Glomerular distribution and gelatinolytic activity of matrix metalloproteinases in human glomerulonephritis

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Abstract
Background. Matrix metalloproteinases (MMPs) have been implicated in the development of glomerular injury in rat experimental glomerulonephritis (GN). However, the significance of MMPs in human GN remains obscure. In order to evaluate the role of MMPs in human GN, we examined the glomerular distribution and gelatinolytic activities of MMP-2 and MMP-9 in human GN.

Methods. We performed immunohistochemistry with polyclonal anti-MMP-2 and MMP-9 antibodies, and analysed gelatin zymograms of five isolated glomeruli from various types of human renal disease. The renal specimens investigated were from normal kidneys (n = 5), IgA nephritis (n = 20), Henoch–Schoenlein nephritis (n = 4), non-IgA mesangial proliferative GN (n = 9), lupus nephritis (n = 6), acute poststreptococcal GN (APSNG) (n = 4) and diabetic nephropathy (DN) (n = 4).

Results. MMP-2 immunoreactivity was not detected in normal controls or in any type of GN. MMP-9 staining, which was almost negative in normal glomeruli, was increased mainly in the mesangial region and corresponded to the level of glomerular cell proliferative changes in mesangial proliferative GN (IgA nephritis, Henoch–Schoenlein nephritis, non-IgA mesangial proliferative GN and lupus nephritis). Positive but weak staining for MMP-9 was observed in mesangial areas in DN. In addition, double immunostaining showed that MMP-9 is colocalized in scattered neutrophils within diseased glomeruli in APSGN. MMP-9 gelatinolytic activity in five normal glomeruli was weakly detected. Consistent with the levels of immunostaining, MMP-9 glomerular activity was dramatically increased in nephritic glomeruli with IgA nephritis, lupus nephritis and DN. The gelatinolytic activity of MMP-2 was occasionally detectable in nephritic glomeruli.

Conclusion. These results strongly suggest that MMP-9 plays an important role in abnormal mesangial proliferative changes in human GN.

Keywords: gelatin zymograms; human glomerulonephritis; immunohistochemistry; matrix metalloproteinases

Introduction

Many forms of progressive glomerular diseases are characterized by sustained cell proliferation and abnormal extracellular matrix (ECM) remodelling by mesangial cells (MC) [1,2]. Therefore, analysis of the factors involved in the regulation of MC proliferation and ECM metabolism is of great importance [2]. Many studies have explored the molecular mechanisms underlying abnormal ECM metabolism leading to the development of glomerular sclerosis [3]. Several lines of evidence indicate that an imbalance between ECM synthesis and degradation is closely associated with abnormal ECM remodelling and subsequent progression of glomerular diseases [3].

The major regulators of ECM degradation in the glomerulus are matrix metalloproteinases (MMPs) [4]. MMPs are a large family of zinc-dependent matrix-degrading enzymes, which include the interstitial collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -7 and -10), membrane-type MMPs (MMP-14, -15, -16 and -17) and others (MMP-7, -11, -12 and -19) [3]. They have been implicated in invasive cell behaviour, embryonic development and tissue fibrosis in the kidney, liver and lung [5,6]. MMPs share several structural and functional properties, including pre-/pro-peptide, hinge, haemopexin-like (except MMP-7) and catalytic zinc-binding domains [3]. They synergistically degrade a broad range of ECM compounds, such as collagens, elastin, laminin, fibronectin and proteoglycan [3].

