Inhibition of Matrix Metalloproteinases Attenuates Anti-Thy1.1 Nephritis

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Abstract. There is accumulating evidence that matrix metalloproteinases (MMP) play a prominent role in glomerular inflammatory diseases. The aim of the present study was to determine the anti-inflammatory effects of the synthetic MMP inhibitor BB-1101 in acute anti-Thy1.1 nephritis. Sixty-three male Wistar rats were studied: healthy rats (n = 9), treated healthy rats (n = 9), nephritic rats (n = 18), and treated nephritic rats (n = 27). BB-1101 therapy (30 mg/kg body wt per d) of nephritic animals was initiated either 2 d before (n = 18) or 2 d after (n = 9) disease induction. Renal histology was analyzed 11 d after induction of the nephritis, at the peak of MMP-2 production and total glomerular cellularity. Pretreatment of nephritic rats by BB-1101 resulted in a significant amelioration of glomerular histology, assessed by glomerular cellularity, extracellular matrix deposition, and size of glomerular cross-sections. These beneficial effects were less pronounced, but in part still significant, in animals treated by BB-1101 after induction of anti-Thy1.1 nephritis. Proteinuria, expressed as area under the curve of the protein:creatinine ratio versus time, was clearly decreased in both groups of treated nephritic rats. Healthy control rats were not affected by MMP inhibitor treatment. In summary, the present study demonstrates for the first time in vivo that mesangial cell proliferation can be effectively suppressed by MMP inhibition. Thus, MMP inhibition by synthetic compounds may represent a new approach to the therapy of mesangial cell-mediated forms of glomerulonephritis. (J Am Soc Nephrol 9: 397–407, 1998)

Many forms of glomerular inflammatory diseases, such as IgA nephritis and membranoproliferative glomerulonephritis, are characterized by excessive proliferation of mesangial cells (MC) and increased deposition of extracellular matrix (ECM) proteins with subsequent alterations in the structure of the glomerular basement membrane and the mesangial matrix (1,2). Therefore, an analysis of the factors responsible for the regulation of MC proliferation and for the metabolism of ECM proteins is of paramount importance.

The quantities of the individual components of the ECM, such as collagens, proteoglycans, and glycoproteins, reflect the balance between their synthesis and degradation. The metabolism of ECM proteins occurs by the action of endopeptidases, such as the matrix metalloproteinases (MMP). The MMP are zinc-dependent proteases that belong to the collagenase supergene family. Primarily based on substrate specificity, they are traditionally classified into three categories (3,4): interstitial collagenases (MMP-1,-8,-13), gelatinases (MMP-2,-9), and stromelysins (MMP-3,-7,-10,-11,-12). The recently described membrane-type (MT)-MMP (MMP-14) created a new category (3).

MMP play an important role in inflammatory diseases, including rheumatoid arthritis and multiple sclerosis (5,6). The irreparable degradation of articular cartilage by MMP is a hallmark of rheumatoid arthritis and osteoarthritis (7,8). Cells of the proliferating synovial tissue may express an increased amount of MMP (8). MMP are also involved in tumor invasion, embryogenesis, bone turnover, uterine involution, ovulation, and wound healing (3,4,9,10).

There is accumulating evidence that MMP may also have a prominent function in glomerular inflammatory diseases. Increased synthesis of MMP was shown to occur in certain forms of human glomerulonephritis, such as pauci-immune, rapidly progressive glomerulonephritis (11) and IgA nephritis (12). Acute anti-Thy 1.1 nephritis, a rat model of mesangial proliferative glomerulonephritis, represents the best-characterized glomerular inflammatory disease with regard to MMP expression. After induction of anti-Thy 1.1 nephritis in rats, mesangiolysis is followed by an increase in activation and proliferation of MC, associated with augmented expression of MMP-2 and accumulation of ECM proteins (13–15). Given the localization of MMP-2 at sites of mesangial disintegration and glomerular basement membrane destruction (14), the increased expression of MMP-2 may have deleterious effects on the integrity of the glomerulus. Inhibition of MMP activity could conceivably interfere with this process.

A broad spectrum of very effective, low molecular weight MMP inhibitors has been developed in recent years by the addition of a zinc-chelator to a peptidyl moiety based on the different MMP substrates (8,16). In addition to MMP inhibition, these compounds may also prevent activation of cell

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membrane-bound, latent tumor necrosis factor-α (17). In the present study, we used a synthetic MMP inhibitor in the anti-Thy1.1 nephritis to investigate for the first time the potential anti-inflammatory effects of such a compound on glomerulonephritis. We hypothesized that inhibition of MMP by BB-1101 demonstrates beneficial effects with regard to glomerular histology and proteinuria.

Materials and Methods

Animals and Antibodies
Male Wistar rats were obtained from the local animal facilities at our hospital. Approval for rat experiments was obtained from the commission for animal studies, a local government agency. Anti-Thy1.1 IgG (OX-7)-producing hybridoma cells (European Collection of Animal Cell Cultures, cell line ECACC no. 84112008) were a gift from Dr. J. Floege (Medizinische Hochschule, Hannover, Germany). Anti-Thy1.1 monoclonal antibodies were isolated and purified according to standard procedures (18–20). Anti-Thy1.1 antibodies were kept in phosphate-buffered saline (PBS) in a concentration of 1 mg/ml and stored in aliquots at −20°C.

