Angiotensin-Converting Enzyme Inhibition but not Angiotensin II Receptor Blockade Regulates Matrix Metalloproteinase Activity in Patients with Glomerulonephritis

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Abstract. Equivalent long-term effects on the kidney are attributed to angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II type 1 receptor blockers (ARB). Nevertheless, it is unknown to which degree effects of these compounds on individual inflammatory mediators, including matrix metalloproteinases (MMP), are comparable. On the basis of structural and functional differences, it was hypothesized that ACEI and ARB differentially regulate MMP activity. In a randomized, prospective crossover trial, the effect of an ACEI (fosinopril; 20 mg/d) and of an ARB (irbesartan; 150 mg/d) on MMP activity was evaluated. Ten hypertensive patients with glomerulonephritis and normal or mildly reduced creatinine clearance were studied. MMP activity and tissue inhibitors of metalloproteinase (TIMP) levels were analyzed in serum and urine: without therapy, with ACEI, with ARB, and with both agents combined. Treatment periods continued for 6 wk separated by periods of 4 wk each without therapy. Untreated patients with glomerulonephritis displayed distinctively higher serum levels of MMP-2 but much lower MMP-1/-8/-9 concentrations compared with healthy control subjects. Immunohistology of MMP-2 and MMP-9 in kidney biopsy specimen was accordingly. However, these patients excreted higher amounts of MMP-2 and MMP-9 in urine than healthy control subjects, possibly reflecting ongoing glomerular inflammation. In patients with glomerulonephritis, ACEI significantly reduced overall MMP serum activity to 25%, whereas ARB did not show any effect. Activities of MMP-1/-2/-8/-9 were also significantly inhibited by fosinopril but not by irbesartan. Levels of TIMP-1/-2 remained unaffected. In conclusion, ACEI and ARB differentially regulate MMP activity, which may ultimately have consequences in certain types of MMP-dependent glomerulonephritis.

Glomerular inflammatory diseases represent frequent and often difficult-to-treat causes of end-stage kidney failure. Hypertension and proteinuria are important independent determinants and risk factors for progression of these diseases (1). Therefore, these two conditions are prime targets for the therapy of all types of glomerulonephritis.

Pharmacologic inhibition of the renin-angiotensin system by angiotensin-converting enzyme inhibitors (ACEI) and angiotensin II type 1 receptor blockers (ARB) has been particularly well demonstrated to treat high BP and proteinuria successfully (1). Moreover, the decline of kidney function occurring in diabetic as well as in nondiabetic glomerulopathies was greatly reduced by these agents in many investigations (2,3). Although ARB have only recently been introduced to common clinical use, several studies reported equivalent renoprotection for both classes of agents, ACEI and ARB (1). However, the issue of long-term effects with regard to the kidney is not yet fully resolved, and the identification of patient groups in which one of these agents demonstrates potential advances over the other one remains of pivotal clinical interest.

Angiotensin II plays a key role in the pathogenesis of chronic injury to the kidneys resulting in loss of function. Beyond hemodynamic effects, angiotensin II also has direct effects on glomerular cells, including induction of cytokine production. Angiotensin II supports mesangial cell proliferation and induces the expression of TGF-β, leading to extracellular matrix (ECM) accumulation (1,4,5). The degradation of the ECM occurs mainly by the action of extracellularly active neutral proteinases, the matrix metalloproteinases (MMP), in conjunction with other proteolytic enzymes (6–8). Importantly, MMP seem to play an increasingly prominent role as inflammatory mediators in the regulation of glomerular ECM proteins and of cell proliferation (6,7,9).

Agents that are given over prolonged periods to patients with inflammatory diseases need to be evaluated for their effect on MMP activity. Taking into account the diverse effects of the renin-angiotensin system on glomerular cells and on ECM regulating cytokines or growth factors, it is conceivable that ACEI and ARB influence MMP activity in patients with glomerulonephritis. ACEI, such as captopril or ramiprilat, have already been demonstrated by us and others to inhibit directly
MMP activity in vitro by chelating the zinc ion in the active center of these proteases (10–12). Moreover, the ACEI cilazapril was shown to decrease microalbuminuria and MMP-9 plasma concentrations in eight patients with non–insulin-dependent diabetes (13). It is interesting that angiotensin II treatment of C57B1/6 female mice did not affect MMP-2 and MMP-9 activity (14). Conclusive data on the effect of ARB on MMP and TIMP are still missing.

MMP expression and activity can be analyzed in tissue sections and in serum or plasma. To our knowledge, there exist no studies conclusively assessing the relationship between circulating and tissue MMP levels, especially in patients with glomerulonephritis. However, to show effects of pharmacologic agents on MMP activity per se, the use of serum seems to be appropriate and the most practicable solution, as in the case of the present study.

It is still unknown whether ACEI and ARB show identical effects on these ECM regulating proteases. However, on the basis of structural and functional differences of ACEI and ARB, we hypothesized that these agents may well regulate MMP activity in a different manner. Thus, it was the prime aim of our investigation to analyze the effect of an ACEI, fosinopril, and an ARB, irbesartan, alone or in combination, on MMP activity in serum of patients with chronic glomerulonephritis. The secondary aim of our study was to compare MMP activity in serum and urine of our untreated patient group with age- and gender-matched healthy control subjects and with a second control group consisting of subjects with noninflammatory renal failure.

