Expression and Response to Angiotensin-Converting Enzyme Inhibition of Matrix Metalloproteinases 2 and 9 in Renal Glomerular Damage in Young Transgenic Rats with Renin-Dependent Hypertension

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ABSTRACT

Extracellular matrix expansion in the glomerular mesangium contributes to the development of glomerulosclerosis and chronic renal disease in arterial hypertension. Transforming growth factor-β1 (TGF-β1), matrix metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs) are involved in this process. Conflicting data are reported on the effects of angiotensin II (Ang II) and the response to angiotensin-converting enzyme inhibition on MMPs and TIMPs in early stages of hypertensive glomerular damage. We therefore investigated the effects of Ang II-dependent hypertension on MMP-2, MMP-9, TIMP-1, and TIMP-2 in isolated glomeruli of 8-week-old homozygous male rats overexpressing the mouse Ren2 gene [TGR(mRen2)27]. At this age, systolic blood pressure was already significantly elevated in Ren2 compared with Sprague-Dawley (SD) rats (197/110 mm Hg versus 125/116 mm Hg, p < 0.01). Ren2 exhibited renal damage as determined by increased urinary albumin excretion, focal glomerulosclerosis, mesangial matrix expansion, and α-smooth muscle actin deposition. Quantification of mRNA levels in isolated glomeruli by real-time polymerase chain reaction showed a significant increase of TGF-β1, a 2.3- and a 2.6-fold increase of MMP-2 and TIMP-1 in Ren2 compared with SD (p < 0.01, respectively) and no strain differences for TIMP-2. In contrast, MMP-9 mRNA expression was markedly suppressed to 10% of control levels in Ren2 (p < 0.01). Early treatment with ramipril completely prevented renal damage in Ren2 and restored mRNA expression of TGF-β1, MMP-2, and TIMP-1 to SD control levels. Interestingly, down-regulation of MMP-9 mRNA, protein, and activity was not affected by ramipril, indicating that the protective effect of this compound is not attributable to restoration of MMP-9 in the glomerulus.

Hypertension is a worldwide public health challenge, with increasing prevalence within the next 2 decades (Kearney et al., 2005). Together with diabetes, hypertension is the most important cause of end-stage renal failure (Atkins, 2005). Moreover, arterial hypertension is also one of the main factors contributing to progression of chronic renal disease toward end-stage failure (Atkins, 2005). The accumulation of extracellular matrix (ECM) in the glomerular mesangium in the hypertensive state represents an important response to injury, which contributes to the development of chronic renal disease (Kriz and LeHir, 2005). The increase in ECM deposition is due to disturbances in the delicate balance between synthesis and degradation of ECM components (Lenz et al., 2000).

Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are the major regulators of ECM synthesis and degradation (Nagase and Woessner, 1999). MMP-2 and MMP-9 are important members of the metalloproteinase family. This work was supported by Grant 01GS0416 from the Bundesministerium für Bildung und Forschung [Nationales Genomforschungsnetz (NGFN) 2], KGCV1 to R.K.

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ABBREVIATIONS: ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of matrix metalloproteinase; Ang II, angiotensin II; RAS, renin-angiotensin-system; Ren2, TGR(mRen2)27; ACE, angiotensin-converting enzyme; MC, mesangial cell; TGF-β, transforming growth factor-β; SD, Sprague-Dawley; SBP, systolic blood pressure; UPE, urinary protein excretion; UAE, urinary albumin excretion; MME, mesangial matrix expansion; FGS, focal glomerular sclerosis; PBS, phosphate-buffered saline; α-SMA, α-smooth muscle actin; PCR, polymerase chain reaction; TBS, Tris-buffered saline.
family because of their capacity to degrade a wide variety of ECM components (Nagase and Woessner, 1999; Sternlicht and Werb, 2001). In contrast to membrane-bound membrane-type MMPs, MMPs such as MMP-2 and MMP-9 are secreted in an inactive form and are subsequently activated extracellularly by other already activated MMPs or serine proteinases (Sternlicht and Werb, 2001; Snoek-van Beurden and Von den Hoff, 2005). In the kidney, MMP-2 and -9 are expressed in the glomerulus, and several reports indicate their importance in controlling ECM turnover in disease states (Lenz et al., 2000; Leloung et al., 2001; Donnelly et al., 2003). Regulation of MMP activity is controlled at three levels: transcription, activation of the proenzyme, and inhibition by TIMPs. Four TIMPs, i.e., TIMP1–4, have been described in vertebrates so far, among which TIMP-1 and -2 are expressed in glomeruli (Donnelly et al., 2003). Changes of TIMP levels are considered to be important because they directly affect the levels of MMP activity since they have the ability to form tight 1:1 complexes with the active MMP enzymes (Sternlicht and Werb, 2001).