MMP Inhibitor
The MMP inhibitor BB-1101 (2S-allyl-4'-hydroxy-3R-isobutyl-N(15-methylcarbamoyl-2-phenylethyl-succinamide; $M_r$ 389.5 g/mol) was obtained from British Biotech Pharmaceuticals (Oxford, United Kingdom). For in vivo studies, BB-1101 was suspended in PBS/0.1% Tween 80 (vol/vol) and sonicated for 5 min before injection. According to the manufacturer’s instructions, BB-1101 should be used for animal experiments at doses between 5 and 30 mg/kg body wt per d, applied intraperitoneally. In a previous study, doses of 10 to 20 mg/kg body wt per d were used successfully (21). Because we administered the compound for a relatively short time period, we opted for the highest approved dose of 30 mg/kg body wt per d. According to an ex vivo bioassay performed by British Biotech Pharmaceuticals, this dose results in blood concentrations that are maintained at multiples of the IC$_{50}$ concentrations for 24 h. The following IC$_{50}$ data for MMP inhibition by BB-1101 were provided by the manufacturer: MMP-1, 10 nM; MMP-2, 5 nM; MMP-3, 30 nM; MMP-8, 3 nM; and MMP-9, 3 nM. As communicated by British Biotech Pharmaceuticals, intraperitoneal administration of 10 mg of BB-1101/kg body wt per d results in peak concentrations of approximately 38 μg/L and trough levels of approximately 15 μg/L after 1 and 24 h, respectively.

Quantitative Zymography
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on a 10-well, 10% polyacrylamide minigel containing 0.1% gelatin (wt/vol), as described by us in detail (22). Briefly, conditioned medium from rat MC cultured for 2 d in the presence of 1% fetal calf serum (FCS) was diluted with an equal volume of double-strength, nonreducing sample buffer (0.125 M Tris-HCl, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate, and 0.02% bromphenol blue). Subsequently, 18-μl aliquots of this mixture were loaded into each of 8 wells and electrophoresed. After a 30-min exposure to renaturing buffer (Triton X-100, 2.5% vol/vol), zymograms were cut into four strips, each containing two samples. Subsequently, gel strips were incubated in developing buffer (50 mM Tris-HCl, pH 8.0, 5 mM CaCl$_2$, and 1 μM ZnCl$_2$) at 37°C for 8 h. For inhibition studies, this buffer was supplemented with 10, 100, or 1000 nM BB-1101 in 0.003% DMSO or, in the case of the control, with 0.003% DMSO only, as the solvent of the inhibitor. Zones of lysis in the zymogram were visualized by staining with Coomassie brilliant blue and quantitatively analyzed by densitometry (22). Inhibition of gelatinolytic activity by BB-1101 was expressed as percentage of the control (absence of inhibitor = 0% inhibition).

Zymograms for the analyses of the effect of BB-1101 on gelatinolytic activity of isolated rat glomeruli were performed essentially as described above. Glomeruli from kidneys of nephritic rats obtained at days 0, +2, +4, +8, +11, and +14 after induction of anti-Thy1.1 nephritis were isolated according to standard procedures, using the sieving technique (11). Aliquots of 20 glomeruli from individual kidneys were homogenized in 0.1 M Tris-HCl, pH 7.5/0.1% Tween 80 (vol/vol) and electrophoresed in a sample volume of 18 μl (23). Gelatinolytic activity was expressed in arbitrary units (1 U of gelatinolytic activity corresponds to the optical density of 0.131, derived from glomeruli of day 0). For inhibition studies, the gels were exposed to 100 nM BB-1101.

Experimental Design
Five groups of male Wistar rats (150 g body wt at day 0) were studied according to the diagram outlined in Figure 1. Group A: healthy rats (n = 9); group B: treated healthy rats (n = 9); group C: nephritic rats (n = 18); group D: pretreated nephritic rats (n = 18); and group E: posttreated nephritic rats (n = 9). Each of the 63 animals was kept in a metabolic cage with free access to food and water. MMP inhibitor BB-1101 (groups B and D) or the respective amount of solvent (groups A and C) was given intraperitoneally once daily from day −2 to +11. Anti-Thy1.1 nephritis was induced at day 0 (groups C, D, and E) by injection of anti-Thy1.1 IgG (1 mg/kg body wt in 500 μl of PBS) into a tail vein; healthy control rats (groups A and B) received the respective amount of PBS only. Finally, BB-1101 treatment of animals from group E was started at day +2 and continued until day +11.

The 24-h urine specimens were collected from day −5 to +6, and ratios of grams of protein per millimolar creatinine were measured. Baseline proteinuria (days −5 to −2) was subtracted from the values obtained during days 0 to +6 for each rat separately. Estimation of the area under the time-curves (AUC) of protein:creatinine ratios was determined according to the trapezoidal rule (24).

The body weight of the rats was assessed at days −5, 0, and +11. Nephrectomy for histology and isolation of glomeruli was performed at day +11. Anesthesia occurred by intraperitoneal injection of a mixture containing 30 mg of ketamine HCl (Parke Davis, Baar, Switzerland) and 4 mg of xylazine HCl (Chassot, Belp, Switzerland). Immediately before removal of the kidneys, blood samples were taken for analyses of creatinine, potassium, sodium, and BB-1101. Measurements of BB-1101 were performed by the manufacturer.