Materials and Methods

Patients and Healthy Control Subjects

Ten consecutive patients (aged 49 ± 3 yr, mean ± SEM; eight men and two women) who had biopsy-proven glomerulonephritis from our renal clinic and met the entry criteria participated in the study (15). Relevant biochemical data are given in Table 1; creatinine clearance was >30 ml/min in all patients. Renal histology was membranous glomerulonephritis (n = 4), focal segmental glomerulosclerosis (n = 4), IgA nephropathy (n = 1), and membranoproliferative glomerulonephritis (n = 1). Pregnant women and patients who had diabetes or were on immunosuppressive therapy were excluded. Before enrollment, all antihypertensive medications were withdrawn for at least 2 wk, except diuretics prescribed at a constant dose to control edema.

Ten age- and gender-matched volunteers (aged 49 ± 3 yr, mean ± SEM; eight men and two women) without any health problems, including the absence of abnormalities with respect to kidney function (creatinine clearance >80 ml/min) or urinalysis, were investigated as healthy control subjects. A second group of untreated patients with documented noninflammatory kidney disease (autosomal dominant polycystic kidney disease [ADPKD]) in the absence of concomitant illness (aged 43 ± 4 yr, mean ± SEM; seven men and three women) were used as an additional control group. These patients had a similar degree of renal failure (creatinine, 147 ± 29 μmol/L; creatinine clearance, 72 ± 10 ml/min, mean ± SD; >30 ml/min in all cases) as the study group.

Study Design

The clinical study was performed in a prospective, randomized, triple-crossover manner, as reported previously (15). After an initial 6-wk run-in control period without antihypertensive agents, our 10 patients with glomerulonephritis were observed during three treatment periods of 6 wk each. During these treatment periods, they received randomly once daily at 8:00 a.m. fosinopril 20 mg, irbesartan 150 mg, or fosinopril 20 mg plus irbesartan 150 mg, in a prospective, randomized, open, blinded end point (PROBE) study. Each of the three active treatment phases was separated by a washout period lasting 4 wk.

At the end of the control run-in phase and of each active treatment phase (weeks 6, 12, 22, and 32), MMP activities were analyzed separately in serum and in morning spot urine samples. Similar analyses of healthy control subjects and of control patients with ADPKD, both groups without therapy, were performed on one occasion.

Blood and Urine Samples

Samples containing 5 ml of blood were collected and centrifuged at 3000 rpm for 10 min. Thereafter, serum was stored in aliquots at −20°C before analyses. Urine spot samples (5 ml) were centrifuged in an identical manner to remove any suspended particles and stored in aliquots at −20°C. All creatinine measurements as well as analyses of serum proteins were performed in the central chemical laboratory of our institution. Urinary protein concentrations were measured using the BCA protein assay reagent kit (Pierce, Lausanne, Switzerland) (16).

Antibodies and Reagents

For Western blot analysis, mouse mAb to human MMP-9 (Ab-3) were obtained from Oncogene (Juro, Lucerne, Switzerland), and the secondary goat anti-mouse horseradish peroxidase–conjugated anti-

<table>
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<tr>
<th>Table 1. Characterization of patients with glomerulonephritis: serum and urine values at baseline without therapy and during different treatment periods (mean ± SD)a</th>
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<tr>
<td></td>
</tr>
<tr>
<td>Total protein (g/L)</td>
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<td>Albumin (g/L)</td>
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<tr>
<td>Creatinine (μmol/L)</td>
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<tr>
<td>Proteinuria (g/d)</td>
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<td>Creatinine clearance (ml/min)</td>
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a ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II type I receptor blocker.  
b P < 0.01, c P < 0.005 versus baseline (15).
bodies were obtained from SantaCruz Biotechnologies (Heidelberg, Germany).

PMSF for inhibition of serine proteinases and the zinc-chelating agent 1,10-phenanthroline were obtained from Sigma-Aldrich (Grogg Chemie, Bern, Switzerland). Purified human recombinant proMMP-2 and proMMP-9 were acquired from Oncogene (Grogg Chemie, Bern, Switzerland). For the assessment of MMP activities by continuously recording fluorescence assay, the quenched broad-spectrum fluorogenic peptide for overall MMP activity [(7-methoxycoumarin-4-yl)Acetyl-Pro-Leu-Gly-Leu-(3,2,4-dinitrophenyl)-L2,3-diaminopropionyl]-Ala-Arg-NH2 (Bachem, Bubendorf, Switzerland); examples are MMP-2 (kcat/Km = 629,000 s⁻¹M⁻¹) and MMP-7 (kcat/Km = 169,000 s⁻¹M⁻¹) or the quenched fluorogenic peptide specific for MMP-2 activity [(7-methoxycoumarin-4-yl)Acetyl-Pro-Leu-Ala-L-Nva-(3,2,4-dinitrophenyl)-L2,3-diaminopropionyl]-Ala-Arg-NH2 (Calbiochem, Lucerne, Switzerland); kcat/Km = 3.97 × 10⁵ M⁻¹s⁻¹] was used.