The MMP gelatinases, MMP-2 (gelatinase A; 72 kDa type IV collagenase) and MMP-9 (gelatinase B: 92 kDa type IV collagenase), are distinct members of the MMP...
family in that they form pro-enzyme complexes with endogenous tissue inhibitors of metalloproteinases (TIMPs) [3]. These enzymes can degrade native type IV, V and VII collagen, as well as gelatin [3]. MMP-2 degrades fibronectin and laminin, whereas MMP-9 has significantly more specificity towards types IV and V collagen [3]. Recent studies have shown that cultured human MC and glomerular epithelial cells secrete both MMP-2 and MMP-9, which are regulated by PMA cytokines, IL-1β and TGF-β [7,8]. These gelatinases were found to play roles in the development of glomerular injury in rat models of experimental glomerulonephritis (GN) [9,10]. For example, increased MMP-2 expression was detected at sites of mesangiolysis and basement membrane disruption in anti-Thy1.1 nephritis [9]. In addition, a study examining passive Heymann nephritis reported a correlation anti-Thy1.1 nephritis [9]. In addition, a study examining passive Heymann nephritis reported a correlation with the appearance of protein-uria [10]. Interestingly, Steinmann-Niggli et al. [11] reported that inhibition of MMP attenuates glomerular changes such as cell proliferation and ECM accumulation in anti-Thy1.1 nephritis, suggesting that MMPs play a role not only in ECM metabolism but also in cell proliferation [11]. In human studies, elevated levels of serum MMP-2 and MMP-9 were found in various forms of GN [12,13]. However, the significance of MMP-2 and -9 in human GN remains obscure.

In order to evaluate the roles of MMPs in human GN, we examined the glomerular distribution and gelatinolytic activities of MMP-2 and -9 by using immunohistochemistry with polyclonal anti-MMP-2 and anti-MMP-9 antibodies, or gelatin zymograms, of five isolated glomeruli in various types of human glomerular diseases. We found that glomerular staining and MMP-9 activity increased in various forms of GN, including IgA nephritis, lupus nephritis and diabetic nephropathy.

Subjects and methods

Kidney specimens

Histologically normal portions of kidney tissues obtained from five patients with renal trauma were used as normal control tissues. In addition, 40 tissue specimens obtained by renal biopsy from patients with renal diseases were used in this study. The specimens included IgA nephritis (n=20), Henoch–Schönlein nephritis (n=4), non-IgA mesangial proliferative GN (n=9), lupus nephritis (n=6), acute post-streptococcal GN (APSGN) (n=4) and diabetic nephropathy (DN) (n=4). The diagnosis was based on tissue studies from light, electron and immunofluorescence microscopy according to the classification of Churg et al. [14].

Antibodies

Rabbit polyclonal antibodies to human MMP-2 and MMP-9 were purchased from Quartett GmbH (Berlin, Germany). The immunostaining reactivity and specificity of these antibodies were confirmed by immunofluorescence analysis using human gastric cancer tissues and western blotting for human recombinant MMP-2 and MMP-9 (CHEMICON International, Inc., Temecula, CA, USA), respectively. To detect the immunolocalization of MMPs in glomeruli, we performed double-staining for MMP-2 and MMP-9 glomerular cells using the following antibodies: a mouse monoclonal antibody (mAb) against α-smooth muscle actin (Sigma, St Louis, MO, USA) as a marker of activated MC, a mAb against podocalyxin (PHM5) (Australian Monoclonal Development, Australia) as a marker of glomerular epithelial cells, a mAb against CD31 (DAKO, Copenhagen, Denmark) as a marker of endothelial cells, a mAb against neutrophil elastase (DAKO) as a marker of neutrophils, and a mAb against CD68 (DAKO) as a marker of macrophages.

Immunofluorescence microscopy

Indirect immunofluorescent stainings of 3-μm cryostat sections using anti-MMP-2 and MMP-9 antibodies were performed as described previously [15]. Fluorescein isothiocyanate (FITC)-coupled donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was used as the secondary antibody. In double-staining experiments of MMPs and markers of glomerular cells (MC, epithelial cells, endothelial cells) and inflammatory cells (neutrophils and macrophages), sections incubated with either anti-MMP-2 or anti-MMP-9 antibodies were further incubated with each marker antibody and then with FITC donkey anti-mouse antibody and tetramethylrhodamine isothiocyanate-coupled donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc.). Negative controls included omission of either of the primary antibodies and produced a lack of staining.

