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Macrophage Metalloelastase as a Major Factor for Glomerular Injury in Anti-Glomerular Basement Membrane Nephritis

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Rat anti-glomerular basement membrane (GBM) nephritis is a model of crescentic glomerulonephritis induced by the administration of anti-GBM antiserum. To elucidate the mechanism of glomerular injury, we analyzed the gene expression patterns in the kidneys of anti-GBM nephritis rats using DNA arrays, and found that macrophage metalloelastase/matrix metalloproteinase (MMP)-12 was one of the highly expressed genes in the kidneys on days 3 and 7 after the injection of anti-GBM antiserum. Enhancement of MMP-12 mRNA expression was confirmed by Northern blot analysis, and in situ hybridization revealed that MMP-12 mRNA was expressed in ED-1-positive macrophages and multinuclear giant cells in the glomeruli with crescent. Moreover, these cells were positive with anti-rat rMMP-12 Ab on the section of the kidneys of anti-GBM nephritis rats on day 7. To clarify the role of MMP-12, we conducted a neutralization experiment using anti-rat rMMP-12 Ab, which had an ability to inhibit rMMP-12 activity of degrading natural substrate such as bovine elastin or human fibronectin in vitro. Anti-rat rMMP-12 Ab or control Ig was injected in each of six rats on days 0, 2, 4, and 6 after the injection of anti-GBM antiserum. Consequently, crescent formation and macrophage infiltration in the glomeruli were significantly reduced in the rats treated with anti-rat rMMP-12 Ab, and the amount of urine protein was also decreased. These results disclosed that MMP-12 played an important role in glomerular injury in a crescentic glomerulonephritis model, and inhibition of MMP-12 may lead to a new therapeutic strategy for this disease. The Journal of Immunology, 2003, 170: 3377–3385.

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2 Address correspondence and reprint requests to Dr. Yoshikatsu Kaneko, Kidney Center, Shinraku-en Hospital, Nishi-ariake-cho 1-27, Niigata 950-2087, Japan. E-mail address: kanekoy@med.niigata-u.ac.jp

3 Abbreviations used in this paper: GBM, glomerular basement membrane; Dig, digoxigenin; MMP, matrix metalloproteinase; PAM, periodic acid-methenamine; PAS, periodic acid-Schiff.

Materials and Methods

Animals

Male Wister-Kyoto rats (7 wk old) weighing 190–210 g were purchased from Charles River Japan (Yokohama, Japan), and maintained in our animal facility. Animal care was in accordance with the guidelines of Niigata University Research Projection.
University (Niigata, Japan). The rats were anesthetized by diethyl ether inhalation and euthanized by exsanguinations after removal of the kidneys. Renal tissue was fixed in 10% Formalin, embedded in paraffin, cut into 2-μm sections, and stained with periodic acid-Schiff (PAS) stain or periodic acid-methenamine (PAM) stain for histological analysis.

Abs and reagents

Rabbit anti-GBM antiserum was prepared as previously described (19). To obtain rabbit anti-rat rMMP-12 antiserum, 1 mg of histidine-tagged rat rMMP-12 catalytic domain was produced (as described below), blended with an equal volume of CFA, and injected s.c. twice in a rabbit. Rabbit preimmune serum, rabbit anti-GBM antiserum, and rabbit anti-rat rMMP-12 antiserum were decomplemented by heating at 56°C for 30 min and absorbed with an excess amount of erythrocytes from Wister-Kyoto rats. For the induction of rat anti-GBM nephritis, anti-rat GBM antiserum (200 μl, containing ~2 mg of IgG) was diluted with 500 μl of pathogen-free PBS and injected i.v. As a control for gene expression analysis by DNA arrays, the same dose of rabbit preimmune serum was diluted and administered the same way. For an experiment of neutralization against MMP-12, anti-rat rMMP-12 antiserum was purified by precipitation with 50% ammonium sulfate, dialyzed against PBS, and adjusted to a concentration of 10 mg/ml. For Western blot, inhibition of in vitro digestion by rat rMMP-12, and immunohistochemistry, anti-rat rMMP-12 antiserum was purified by immunoaffinity chromatography using HiTrap H-hydroxysuccinimide-activated HP (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). To avoid the contamination of Abs reacting to histidine tag, rGST-tagged MMP-12 catalytic domain was used as a ligand. The specificity was tested against the catalytic domains of other MMP families, such as recombinant rat MMP-7, MMP-8, MMP-9, and MMP-11 by Western blot, and no cross-reaction was observed.