Following the same protocol, a limited number of animals were added to three of the five experimental groups (groups A, C, and D) to analyze the effects of BB-1101 pretreatment at other time points. In each of these three groups, 12 rats were studied specifically to determine the total cell count of glomerular cross-sections and the gelatinolytic activity in isolated glomeruli at days +4 (n = 4), +8 (n = 4), and +14 (n = 4) after induction of nephritis. Furthermore, five rats per group were used to analyze glomerular antibody deposition, monocyte/macrophage influx, glomerular cellularity, and gelatinolytic activity in isolated glomeruli at days 0 and +2, as described in a following section.
Renal Morphology and Histochemistry

Renal tissues were fixed for 24 h in 5% buffered formalin, dehydrated, and embedded in paraffin. Subsequently, kidneys were cut longitudinally into 2-μm sections for periodic acid-Schiff reaction.

All morphometric analyses of equally distributed glomerular cross-sections were performed by one blinded observer (Dr. Steinmann-Niggli) on periodic acid-Schiff-stained tissue sections. Glomerular cross-sections containing only a minor portion of the glomerular tuft were not included in the analysis. The investigations were performed using a video camera mounted on a Leitz microscope (Leitz Wetzlar, Dialux 20, Wetzlar, Germany) and a color monitor. For point-count morphometry (25), the image of the glomerulus (×400 magnification under the microscope) was overlaid on the screen of the monitor with a grid of 16 vertical and 12 horizontal lines (192 intersecting points forming squares of 3.375 cm²; 6.1 cm on the screen are equal to 0.05 mm on the kidney specimen). When an intersecting point fell on the border of two tissue elements, the object on the left side of the point was counted.

Three different histologic parameters were studied. Cell nuclei were counted manually to determine the total cell number per glomerular cross-section (n = 63 per animal group). Point-count morphometry was used to quantify the ECM content in glomerular cross-sections (n = 63 per animal group). ECM was operationally defined as all glomerular components not representing capillary lumen and cell nuclei. The maximal diameter of 180 glomerular cross-sections per animal group was measured on the monitor-screen, classified in three different groups (<100 μm, 100 to 125 μm, and >125 μm), and depicted in a histogram.

Fluorescence-Activated Cell Sorting Analyses of Anti-Thy1.1 Antibody Binding

Rat mesangial cells (8th passage) were propagated in culture medium consisting of RPMI 1640 (Life Technologies, Basel, Switzerland) supplemented with 1% nonessential amino acids (Life Technologies), 2 mM l-glutamine (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies), 10 mM Hepes, pH 7.4 (Life Technologies), and 10% FCS (Inotech, Dottikon, Switzerland), as described by us previously (22).

Fluorescence-activated cell sorting (FACS) analyses of the binding of anti-Thy1.1 antibodies to MC were performed according to standard methods (22,26). Cells were propagated in culture medium containing 10% FCS for 2 d. Subconfluent MC were rendered quiescent by exposure to culture medium supplemented with 1% FCS for 24 h. Subsequently, the cells were exposed to either 1 μM BB-1101 in 0.003% DMSO or to 0.003% DMSO only in culture medium containing 1% FCS for 3 d. Thereafter, MC were rinsed with PBS, detached with dispase (Boehringer Mannheim, Rotkreuz, Switzerland), and resuspended in PBS.

MC (10⁶ cells/sample) were washed twice in ice-cold PBS with 1% goat serum (Life Technologies) and then incubated in 10 μg/ml anti-Thy 1.1 IgG for 1 h at 4°C. Subsequently, MC were washed twice with PBS, and nonspecific binding was blocked with 1% goat serum. Thereafter, MC were incubated with FITC goat anti-mouse IgG in a dilution of 1:100 (stock solution of 1 mg/ml; Sigma) for 30 min at 4°C. After two washes in PBS, the MC were resuspended in 1 ml of PBS, and the cells were analyzed by FACS (Becton Dickinson, San Jose, CA).

Glomerular Antibody Deposition, Monocyte/Macrophage Influx, and Mesangiolysis

A separate experiment was performed for analysis of possible effects of BB-1101 on glomerular deposition of anti-Thy1.1 IgG (27), on complement-dependent loss of MC (28), and on infiltration of monocytes/macrophages (27). A total of 15 male Wistar rats was divided into three groups consisting of healthy (n = 5), nephritic (n = 5), and pretreated nephritic (n = 5) rats, with BB-1101 treatment initiated 2 d before disease induction. Nephrectomy in three rats per group was performed 4 h after initiation of nephritis. The remaining animals were nephrectomized 2 d after induction of the nephritis.

Immunofluorescence analyses for glomerular deposition of anti-Thy1.1 antibodies 4 h after induction of nephritis were adapted from a previously published method (22,29). The 2-μm renal sections were
stained with an FITC-coupled caprine anti-mouse IgG (1:50; Sigma, Buchs, Switzerland) for 3 h at room temperature. All kidney sections were stained simultaneously and photographed under identical conditions, such as light intensity, and exposure or development times. Immunofluorescence intensity of 90 individual glomeruli per group was graded semiquantitatively (on a scale of 0 to 4), adapting a previously published method (15): 0 = very weak or absent staining; 1 = 1 to 25%; 2 = 25 to 50%; 3 = 50 to 75%; and 4 = 75 to 100% of glomerular tuft showing positive staining.