A key role for angiostatin II (Ang II) as the crucial mediator of the renin-angiotensin system (RAS) has been firmly established (Bader et al., 2001), and with regard to glomerular damage, Ang II is known to induce glomerular growth and sclerosis (Mezzano et al., 2001). In double-transgenic rats harboring the human renin and angiotensinogen gene, respectively, elevated renal Ang II levels are associated with albuminuria and strikingly elevated glomerular collagen, and fibronectin expression reflects the pronounced renal damage in this model (Muller et al., 2002). In addition, we have shown the effects of elevated Ang II levels on the induction of renal fibrosis in the Ren2 model (Rothermund et al., 2003). Several reports indicate that blockade of the RAS is a valuable target to prevent progression of kidney disease (Brewster and Perazella, 2004). Furthermore, treatment with the AT1 receptor antagonist losartan was able to reverse the observed increases in collagen I and IV gene and protein expression (Boffa et al., 2003), and Adamczak et al. showed a reversal of glomerulosclerosis, tubulointerstitial, and vascular indices in subtotally nephrectomized rats by treatment with the ACE inhibitor enalapril (Adamczak et al., 2003).

In cultured rat mesangial cells (MC), Ang II was shown to induce TGF-β mRNA expression and activity, thereby leading to MC hypertrophy and increased synthesis of fibronectin and collagen type I (Kagami et al., 1994). It is generally accepted that the Ang II–induced increases in the expression and synthesis of ECM proteins are mainly mediated by TGF-β (Kim and Iwao, 2000; Mezzano et al., 2001). Furthermore, the ACE inhibitor enalapril as well as the Ang II type 1 receptor blocker losartan reduced TGF-β overproduction in a rat model of Thy 1.1 glomerulonephritis and decreased glomerular matrix accumulation (Peters et al., 1998). Regarding a possible modulation of ECM-degrading enzymes by TGF-β, Mozes et al. (1999) observed an increased expression of MMP-2 and TIMP-1 in mice transgenic for TGF-β1.

The present study was therefore conducted to investigate the effects of Ang II–dependent hypertension on MMP-2 and MMP-9 and their endogenous regulators TIMP-1 and TIMP-2 in the course of early hypertensive glomerular damage. To this end, we investigated young homozygous transgenic rats with overexpression of the murine Ren2 gene, i.e., TGR(mRen2)27 rats, in comparison with nontransgenic Sprague-Dawley (SD) control rats (Rothermund et al., 2003). To elucidate the effect of elevated Ang II on glomerular expression of its known mediator TGF-β in this experimental model, TGF-β1 mRNA expression was determined. In addition, we analyzed the effect of early treatment with ACE inhibition on these molecules involved in ECM modulation.

