Glomerular Plasmin-Like Activity in Relation to Nephritis-Associated Plasmin Receptor in Acute Poststreptococcal Glomerulonephritis

Takashi Oda,* Kazuo Yamakami,* Fumihiro Omasu,* Shigenobu Suzuki,† Soichiro Miura,† Tetsuzo Sugisaki,‡ and Nobuyuki Yoshizawa*

*Department of Public Health and †Second Department of Internal Medicine, National Defense Medical College, Saitama; and ‡Department of Nephrology, Showa University School of Medicine, Tokyo, Japan

A nephritogenic antigen for acute poststreptococcal glomerulonephritis (APSGN) was isolated recently from group A streptococcus and termed nephritis-associated plasmin receptor (NAPlr). In vitro experimental data indicate that the pathogenic role of NAPlr occurs through its ability to bind to plasmin and maintain its proteolytic activity. However, the mechanism whereby this antigen induces glomerular damage in vivo has not been fully elucidated. Renal biopsy tissues from 17 patients with APSGN, 8 patients with rapidly progressive glomerulonephritis, and 10 normal kidneys were analyzed in this study. Plasmin-like activity was assessed on cryostat sections by in situ zymography with a plasmin-sensitive synthetic substrate. Serial sections were simultaneously assessed for NAPlr deposition by immunofluorescence staining. Glomerular plasmin-like activity was absent or weak in normal controls and in patients with rapidly progressive glomerulonephritis, although tubulointerstitial activity was occasionally detected. Prominent glomerular plasmin-like activity was found in patients who had APSGN and in whom glomerular NAPlr was positive, whereas it was absent or weak in patients who had APSGN and in whom glomerular NAPlr was negative. The distribution of glomerular plasmin-like activity was identical to that of NAPlr deposition but was generally different from that of fibrinogen deposition as assessed by double staining. The activity was abolished by the addition of aprotinin to the reaction mixture but was not altered by the addition of a matrix metalloprotease inhibitor, a cysteine protease inhibitor, or inhibitors of plasminogen activators. Thus, upregulated glomerular plasmin-like activity in relation to NAPlr deposition in APSGN was identified. This result supports the nephritogenic character of NAPlr and offers insight into the mechanism whereby this antigen induces nephritis.

Received April 30, 2004. Accepted September 28, 2004.

Published online ahead of print. Publication date available at www.jasn.org.

Address correspondence to: Dr. Takashi Oda, Department of Public Health, National Defense Medical College, 3-2 Namiki, Tokorozawa-shi, Saitama 359-8513, Japan. Phone: +81-4-2995-1575; Fax: +81-4-2996-5196; E-mail: takashio@cc.ndmc.ac.jp

Copyright © 2005 by the American Society of Nephrology

ISSN: 1046-6673/1601-0001

Copyright © 2005 by the American Society of Nephrology
plasminogen activators, urokinase type (uPA) and tissue type (tPA). This conversion is inhibited by a primary physiologic inhibitor, plasminogen activator inhibitor-1 (PAI-1) (17). The central role of the plasminogen activator/plasmin cascade in fibrinolysis has been well characterized. However, recent studies (17,18) suggest more broad pathophysiologic roles of this cascade in various processes such as embryonic development, ovulation, cell migration, wound healing, angiogenesis, and neoplasia. In the renal field, this pathway is attracting considerable attention as a potent modulator of renal fibrosis in relation to its effect on extracellular matrix turnover (19–23). Indeed, plasmin can degrade laminin and fibronectin and can activate latent matrix metalloprotease (MMP) in vitro. However, it is rapidly inhibited and tightly regulated by physiologic inhibitors such as α2-AP and hence is not normally found in an active form in vivo (14,18,24). Probably because of such unstable characteristics, few researchers have attempted to detect plasmin activity in vivo, despite the considerable interest in it.