Monocytes/macrophages were identified by positive staining for ED-1 4 h after induction of nephritis (Chemicon, Temecula, CA) (27,30,31). Loss of mesangial cells during the stage of mesangiolysis was reflected by changes in the total glomerular cell number 4 h and 2 d after induction of nephritis.

**Complement Depletion**

Blood levels of complement factors CH₅₀ and C₃ were measured in another separate study. Blood samples of three rats were analyzed before and after a 2-d application of MMP inhibitor. Analyses of CH₅₀ were performed by a hemolytic assay and of C₃ by rocket immunoelectrophoresis (C3 protein), as well as by a hemolytic assay (C3 function). These analyses were done in the laboratory of M. Kirschfink (Institut für Immunologie, Ruprecht-Karls-Universität Heidelberg, Heidelberg, Germany) (32,33).

**Statistical Analyses**

Results are expressed as mean ± SD. Statistical analyses were performed by ANOVA, and comparison of means was done by the Student-Newman-Keuls test. For all experiments, P values <0.05 were considered significant. Data of proteinuria are given as median (percentile P₂₅ to percentile P₇₅), with statistical analyses performed by the Wilcoxon rank sum test.

**Results**

**Inhibition of MMP Activity In Vitro**

First, we evaluated BB-1101 with regard to inhibition of mesangial cell MMP in vitro. Zymography represents a very sensitive and specific method for the detection of MMP activity (22). Conditioned medium from our rat MC contained gelatinolytic activity derived from MMP-2 (22,34). The zones of lysis in the zymogram were inhibited in a dose-dependent manner by BB-1101, as demonstrated in Figure 2. Gelatinolytic activity significantly decreased after exposure of a gel strip to as little as 10 nM inhibitor (81% inhibition), and it almost completely disappeared after incubation with 1000 nM BB-1101 (99% inhibition). Analyses were performed in duplicate, with two identically treated samples on each gel strip. As expected, there was no visible difference in gelatinolytic activity between the two identical samples on a single piece of gel. According to these findings, BB-1101 represents a powerful inhibitor of rat mesangial cell MMP-2.

The effect of BB-1101 on other MMP was confirmed by zymography demonstrating inhibition of purified human MMP-2, -3, and -9 (ANAWA Trading SA, Wangen, Switzerland) by this compound (data not shown).

**Effects of MMP Inhibition on Glomerular Histology at Day +11**

Anti-Thy1.1 nephritis is characterized by an approximately 2-d initial phase of complement-dependent mesangiolysis that is followed by a marked proliferative response of residual MC, associated with accumulation of ECM proteins (28). Preliminary experiments showed that under our conditions, the latter two features were observed with a maximum at day +11. Therefore, this day was chosen primarily for nephrectomy and analyses of renal histology. The treatment of nephritic rats with BB-1101 resulted in an obvious amelioration of the glomerular histology, as shown in Figure 3. Subsequently, the beneficial effects of BB-1101 treatment were quantified by morphometric analyses.

Mesangial cell proliferation was reflected by changes in total glomerular cell numbers that were calculated by counting all nuclei present in the glomerular cross-sections (Figure 4). Healthy rats (group A) demonstrated 53 ± 11 cells, and the exposure of these rats to BB-1101 (group B) did not significantly alter glomerular cellularity, as reflected by a cell number of 58 ± 9 (P > 0.05). As expected, compared with these two groups, total cell number in glomerular cross-sections of nephritic rats (group C) significantly increased to 85 ± 12 (P < 0.001). Pretreatment of nephritic animals (group D) with BB-1101 resulted in a decreased number of cells (66 ± 8; P < 0.001), and posttreatment of nephritic rats (group E) resulted in a cell number of 78 ± 9 (P < 0.01). Results of groups A, C, and D were also incorporated in Figure 6A.

Glomerular size was assessed by measurements of the maximal glomerular diameter of 180 glomerular cross-sections per experimental group. The two healthy control groups (groups A and B) demonstrated in 46% of the analyzed glomeruli a diameter between 100 and 125 μm (Figure 5). As expected, the most frequent diameter (57%) in the nephritic animals (group C) was larger than 125 μm. BB-1101 treatment of nephritic animals, whether initiated before (group D) or after (group E) disease induction, resulted in a significant decrease of the
maximal glomerular diameter (43 and 52% of the glomeruli between 100 and 125 μm, respectively).

Using point-count morphometry, ECM accumulation was assessed by counting in glomerular cross-sections the percentage of intersecting points that fell on components not consisting of capillary lumen and of cell nuclei, as summarized in Table 1. This simplified definition of ECM was necessary for objective and consistent counting, thereby compensating for the resulting systematic error in the analyses. In addition, the morphometric investigations focused on the differences between the various animal groups and not on the absolute ECM surface area. In this respect, glomerular cross-sections of healthy rats (group A) showed 38.7% ± 4.5 almost identical to the 39.4% ± 1.4 found in treated healthy animals (group B; P > 0.05). As expected, nephritic animals (group C) were characterized by increased ECM deposition reflected by 46.6% ± 2.1 (P < 0.001). Pretreatment of nephritic animals (group D) by BB-1101 almost completely prevented the accumulation of ECM proteins and resulted in a value of 40.4% ± 4.5, significantly less than untreated nephritic animals (P < 0.001). Posttreated animals (group E) showed a tendency, although not statistically significant, toward an attenuation of ECM deposition (44.4% ± 4.0; P > 0.05). However, according to our operational definition of ECM, all of these numbers overestimated the true matrix content in glomerular cross-sections. Nevertheless, point-count morphometry is still an objective and valuable alternative to the semiquantitative matrix scoring system described by others (15).