To evaluate the glomerular expression of MMP-2 or MMP-9, the intensity of glomerular stainings with either anti-MMP-2 or anti-MMP-9 antibodies was graded as follows: negative, with very weak or absent mesangial staining (0); weak, diffuse staining with 1–25% focally increased mesangial staining (1+); mild, with 25–50% of the glomerular tuft demonstrating strong mesangial staining (2+); and strong, with ≥50% of the glomerular tuft strongly stained (3+).

Glomerular cell proliferation grading

All of the glomeruli in each section (usually 6–30) were examined by light microscopy. The number of cells in at least four equatorially cut glomeruli in each section stained with periodic acid-Schiff (PAS) was counted, and the average number of cells was used as an indicator of glomerular cell proliferation, as described previously [2].

Human mesangial cell culture

Cultured human MC, obtained from intact glomeruli from a patient with renal trauma using the graded sieving technique, were characterized as described previously [17]. MC were used between passages 4 and 5 in these experiments and were maintained in RPMI medium supplemented with 18% fetal bovine serum (Gibco BRL, Orland Island, NY, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 U/ml insulin and 25 mM HEPES buffer at 37°C in a 5% CO₂ incubator. MC grown to confluence in 75-cm² flasks (Falcon, NJ, USA) were incubated in serum-free RPMI 1640 medium for 24 h, and the conditioned media were collected, concentrated 10-fold by ultra dialition and stored at −20°C until examination.
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Culture and isolation of glomeruli from renal biopsy specimens

Isolation of human glomeruli from normal kidney tissues (n = 4) and from biopsy specimens with IgA nephritis (n = 9), lupus nephritis (n = 3) and diabetic nephropathy (DN) (n = 4) were performed as described previously [18]. Briefly, five glomeruli were dissected free from the surrounding tissues under a stereoscopic microscope (SZH; Olympus, Tokyo, Japan) using thin steel needles and sharpened forceps (N0.5; Dumont, Switzerland) at 4°C. The five isolated glomeruli were incubated in 50 μl of serum-free RPMI 1640 medium (Sigma Chemical Co.) for 24 h at 37°C, and the conditioned media were then centrifuged to remove debris and stored at −20°C until assayed for enzymatic activity. The large number of glomeruli were isolated from normal portions of kidney tissues obtained from patients with renal trauma, and were then cultured for 24 h. The culture supernatant obtained was used for characterization of gelatinolytic bands using zymography.

Zymography

Gelatinase activity in each conditioned medium was assayed using the degradation of gelatin. Degraderen tissue was obtained from heat-denatured (60°C, 30 min) rat tail tendon collagen, prepared as described previously [7]. Briefly, 20 μl of each sample was incubated with 5 μl of sample buffer (5×) (1.25 M Tris–HCl, pH 6.8, 20% SDS) for 30 min at room temperature prior to electrophoresis. The non-reduced samples were run on 10% SDS polyacrylamide gels containing gelatin at a final concentration of 1 mg/ml at 4°C. After washing with 2.5% Triton X-100 for 1 h, the gels were incubated for 24 h at 37°C in 50 mM Tris–HCl (pH 8.0), 50 mM CaCl2 and 1 mM ZnCl2. Thereafter, the gels were stained with 1% Coomassie Blue R-250, and destained by mixing. The resulting slurries were centrifuged and the corresponding to MMP-9. Briefly, the supernatants (100 μl) obtained from cultured glomeruli were incubated with either anti-MMP-9 antibodies (10 μl) or rabbit IgG overnight at 4°C. Protein A-Sepharose (50 μl) was added to the mixtures, which were then incubated for 60 min at 4°C with occasional mixing. The resulting slurries were centrifuged and the supernatants were analysed by gelatin zymography. As a positive control, 8 ng of human recombinant MMP-9 (CEMICON International Inc., Temecula, CA, USA) was immunodepleted with anti-MMP-9 antibodies as described above.

Statistical analysis

Statistical significance (P < 0.05) was evaluated using Pearson’s or Spearman’s correlation coefficients.