For in vitro studies, fosinopril and fosinoprilat (Bristol-Myers Squibb, Baar, Switzerland) as well as irbesartan (Sanofi-Synthélabo, Meyrin, Switzerland) were prepared and used according to instructions of the manufacturers. The MMP inhibitor RO111-3456 (MMP inhibitor) was included (10,17).

**Immunohistologic Analyses of MMP-2 and MMP-9**

Tissue samples from patients (n = 10) and control subjects (n = 5), namely normal renal cortex from nephrectomy specimen of patients with well-circumscribed kidney tumors, were routinely fixed in neutral buffered 4% formaldehyde solution and embedded in paraffin. For routine analysis, hematoxylin and eosin stainings were performed according to standard practice.

For immunohistochemistry, 2- to 3-μm paraffin-embedded sections were dewaxed, rehydrated, and pretreated by boiling in 100 mM Tris with 5% urea (pH 9.5) in a microwave oven. Sections were then (and after all subsequent steps) washed in Tris-buffered saline (TBS) and incubated with the primary antibody, either a mouse anti–MMP-2 antibody, clone A-Cell VC2 (working concentration, 2 μg IgG/ml; NeoMarkers, Fremont, CA), or a mouse anti–MMP-9 antibody, clone 56-2A4 (1 μg IgG/ml; Oncogene, San Diego, CA), diluted in TBS with 0.5% casein and 5% normal goat serum, for 60 min at room temperature. Sections were then incubated for 45 min with a 1:300 dilution (same buffer as primary antibody) of a biotinylated goat anti-mouse Ig antiserum (DakoCytomation, Glostrup, Denmark). Thereafter, a streptavidin-biotin-complex/alkaline phosphatase (1:200 in TBS; DakoCytomation) was applied for 60 min. Finally, sections were developed in new fuchsin-naphthol AS-BI (Sigma) for 30 min, counterstained with hematoxylin, and mounted. Histology slides were analyzed and selected by a single, blinded pathologist (A.K.).

**MMP-1, MMP-8, and MMP-9 Serum Activities**

Endogenous MMP-1, MMP-8, and MMP-9 activities were measured by the use of respective Fluorokine activity assay kits (R&D Systems, Wiesbaden-Nordenstadt, Germany), according to the instructions of the manufacturer. After subtraction of background activities, concentrations of active MMP were determined by interpolation from a standard curve. After adjustments to the total protein content, results were expressed in ng/g protein (MMP-1, MMP-8) or in ng/mg protein (MMP-9). Endogenous MMP activity refers to the naturally occurring MMP serum activity, without exogenous in vitro activation of pro-MMP forms by substances such as p-aminophenylmercuric acetate.

**Overall MMP and MMP-2 Serum Activities**

Analyses of overall MMP activity and of MMP-2 activity in serum were performed by a continuously recording fluorescence assay, as published previously (17,18). Serum samples were analyzed in the presence or absence of the serine protease inhibitor PMSF (2 mM) or the zinc chelator 1,10-phenanthroline (5 mM). The quenched broad-spectrum fluorogenic peptide assessing overall MMP activity or the quenched fluorogenic peptide specific for MMP-2 was added at the final concentration of 5 μM (17,18). Emission of fluorescence occurred by MMP-induced cleavage of the Gly-Leu bond in the former or of the Ala-Nva bond in the latter peptide. Fluorescence was quantified by subtracting the increase in emission of the blank samples consisting of reagent buffer only from the samples containing patient serum. The kinetic of fluorescence activity demonstrating linearity of cleavage rates over time was measured (excitation wavelength, 328 nm; emission wavelength, 393 nm) every 2 min for 1 h at 37°C by Spectra max Gemini XS (Paul Bucher Company, Basel, Switzerland). Results were expressed as Vmax (units/s) using linear regression for MMP activity. Thereafter, results were adjusted to the respective total protein concentration. Thus, MMP activity was expressed as Vmax/g total protein. A nonspecific interference of differences in serum protein concentrations per se with the test results has been excluded by separate experiments (data not shown).

**TIMP-1, TIMP-2, and TGF-β Serum Levels**

Serum concentrations of TIMP-1, TIMP-2, and TGF-β were measured by respective Quantikine assay kits (R&D Systems), according to the instructions of the manufacturer. The optical density resulting from substrate cleavage was read at 450 nm, with a correction at 540 nm to remove any background activity, by a spectrophotometer (Spectra Rainbow Thermoo; Tecan, Hombrechtikon, Switzerland). Serum concentrations were determined by interpolation from a standard curve. After adjustments to the respective total protein content, results were expressed in ng/mg protein (TIMP-1/2) or in pg/mg protein (TGF-β).