RNA extraction and array hybridization

Cortices of the kidneys were homogenized immediately after removal in TRIzol reagent (Life Technologies, Grand Island, NY), and the total RNA was extracted. Poly(A) mRNA was isolated from total RNA with an Oligotex Direct mRNA Kit (Qiagen GmbH, Hilden, Germany). cRNA synthesis, array hybridization, and gene expression analysis were conducted, as described previously (20). Hybridization and following washing and staining procedures were performed with oligonucleotide arrays (rat genome U34A; Affymetrix, Santa Clara, CA) composed of 8800 rat genes and expressed sequence tags, according to the manufacturer’s instructions. Expression analysis was implemented using statistical algorithms in Microarray Suite version 5.0 (Affymetrix). Average difference was expressed by the average of differences between the intensities of perfect match probe cells and those of mismatch probe cells. Discrimination score was calculated by the target-specific intensity difference of the probe pair (perfect match probe cells minus mismatch probe cells) relative to its overall hybridization intensity (perfect match probe cells plus mismatch probe cells). Then detection p value was calculated by one-sided Wilcoxon’s signed rank test to compare each discrimination score. When the detection p value was >0.05, it was determined to be absent.

Template preparation

To amplify cDNA of rat MMP-12 coding region (857 bp) by PCR, two primers, 5’-CTCCATGAAGCAGGACGGAAAC-3’ as a forward primer, and 5’-CACCTCCACGAGAAGACC-3’ as a reverse primer, were used. The PCR product was inserted into a pCR2.1-TOPO (Invitrogen, Carlsbad, CA) cloning vector for Northern blot, or a pGEM-T (Promega, Madison, WI) cloning vector for in situ hybridization.

Northern blot analysis

Twenty micrograms of total RNA was loaded per lane and electrophoresed on a 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane. Alkaline phosphatase-labeled cDNA probe was synthesized, hybridized with the RNA, washed, and detected using AlkPhos Direct Labeling and Detection System with CDP-Star (Amersham).

In situ hybridization

In situ hybridization with rat MMP-12 mRNA was conducted as previously described (21). For digoxigenin (Dig)-labeled probe synthesis, in vitro transcription was performed using a Dig RNA labeling kit (SP6/T7; Roche, Mannheim, Germany). MMP-12 antisense cRNA probe and sense probe as a negative control were synthesized from the same template and used for hybridization performed at 52°C for 16 h. The following immunological detection procedure was performed using Dig nucleic acid detection kit (Roche).

Recombinant protein

Full length of rat MMP-12 catalytic domain cDNA (546 bp) was prepared by PCR with two primers: 5’-GGATCCCTTAGAGCAGTGCCCCAGAG-3’ as a forward primer, and 5’-CAGCCTCACCAGAAGACC-3’ as a reverse primer, were used. The PCR product was inserted into a pCR2.1-TOPO (Invitrogen, Carlsbad, CA) cloning vector for Northern blot, or a pGEM-T (Promega, Madison, WI) cloning vector for in situ hybridization.

Table I. Genes with elevated expression in the kidney of anti-GBM nephritis rats more than 6-fold compared with those in control rats on day 1

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession Number</th>
<th>AVD of Control</th>
<th>Day 1-1</th>
<th>Day 1-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig germ line κ-chain C region</td>
<td>M18529</td>
<td>254.8, A</td>
<td>6.3</td>
<td>6.4</td>
</tr>
</tbody>
</table>

* Expression levels of the two individual rats are presented as fold change of average differences compared with those of control rats. When the detection p value was >0.05, it was determined to be absent (A). The accession number is the GenBank entry. AVD, average difference.
incubated with 0.2 mg of rat rMMP-12 catalytic domain for 16 h at room temperature. AVD, average difference.