Results

Immunolocalization of MMP-9 and MMP-2 in normal and nephritic glomeruli

We first examined the localization of MMP-9 and MMP-2 in glomeruli from renal biopsy specimens. In control biopsies from patients without renal disease, immunofluorescence stainings for MMP-9 in glomerular cells were almost negative (Figure 1A). In renal disease, glomerular MMP-9 stainings were increased mainly in the mesangial region, and varied according to the level of mesangial proliferative changes in IgA nephritis, Henoch–Schönlein nephritis and lupus nephritis (Figure 1B–D). Positive but weak MMP-9 stainings were found in glomerular mesangial areas of some DN patients (Figure 1E). As a negative control, we added PBS and rabbit IgG to all specimens and found no staining in these sections. In contrast, immunoreactivities to MMP-2 were not detected on glomerular tufts in controls or in patients with any types of GN.

To investigate further the localization of MMP-9 in nephritic glomeruli, we double-stained biopsy sections with anti-MMP-9 antibodies and antibodies to each resident glomerular cell. In most cases, there were strong stainings for MMP-9 in MC expressing α-smooth muscle actin, but few stainings in glomerular endothelial and epithelial cells (Figure 2A–C). These observations indicated that MMP-9 was generally present in mesangial areas with proliferative GN. We also examined the association between MMP-9 staining and inflammatory cells infiltrating the glomeruli in APSGN biopsies. Although many neutrophils infiltrating the glomeruli showed strong immunoreactivity for MMP-9, CD68 positive macrophages had very weak reactivity (Figure 2D and E).

Relationship between MMP-9 expression and glomerular cell proliferation in nephritic kidneys

Since glomerular expression of MMP-9 was mostly found in α-smooth muscle actin positive cells shown to be activated MC in various diseased states [19], we examined the relationship between MMP-9 expression and glomerular cell proliferation in mesangial proliferative GN, such as IgA nephritis, Henoch–Schönlein nephritis, non-IgA mesangial proliferative GN and lupus nephritis (pure mesangial proliferative GN, WHO class II). The level of glomerular MMP-9 staining correlated significantly with the level of glomerular cell proliferation, regardless of the type of GN (P < 0.01) (Figure 3). Because MC proliferation is reflected by the level of total glomerular cell numbers in mesangial proliferative GN [2], this result suggests that enhanced glomerular expression of MMP-9 may be associated with MC proliferation.

Gelatinolytic activities of conditioned medium from isolated glomeruli by zymography analysis

Gelatin zymography was performed on human MC and human glomerular cultures for characterization of gelatinases produced by 24 h culture of isolated glomeruli. Cultured human MC have been shown to produce 72 and 92 kDa MMPs [7]. A major gelatinolytic band at 72 kDa and a small band at 92 kDa were observed in the MC culture supernatant (Figure 4, lane 1). In contrast, a major gelatinolytic band at
92 kDa and a faint band at 72 kDa were observed in the supernatant from $3 \times 10^3$ cultured glomeruli (Figure 4, lane 2). When the same samples were applied to a casein substrate gel, no lytic bands were seen (data not shown). To confirm whether the 92 kDa gelatinolytic band reflected MMP-9 activity, MMP-9 protein in glomerular culture supernatant was immuno-depleted with an anti-MMP-9 antibody, followed by gelatin zymography (Figure 5). The 92 kDa gelatinolytic band was diminished in a sample treated with anti-MMP-9 antibody (Figure 5, lane 2), but was not in a rabbit IgG sample (Figure 5, lane 3). A parallel experiment using human recombinant MMP-9 and the anti-MMP-9 antibody showed similar results (Figure 5, lanes 4–6). These results indicated that the 92 kDa gelatinolytic band was due to MMP-9 activity. Therefore, the lower 72 kDa gelatinolytic band appears to correspond to MMP-2 activity produced by human glomeruli.