**Inhibition of MMP-2 and MMP-9 In Vitro**

Inhibition of MMP-2 and MMP-9 in vitro was assessed by gelatin substrate zymography, as described previously (17,19). MMP-1 and MMP-8 were not included, because gelatin does not represent their preferred substrate. SDS-PAGE of aliquots of 10 μl of serum adjusted with water to a protein content of 30 μg from untreated patients and healthy control subjects was performed on a 10-well, 10% polyacrylamide gel with a 4–15% linear gradient. Serum samples were normalized according to their protein content and subjected to electrophoresis. After electrophoresis, gelatin was subjected to a staining reaction with Coomassie blue R 250 (according to manufacturer’s instructions). Gelatinolytic activity was detected as zones of clearing in the gel and was measured using a densitometric scanner (Gel Doc 2000;uehren). Densitometric analysis was carried out using the ImageJ program (Wayne Rasband, National Institutes of Health, Bethesda, Maryland), and gelatinolytic activity was expressed as intensity of gelatinolytic activity in arbitrary units.

**Western Blot Analysis of MMP-9 in Serum**

Results of MMP-9 analyses were confirmed and extended by Western blot analysis of serum. Aliquots of 10 μl of serum, adjusted...
with water to a protein content of 10 μg, were diluted in 10 μl of 2× sample buffer (0.06 M Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.025% bromophenol blue) according to Laemml (20). The samples were heated to 95°C and centrifuged at 13,000 rpm for 1 min, followed by 10% SDS-PAGE. Subsequently, the gel was transferred to a polyvinylidene fluoride microporous membrane (Millipore, Volketswil, Switzerland) for 2 h at 250 mA. Thereafter, the membrane was blocked for 30 min at 37°C or for 2 h at room temperature by gently shaking in 5% nonfat dry milk (wt/vol) in TBS-T. Subsequently, the membrane was incubated with goat anti-mouse horseradish peroxidase conjugated secondary antibody. Finally, the membrane was incubated with MMP-9 antibodies diluted 1:1000 in TBS-T. Subsequently, the blot was washed extensively in TBS-T during 1 h (6 × 10 min) and was incubated with goat anti-mouse horseradish peroxidase–conjugated secondary antibody. Finally, the membrane was incubated with enhanced chemiluminescent reagents for 1 min (Amersham Pharmacia Biotech, Dübendorf, Switzerland), followed by autoradiography (Hyperfilm ECL; Amersham Pharmacia Biotech, Dübendorf, Switzerland).

MMP-2 and MMP-9 Urine Activities

Endogenous, naturally occurring MMP-2 and MMP-9 activities in aliquots of 100 μl of urine were measured by use of Biotrak activity assay kits (Amersham Pharmacia Biotech), according to the instructions of the manufacturer. The cleavage of the chromogenic peptide substrate resulted in a color change, read at 405 nm by a spectrophotometer (Spectra Rainbow Thermo). Concentrations of active MMP were determined after subtraction of background activities by interpolation from a standard curve and were expressed in μg/μmol creatinine.

Statistical Analyses

Differences between means were assessed by t test or ANOVA for analysis of continuous variables and by nonparametric Mann-Whitney U test for variables that were not normally distributed. All statistical tests were two sided. Statistical analysis was performed with the GraphPad Prism Software version 3.02 for Windows (Graphpad Software, San Diego, CA). Results are in mean ± SD for continuous variables. For all experiments, P values were calculated; values of P < 0.05 were regarded as significant. In vitro experiments were performed three times in duplicate.

Results

The results obtained from the investigations of MMP and TIMP were analyzed and reported separately with respect to the comparison of untreated patients with glomerulonephritis versus age- and gender-matched healthy control subjects and control patients with noninflammatory renal failure (ADPKD) and regarding the differential effects of the ACEI fosinopril and the ARB irbesartan on MMP/TIMP in patients during the different treatment phases. Finally, the direct inhibitory effect of the ACEI fosinopril was confirmed in vitro.

Untreated Patients versus Healthy Control Subjects and versus Subjects with Noninflammatory Renal Failure

Overall MMP, MMP-1, and MMP-8 Activities in Serum.

Results of overall MMP, MMP-1, and MMP-8 activities in serum are summarized in Table 2. MMP activities in groups of healthy control subjects and untreated patients were of a similar magnitude. Probably as a result of a higher variability of the activities observed in patients, the higher MMP activity in untreated patients of 25% versus control subjects did not reach statistical significance (P > 0.05). As summarized in Table 2, there were differences between patients and healthy control subjects with respect to serum MMP-1 and MMP-8 activities. Both MMP were notably decreased in patients’ serum: MMP-1 by 52% and MMP-8 by 32%, as compared with control subjects. MMP activities of subjects with ADPKD were not different as compared with healthy control subjects.

MMP-2/TIMP-2 and MMP-9/TIMP-1 in Serum.

There were distinct differences between untreated patients and healthy control subjects regarding MMP-2 and its main inhibitor TIMP-2, as depicted in Figure 1 and Table 2. Patients with glomerulonephritis displayed clearly increased serum MMP-2 activity by one third (32.5 units versus 21.3 units/s per g protein; P = 0.001). Levels of TIMP-2 demonstrated a clear tendency toward an increase but just did not differ to a significant degree (P = 0.05).