Table II. Genes with elevated expression in the kidney of anti-GBM nephritis rats more than 6-fold compared with those in control rats on day 3

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession Number</th>
<th>AVD of Control</th>
<th>Day 3–1</th>
<th>Day 3–2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage metalloelastase</td>
<td>X98517</td>
<td>508.6,A</td>
<td>15.9</td>
<td>18.1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>M98820</td>
<td>782.4,A</td>
<td>9.0</td>
<td>6.7</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-1</td>
<td>M24067</td>
<td>772.0,A</td>
<td>8.8</td>
<td>10.2</td>
</tr>
<tr>
<td>Wee 1 tyrosine kinase</td>
<td>D51838</td>
<td>221.3,A</td>
<td>8.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Immediate-early serum-responsive JE gene</td>
<td>X17053</td>
<td>1920.5</td>
<td>7.2</td>
<td>6.8</td>
</tr>
<tr>
<td>UDP glucosyltransferase</td>
<td>D38062</td>
<td>288.4,A</td>
<td>6.6</td>
<td>7.2</td>
</tr>
<tr>
<td>EST232720</td>
<td>AI236158</td>
<td>753.2</td>
<td>6.1</td>
<td>7.3</td>
</tr>
</tbody>
</table>

a Expression levels of the two individual rats are presented as fold change of average differences compared with those of control rats. When the detection p value was >0.06, it was determined to be absent (A). The accession number is the GenBank entry. AVD, average difference.

GTCA-3′ as a forward primer, and 5′-GGATCCCTACACGTTGATG GTGGACTTCC-3′ as a reverse primer. The PCR product was inserted into a pCR2.1-TOPO cloning vector, digested with BamHI, and ligated into a pQE-30 vector (Qiagen). This vector was transformed into Escherichia coli strain JM109 (Toyobo, Osaka, Japan), and the production of 6× histidine-tagged rat MMP-12 recombinant protein was induced by 1 mM of isopropyl β-D-thiogalactopyranoside in 500 ml of Lennox Broth medium at 37°C for 3 h, and then the cells were lysed by sonication. After centrifugation at 15,000 rpm for 30 min, the insoluble fraction was resuspended, and affinity purification was performed using QIAexpress Type IV Kit (Qiagen). For GST-tagged rat rMMP-12, a pGEX-6P-1 vector (Amersham) was used as an expression vector. The production of GST-tagged rat rMMP-12 catalytic domain was conducted the same way, and affinity purification was performed using GSTrap FF (Amersham).

Western blot

Five hundred nanograms of rat rMMP-12 catalytic domain was resolved by SDS-PAGE using gradient gel (5–20%) under reducing conditions. The proteins were transferred to a polyvinylidene difluoride membrane, blocked with 3% powdered milk in TBST (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.5% Tween 20), then incubated with rabbit anti-rat rMMP-12 Ab (1.5 μg/ml) for 40 min at room temperature. Peroxidase-conjugated goat anti-rabbit IgG Ab (Bio-Rad Laboratories, Richmond, CA) was used as the second Ab at a dilution of 1/2000, and the immune complex was visualized using ECL Western blotting detection reagents (Amersham). Normal rabbit IgG (Chemicon, Temecula, CA) was used as a control, instead of anti-rabbit rMMP-12 Ab.

Natural substrate digestion assay

Ten micrograms of human fibronectin (Chemicon) in 10 μl of 50 mM HEPES buffer (pH 7.5) containing 150 mM NaCl and 5 mM CaCl₂ was incubated with 0.2 μg of rat rMMP-12 catalytic domain for 16 h at room temperature. For blocking of rat rMMP-12 digestion, 2, 0.6, or 0.2 μg of anti-rat rMMP-12 Ab, or 2 μg of normal rabbit IgG as a control, in 10 μl PBS was incubated with fibronectin and rat rMMP-12 together. These substrates were resolved by SDS-PAGE using 5% gel under reducing conditions and stained with Coomassie brilliant blue. Fifty micrograms of bovine solubilized elastin (Elastin Products, Owensville, MO) in 50 μl of HEPES buffer was incubated with 4 μg of rat rMMP-12 in the same condition. For blocking, 10, 3, or 1 μg of anti-rat rMMP-12 Ab, or 10 μg of normal rabbit IgG, in 50 μl PBS was incubated together. Released peptides were quantitatively determined by the method of Keller and Mandl (22). Briefly, 40% (w/v) trichloroacetic acid solution (15 μl) was added to each tube chilled on ice after the digestion, followed by centrifugation for 10 min at 15,000 rpm. The supernatant (100 μl) was blended with 100 μl of ninhydrin reagent (Sigma-Aldrich, St. Louis, MO) and incubated for 5 min at 100°C. After 800 μl of 50% (v/v) ethanol was added, absorbance was read at 570 nm. Buffer control was used as the blank.