Materials and Methods

Animals. Homozygous male TGR(mRen2)27 (Ren2) rats were obtained from our colony at the Campus Benjamin Franklin in Berlin, Germany, and male SD rats were purchased from Charles River Laboratories (Sulzfeld, Germany) (n = 7–10, respectively). Animals were studied in compliance with institutional regulations. Rats were grouped under conditions of regular 12-h diurnal cycles using an automated light switching device and climate-controlled conditions at a room temperature of 22°C. The rats were fed a normal pelleted diet containing 0.2% NaCl and had free access to food and water. Ren2 rats were randomly assigned to treatment groups at birth. After weaning at the age of 4 weeks, homozygous Ren2 rats were treated until the end of the observation period at 8 weeks of age with the ACE inhibitor ramipril (1 mg/kg/day) in drinking water (Ren2-ramipril) (Aventis Pharma Deutschland GmbH, Frankfurt am Main, Germany) (Linz et al., 1999).

Blood Pressure Measurement. Systolic blood pressure (SBP) was measured in male Ren2, Ren2-ramipril, and SD at the age of 8 weeks under slight ether anesthesia by a noninvasive tail-cuff method using a computer-assisted oscillatory detection device (TSE, Bad Homburg, Germany) (Rothermund et al., 2003).

Urine Analysis. Animals were placed in metabolic cages for 24 h, and urine samples were collected for determination of urinary protein (UPE) and albumin excretion (UAE) (Rothermund et al., 2003).

Organ Preparation. At the age of 8 weeks, rats were sacrificed. The kidneys were rapidly excised, rinsed, and dried. A cross section of the left kidney was fixed in methacarn solution (60% methanol, 30% chloroform, and 10% acetic acid) for 24 h, which was then changed to 80% ethanol until further dehydration and embedding in paraffin. The rest of the kidney was separated into cortex and medulla and immediately frozen in liquid nitrogen and stored at −80°C until further analysis. The other kidney was used for isolation of glomeruli by differential sieving as described (Ketteler et al., 1996). Pellets of isolated glomeruli were immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Morphological Investigation of the Kidney. Midecoronal sections of kidneys, routinely stained with periodic acid-Schiff, were scored for glomerular mesangial matrix expansion (MME) and focal glomerular sclerosis (FGS). MME was scored positive if the broadening of the mesangial areas was 2 to 3 times of that seen in unaffected glomeruli. FGS was scored positive when collapse of capillaries, mesangial matrix expansion, and adhesion formation were simultaneously present. Glomeruli were scored as follows: unaffected glomeruli were scored as 0; if one quadrant was affected with MME or FGS, a score of 1 was adjusted; two quadrants affected was scored as 2; three quadrants as 3; and if all quadrants were positive for MME and FGS, a score of 4 was given. The ultimate score was then obtained by multiplying the degree of change by the percentage of glomeruli with the same degree of injury and addition of these scores. A total number of 40 to 50 glomeruli per animal were scored moving from cortex to medulla and vice versa. The means for MME and FGS of all scored glomeruli per animal were calculated.

Immunohistochemical Staining Procedures. For immunohistochemical staining procedures, paraffin sections (4 μm) were dewaxed and rehydrated, and endogenous peroxidase was blocked with 0.3% H2O2 in phosphate-buffered saline (PBS; pH 7.4) for 30 min. α-SMA (smooth muscle actin; α-SMA) was detected using a murine monoclonal antibody (clone 1A4; Sigma-Aldrich, St. Louis, MO) for 60 min at room temperature. Binding was detected using sequential incu-
bations with peroxidase-labeled rabbit anti-mouse antibody (Dakopatts; DAKO, Glostrup, Denmark) for 30 min. Antibody dilutions were made in PBS supplemented with 1% bovine serum albumin, and 1% normal rat serum was added to the secondary antibodies. Peroxidase activity was developed by using 3,3'-diaminobenzidine tetrachloride containing 0.03% H2O2 for 10 min. Counterstaining was performed using Mayer’s hematoxylin. The expression of glomerular α-SMA was measured using computerized morphometry (average of 50 glomeruli per kidney). The image of a given glomerulus present on the screen was traced with a cursor along Bowman’s capsule. The renal sections were evaluated moving from cortex to medulla and vice versa at a magnification of 200×. The total staining was divided by the total area surface and expressed as a percentage.