**Effects of MMP Inhibition on Glomerular Cellularity at Days +4, +8, and +14**

At days +4, +8, and +14, glomerular cross-sections of rats demonstrated the following total cell counts: healthy rats (group A, n = 4): 56 ± 1, 55 ± 16, and 57 ± 3 cells, respectively; nephritic rats (group C, n = 4): 51 ± 9, 77 ± 12, and 66 ± 4 cells, respectively; and BB-1101 pretreated ne-
groups reached statistical significance. In addition, the body
± 0.39 0.04%.
animals (group C) demonstrated an increased value of 0.42 ± 0.04% in posttreated (group E) nephritic animals. Compared with these animals, MMP inhibitor treat-

Time Course of MMP Induction in the anti-Thyl.1 Nephritis

The synthesis of MMP as a function of time was analyzed in isolated glomeruli. Homogenizates of glomeruli from nephritic rats (group C) obtained at days 0, +2, +4, +8, +11, and +14 were investigated by zymography (23). A representative gel is depicted in Figure 6B. Quantitative analysis of this zymogram by densitometry demonstrated the following results, expressed in arbitrary units (1 U corresponds to the optical density of 0.131, obtained at day 0): day 0: 1 U; day +2: 4 U; day +4: 3 U; day +8: 19 U; day +11: 43 U; and day +14: 15 U (Figure 6B). Maximal induction of MMP-2 was observed at day +11, parallel to the degree of glomerular cell proliferation.

BB-1101 in a concentration of 100 nM, comparable to the 24-h trough blood levels achieved in vivo, led to a complete inhibition of gelatinolytic MMP-2 activity at all analyzed time points (Figure 6B).

Effect of BB-1101 on Kidney Weight

The kidney weight of each kidney at day +11 was expressed as a percentage of the total body weight. Healthy rats (group A) showed a mean of 0.38 ± 0.05%, and treated healthy animals (group B) were in the same range of 0.38 ± 0.04%. Nephritic animals (group C) demonstrated an increased value of 0.42 ± 0.04%. Compared with these animals, MMP inhibitor treatment demonstrated 0.39 ± 0.02% in pretreated (group D) and 0.39 ± 0.03% in posttreated (group E) nephritic animals. However, none of the differences between any of the five groups reached statistical significance. In addition, the body weight of the rats belonging to the five experimental groups did not significantly differ from one another (224 ± 9 g).

Table 1. Effect of BB-1101 on glomerular ECM accumulation and proteinuria

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>n</th>
<th>ECM^a day +11</th>
<th>Proteinuria^c AUC day (0-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy rats</td>
<td>9</td>
<td>38.7 ± 4.5</td>
<td>0.1 (0.0/0.2)</td>
</tr>
<tr>
<td>Treated healthy rats</td>
<td>9</td>
<td>39.4 ± 1.4</td>
<td>0.0 (0.0/0.2)</td>
</tr>
<tr>
<td>Nephritic rats</td>
<td>18</td>
<td>46.6 ± 2.1</td>
<td>1.5 (1.1/2.6)</td>
</tr>
<tr>
<td>Pretreated nephritic</td>
<td>18</td>
<td>40.4 ± 4.5^d</td>
<td>0.6 (0.2/1.1)</td>
</tr>
<tr>
<td>Posttreated nephritic</td>
<td>9</td>
<td>44.4 ± 4.0</td>
<td>0.3 (0.2/1.4)</td>
</tr>
</tbody>
</table>

^a Effect of MMP inhibition on glomerular ECM accumulation and on proteinuria. The data of ECM accumulation in glomerular cross-sections at day +11 are expressed as mean ± SD of the percentage of intersecting points that fell on glomerular components not consisting of capillary lumen and of cell nuclei, using point-count morphometry (*P < 0.01 nephritic versus pretreated nephritic rats). Proteinuria is reflected by the area under the protein:creatinine ratios versus time curve in 24-h urine specimens during days 0 to +6 (AUC_day+6), and data are expressed as median (percentile P25 to P75). ECM, extracellular matrix; AUC, area under the curve; MMP, matrix metalloproteinase.

^b Values represent mean ± SD.

^c Values represent median (P25/P75).

^d P < 0.01 significant difference versus untreated nephritic rats.

Blood Concentrations of Creatinine, Sodium, Potassium, and BB-1101

At day +11, mean blood levels of creatinine (43 ± 3 μM/L), sodium (141 ± 3 mM/L), and potassium (4.8 ± 0.8 mM/L) were almost identical in all animals, without significant differences in any of the investigated groups (data not shown). The 24-h trough blood concentration of BB-1101 at the time of nephrectomy (groups B, D, and E; n = 36) showed a mean of 38 ± 25 μg/L (approximately 100 nM), equivalent to multiples of the IC_{50} concentrations for inhibition of MMP-2 and other MMP. No correlation could be established between BB-1101 blood concentrations and beneficial effects, such as amelioration of renal histology or proteinuria.