Table III. Genes with elevated expression in the kidney of anti-GBM nephritis rats more than 6-fold compared with those in control rats on day 7

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession Number</th>
<th>AVD of Control</th>
<th>Fold Change of AVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage metalloelastase</td>
<td>X98517</td>
<td>184.0,A</td>
<td>37.3</td>
</tr>
<tr>
<td>SM22</td>
<td>M83107</td>
<td>288.3</td>
<td>16.7</td>
</tr>
<tr>
<td>Kidney injury molecule-1</td>
<td>AP035963</td>
<td>231.3,A</td>
<td>29.2</td>
</tr>
<tr>
<td>Major acute phase α-1 protein</td>
<td>K02814</td>
<td>653.6,A</td>
<td>15.7</td>
</tr>
<tr>
<td>Fc-γ receptor</td>
<td>X73371</td>
<td>239.3</td>
<td>8.0</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-1</td>
<td>M24067</td>
<td>1037.4</td>
<td>6.0</td>
</tr>
<tr>
<td>Complement protein Clq β-chain</td>
<td>X71127</td>
<td>1071.2,A</td>
<td>12.1</td>
</tr>
<tr>
<td>Immediate-early serum responsive JE gene</td>
<td>X17053</td>
<td>1630.4</td>
<td>9.5</td>
</tr>
<tr>
<td>MPP-9</td>
<td>U24441</td>
<td>509.3,A</td>
<td>6.5</td>
</tr>
</tbody>
</table>

a Expression levels of the two individual rats are presented as fold change of average differences compared with those of control rats. When the detection p value was >0.06, it was determined to be absent (A). The accession number is the GenBank entry. AVD, average difference.

Immunohistochemistry

Serial sections of kidney tissue (4 μm) were deparaffinized and rehydrated in PBS. Slides were quenched with 0.3% hydrogen peroxide, heated with a microwave, and stained using an RTP-350 system (Ventana Medical Systems, Tucson, AZ). For detection of MMP-12, the slides were incubated with anti-rat rMMP-12 Ab (3 μg/ml) for 30 min at room temperature, and after being treated with alkaline phosphatase-conjugated goat anti-rabbit IgG Ab (DAKO, Carpentry, CA), the immune complex was detected with Fast Red (Ventana) before counterstaining with hematoxylin. Control sections were incubated with normal rabbit IgG instead of anti-rat rMMP-12 Ab. For double-label immunohistochemistry, the sections were incubated for 30 min at room temperature with mouse anti-rat ED-1 mAb (Serotec, Oxford, U.K.), followed by peroxidase-conjugated goat anti-mouse IgG Ab (DAKO). After detection of the immune complex with 3,3′-diaminobenzidine tetrahydrochloride, slides were heated with a microwave again to denature and remove the applied Abs, followed by staining with anti-rat rMMP-12 Ab. For detection of rat CD8+ cell, mouse anti-rat CD8 mAb (Cosmo Bio, Tokyo, Japan) was used.
Neutralization with anti-rat recombinant macrophage metalloelastase Ab

Twelve rats were injected i.v. with anti-GBM antiserum (200 μl) on day 0, then 6 of 12 rats were injected i.v. with 5 mg of anti-rat rMMP-12 Ab (10 mg/ml) on days 0, 2, 4, and 6. Another 6 rats were injected with the same dose of rabbit preimmune Ig on the same days as controls. On day 6–7, the rats were housed in metabolic cages and urine was collected for 24 h to measure urine protein (Pyrogallol Red method; Wako, Osaka, Japan) and urine creatinine (enzymatic method; Hitachi 7600, Tokyo, Japan). Creatinine clearance (ml/min) was calculated as urine creatinine (mg/ml) × urine volume (ml)/serum creatinine (mg/ml) × 1440 (min). On day 7, the rats were anesthetized and the kidneys were removed to assess glomerular injuries. Sera were also collected on day 7 to measure serum creatinine level and circulating anti-rabbit Ig Ab titer.

