obtained from our department of pathology. The control was stained with an irrelevant antibody and gave a negative result. MGP is abundantly present in the epithelial cells of the distal tubules (Fig. 4). This localization suggests local production of MGP because there is virtually no protein reabsorption in the distal tubules. MGP can also be seen in the small arterioles of the kidney, mainly aligned along the elastic lamina. Therefore, it is possible that part of the venous MGP is produced by the kidney. If this is truly the case, the actual extraction of MGP by the kidney would be even greater than what was measured in this study.

Because it cannot entirely be excluded that some MGP is produced in the kidney, thus influencing actual clearance as assessed by our method, we also measured OC concentrations in samples from renal arterial blood and the left renal vein (*25*, *27*). OC is comparable to MGP with respect to electrical charge and is only slightly different in size (5.8 kDa *vs*. 10 kDa). Therefore, it is assumed to have the same clearance characteristics as MGP. Since OC is exclusively produced by osteoblasts and odontoblasts, the renal clearance of this peptide is not influenced by its production (*28*). To estimate MGP clearance assuming that there is no production, we measured the clearance of OC. Similar to MGP, only fragments from OC can be measured in the urine (*29*, *30*). There was an approximately 19% difference in clearance between MGP and OC, suggesting that this percentage of venous MGP is added to the blood flowing through the kidney through the production of MGP.

Renal calcification, inflammation or oxidized low-density lipoprotein are among the factors that stimulate osteogenic regulatory genes, which may induce up-regulation of renal MGP expression (*31*). Some authors have speculated that if calcification is totally absent, most of the MGP produced by vascular smooth muscle cells in the matrix spills over into the plasma, thereby increasing its concentration in the venous effluent relative to arterial levels (*6*, *10*). It is unknown whether hypertension and associated oxidative stress cause increased vascular calcification and therefore less "spillover" of MGP to the circulation, or *vice versa*. It is also unknown whether lower MGP levels cause arterial calcification with increased arterial stiffening, eventually resulting in hypertension. The fact that the majority of hypertensive patients have no obvious vascular calcifications together with our finding that venous MGP levels are comparable between subjects with or without renal artery stenosis speaks in favour of blood pressure–related "trapping" of MGP in the vessel wall.

Clearly, our study has some limitations. Amongst others, the test used to measure MGP detects total MGP antigen. At present, it is impossible to distinguish between different conformations of MGP, or between free MGP and MGP bound to fetuin in the mineral complex. Since the latter is too large to be removed by filtration in the kidney, the amount of MGP that is actually extracted most likely represents the unbound fraction of MGP. This may, in fact, be another explanation for the small differences between arterial and renal venous MGP levels. Another limitation of our study may be the exclusion of 43 out of the 133 consecutive patients because they were unable to stop their medication prior to angiography. However, since their clinical characteristics were comparable to the patients who did participate, it is unlikely that this has caused significant bias.

In conclusion, we demonstrated that the kidney is able to extract MGP from the circulation and that this extraction is not impaired when renal function is jeopardized.

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