Proteinuria

Proteinuria was analyzed in the rats that were subsequently nephrectomized at day +11. Protein excretion in 24-h urine specimens was expressed as protein:creatinine ratios (g/mL) from day −5 to +6. Before induction of nephritis, baseline proteinuria from day −5 to −2 was minimal and almost identical in all animals (0.06 ± 0.02 g/mL). The baseline proteinuria was subtracted from all subsequent measurements. The AUC of the protein:creatinine ratio versus time was calculated from days 0 to +6 for each rat separately. As shown in Table 1, from the AUC(0-6) of the individual rats, a median AUC(0-6) for each animal group was obtained (percentile P25 to P75).

In healthy rats (group A), the median AUC(0-6) was only 0.1 (0.0 to 0.2) g/mL × d, almost identical to the value of 0.0 (0.0 to 0.2) g/mL × d obtained from the group of healthy rats exposed to MMP inhibitor (group B). As expected, the median
cell counts of glomerular cross-sections at days 0, +2, +4, +8, +11, and +14. The three curves demonstrate total Nonspecific Effects of BB-1101.

0.6 (0.2 to 1.0) g/mM X d. Posttreated rats (group E) also demonstrated complete inhibition of gelatinolytic activity by exposure of the gel strips to 100 nM BB-1101. Columns represent the results of pretreatment of nephritic rats by BB-1101 (group D) demonstrated a clear tendency toward a reduced median AUC(0-6) of 0.13 g/mM obtained at day 0). The top panel shows zymography in the absence of inhibitor, and the bottom panel demonstrates complete inhibition of gelatinolytic activity by exposure of the gel strips to 100 nM BB-1101. Columns represent the results of the quantitative analysis of the upper zymogram expressed in arbitrary units (1 U of gelatinolytic activity corresponds to the optical density of 0.131 obtained at day 0).

AUC(0-6) of nephritic rats (group C) increased to 1.5 (1.1 to 2.6) g/mM X d, with a maximum around day +2 to +3 and a return toward baseline around day +5 to +6. Interestingly, pretreatment of nephritic rats by BB-1101 (group D) demonstrated a clear tendency toward a reduced median AUC(0-6) of 0.6 (0.2 to 1.1) g/mM X d. Posttreated rats (group E) also showed an amelioration of proteinuria, as reflected by a decrease of the median AUC(0-6) to 0.3 (0.2 to 1.4) g/mM X d.

Nonspecific Effects of BB-1101

BB-1101 administered before induction of nephritis may interfere with anti-Thy1.1 nephritis in a nonspecific way by reducing complement factors or glomerular deposition of anti-Thy1.1 IgG. During the initial phase of mesangiolysis after induction of nephritis, such effects may result in attenuation of loss of MC and in decreased infiltration of monocytes/macrophages. Therefore, these issues were addressed extensively.

FACS analyses of binding of anti-Thy1.1 IgG were performed with cultured rat MC. The MC were either exposed to 1 μM BB-1101 or to 0.003% DMSO for 2 d, incubated with anti-Thy1.1 IgG, and labeled with FITC-labeled goat antimouse antibody. The FACS analyses showed identical fluorescence in both samples (Figure 7). Therefore, in vitro, no interference of MMP inhibitor with the binding of anti-Thy1.1 antibodies was found.

Immunofluorescence analyses of glomerular binding of anti-Thy1.1 IgG were accomplished in vivo. To investigate whether pretreatment with BB-1101 reduces the amount of glomerular deposition of anti-Thy1.1 IgG, renal tissues were harvested 4 h (27) after induction of the nephritis. Kidney sections of healthy rats, nephritic rats, and pretreated nephritic rats were stained for the presence of anti-Thy1.1 IgG with FITC-coupled anti-mouse IgG. Micrographs of representative glomeruli are shown in Figure 7. No immunofluorescence staining was detected in normal glomeruli, whereas both nephritic groups demonstrated equal amounts of anti-Thy1.1 IgG deposition. Subsequently, immunofluorescence intensity of 90 glomeruli per animal group was quantified by a semiquantitative scoring system in grades 0 to 4 (15). Healthy animals showed a negligible mean score of 0.1 ± 0.3, whereas nephritic and pretreated nephritic rats were characterized by almost identical mean scores of 2.3 ± 0.8 and 2.4 ± 0.9, respectively (P > 0.05).

Monocyte/macrophage infiltration was analyzed in 20 glomerular cross-sections per animal 4 h after induction of nephritis (27). Nephritic animals (n = 3) displayed 9.1 ± 2.8 cells positive for ED-1, a well described monocyte and macrophage marker (30). As expected, pretreated nephritic rats (n = 3) demonstrated a practically identical cell number of 9.8 ± 2.1 (P > 0.05). In contrast, healthy animals (n = 3) showed only 2.3 ± 1.9 ED-1-positive cells per glomerular cross-section. Therefore, administration of BB-1101 did not seem to affect glomerular influx of monocyte and macrophages in nephritic animals (Table 2).