Assessment of glomerular injury

The proportion of crescent formation (defined as the presence of three or more layers of cells in Bowman’s space) was determined on 100 glomeruli/kidney, and number of macrophages or CD8+ cells infiltrating into the glomerulus (detected with anti-rat ED-1 mAb, or anti-rat CD8 mAb, respectively) was determined on 30 glomeruli/kidney (23) from each of the six rats treated, as described above.

Estimation of serum Ab levels

Rat serum anti-rabbit IgG titer was determined by ELISA. Normal rabbit IgG (500 ng) was coated in each well of 96-well plates. After being blocked with 0.5% BSA, the wells were incubated with 100 μl of 1/100-diluted test sera, washed, and incubated with 1/4000-diluted HRP-conjugated goat anti-rat IgG + IgM Ab (Southern Biotechnology Associates, Birmingham, AL). The 3,3′,5,5′-tetramethylbenzidine product reacted with peroxidase was quantified by measuring the absorbance at 450 nm.

FIGURE 2. Northern blot analysis of rat MMP-12 mRNA in the kidneys of anti-GBM nephritis rats. Total RNA (20 μg) purified from kidneys in untreated normal rat, anti-GBM nephritis rat killed on days 1, 3, and 7, or control serum-administered rat killed on days 1, 3, and 7 was hybridized with alkaline phosphatase-labeled rat MMP-12 cDNA probe.

FIGURE 3. In situ hybridization of rat MMP-12 mRNA in the kidneys of anti-GBM nephritis rats. RNA in the kidneys on paraffin sections from anti-GBM nephritis rats killed on days 7 (A–D, H, and I), 3 (E), and 1 (F), or from untreated normal rat (G) was hybridized with Dig-labeled rat MMP-12 cRNA probe, and the macrophage marker was detected with anti-rat ED-1 mAb. A, mRNA in multinuclear giant cells forming crescent in the glomerulus was hybridized with antisense cRNA probe (blue). B, Sense cRNA probe was used as a control. C, Macrophages and multinuclear giant cells were stained with anti-rat ED-1 mAb (brown) after the hybridization with antisense cRNA probe (blue). D, Serial section was stained with PAS. MMP-12 mRNA was mildly expressed in macrophages infiltrated into some glomeruli on day 3 (E), but not in the kidney on day 1 or normal control (F, G). H, MMP-12 mRNA was detected in most of the glomeruli with crescent (arrow), but not in the glomeruli, with only mild proliferative change (arrowhead). I, MMP-12 mRNA was not detected in the macrophages infiltrated within the interstitium. (Original magnifications: A–G, ×200; H and I, ×100.)
after addition of 1 N HCl. Normal rat serum and serum from rat immunized with rabbit Ig with CFA, followed by immunization with rabbit Ig with IFA 2 wk later, were used as negative and positive controls, respectively.

Statistical analysis

The results are expressed as mean ± SEM. Significances of differences on histological changes or urinary parameters between the rats treated with anti-rat rMMP-12 Ab and the control rats were determined by the Mann-Whitney U test. Difference of the amount of released peptides by in vitro digestion, or differences of rat serum anti-rabbit Ig Ab titer were tested with Student’s t test.

Results

Histological features of anti-GBM nephritis

Fig. 1 shows the development of crescentic glomerulonephritis after the injection of anti-GBM antiserum. On day 1, no remarkable change except for marginal endocapillary proliferation was seen.
On day 3, mild proliferative changes were observed in some glomeruli. On day 7, severe crescent formations with proliferation of mesangial cells were observed in 50% of the glomeruli, and the structure of the glomerular tuft was damaged mainly in glomeruli with crescent. Fibrin deposits and multinuclear giant cells were also observed in the crescent in Bowman’s space, and rupture of Bowman’s capsule and infiltration of large cells into Bowman’s space were seen in some glomeruli. No remarkable changes were found in the kidneys of the rats injected with preimmune serum as the controls and killed on days 1, 3, or 7 (data not shown).