**Expression Analysis.** Total RNA of isolated glomeruli was obtained by using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturers protocol. An on-column DNase digestion step was included in the protocol with RNase-Free DNase Set (QIAGEN GmbH) to minimize genomic contamination. One microgram of total glomerular RNA was reverse transcribed in a total volume of 20 μl using the First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer’s recommendations. To quantify expression levels of genes of interest, we used the real-time quantitative reverse transcription (“TaqMan”)-PCR. Appropriate primers and fluorogenic probes were designed using the Primer Express software. The ABI PRISM 7000 SDS instrument, in conjunction with the ABI TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, US), was used to perform the assays. The reaction volume was 25 μl, with a final concentration of 300 nM for the primers and 100 nM for the probes, except for detection of MMP-2 where final concentration was 800 nM for the primers and 300 nM for the probes. PCR conditions were used as recommended by the manufacturer. Fluorogenic probes were synthesized by TIB Molbiol (Berlin, Germany), except for the collagen type III probe (Applied Biosystems, Darmstadt, Germany) primers were obtained from Prologol (Paris, France). For details of primer and probe sequences see Table 1. Specificity of the products was confirmed by sequencing. Relative quantitation was done using the standard curve method. For each gene, a PCR fragment containing the sequence of the TaqMan system was generated. Seven serial 1:10 dilutions of this fragment served as a standard curve that was assayed together with the corresponding unknown samples on each plate. Every sample was measured in triplicate. To normalize our expression data, we used porphobilinogen-deaminase as a housekeeping gene as previously described (Fink et al., 1999).

**Protein Isolation.** Total protein of frozen glomeruli was isolated after tissue homogenization in ice-cold 1× PBS containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS), Complete Mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) using an Ultra-Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany). After subsequent centrifugation (14,000 rpm, 30 min, 4°C), the supernatant was recovered and protein concentration was measured using the DC Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany).

**Western Blot.** Forty micrograms of glomerular protein were diluted in 1× Laemmli buffer containing β-mercaptoethanol, boiled (95°C, 5 min.), subjected to SDS-polyacrylamide gel electrophoresis in 25 mM Tris base, 192 mM glycin, and 0.1% SDS, and equilibrated in transfer buffer. Proteins were transferred to nitrocellulose membranes by semidyby blotting (5 mA/cm²). After blocking with 5% nonfat dry milk in 1× Tris-buffered saline (TBS), blots were incubated with primary antibody (MMP-9, 1:500; Chemicon International, Temecula, CA; TIMP-1, 1:200; Oncogene Research Products, San Diego, CA) in 1× TBS, 0.01% Tween, and 5% nonfat dry milk at 4°C overnight, followed by a washing in 1× TBS and 0.01% Tween and incubation at room temperature using 1:10,000-diluted horseradish peroxidase-conjugated secondary antibody for MMP-9 detection (goat anti-rabbit; Calbiochem, Darmstadt, Germany) and 1:5000 for TIMP-1 detection (goat anti-mouse; Calbiochem) for 1 h in 3% nonfat dry milk. Chemiluminescence was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and exposure to Hyperfilm (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

**Gelatin Zymography.** Determination of proteolytic activity in glomeruli was performed with zymography according to Woessner (1992). Briefly, 7.5% running gels containing 1 mg/ml gelatin were overlaid with a 5% stacking gel. Samples were loaded with equal amounts of Tris-glycine SDS sample buffer (Novex; Invitrogen GmbH, Karlsruhe, Germany), and electrophoresis was performed at 200-V constant voltage. Gels were removed from glass plates and soaked for 2×15 min in 2.5% Triton X-100 on a rocker, followed by 30-min incubation in 1× developing buffer + 0.02% NaN3 (Zymogram Developing buffer 10×, Novex; Invitrogen GmbH). After exchange of developing buffer, gels were incubated for 16 to 18 h at 37°C. After a brief rinse in distilled water, gels were stained in a stain-working solution prepared by mixing 5 ml 0.2% Brilliant Blue G (Sigma-Aldrich) and 100 ml destain (1:3:6, glacial acetic acid/methanol/distilled water) on a rocker at room temperature. After completion of staining for 2 to 3 h, gels were briefly rinsed in distilled water. A mixture of MMP-9 and MMP-2 (Chemicon International) served as a positive control and molecular standard. Gels were scanned using an EPSON perfection 1240U scanner (EPSON, Meckenbeuren, Germany) and Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA) in gray-scale mode at 600 dpi. Images were analyzed using the NIH Image J software (Bethesda, MD).