In the present study, we attempted to confirm our hypothesis that renal glomerular plasmin activity is upregulated in relation to NAPPr deposition in patients with APSGN. For this purpose, we used an in situ zymography method with a plasmin-sensitive synthetic substrate that we found to be a simple, stable, and highly sensitive procedure.

Materials and Methods

Patients

Renal tissues that were collected from 17 patients with APSGN were used in this study. Patient characteristics are given in Table 1. All patients showed overt symptoms of APSGN, such as facial edema, hypertension, and hematuria. Percutaneous needle biopsies were performed for diagnostic purposes over a period of 24 yr (1979 to 2003) at the National Defense Medical College (Saitama, Japan) to rule out progressive renal disease with acute nephritic syndrome (e.g., IgA nephropathy, lupus nephritis, RPGN). Informed consent was obtained from each patient. A diagnosis of APSGN was made according to serologic and bacteriologic evidence of acute streptococcal infection before the onset of nephritis as well as from characteristic histologic features of the renal tissue under light microscopy, immunofluorescence (IF), and electron microscopy. Eight patients with RPGN (defined as the presence of crescents in >60% of glomeruli) and 10 normal kidneys that had been removed for localized tumors served as disease controls and normal controls, respectively.

In Vitro Zymography for Plasmin Activity

To confirm the suitability of a zymographic assay for evaluation of plasmin activity, we conducted preliminary in vitro experiments. Ten micromolars of plasmin (Sigma Chemical Co., St. Louis, MO) at increasing concentrations (0, 1, 2, 5, and 10 × 10⁻² µg/µl) in 0.067 M sodium phosphate buffer (PB; pH 7.1) were incubated in 96-well microtiter plates with 100 µl of reaction mixture that contained 0.1% Fast Violet B and 0.5 mM p-toluenesulfonyl-l-lysine α-naphthyl ester (Tos-Lys-NE) in 0.067 M PB for 10 min at 27°C. Tos-Lys-NE was obtained from Torii Pharmaceutical Co., Ltd. (Tokyo, Japan). Reaction color as a result of cleavage of Tos-Lys-NE by plasmin was evaluated by absorbance at 500 nm with a microplate reader after addition of 0.1% SDS to dissolve the precipitated reaction product. A control assay in which the substrate was omitted was performed. In addition, the effects of α2-AP (5 µg/ml; Merck, Darmstadt, Germany) and aprotinin (0.1 µU/µl) as plasmin inhibitors (16,20), EDTA as an MMP inhibitor (25), and E-64 as a cysteine protease inhibitor were examined by running the same reaction with the addition of these inhibitors. To determine the sensitivity of Tos-Lys-NE to plasminogen activators, we performed similar in vitro zymographic assays in which plasmin was replaced by either tPA or uPA (Merck) at comparable concentrations. All chemicals, unless otherwise stated, were purchased from WAKO Pure Chemical Industries (Osaka, Japan).

In Situ Zymography for Plasmin-Like Activity

Plasmin-like activity in cryostat sections (4 µm) of renal tissues was assessed by in situ zymography according to the method of Takuma et al. (26) with a few modifications. Briefly, after being washed with PBS, the sections were incubated for 30 min at 27°C with the same reaction mixture used for the in vitro assay. The sections were then counterstained with methylgreen. The specificity of the reaction was investigated by including the same protease inhibitors used for the in vitro assay (aprotinin, α2-AP, EDTA, or E-64) in the reaction mixture. To rule out the possibility that plasminogen activators contribute to the reaction by generating plasmin in situ during the incubation period, we also investigated the effect of a tPA inhibitor (rabbit anti-human tPA inhibiting antibody; ICN, Irvine, CA) and a uPA inhibitor (1 mM amlodipine; Sigma Chemical Co.) on the in situ zymographic reaction by including these inhibitors in the reaction mixture.