Analysis of gene expression by DNA arrays
To investigate the pattern of gene expression with the progress of anti-GBM nephritis, we compared the gene expression profile in the kidneys of anti-GBM nephritis rats on days 1, 3, and 7 after the injection of anti-GBM antiserum using DNA arrays. Tables I, II, and III show the list of genes with increased expression at least 6 times as much as control in both of the two anti-GBM nephritis rats. On day 1, the expression level of only one gene was enhanced ≥6 times. On days 3 and 7, the expression levels of seven and nine genes were increased at least 6 times more than control, respectively.

Northern blot and in situ hybridization
Among the genes with elevated expression, we focused particularly on the function of MMP-12, because its expression was extremely elevated in the nephritis kidney on both days 3 and 7, whereas its role in glomerular injury has not been reported to date.

Northern blot analysis confirmed that MMP-12 mRNA was expressed on days 3 and 7 in the kidneys of anti-GBM nephritis rats, but not in the kidneys from untreated normal rat, anti-GBM nephritis rats on day 1, or rat injected with rabbit control serum (Fig. 2). Subsequently, we used an in situ hybridization technique with rat MMP-12 cRNA probe to detect which components of the kidney expressed MMP-12 mRNA. Fig. 3 shows that MMP-12 mRNA was highly expressed around the nuclei in the ED-1-positive macrophages infiltrated into the glomeruli with crescent formation and multinuclear giant cells. These MMP-12 mRNA-positive cells were observed in most of the glomeruli with crescent, which were ~40% of all the glomeruli, but not in the glomeruli with only mild proliferative change on day 7. MMP-12 mRNA was not detected in the ED-1-positive macrophages infiltrated into the interstitium either. In the day 3 kidney of anti-GBM nephritis rat, expression of MMP-12 mRNA was observed in the ED-1-positive macrophages in some glomeruli, but in the day 1 kidney of anti-GBM nephritis rat or normal kidney, the expression of MMP-12 mRNA was not detected.

Western blot and natural substrate digestion assay
To verify the production of MMP-12 more definitively, we prepared anti-rat rMMP-12 Ab to detect the production of MMP-12 protein in the macrophages expressing MMP-12 mRNA. Anti-rat rMMP-12 antiserum was collected from the rabbit immunized with histidine-tagged rat rMMP-12 catalytic domain, and affinity-purified anti-rat rMMP-12 polyclonal Ab was tested for its reactivity by Western blot analysis and was revealed to react with rat...
rMMP-12 (Fig. 4A). We also investigated the blocking effect of anti-rat rMMP-12 Ab. Rat rMMP-12 had an ability to digest natural substrate, such as human fibronectin or bovine elastin, in vitro incubation, and the digestion activity of rMMP-12 was inhibited by anti-rat rMMP-12 Ab in a dose-dependent manner (Fig. 4, B and C).

Immunohistochemistry

Subsequently, we performed immunohistochemical studies using anti-rat rMMP-12 Ab on the sections of the kidneys from anti-GBM nephritis rats, and confirmed the production of MMP-12 protein in the ED-1-positive macrophages and multinuclear giant cells infiltrated in the crescent in the damaged glomerulus (Fig. 5). Large granules were stained with anti-rat rMMP-12 Ab mainly in the cytoplasm of the ED-1-positive macrophages and multinuclear giant cells in the crescents, but not in the ED-1-positive cells in the interstitium. The pattern of the distribution of MMP-12-producing cells was almost the same as that for MMP-12 mRNA-expressing cells. In the kidneys from untreated normal rat or anti-GBM nephritis rats killed on day 1 or 3, MMP-12 production was not detected (data not shown). Finally, to elucidate the role of MMP-12 in the crescentic glomerulonephritis model, we conducted experiments aimed at neutralization of MMP-12 using anti-rat rMMP-12 Ab.