**Statistical Evaluation.** All data are expressed as means ± S.D. unless stated otherwise. Statistical analysis was performed by one-way analysis of variance followed by Bonferroni’s adjustment and by
nonparametric Mann-Whitney U test. Differences were considered significant at the level of \( p < 0.05 \).

**Results**

**Characteristics of Animals.** Body weight was similar in SD (231 ± 5 g) and Ren2 rats after ramipril treatment (230 ± 19 g) and significantly higher compared with Ren2 animals (173 ± 45 g, \( p < 0.05 \), respectively). Relative heart weight was markedly increased in Ren2 compared with SD (6.9 ± 0.9 mg/g versus 3.6 ± 0.3 mg/g, \( p < 0.001 \)), whereas ramipril normalized relative heart weight in Ren2-ramipril (3.1 ± 0.2 mg/g, \( p < 0.001 \) compared with Ren2).

The data for SBP are shown in Fig. 1A. SBP was significantly increased in Ren2 compared with SD rats (\( p < 0.01 \)) and normalized after treatment with ramipril (\( p < 0.01 \) versus Ren2). Data for UPE and UAE are presented in Fig. 1, B and C, respectively. UPE was 4.3-fold and UAE was 67-fold increased in Ren2 compared with SD rats (\( p < 0.01 \), respectively). The increased UPE and UAE clearly decreased by ramipril treatment in Ren2-ramipril (\( p < 0.01 \), respectively).

In Ren2-ramipril animals, UAE levels were even significantly lower than those observed in normal SD animals (\( p < 0.05 \), Fig. 1C).

**Characterization of Glomerular Damage.** Data for FGS and MME are shown in Fig. 2, A and B. The FGS and MME scores were 6.5- and 5.8-fold elevated in Ren2 rats compared with SD rats (\( p < 0.01 \), respectively). Early treatment with ramipril fully prevented the development of glomerulosclerosis in young Ren2 rats. Representative histology photographs of glomeruli from all groups are presented in Fig. 3, A, B, and C, respectively. Glomerular morphology was normal in SD (Fig. 3A) and ramipril-treated Ren2 rats (Fig. 3C), whereas untreated Ren2 rats (Fig. 3B) showed abundant vascular hypertensive lesions and glomerular fibrosis. A significant increase of both glomerular \( \alpha \)-SMA protein and mRNA levels was observed in Ren2 compared with SD (Fig. 4, A and B, \( p < 0.01 \), respectively). Treatment with ramipril completely prevented this increase. Representative photographs of \( \alpha \)-SMA staining are presented in Fig. 3, D, E, and F, respectively. In SD rats (Fig. 3D) and ramipril-treated Ren2 rats (Fig. 3F), only arterial \( \alpha \)-SMA staining was evi-

![Fig. 1. A, SBP; **, \( p < 0.01 \) versus SD and Ren2-ramipril. B, UPE; **, \( p < 0.01 \) versus SD and Ren2-ramipril. C, UAE; **, \( p < 0.01 \) versus SD and Ren2-ramipril; *, \( p < 0.05 \) versus SD.](image)