The loss of mesangial cells during the initial phase of anti-Thy1.1 nephritis was assessed by counting all of the cells in 20 glomerular cross-sections per animal at 4 h (27) and 2 d (28) after disease induction (Table 2). A total of three animals per group was analyzed at the former and two rats per group at the latter time point. The total number of cells present at 4 h in nephritic and in pretreated nephritic animals was not significantly different, as reflected by 47 ± 3 and 44 ± 4, respectively (P > 0.05). As expected, cell number of 58 ± 6 was significantly higher in healthy rats (P < 0.05 versus nephritic animals). These results indicate that BB-1101 pretreatment did not interfere with the process of mesangiolysis. These findings were confirmed and extended by investigations performed at day +2, demonstrating mean cell numbers of 46 and 47 in nephritic animals and of 43 and 45 in pretreated nephritic
Figure 7. Binding of anti-Thy1.1 IgG to MC: Fluorescence-activated cell sorting (FACS) analyses and immunofluorescence micrographs. FACS analyses of untreated cultured rat MC (a) and of cells exposed to 1 μM BB-1101 for 2 d (b) demonstrate identical fluorescence intensity. Immunofluorescence micrograph of a glomerulus from a healthy rat that did not reveal any fluorescence (c) and of a glomerulus from a nephritic rat that demonstrated identical fluorescence (d) compared with Panel e, a glomerulus from a pretreated nephritic rat. Nephrectomy was performed 4 h after injection of anti-Thy1.1 IgG or phosphate-buffered saline. Tissue sections were photographed under identical exposure conditions. Magnification, ×125.

Table 2. Effect of BB-1101 on total cell count and on ED-1-positive cells per glomerular cross-section

<table>
<thead>
<tr>
<th>Group</th>
<th>ED-1+</th>
<th>Total Cell Count</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>4 h (n = 3)</td>
<td>2 d (n = 2)</td>
</tr>
<tr>
<td>A: Healthy rats</td>
<td>2.3 ± 1.9</td>
<td>55/58</td>
</tr>
<tr>
<td>C: Nephritic rats</td>
<td>9.1 ± 2.8</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>D: Pretreated rats</td>
<td>9.8 ± 2.1</td>
<td>44 ± 4</td>
</tr>
</tbody>
</table>

*Effect of BB-1101 pretreatment on loss of mesangial cells and on invasion of ED-1-positive monocytes/macrophages. Animals were divided into three equal groups consisting of healthy (n = 5), nephritic (n = 5), and pretreated nephritic (n = 5) rats. Twenty glomerular cross-sections per animal were analyzed. After induction of nephritis and during the phase of mesangioplasia, three animals in each group were studied after an interval of 4 h, and the remaining two rats per group after a period of 2 d.

Discussion

The present study describes the anti-inflammatory effects of the MMP inhibitor BB-1101 on acute anti-Thy1.1 nephritis in the rat (15,30,35). This nephritis represents an animal model of immune complex-mediated, mesangial proliferative glomerulonephritis. In humans, mesangial proliferative glomerulonephritis such as IgA nephritis is an important cause of end-stage renal failure (36,37). At present, treatment of this disease is still controversial because none of the current treatment modalities has shown a consistent and convincing benefit (36,37). Therefore, investigations targeted at the disordered mesangial cell biology in mesangial proliferative glomerulonephritis are of considerable clinical interest.
First, we investigated the effect of BB-1101 on MMP activity in vitro, using conditioned medium from cultured rat MC. Zymography demonstrated strong mesangial cell MMP-2 inhibition by BB-1101. Thereafter, we administered the MMP inhibitor to nephritic animals. As therapeutic end points, we chose MC proliferation, ECM accumulation, glomerular hypertrophy, and proteinuria. The main aim of the study was to determine whether the application of a synthetic MMP inhibitor exerts anti-inflammatory effects in acute anti-Thy1.1 nephritis, regardless of the cell source of the inhibited MMP. Therefore, intraperitoneal applications of BB-1101 were initiated before induction of nephritis. In this way, MMP expressed by intrinsic glomerular cells and of invading hemopoietic cells, mainly monocytes and macrophages, were targeted by the inhibitor. Pretreatment of animals was justified because the MMP inhibitor did not display unwanted side effects, such as interference with binding of anti-Thy1.1 antibodies to MC, complement depletion, or inhibition of monocyte/macrophage infiltration.

The synthesis of MMP-2 as a function of time showed a close correlation with the degree of glomerular cell proliferation, as published previously (14). MMP inhibition by BB-1101 attenuated all investigated inflammatory features. Total glomerular cell counts reflecting the degree of MC proliferation, ECM accumulation, and maximal glomerular diameter were significantly reduced. In addition, proteinuria showed a clear tendency toward a decrease. The observation that BB-1101 inhibits proteinuria is consistent with blocking the MMP-mediated degradation of the glomerular basement membrane (14). All of these results indicate that MMP play a significant role in the pathogenesis of acute anti-Thy1.1 nephritis.

To more specifically target MMP from MC, we extended the study and included an animal group treated by BB-1101 after induction of disease. As expected, the anti-inflammatory effects of MMP inhibition were somewhat less pronounced, because MMP secreted by invading and activated monocytes/macrophages were not affected during the first days of the disease. Nevertheless, all analyzed inflammatory features of anti-Thy1.1 nephritis were again ameliorated by BB-1101. In these animals with late treatment, the still significant reduction in MC proliferation and the tendency toward a reduction of ECM deposition resulted in protective effects on glomerular hypertension. Therefore, MMP represent a new target in the therapy of mesangial proliferative glomerulonephritis.