There were even more distinct differences between untreated patients and healthy control subjects regarding MMP-9 and its main inhibitor TIMP-1, as depicted in Figure 1 and Table 2. Patients with glomerulonephritis displayed an approximately sixfold lower serum MMP-9 activity (21.1 ng versus 125 ng/mg serum protein; P = 0.0003). In addition, TIMP-1 in patients was increased by almost 50% (4.2 ng versus 2.96 ng/mg protein; P = 0.007). It is interesting that in healthy control subjects as well as in patients, TIMP-1 levels were

Table 2. Serum levels of MMP/TIMP in healthy control subjects and in control subjects with noninflammatory renal failure (ADPKD) versus untreated patients with GN (mean ± SD)¢

<table>
<thead>
<tr>
<th>MMP/TIMP</th>
<th>Control subjects</th>
<th>ADPKD</th>
<th>Patients with GN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall MMP (Vmax/g protein)</td>
<td>7.0 ± 0.2</td>
<td>6.8 ± 0.5</td>
<td>8.8 ± 1.1</td>
</tr>
<tr>
<td>MMP-1 (ng/g protein)</td>
<td>4.5 ± 0.3d</td>
<td>5.7 ± 0.9d</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>MMP-8 (ng/g protein)</td>
<td>28.9 ± 2.9b</td>
<td>32.7 ± 1.7b</td>
<td>19.7 ± 1.8</td>
</tr>
<tr>
<td>TIMP-1 (ng/mg protein)</td>
<td>2.9 ± 0.2c</td>
<td>2.9 ± 0.3c</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>TIMP-2 (ng/mg protein)</td>
<td>2.0 ± 0.1</td>
<td>1.75 ± 0.08</td>
<td>2.7 ± 0.4</td>
</tr>
</tbody>
</table>

¢MMP, Matrix Metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ADPKD, autosomal dominant polycystic kidney disease; GN, glomerulonephritis. P < 0.05, c P < 0.01, d P < 0.001 versus patients with GN.
higher than the respective TIMP-2 concentrations ($P < 0.03$ for all comparisons).

The decrease in the serum level of MMP-9 in untreated patients compared with healthy control subjects was confirmed by gelatin zymography and by Western blot analysis. Figure 2A shows results of gelatin zymography of three representative patients, each with the matched healthy control subject. Densitometric analysis of all respective gelatinolytic bands revealed a decrease by one third of total MMP-9 activity in the 10 patients with glomerulonephritis compared with the 10 healthy control subjects (i.e., mean 41 versus 63 arbitrary units; $P = 0.001$). Similarly, densitometry of Western blot analysis demonstrated a relevant decrease of MMP-9 protein in patients in the order of 35% (388 versus 584 arbitrary units; $P = 0.002$), as demonstrated in Figure 2B. MMP-2/-9 activities and TIMP-1/-2 levels of patients with ADKPD were very similar to healthy control subjects, as also shown in Figure 1 and Table 2.

### Concentration of Active MMP-2 and MMP-9 in Urine

Untreated patients excreted in their urine distinctively higher levels of both MMP-2 and of MMP-9, as compared with healthy control subjects (Figure 3). The respective values for

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**Figure 1.** Matrix metalloproteinase-2 (MMP-2) and MMP-9 serum levels of healthy control subjects (□), control subjects with non-inflammatory renal failure as a result of autosomal dominant polycystic kidney disease (■), and untreated patients with glomerulonephritis (□). *$P < 0.05$ compared with both control groups.

**Figure 2.** (A) Zymography of MMP-9 in serum. Results of three representative patients (P1 to P3) with their age- and gender-matched control subjects (C1 to C3) are depicted; M, 100 ng of purified human MMP-9, used as positive control. Densitometry of gelatinolytic activity in 10 healthy control subjects (□) versus 10 untreated patients with glomerulonephritis (■) is depicted below. (B) Western blot analysis of MMP-9 in serum. Again, results of three representative patients (P1 to P3) with their matched control subjects (C1 to C3) are given; M, 100 ng of purified human MMP-9. Densitometry of bands in 10 healthy control subjects (□) versus 10 untreated patients with glomerulonephritis (■) is given below. *$P < 0.05$ compared with control group.
MMP-2 were 69.8 \textit{versus} 21 \, \mu g/\mu mol creatinine ($P = 0.007$) and for MMP-9 amounted to 227 \textit{versus} 23.9 \, \mu g/\mu mol creatinine ($P = 0.008$). Importantly, there was no clear correlation between the amount of proteinuria and the level of activity derived from either MMP-2 or MMP-9 (data not shown).

Urinary MMP levels in subjects with ADPKD may be difficult to interpret, because they have a tubular disease. In this respect, tubular MMP-2 was reduced in an animal model of polycystic kidney disease (21). Therefore, we did not investigate urinary MMP-2 levels in subjects with ADPKD. However, the urinary MMP-9 level of 28 ± 4 \, \mu g/\mu mol creatinine was almost identical to the level of healthy control subjects reported above.

Immunohistology of MMP-2 and MMP-2 in Kidney Biopsies. Immunohistologic analyses of MMP-2 and MMP-9 were performed in the kidney biopsies of all of our patients with glomerulonephritis and in normal-appearing renal cortex from tumor nephrectomy specimen. There was distinctly stronger staining for MMP-2 but a clearly weaker detection of MMP-9 in essentially all glomeruli of each patient investigated, as compared with the control tissue. Representative histologic examples are depicted in Figure 4.