Light microscopic images of all glomeruli in each renal section were acquired with a digital camera. Measurement of the positive area relative to the total area of each glomerulus was calculated with Luminet Vision Ver. 2.04 image analysis software (Mitani Corp., Fukui, Japan), and the average numbers were regarded as the relative glomerular plasmin-like activity for each patient. The number of glomeruli analyzed for each APSGN patient ranged from 2 to 11 (4.1 ± 2.7), and the number of glomeruli analyzed for each RPGN patient ranged from 3 to 17 (8.0 ± 5.5). In normal control kidneys, 10 glomeruli were selected randomly and were analyzed similarly.

Table 1. Characteristics of APSGN patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>From Onset to Biopsy (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>F</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>M</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>M</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>F</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>M</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>41</td>
<td>F</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>M</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>F</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>33</td>
<td>F</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>M</td>
<td>15</td>
</tr>
<tr>
<td>11</td>
<td>27</td>
<td>M</td>
<td>16</td>
</tr>
<tr>
<td>12</td>
<td>49</td>
<td>M</td>
<td>18</td>
</tr>
<tr>
<td>13</td>
<td>10</td>
<td>M</td>
<td>20</td>
</tr>
<tr>
<td>14</td>
<td>19</td>
<td>M</td>
<td>24</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>F</td>
<td>26</td>
</tr>
<tr>
<td>16</td>
<td>27</td>
<td>F</td>
<td>26</td>
</tr>
<tr>
<td>17</td>
<td>34</td>
<td>F</td>
<td>44</td>
</tr>
</tbody>
</table>

aPSGN, acute poststreptococcal glomerulonephritis.
Preparation of FITC-conjugated rabbit anti-NAPlr antibody and direct IF staining were performed as reported previously (12). Briefly, serial sections were washed with PBS and incubated with anti-NAPlr antibody for 30 min at 27°C. After being washed with PBS, the sections were mounted and observed by fluorescence microscopy. To evaluate the relation between NAPlr deposition and plasmin activity, we assigned APSGN patients to either of two groups, depending on the glomerular positivity for NAPlr: NAPlr positive (14 patients) and NAPlr negative/weak (3 patients).

Double Staining for Plasmin-Like Activity and NAPlr IF or Plasmin(ogen) IF

To verify the co-localization of plasmin-like activity and NAPlr deposition, we performed the zymographic assay and NAPlr IF staining sequentially on the same sections obtained from several NAPlr-positive APSGN patients. To clarify the relation between plasmin(ogen) deposition and plasmin-like activity, we also performed double staining for plasmin-like activity and plasmin(ogen) IF on sections from several NAPlr-positive APSGN patients. After incubation with the reaction mixture for the plasmin assay, sections were washed with PBS without counterstaining and then stained for either NAPlr or plasmin(ogen) with an FITC-conjugated rabbit anti-NAPlr antibody or a rabbit anti-human plasmin(ogen) antibody (Nordic Immunological Laboratories, Tilburg, Netherlands) that was prelabeled with fluorescein. Labeling of the anti-plasmin(ogen) antibody with fluorescein was performed with the EZ-Label Fluorescein Protein Labeling Kit (Pierce Biotechnology, Rockford, IL) by following the manufacturer’s instructions. Sections were incubated with the antibodies for 30 min at 27°C. Sections were then washed with PBS, mounted, and observed by light microscopy for plasmin-like activity and by fluorescence microscopy for either NAPlr or plasmin(ogen) staining.

Double Staining for Fibrin(ogen) IF and NAPlr IF or Plasmin-Like Activity

The relation between fibrin(ogen) deposition and NAPlr deposition or plasmin-like activity was evaluated by double staining. For co-localization of fibrin(ogen) and NAPlr, we labeled the anti-NAPlr antibody with Alexa Fluor 594 (Molecular Probes, Eugene, OR), according to the manufacturer’s instructions, and applied the labeled antibody and FITC-conjugated goat anti-human fibrin(ogen) antibody (ICN) simultaneously to sections from several NAPlr-positive APSGN patients. Double staining for plasmin-like activity and fibrin(ogen) IF was performed in the same manner as the double staining for plasmin-like activity and NAPlr IF.