![Fig. 2. A, FGS; **, \( p < 0.01 \) versus SD and Ren2-ramipril. B, MME; **, \( p < 0.01 \) versus SD and Ren2-ramipril.](image)
Fig. 4. A, histological evaluation of α-SMA content in 50 glomeruli per animal; **, *p < 0.01 versus SD and Ren2-ramipril. B, α-SMA mRNA analysis in isolated glomeruli; **, *p < 0.01 versus SD and Ren2-ramipril. C, collagen III mRNA analysis in isolated glomeruli; **, *p < 0.01 versus SD and Ren2-ramipril. D, TGF-β1 mRNA analysis in isolated glomeruli; **, *p < 0.01 versus SD and Ren2-ramipril; *, *p < 0.05 versus SD.
dent, whereas in untreated Ren2 rats (Fig. 3E), there was a massive induction of α-SMA in glomerular and vascular structures. A similar pattern was found for collagen III mRNA (Fig. 4C) and transforming growth factor-β1 (TGF-β1) mRNA expression (Fig. 4D), respectively. Isolated glomeruli of Ren2 showed a 40% increase of TGF-β1 expression compared with SD rats (p < 0.01), whereas TGF-β1 expression was even significantly lower than in SD animals after ramipril treatment (p < 0.05).

Expression of Glomerular MMP-2 and TIMP-2 mRNA. Isolated glomeruli of Ren2 showed a 2.2-fold increase of MMP-2 mRNA expression compared with SD rats (100 ± 22 versus 226 ± 40%, p < 0.01), whereas MMP-2 expression was similar to SD animals after ramipril treatment (65 ± 16%, p = 0.173). A significant correlation between MMP-2 mRNA levels and FGS (r = 0.72, p < 0.001) and MME was found (r = 0.68, p < 0.01). TIMP-2 mRNA expression showed no significant differences between groups (data not shown).

Expression Analysis of Glomerular MMP-9 and TIMP-1 mRNA. Data for MMP-9 mRNA expression in isolated glomeruli are shown in Fig. 5A. Glomeruli of Ren2 showed an 85% decrease of MMP-9 expression compared with SD rats (p < 0.01). In Ren2-ramipril rats, MMP-9 mRNA expression was unchanged compared with untreated Ren2 rats, and a similar 94% down-regulation compared with SD rats was observed (p < 0.01). MMP-9 expression was not correlated with glomerular damage indices. Data for TIMP-1 mRNA expression in isolated glomeruli are shown in Fig. 5C. Glomeruli of Ren2 showed a 2.6-fold increase in TIMP-1 mRNA levels compared with SD rats (p < 0.01), which was completely prevented by ramipril treatment. Western blot analysis of MMP-9 (Fig. 5B) and TIMP-1 (Fig. 5D) extended these findings on the protein level.

Glomerular Gelatinolytic Activities. Gelatin zymography revealed two bands at 92 and 72 kDa from isolated glomeruli of SD (Fig. 6A, lanes 1 and 2) due to the latent (pro-) forms of MMP-9 (92 kDa) and MMP-2 (72 kDa). Semiquantitative analysis of relative MMP-2 activity of four zymograms with a total of n = 10 animals per group revealed an increase of activity in Ren2 (Fig. 6C), whereas no bands of MMP-9 activity at 92 kDa could be detected in Ren2 (Fig. 6A, lanes 3 and 4, and B). In Ren2-ramipril rats, MMP-2 activity was comparable with that of SD. In contrast, MMP-9 activity remained undetectable (Fig. 6A, lanes 5 and 6, and B).