Gelatin zymography of five isolated glomeruli without glomerular disease revealed an MMP-9 activity band that was faint or non-detectable (Figure 6A). In contrast, a striking increase in MMP-9 gelatinolytic activity was seen in samples from nephritic glomeruli with IgA nephritis, lupus nephritis (pure mesangial proliferative GN, WHO class II) or DN (Figure 6B–D). Renal tissues from these patients had enhanced MMP-9 gelatinolytic activity and tended to show not only strong MMP-9 staining but also increased glomerular cell counts. However, the 72 kDa gelatinolytic band, presumably corresponding to MMP-2, was almost negative, even in nephritic glomeruli. This band was not present in samples from five normal control glomeruli. Finally, gelatinolytic bands other than 92 and 72 kDa were not detected.

Discussion

Our study, using immunohistochemistry or gelatin zymography of isolated glomeruli from various types of human renal disease, indicated a possible role for glomerular MMP-9 expression in the pathogenesis of glomerulonephritis. To our knowledge, this is the first investigation using MMP-9 activities derived from diseased glomeruli to study the role of gelatinases in human GN.

Recently, many studies have revealed that two gelatinases, MMP-2 and MMP-9, are involved in the pathophysiology of GN in different rat models.

Fig. 1. Immunofluorescence staining of glomerular MMP-9 in normal control (A), IgA nephritis (B), Henoch–Scho¨nlein nephritis (C), lupus nephritis (D) and DN (E). Glomerular staining of MMP-9 is negative in the control (A). Increased staining of MMP-9 is seen mainly in glomerular mesangial areas in IgA nephritis (B), Henoch–Scho¨nlein nephritis (C) and lupus nephritis (D). Weak staining for MMP-9 is present in mesangial areas in DN. Magnification: 3200 in (A–D); 4000 in (E).
Enhanced expression of MMP-2 was observed at the site of mesangial disintegration and glomerular basement membrane destruction in acute anti-Thy 1.1 nephritis [2,11]. Induction of visceral glomerular epithelial cell injury in the passive Heymann nephritis model led to rapid increases in MMP-9 synthesis that were temporarily associated with maximal proteinuria, suggesting a possible link between glomerular epithelial cell proteolytic activities and loss of glomerular permselectivity [10]. Furthermore, treatment of anti-Thy 1.1 nephritics with a synthetic MMP inhibitor produced reductions in MMP-2, MC proliferation, ECM deposition and proteinuria, indicating a critical role for MMP-2 in the development of glomerular injury [2,11]. In a very recent study using MMP-9-deficient mice, MMP-9 protected against the development of fibrin-induced glomerular lesions in anti-glomerular basement membrane nephritis [26].

In the present study, increased MMP-9 expression was mainly found in activated MC (+α-smooth muscle actin) from various types of GN. MMP-9 was also expressed in neutrophils influxing into nephritic glomeruli of APSGN and Henoch-Schönlein nephritis, suggesting that neutrophil-derived MMP-9 may be involved in the development of acute glomerular injury. In addition, the significant correlation ($P<0.01$) between MMP-9 staining intensity and total glomerular cell counts may reflect the degree of MC proliferation in mesangial proliferative GN [2]. We additionally detected increased gelatinolytic activity of MMP-9 in the supernatants from cultured nephritic glomeruli compared with normal controls. These findings suggest that the increased glomerular
MMP-9 activity reflects GN-induced changes, including MC proliferation and inflammatory infiltration of neutrophils.

IL-1, PMA and a specific matrix protein, fibronectin, in cultured human MC have been reported to stimulate the induction and activation of MMP-9 [7,20]. Therefore, it is likely that activated MC may induce MMP-9 activation under pathological conditions, such as during influx of cytokine-producing inflammatory cells into the glomerulus during compositional mesangial changes, which together contribute to further glomerular damage [3,20].

In addition to ECM degradation, there is accumulating evidence from cell biology that MMP plays a critical role in cell proliferation, migration and invasion [21,22]. Growth factor-induced cell proliferation have often been shown to accompany increased expression of MMPs in several cell lines [23,24]. It has been demonstrated that synthetic MMP inhibitors inhibit not only ECM degradation but also cell growth in cultured cells [22,25]. Interestingly, Steinmann-Niggli et al. [2] reported that an MMP inhibitor, BB-1101, attenuated MC proliferation as well as ECM accumulation in an anti-Thy-1.1 model of GN, suggesting that MC proliferation may be suppressed by MMP inhibition. Thus, this result, together with our observation of a close relationship between glomerular MMP-9 expression and cellularity in human GN, provides further evidence that MMP-9 plays a role in MC proliferation.