Statistical Analyses

Data are expressed as the mean ± SD. A t test (parametric assay) or the Mann-Whitney U test (nonparametric assay) was used to evaluate differences between mean values. Differences were considered significant when the two-tailed P value was <0.05.

Results

In Vitro Zymography for Plasmin Activity

In vitro assay confirmed that the Tos-Lys-NE substrate was sensitive for plasmin activity (significant positive linear regression of the assay for plasmin, Y = 0.042 + 0.017 X, r = 0.998, P < 0.0001) and that the assay system was stable (no reaction was observed in the absence of either substrate or plasmin; Figure 1). Hydrolyzing activity was completely suppressed by the addition of aprotinin or α2-antiplasmin (α2-AP).

In Situ Zymography for Plasmin-Like Activity and IF Detection of NAPlr

Weak or no plasmin-like activity was found in the glomeruli of normal control subjects. However, occasional tubulointerstitial activity was identified (Figure 2A). In RPGN patients, occasional glomerular plasmin-like activity was identified, predominantly in crescents, but in general, glomerular activity was weak or absent. However, strong tubulointerstitial activity was present in tubular epithelial cells and in interstitial infiltrating cells (Figure 2B). In NAPlr-positive APSGN patients, prominent activity was identified in glomeruli (Figure 2C). Activity was localized predominantly in the mesangium and infiltrating leukocytes and partially along the glomerular basement membrane (GBM); the distribution was almost identical to that of NAPlr deposition as assessed by IF staining of serial sections (data not shown). The activity was completely abolished by the addition of aprotinin (Figure 2D), whereas addition of α2-AP (Figure 2E), EDTA, E-64, or inhibitors of plasminogen activators (anti-tPA antibody, amiloride) did not affect the reaction color (data not shown). Glomerular plasmin-like activity was weak or absent in APSGN patients who were negative for glomerular NAPlr (Figure 2F). Relative glomerular plasmin-like activity as assessed by the measurement of positive glomerular areas was significantly greater in NAPlr-positive APSGN (9.9 ± 3.4%) than in NAPlr-negative APSGN (0.84 ± 0.95%; P < 0.01) or in RPGN (0.57 ± 0.33%; P < 0.0001) or in normal control tissues (0.30 ± 0.19%; P < 0.0001) (Figure 3).

Double Staining for Plasmin-Like Activity and NAPlr IF or Plasmin(ogen) IF

The similar distribution of glomerular plasmin-like activity and NAPlr deposition in serial sections was examined further.
by double staining in the same sections. The merged image (Figure 4C) clearly showed that the distributions of the activity (Figure 4A) and NAPlr deposition (Figure 4B) were almost identical, although zymography tended to detect a slightly larger area than did NAPlr IF staining. This slight difference in distribution may be due to a difference in the sensitivities of these methods.

A plasmin-like activity and plasmin(ogen) IF co-localization assay was performed to confirm that the activity detected by in situ zymography truly represented that of plasmin(ogen). The glomerular staining pattern of in situ zymography (Figure 4D) differed from that of plasmin(ogen) IF (Figure 4E), but the overall distribution was similar; the staining pattern of plasmin(ogen) was generally diffuse, whereas that of in situ zymography was punctate and relatively restricted. The merged image showed that glomerular plasmin-like activity was localized within plasmin(ogen)-positive sites (Figure 4F). This result is reasonable because the anti-plasmin(ogen) antibody should detect both active and inactive plasmin as well as plasminogen, whereas zymography should detect only active plasmin.

Double Staining for Fibrin(ogen) IF and NAPlr IF or Plasmin-Like Activity

Merged images (Figure 5, C and F) showed that the distributions of fibrin(ogen) deposition (Figure 5, B and E) and of NAPlr deposition (Figure 5A) or plasmin-like activity (Figure 5D) were generally different. Positive areas for NAPlr deposition or plasmin-like activity seemed to be free of fibrin(ogen) deposition.