Patients Who Were Treated with the ACEI Fosinopril and/or the ARB Irbesartan

Overall MMP Activity in Serum. Overall MMP activities in untreated patients and in patients who were treated with irbesartan were of the same magnitude (Figure 5). Treatment with the ACEI fosinopril reduced MMP activity by a mean value of approximately 60\% ($V_{\text{max}} = 3.2$ units/s per g total protein) in comparison with the untreated patient group ($P = 0.0003$), as measured in the assay using reagent buffer alone. Such a decline was observed in every individual patient. In comparison with ACEI alone, there was no further significant reduction of MMP activity by the combined use of ACEI with the ARB irbesartan ($V_{\text{max}} = 2.5$ units/s per g total protein; $P > 0.05$), as depicted in Figure 5.

For confirming that cleavage of fluorogenic peptide occurred by MMP, the activity of each sample was measured in presence of the serine proteinase inhibitor PMSF and the zinc-chelating agent 1,10-phenanthroline. As expected, the ad-
dition of 2 mM serine proteinase inhibitor demonstrated no significant effect within the respective groups (P > 0.05), whereas 5 mM zinc chelator lead to a complete inhibition of cleavage of fluorogenic substrate (Vmax = 0). Importantly, analyses of enzymatic activity in the presence of PMSF to further enhance assay specificity for MMP by the elimination of any intervening serine proteases (22) showed that ACEI reduced MMP activity to approximately 75% as compared with the untreated phase (Vmax = 9.2 units/s per g protein versus 2 units/s per g protein; P < 0.0001). Overall MMP activity could not be compared with MMP-2 activity because of different fluorescence substrates containing different cleavage sites.

MMP-1, MMP-2, MMP-8, and MMP-9 Serum Activities. Figure 6 depicts serum activities of interstitial collagenases (MMP-1, MMP-8) and of gelatinase B (MMP-9). Notably, MMP-9 activity was 1000-fold higher than MMP-8 and 10,000-fold higher than MMP-1 activity. Compared with the phase without any therapy, the ARB (irbesartan) showed no significant effect on MMP activity. The ACEI (fosinopril) reduced MMP-1 activity by 21% and MMP-8/-9 activity by 28% (P < 0.04), and the addition of irbesartan did not change these results (Figure 6). Figure 7 shows the results of MMP-2 activity by the continuously recording fluorescence assay (17). Again, irbesartan had no effect on MMP-2 activity, whereas fosinopril reduced MMP-2 activity by 40% (Vmax = 19.7) compared with the untreated phase (Vmax = 32.5; P = 0.04).

Serum Levels of TIMP-1 and TIMP-2. Serum concentrations of TIMP-1 and TIMP-2 remained unaffected by all drug regimens, as demonstrated in Figure 8. TIMP-1 levels remained stable at approximately 4 ng/mg total protein, and TIMP-2 levels stayed at approximately 2.85 ng/mg total protein. TIMP-1 levels were significantly higher than TIMP-2 during all four study phases (P < 0.03 for all comparisons).

Serum Levels of TGF-β. To extend the findings of ACEI and ARB on inflammatory mediators, we investigated the serum levels of TGF-β, a key mediator of chronic glomerular inflammation. As reported in Table 3, there was a clear trend toward a decrease of circulating TGF-β concentrations in treated patients. The magnitude of TGF-β inhibition achieved by ACEI and ARB and their combination was very similar, although only therapy with ACEI just reached statistical significance.

Inhibition of MMP Activity In Vitro by ACEI Fosinoprilat

Exposure of Purified MMP-2 and MMP-9 to ACEI and ARB. The direct effect of ACEI and ARB on MMP-2 and MMP-9 activities in vitro is shown in Figure 9. Fosinoprilat significantly inhibited both MMP-2 and MMP-9 activities by approximately 50% at a dose of 500 nM, and an even higher MMP inhibition occurred at 5 μM. Fosinopril showed a similar effect but only at very much higher concentrations. In accordance with the results described above, irbesartan failed to exert any effect on either of the gelatinases. The known MMP inhibitor RO111-3456 (10) was used as positive control and demonstrated inhibition of both gelatinases in the order of 80%.

Exposure of Serum from Control Subjects and Patients to ACEI and ARB. The direct inhibitory effect of ACEI on MMP activity was confirmed in the case of MMP-9 by exposure of serum to fosinoprilat in vitro. Quantitative densitometry of gelatinolytic bands reflecting MMP-9 activity is summarized in Figure 10. MMP-9 inhibition in the order of 50% was
achieved by the use of 1 μM fosinoprilat. Therefore, purified MMP-9 and MMP-9 in serum were inhibited by fosinoprilat to a similar extent. As expected, irbesartan did not influence MMP activity.