Discussion

The major finding of the current study is the remarkable differential regulation of MMP-2 and MMP-9 during the development of early glomerular damage in young homozygous transgenic Ren2 rats with activated RAS. That is, although MMP-2 expression and activity was significantly increased in damaged glomeruli of Ren2 rats and normalized after ramipril treatment, MMP-9 expression and activity was markedly suppressed in isolated glomeruli of Ren2 animals and unaffected by ACE inhibition. Alterations of renal MMP expression have been documented in a variety of kidney diseases (Lelongt et al., 2001). MMPs are proteases, and it is generally accepted that they contribute to matrix degradation with prevention of matrix accumulation (Nagase and Woessner, 1999; Lenz et al., 2000). However, besides their ability to degrade basement membrane and ECM components, MMPs have been shown to influence the local availability of growth factors and the behavior of glomerular cells in part by cleaving cell surface molecules and nonmatrix proteins (Lelongt et al., 2001; Sternlicht and Werb, 2001; Visse and Nagase, 2003).

Regarding our observed increase of MMP-2, the finding that MMP-2 promotes MC differentiation into the activated phenotype is of interest (Johnson et al., 1992; Turck et al., 1996). In vitro, this activation is reflected by abnormal α-SMA expression and is accompanied by high expression of collagen III, i.e., changes that we also observed in the Ren2 model. Therefore, up-regulation of MMP-2 in Ren2 might play a causative role in the development of pathological ECM accumulation and early onset of glomerular disease, which is prevented by treatment with ramipril. This view is supported by the report of Camp et al. who detected elevation of MMP-2 and MMP-9 in the renal cortex of 6-week old spontaneously hypertensive rats. The authors speculated that this increase led to a relatively greater elastin than collagen degradation,

Fig. 5. A, MMP-9 mRNA analysis in isolated glomeruli; **, p < 0.01 versus SD. B, evaluation of MMP-9 protein level in isolated glomeruli. C, TIMP-1 mRNA analysis in isolated glomeruli; **, p < 0.01 versus SD and Ren2-ramipril. D, evaluation of TIMP-1 protein level in isolated glomeruli.
resulting in glomerular stiffness and thus contributing to intraglomerular hypertension (Camp et al., 2003). A recent study by Brassard et al. (2005) underlines this view assuming that the beneficial effects of \( \text{AT}_2 \) receptors are in part due to increases of elastin deposition and are attributable to a decline in MMP-2 activity. In this context, it is important to note that MMP-2 and MMP-9 are particularly efficient in degrading collagen IV—the major structural component of the physiologic mesangial matrix and glomerular basement membrane—and elastin (Lenz et al., 2000; Donnelly et al., 2003), whereas collagen III is degraded by interstitial collagenases. Thus, assuming the up-regulation of MMP-2 as a reaction to and not a cause of fibrosis the increase would not help to counteract the increase of collagen III. We cannot exclude, however, that additional MMPs—such as MMP-1—which have been shown to be up-regulated by Ang II in other tissues or cells, might have been up-regulated in Ren2 as well to antagonize the observed glomerular damage (Kim et al., 2005).

Regarding the activity of MMP-2, it has to be kept in mind that the observed increase in TIMP-1 mRNA and protein might prevent activation of synthesized MMP-2 in the in vivo setting. The in vivo net effect of increased MMP-2 and TIMP-1 is difficult to determine. Although activity was measured in our study and was shown to be elevated, zymography is not able to detect the possible interaction of TIMP-1 as SDS separates the molecules during electrophoresis, and only the proteolytic activity of "free" MMP-2 is measured (Snoek-van Beurden and Von den Hoff, 2005).

Overactivity of the RAS at the tissue level has been shown in Ren2 rats in several organs, including the kidney, and was associated at least in part with the development of blood pressure-independent end-organ damage (Lee et al., 1996; Rothermund et al., 2003). In the current study, we had to rely on indirect blood pressure measurements by the tail-cuff method since we studied homozygous Ren2 rats at an early age of 8 weeks. At this age, the rats are already considerably hypertensive and vulnerable during surgical procedures that are performed to obtain direct measurements, e.g., during radiotelemetry. Nevertheless, the goal in the current study...
was to detect the expected substantial group differences in systolic blood pressure, which can be achieved by the indirect tail-cuff method in agreement with recent recommendations (Kurtz et al., 2005). Besides this increase in blood pressure, Ren2 rats showed signs of renal damage with increased UAE, FGS, MME, and α-SMA deposition. As a limitation of this study, we cannot distinguish between blood pressure-dependent or -independent effects of the activated RAS because treatment with ramipril effectively lowered blood pressure in Ren2. However, only recently it was shown that Ang II can directly enhance MMP-2 activity and protein expression via the p47phox containing NAD(P)H-oxidase system in murine smooth muscle cells (Luchtefeld et al., 2005). Therefore, the observed up-regulation of MMP-2 could have been directly caused by elevated glomerular Ang II levels in Ren2 rats.