Neutralization by anti-rat recombinant macrophage metalloelastase Ab

Each of six rats was injected with 5 mg of anti-rat rMMP-12 Ab or control Ig on days 0, 2, 4, and 6 after the injection of anti-GBM antiserum. Fig. 6 shows the typical histological features of the kidney in the two groups. The number of glomeruli with crescent and the number of ED-1-positive cells per glomerulus were obviously reduced in the kidney of the rat treated with anti-rat rMMP-12 Ab. As demonstrated in Fig. 7A, crescent formation was reduced at ~60% level in the kidneys of the rats treated with anti-rat rMMP-12 Ab, and consequently the numbers of ED-1-positive macrophages per glomerulus were also reduced at ~50%. CD8+ cells in glomeruli were also decreased in the rats treated with anti-rMMP-12 Ab, but not significantly (p = 0.0542). Fig. 7B shows that the amount of urinary protein was significantly reduced, and renal function was also preserved on day 7 in the rats treated with anti-rat rMMP-12 Ab. Taken together, the development of glomerular injury was significantly inhibited by the injection of anti-rat rMMP-12 Ab. Sera were also collected on day 7 from anti-GBM nephritis rats treated with anti-rMMP-12 Ab, control Ig, or those without any further administration of rabbit Ig except for anti-GBM antiserum to test serum anti-rabbit Ig Ab titer, and no significant differences were observed between each group of rats (Table IV).

**Table IV. Serum anti-rabbit Ig Ab levels in the anti-GBM nephritis rats treated with anti-rMMP-12 Ab, with control Ig, or without any further administration of rabbit Ig**

<table>
<thead>
<tr>
<th>Group</th>
<th>ELISA (OD450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-rMMP-12 Ab</td>
<td>0.094 ± 0.015</td>
</tr>
<tr>
<td>Control Ig</td>
<td>0.134 ± 0.016</td>
</tr>
<tr>
<td>No further treatment</td>
<td>0.158 ± 0.002</td>
</tr>
</tbody>
</table>

*p* values were compared with those of control Ig-treated rats (antiserum). *p* values were compared with those of control rats (antiserum). **Table IV. Serum anti-rabbit Ig Ab levels in the anti-GBM nephritis rats treated with anti-rMMP-12 Ab, with control Ig, or without any further administration of rabbit Ig**

Discussion

The results in this study show that MMP-12 produced by macrophages that infiltrated into the glomeruli and formed a crescent in Bowman’s space was a major factor for the development of crescentic glomerulonephritis.

To elucidate the mechanism of glomerular injury in the crescentic glomerulonephritis model, we compared the levels of ~8800 kinds of mRNA expression in the kidneys of anti-GBM nephritis rats with those of control rats using DNA arrays, and revealed that the expression of MMP-12 mRNA was dramatically elevated 3 and 7 days after the injection of anti-GBM antiserum (Tables II and III). To date, other members of the MMP family,
MMP-2 and MMP-9, are reported to be capable of degrading the constituents of the GBM or extracellular matrix in the kidney, and hence were involved not only in the regular maintenance of the extracellular matrix, but also in any damage to these components that occurs in diseased states of the kidney (24–26). Especially, MMP-9 is reported to play important roles in several models of renal diseases, such as an anti-GBM nephritis model (23) or a membranous nephropathy model (27). However, little has been reported with respect to MMP-12.

MMP-12 is mainly produced from macrophages infiltrating into the tissues in which tissue injury or remodeling is occurring, and contributes to degenerate the extracellular matrix (14). In this crescentic glomerulonephritis model, macrophages accumulate in the glomeruli by monocyte chemoattractant protein-1 secreted from mesangial cells (28), endothelial cells (29), or infiltrating CD8-positive lymphocytes or macrophages (30) in the diseased glomeruli. After the adhesion of monocytes/macrophages to glomerular endothelial cells through LFA-1 on the monocytes/macrophages (31) and ICAM-1 on the endothelial cells, subsequent progression of crescentic glomerulonephritis is induced (32). However, the precise mechanism of glomerular injury after the accumulation of macrophages in the glomeruli has not been elucidated completely.

In this study, in situ hybridization and immunohistochemical analysis revealed that ED-1-positive macrophages and multinuclear giant cells produced MMP-12 in the damaged capillary tuft or crescent in the diseased glomeruli (Figs. 3 and 5). Given that MMP-12 mRNA was already expressed on day 3 when ED-1-positive macrophages had already infiltrated into the capillary tuft, but little glomerular injury or crescent formation was observed, we hypothesized that the role of MMP-12 was destructive, i.e., MMP-12 was critical for induction of the glomerular injury and crescent formation. The results of the experiment to neutralize MMP-12 activity with anti-rat rMMP-12 Ab verified our hypothesis (Figs. 6 and 7).