**Discussion**

The present study describes for the first time the differential effects of ACEI and ARB on MMP activity in a prospective, randomized, clinical trial analyzing patients with biopsy-proven glomerular inflammatory disease. Our findings indicate that in serum of patients with glomerulonephritis, the ACEI fosinopril significantly reduced broad-spectrum endogenous MMP activity by 75% and that the individual MMP-1, MMP-2, MMP-8, and MMP-9 were also significantly inhibited by fosinopril but not by irbesartan. The degree of inhibition of these individual MMP did not reach the extent of the decrease in overall MMP activity. As it can be expected, this may indicate that our global test of MMP activity included additional MMP not represented in our selection of individual proteases. Moreover, levels of TIMP-1 and TIMP-2 were not affected by either ACEI or ARB. Direct inhibition of MMP activity by ACEI but not by ARB was shown by straight exposure of purified MMP and of serum to fosinoprilat, the active metabolite of fosinopril and to irbesartan. In our *in vitro* assay, fosinoprilat showed a 50% inhibitory effect on MMP activity in concentrations within the clinically observable range of blood levels, but irbesartan failed to have this effect even in concentrations clearly above the therapeutic range (23,24).

Progression of chronic forms of glomerulonephritis to end-stage kidney disease is closely related to the accumulation of ECM, leading to fibrosis and sclerosis (6). Therefore, MMP are increasingly recognized to play an important role in the pathogenesis of glomerulonephritis. Thus, the effects of ACEI and ARB on MMP are of pivotal clinical interest, especially in the case of glomerulonephritis, because MMP regulate physiologic and pathologic turnover of the ECM and of cell surface proteins (6,7). These proteolytic enzymes remodel the ECM in the extracellular space mainly in conjunction with serine proteinases and with a recently characterized gene family, the metalloproteinase disintegrins (8). MMP activity is regulated and contained by natural inhibitors, mainly TIMP-1 to -4 (7). Furthermore, the complex regulation of MMP activity and expression involves many cytokines or growth factors and also the renin-angiotensin system. The renin-angiotensin system is involved in the regulation of MMP expression and activity in several ways. First, angiotensin II induces the synthesis of plasminogen activator inhibitor-1 (PAI-1) (1,25). PAI-1 itself inhibits the plasmin-mediated activation of MMP (1,26). Second, ACEI have been demonstrated by us and others to inhibit MMP activity directly (10–12). ACEI are chelators of the crucial zinc ion located in the active site of zinc metalloproteinases such as ACE and MMP (12,27,28).

Given the potentially important therapeutic role of ACEI and ARB in glomerulonephritis, their effects on MMP were the prime focus of the present investigation. In serum of patients with glomerulonephritis, the ACEI fosinopril significantly re-

**Table 3.** Serum levels of TGF-β in patients with glomerulonephritis at baseline without therapy and during different treatment periods (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>ACEI</th>
<th>ARB</th>
<th>ACEI + ARB</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β (pg/mg total protein)</td>
<td>735 ± 132</td>
<td>455 ± 54*</td>
<td>512 ± 60</td>
<td>537 ± 91</td>
</tr>
</tbody>
</table>

*P < 0.05 for ACEI versus baseline.
duced broad-spectrum endogenous MMP activity to a level of only 25%, whereas the ARB irbesartan did not show any effect. The gelatinases (MMP-2, MMP-9) and the interstitial collagenases (MMP-1, MMP-8) represent particularly important MMP (6,7). In accordance with the analyses of the overall MMP activity, the individual MMP-1, MMP-2, MMP-8, and MMP-9 were also significantly inhibited by fosinopril but not by irbesartan. A clear direct inhibition of MMP activity by ACEI but not by ARB was shown by exposure of purified MMP and of serum to these agents. Therefore, MMP inhibition most likely occurred in a direct and therefore angiotensin II–independent manner. Moreover, for these in vitro studies, we have used concentrations of fosinopril that were within the range of levels attained in human subjects (24). The constant levels of TIMP-1 and TIMP-2 that were not affected by either ACEI or ARB support the specific effect of ACEI on MMP. It is interesting that TIMP-1 levels were consistently higher than the respective TIMP-2 concentrations in treatment phases.

The magnitude of MMP inhibition in our study was similar as reported in the case of trandolapril (29). This agent reduced plasma MMP-9 activity in a subgroup of patients with diabetes in the order of 37% (29). ARB showed only a mild trend toward a decrease in MMP activity. One can only speculate that this finding might have been the result of an angiotensin II–dependent limited inhibition of MMP expression (30). From our studies, we were not able to determine to which extent MMP expression was also influenced by ACEI (31).

The effects of fosinopril and irbesartan on MMP were not related to different effects on BP, total serum protein, kidney function, and proteinuria, because both agents displayed identical effects on these four parameters, as reported previously (15). In addition, the differential effects of ACEI and ARB on inflammatory mediators may well be somehow restricted to MMP, because TGF-β was downregulated to a similar extent by both agents, in accordance with previous publications (32–34).

It is interesting that in comparison with age- and gender-matched healthy control subjects or with control subjects with comparable noninflammatory renal failure, untreated patients displayed a distinctively lower serum MMP-9 activity but a higher MMP-2 activity. The significance of the lower MMP-9 levels in our patients was even enhanced by the concomitant increase in TIMP-1. The pathogenetic relevance of these findings remains to be defined in more detail, for instance by concurrent measurements of MMP activity in the respective renal tissue. However, our immunohistologic analyses were compatible with an increase of MMP-2 and a decrease of MMP-9 within the glomeruli of our patients. Therefore, circulating MMP levels may reflect tissue activities to a certain extent. Naturally, our studies that comprise only a limited number of patients do not allow any definite conclusions on MMP expression and activity in different types of human glomerulonephritis, as it was not the prime aim of our study.