Another collagenase, MMP-2, has been detected in nephritic glomeruli from a rat model of GN [27]. In addition, Turck et al. [28] reported that MMP-2 is required for the α-smooth muscle actin positive proliferative MC phenotype, suggesting that MMP-2 plays an important role in inflammatory processes. However, MMP-2 immunoreactivity was not detected in human glomeruli with or without GN. In contrast to MMP-9 activity, MMP-2 activity was not detected in culture supernatants from five normal glomeruli. In nephritic glomeruli, it was absent or weakly detected, despite prominent increases in MMP-9 activity. We speculate that glomeruli produce small amounts of MMP-2 compared with MMP-9, making it difficult to confirm the significance of MMP-2 in the development of human GN in the present study.

In conclusion, we found that glomerular MMP-9 staining intensity increased in parallel with levels of mesangial proliferative changes in IgA nephritis, Henoch-Shönlein nephritis, non-IgA mesangial proliferative GN and in lupus nephritis GN. Increased gelatinolytic glomerular MMP-9 activity was observed

![Fig. 3](image1.png)

**Fig. 3.** The relationship between the staining intensity of MMP-9 and the average number of glomerular cells in mesangial proliferative GN, such as IgA nephritis (○, n = 20), Henoch–Shönlein nephritis (●, n = 4), non-IgA nephritis (●, n = 9) and lupus nephritis (●, n = 6). The vertical axis represents the staining intensity of MMP-9 and the horizontal axis represents number of glomerular cells. The expression of MMP-9 correlates with the increase in the number of glomerular cells, which reflects mesangial cell proliferation in mesangial proliferative GN, regardless of the type of disease (P < 0.01).

![Fig. 4](image2.png)

**Fig. 4.** Gelatin zymography of concentrated culture supernatants from a human mesangial cell culture (lane 1) and from normal human glomerular culture (isolated 3 × 10^5 glomeruli) (lane 2). A broad major gelatinolytic band at 72 kDa and a small band at 92 kDa were seen in human mesangial cell culture (lane 1). The normal human glomerular culture (isolated 3 × 10^5 glomeruli) showed a major band of gelatinolytic activity at 92 kDa and a small band at 72 kDa in the culture supernatant (lane 2).

![Fig. 5](image3.png)

**Fig. 5.** Gelatin zymography after immunodepletion using an anti-MMP-9 antibody. To confirm whether the 92 kDa gelatinolytic band was due to MMP-9 activity, the supernatant from cultured glomeruli was subjected to immunodepletion experiments using either an anti-MMP-9 antibody or a normal control rabbit IgG. The 92 kDa gelatinolytic band was seen in the culture supernatant (lane 1), was not seen after immunodepletion using an anti-MMP-9 antibody (lane 2), and was still visible in a culture supernatant treated with a control rabbit IgG (lane 3). Immunodepletion of human recombinant MMP-9 protein activity (lane 4) was performed using either an anti-MMP-9 antibody (lane 5) or rabbit IgG (lane 6) as a control experiment.
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Case

A

B

C

D

Fig. 6. Representative gelatin zymography of conditioned media from five glomeruli with or without glomerular disease. The bands of MMP-9 activity were undetectable or slightly detected in control samples (A). MMP-9 activity in nephritic glomeruli with IgA nephritis (B), lupus nephritis (C) and DN (D) clearly increased compared with controls. MMP-2 activity was occasionally detectable in nephritic glomeruli (B and D).

in nephritic glomeruli with IgA nephritis, lupus nephritis and DN. These results strongly suggest that MMP-9 plays important roles in abnormal mesangial proliferative changes in human GN.

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