Apart from MMP inhibition, a wide range of other treatment modalities were investigated in anti-Thy1.1 nephritis. Transforming growth factor-β1 (TGF-β1) was found to be a particularly effective target. Administration of neutralizing antibodies raised against TGF-β1 (38) or of antisense oligonucleotides to inhibit TGF-β1 expression (2) resulted in a substantial decrease in excess ECM deposition. The prominent role of TGF-β in this disease was further elucidated by the use of the proteoglycan decorin, a natural inhibitor of TGF-β activity (39,40). Increased levels of this matrix component, achieved either by intravenous application (39) or by overexpression (40), also led to a decrease in ECM accumulation. In addition, the same beneficial effect was observed by therapy of nephritic rats with N⁵-monomethyl-L-arginine, a nitric oxide synthase inhibitor (27). On the contrary, overall matrix accumulation was unaltered by subcutaneously administered interferon-γ, which nevertheless inhibits MC proliferation to a highly significant extent (1). A combined reduction in MC proliferation and ECM accumulation was achieved by heparin (28) and neutralizing antibodies to platelet-derived growth factor (41). Agents exerting other beneficial effects in anti-Thy1.1 nephritis were the platelet activating factor receptor antagonist WEB 2170 (42) and the prostacyclin analog Iloprost, as well as the adenosine analog 2-chloroadenosine (43).

Apart from the histologic changes, proteinuria also represents a clinically relevant result of glomerular damage caused by inflammatory processes. In previous studies by other groups, a reduction in proteinuria of nephritic rats was also documented by the use of decorin (39,40), N⁵-monomethyl-L-arginine (27), Iloprost, and 2-chloroadenosine (43). However, the additional reduction of MC proliferation is a distinguishing feature of MMP inhibition by BB-1101.

The exact mechanism by which the MMP inhibitor exerts its beneficial effects remains to be defined. At this stage, we do not know to what extent a possible interference of BB-1101 with activation of latent tumor necrosis factor-α (17) plays a role in our study. However, the antiproliferative action of MMP inhibition may well be the result of a direct effect on the MC. The biological role of MMP-2 beyond degradation of ECM proteins was analyzed previously in cultured rat MC (44). The constitutive synthesis of MMP-2 was greatly reduced with antisense RNA expressed by an episomally replicating vector or with specific anti-MMP-2 ribozymes expressed by a retroviral transducing vector (44). The transfected or retrovirally infected MC reverted from a proliferative, inflammatory phenotype to the quiescent state that occurs in the normal renal glomerulus. The noticeable differences included changes in synthesis of ECM proteins, loss of activation markers, and an almost total stop of proliferation. Reconstitution with exogenous active MMP-2 caused a rapid return to the inflammatory phenotype. Furthermore, Turck et al. showed that infusion of MMP-2 into rat kidneys led to focal areas of MC proliferation and expansion (45).

Previously, we have investigated the in vitro effects of an MMP inhibitor on cultured rat MC (22). Exposure of MC to Ro 31-9790 resulted in a dose-dependent decrease in cell proliferation, associated with a reduced expression of α-smooth muscle actin, a well described MC activation marker (1,46). These results, together with the present in vivo findings, provide further evidence that MMP-2 acts directly on MC to induce or to maintain an inflammatory cell phenotype. Therefore, the beneficial effects of BB-1101 on ECM accumulation is compatible with the hypothesis that MMP regulate matrix remodeling not only by their protease activity, but in certain circumstances even more by their proinflammatory action as growth factors.

MMP inhibitors have already been evaluated for their anti-inflammatory effects in various disease models, such as experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis, and in adjuvant arthritis, a rat model of
rheumatoid arthritis. GM 6001 administered daily, either from the time of disease induction or from the onset of clinical signs, suppressed the evolution or reversed clinical experimental autoimmune encephalomyelitis in a dose-dependent manner (6). Treatment with GI168, initiated 8 d after induction of adjuvant arthritis, provided protection against tissue edema, cartilage, and bone destruction, as well as pannus formation (5). In addition, the effect of the MMP inhibitor BB-1101, also used in the present study, was analyzed on a delayed-type hypersensitivity response in the central nerve system of rats. Therapy of such animals by BB-1101 strongly reduced breakdown of the blood–brain barrier, infiltration of macrophages and T cells, and damage to myelin (21). Therefore, MMP inhibitors may be successfully used to treat inflammatory diseases.

Despite the encouraging results of the present study, the complete therapeutic potential of MMP inhibition in glomerulonephritis has not yet been definitively elucidated. Additional studies, using MMP inhibitors that can be given by oral application, may help to further determine the therapeutic value of these synthetic compounds. Further research also will be needed to address the consequences of MMP inhibition on the ECM in more detail, such as accumulation of laminin, fibronectin, and various types of collagens. Quantitative analyses of these individual matrix components may provide information on further beneficial effects of MMP inhibition. In addition, a better definition of the role of MMP in inflammatory diseases may eventually lead to the development and promotion of new research areas. Gene therapy is one of the most prominent examples, which may be realized by modifying MC to overexpress naturally occurring tissue inhibitors of metalloproteinases (TIMP), such as TIMP-1 or TIMP-2 (47).

In summary, MMP play a prominent role in vivo beyond degradation of ECM proteins, as shown in acute anti-Thy 1.1 nephritis. Treatment of nephritic animals with a synthetic MMP inhibitor resulted in a reduction of MC proliferation, ECM deposition, glomerular hypertrophy, and proteinuria. Therefore, MMP play an important role in the pathogenesis of acute anti-Thy.1.1 nephritis. For the first time, it was demonstrated in vivo that MC proliferation can be suppressed by MMP inhibition. In conclusion, MMP inhibition may represent a new approach to the treatment of MC-mediated forms of glomerulonephritis.

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