GBM is composed of laminin, fibronectin, heparan sulfate proteoglycans, entactin, collagen type IV (33), and collagen type V (34), and the basement membrane of Bowman’s capsule includes elastin (35); part of them can be degenerated by MMP-12. MMP-12 produced by accumulated macrophages may contribute to rupture of the GBM or basement membrane of Bowman’s capsule, and may lead to the infiltration of macrophages into Bowman’s space. Because production of MMP-12 protein was not detected on day 3, although MMP-12 mRNA was already expressed, anti-rat rMMP-12 Ab may have inhibited rupture of the basement membrane through neutralization of MMP-12 from macrophages accumulated in the capillary after day 3 in the neutralization experiment. As a result, the number of macrophages that accumulated in the glomerulus by accelerated inflammatory reaction induced by glomerular injury with MMP-12 may have decreased in the rats treated with anti-rat rMMP-12 Ab as well as the number of glomeruli with crescent. CD8+ lymphocytes are also crucial for development of anti-GBM nephritis through their direct killer activity or their secretion of various chemokines (30, 32, 36). CD8+ cells were also decreased in the rats treated with anti-rMMP-12 Ab, but not statistically significantly (Fig. 7A).

In this study, we used rabbit anti-rat rMMP-12 Ab for neutralization, which was from the same species as the disease initiating anti-GBM antiserum. The development of anti-rabbit Ig Ab might affect the immune response in the anti-GBM nephritis rats by a series of administrations of rabbit anti-rat rMMP-12 Ab. Therefore, we used rabbit preimmune Ig as the control, and confirmed that the circulating titers of rat anti-rabbit Ig Ab did not differ significantly between the two groups, rats treated with anti-rat rMMP-12 and those treated with control Ig. Furthermore, the titers of rat anti-rabbit Ig Ab on day 7 did not differ between the anti-GBM nephritis rats treated with anti-rMMP-12 Ab or control Ig, and those without any further administration of rabbit Ig (Table IV). However, if administration of rabbit Ig continues after day 7, the production of rat anti-rabbit Ig Ab will be accelerated and may affect the development of kidney lesion.

Our results show that neutralization of MMP-12 could prevent the crescent formation and infiltration of macrophages partly, but not completely. It is possible that the dose of anti-rat rMMP-12 Ab was not enough, and MMP-12-deficient mice, which lack production of MMP-12 genetically (14), should be used to elucidate the role of MMP-12 more clearly in further study. However, given that glomerular injury is also induced by several proteinases, such as serine proteinase, elastase, and cathepsin G, or reactive oxygen metabolites from neutrophils (37–39), reactive oxygen species from macrophages (40), or also induced by direct killer activity of CD8+ cells (32), our results on the effect of MMP-12 neutralization may be compatible with the fact that MMP-12 is one of the major factors, but not the absolute factor, of glomerular injury. In the present study, some of these other important mediators of glomerular injury were not detected by the gene expression profiling analysis. It is obvious that several molecules, although their mRNA expressions were not enhanced >6 times, also play functionally important roles in the pathogenesis of glomerular injury. The reduction of mRNA expression of some protective molecules against glomerular injury may also be causes of development of the disease. In addition, the DNA arrays used in the present study do not cover all the known rat genes. Therefore, other molecules, which were not detected in the present study, could also be vital for the development of crescentic glomerulonephritis.

It may be possible that a similar mechanism occurs in the development of human crescentic glomerulonephritis. In addition, collagen type IV and laminin, which are degenerated by human MMP-12 (13), but not by rat MMP-12 (11), are major components of GBM. Therefore, MMP-12 might contribute to degrading also collagen type IV and laminin in human crescentic glomerulonephritis. Corticosteroid or immunosuppressive drugs, or plasma exchange have been used to treat the disease, but these therapies are not sufficient to control the disease completely, and the method of treatment is still controversial (1). To date, several studies have been conducted to treat anti-GBM nephritis or other kinds of nephritis by administration of proteinase inhibitors (26, 41–43), but administration of nonspecific proteinase inhibitors could also prevent healing process through the degeneration of excess extracellular matrix, such as the fibrinolytic activity of MMP-9 (23). The administration of a specific regulator of MMP-12 could provide a new therapeutic strategy for the treatment of crescentic glomerulonephritis.

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References