As stated above, the known actions of Ang II on initializing MC proliferation and hypertrophy and the stimulation of mesangial matrix expansion are mainly mediated via TGF-β1 induction (Kim and Iwao, 2000; Mezzano et al., 2001). In the course of purumycin aminonucleoside nephropathy in rats, the observed increase of MMP-2 was prevented by treatment with TGF-β1 antibody (Ma et al., 2004). It is also known that TGF-β1 induces MMP-2 gene expression (Overall et al., 1991) while it exerts an inhibitory effect on the expression of MMP-9 (Ogawa et al., 2004). This is in keeping with our finding of up-regulated TGF-β1 mRNA accompanying by a rise in MMP-2 mRNA in Ren2 while MMP-9 expression was diminished. In accordance with the current report, McLennan et al. reported an increase of MMP-2 and TIMP-1 mRNA, whereas MMP-9 showed a reduced expression in a rat model of streptozotocin-induced diabetes (McLennan et al., 2002). More recently, similar divergent findings concerning MMP-2 and MMP-9 mRNA expression were reported by Ma et al., who showed an up-regulation of MMP-2 and TIMP-1 in the remaining damaged kidneys 12 weeks after 5/6 nephrectomy of SD rats, which was reversed by high-dose losartan or enalapril treatment (Ma et al., 2005). In contrast, MMP-9 mRNA was significantly lower in the kidney after 5/6 nephrectomy, and this suppression was, however, not affected by either ACE inhibition with enalapril or AT1 receptor blockade by losartan although both drugs were renoprotective (Ma et al., 2005). We observed the same in our study: MMP-9 mRNA, protein expression, and activity were strikingly lowered during development of glomerulosclerosis in Ren2 but not restored by ramipril treatment, although TGF-β1 mRNA levels as well as blood pressure, albuminuria, proteinuria, α-SMA content, and collagen III in treated rats were similar to nontransgenic SD control rats. Thus, the observed down-regulation of MMP-9 cannot be solely explained by a differential effect of TGF-β1 on this metalloproteinase.

Our finding on MMP-9 appears compatible with a study demonstrating that ACE inhibitors are able to directly inhibit MMP activity in vitro by chelating the zinc ion in the active center of these proteases (Sorbi et al., 1993). Thus, this would suggest an ACE inhibitor-specific suppressive effect on MMP-9 that is independent from the inhibitory effect of the compound on the RAS and kinin system but is rather related to the chemical structure. Moreover, Lods et al. could show that ACE inhibition also significantly reduced overall MMP serum activity in hypertensive patients with glomerulonephritis besides the fact that untreated patients displayed higher serum levels of MMP-2 but much lower MMP-1, -8, and -9 concentrations compared with healthy subjects (Lods et al., 2003). The finding of maintained down-regulation of MMP-9 in the kidney is probably not specific to ACE inhibitor treatment, since after both enalapril and losartan treatment MMP-9 suppression remained unchanged (Ma et al., 2005).

Therefore, MMP-9 does not seem to be a crucial modulator involved during the development of early hypertensive glomerular damage or in the protection conferred by ACE inhibition in RAS-dependent hypertension. Nevertheless, the up-regulation of MMP-2 in Ren2 might play a causative role in development of pathological ECM accumulation and early onset of glomerular disease in this model, which is prevented by treatment with ramipril.

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