In addition to MMP-9, untreated patients with glomerulonephritis displayed distinctly decreased activities of the interstitial collagenases MMP-1 and MMP-8. One of the reasons for the lower MMP-9 activity may be an increased angiotensin II–mediated stimulation of PAI-1 synthesis (25), which leads to a decrease in the plasmin-mediated MMP-9 activation (26,35). Furthermore, differential regulation of MMP has previously been shown. In human mesangial cells, MMP-2 seems to be constitutively secreted, whereas MMP-9 is affected by cytokines such as IL-1 (36).

![Figure 9. Zymography of purified MMP-2 and MMP-9.](image)

**Figure 9.** Zymography of purified MMP-2 and MMP-9. (A) In presence of ACEI (fosinopril), ARB (irbesartan), or synthetic MMP inhibitor (RO 111-3456), as positive control, showing dose-dependent inhibition of MMP-2 and MMP-9 by ACEI and also inhibition by RO 111-3456. (B) MMP-2 and MMP-9 inhibition by fosinoprilat in much lower doses than the parent drug fosinopril.

![Figure 10. Densitometric analysis of zymograms of MMP-9 from serum of healthy controls (□) and of untreated patients (■), in the absence or presence of ACEI (fosinoprilat) or ARB (irbesartan).](image)

**Figure 10.** Densitometric analysis of zymograms of MMP-9 from serum of healthy controls (□) and of untreated patients (■), in the absence or presence of ACEI (fosinoprilat) or ARB (irbesartan). *P < 0.05 compared with no ACEI/ARB.
Differences of MMP activities between patients and healthy control subjects were not a consequence of the difference in kidney function per se, because our patients with glomerulonephritis experienced only mild renal failure and because subjects with comparable noninflammatory kidney failure as a result of ADPKD displayed similar results as the healthy control group. In this respect, the slightly higher levels MMP-1 and of MMP-9 were in line with a previous investigation of a somewhat older group of predominantly female patients with ADPKD (37). Moreover, our patients with glomerulonephritis excreted significantly higher amounts of both MMP-2 and MMP-9 in their urine than healthy control subjects, possibly reflecting ongoing glomerular inflammation. Also, urinary MMP-9 was much higher compared with MMP-2, especially taking into account the lower MMP-9 serum levels, although there was no correlation between proteinuria and MMP activity of any of the two proteases. Therefore, increased urinary excretion of MMP may not just represent augmented MMP loss accompanying increased proteinuria.

In the early stage of diabetic nephropathy in humans, an increased plasma MMP-9 activity was found to precede even the evolution of microalbuminuria in type 2 diabetes (13,38). An increased plasma level of MMP-9 was also found in type 1 diabetes in the absence of significant vascular disease (39). Beyond diabetes, there are reports about increased monocytic expression of MMP-9 in patients with IgA nephropathy and increased serum levels of MMP-3 in patients with lupus nephritis (40,41). Nevertheless, the exact role of MMP in human types of glomerulonephritis remains to be defined, and there are no established blood levels for any MMP in such patients. In this respect, our studies also helped to determine serum MMP concentrations in populations of patients and healthy control subjects.

In animals, increased MT1-MMP and MMP-2 expression in experimental mesangial proliferative glomerulonephritis has been found by us and others in the past (19,42,43). Consequently, the inflammatory features of this disease, such as increased mesangial cell proliferation and ECM deposition, were significantly attenuated by the use of an MMP inhibitor (19). Furthermore, increased expression of MT1-MMP and MMP-2 were also found in rat anti–glomerular basement membrane disease (44). In addition, MMP-9 showed an enhanced expression in Heymann nephritis, a model of membranous nephropathy (45). It is interesting that MMP-9 may also be protective, as shown for the development of fibrin-induced glomerular damage (46). In this study, susceptibility to the accelerated model of anti–glomerular basement membrane nephritis was enhanced in MMP-9(−/−) compared with MMP-9(+/-) mice. In nephrotic rats, it was suggested that metalloproteinases from different sources, such as intrinsic renal cells and infiltrating phagocytes, may play a role in the development of proteinuria by their direct action on the glomerular filtration barrier (47).

The ultimate consequences of the differential action of ACEI and ARB on MMP activity remain to be determined. MMP have a dual role: maintenance of accurate, physiologic catabolism of matrix proteins and prevention of pathogenic ECM accumulation (48). Therefore, it is highly likely that MMP inhibition over a prolonged period may interfere with the course of the disease.

Subject to the actual stage of a given glomerular inflammatory disease, too much or too little MMP activity may occur, and both of these features can be detrimental. Therefore, any recommendations on a preferential use of ACEI or ARB in glomerulonephritis would greatly depend on at least some knowledge of MMP activity in the respective tissue.

In conclusion, ACEI and ARB have a differential effect on MMP activity that may have long-term consequences in certain types of glomerulonephritis depending on accurate MMP activity for prevention of disease progression.

Acknowledgments

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