Discussion

Our previous finding that the nephritogenic antigen for APSGN is the same entity as Plr of GAS led us to speculate that the antigen might be able to maintain the proteolytic activity of plasmin in the same way as shown in vitro and contribute to the development of APSGN in vivo (11). Poon-King et al. (27) reported the in vitro plasmin(ogen)-binding ability of nephritis plasmin binding protein, which is identical to SPEB, another potent nephritogenic antigen of GAS, and suggested a similar mechanism for the development of APSGN. However, to the best of our knowledge, there have been no reports showing...
Val-Leu-Lys-substrate is completely specific for plasmin. Tos-Lys-NE, D-Leu-Lys-\(p\)-nitroanilide (25) and Tos-Gly-Pro-Lys-\(p\)-nitroanilide (19,21) are also commercially available synthetic peptides for the general difference in distribution between fibrin(ogen) deposition and NAPiR deposition or plasmin-like activity is not surprising, because plasmin, which is suspected to be bound by and co-localized with NAPiR, should have the ability to degrade fibrin(ogen), thereby decreasing the fibrin(ogen) deposition. Fibrin deposition has been shown to be an important mediator of glomerular injury in progressive renal diseases, particularly crescentic glomerulonephritis (34). In this sense, the fibrin(ogen)ysis in situ shown in this study may indicate the contribution of plasmin also to the resolution of APSGN, although further investigation will be required to confirm this.

APSGN is believed to be mediated by an immune complex that may be formed either in situ or in the circulation (1). NAPiR is a potential candidate for the nephritogenic antigen because it is highly antigenic (12). However, this study clearly showed that NAPiR not only acts as a component of the immune complex but also has a direct, nonimmunologic function as a plasmin receptor and contributes to pathogenesis by maintaining proteolytic activity in situ. This is somewhat consistent with previous clinical findings that proteinuria and microscopic hematuria are occasionally found in the dormant phase of the disease, when antibody against the nephritogenic antigen has not yet developed. Patients with streptococcal infection and the above manifestations have a higher incidence of APSGN (35).

We selected RPGN as a disease control because it is one of the few types of human glomerulonephritis with prominent glomerular infiltration of neutrophils and macrophages, similar to APSGN (28,29). Glomerular infiltrating cells are suspected to affect the results of the zymographic assay because these cells are known to secrete uPA and to express uPA receptor (17,30). However, glomerular plasmin-like activity was minimal in RPGN patients. This may be due to the instability of plasmin or to the upregulated expression of PAI-1 in the glomeruli of RPGN patients, as reported previously (31–33).

The general difference in distribution between fibrin(ogen) deposition and NAPiR deposition or plasmin-like activity is not surprising, because plasmin, which is suspected to be bound by and co-localized with NAPiR, should have the ability to degrade fibrin(ogen), thereby decreasing the fibrin(ogen) deposition. Fibrin deposition has been shown to be an important mediator of glomerular injury in progressive renal diseases, particularly crescentic glomerulonephritis (34). In this sense, the fibrin(ogen)ysis in situ shown in this study may indicate the contribution of plasmin also to the resolution of APSGN, although further investigation will be required to confirm this.

APSGN is believed to be mediated by an immune complex that may be formed either in situ or in the circulation (1). NAPiR is a potential candidate for the nephritogenic antigen because it is highly antigenic (12). However, this study clearly showed that NAPiR not only acts as a component of the immune complex but also has a direct, nonimmunologic function as a plasmin receptor and contributes to pathogenesis by maintaining proteolytic activity in situ. This is somewhat consistent with previous clinical findings that proteinuria and microscopic hematuria are occasionally found in the dormant phase of the disease, when antibody against the nephritogenic antigen has not yet developed. Patients with streptococcal infection and the above manifestations have a higher incidence of APSGN (35).

We previously suggested a mechanism for glomerular damage by the nephritogenic antigen through direct activation of the complement system in situ (7). However, in our recent study,
we found different distributions of C3 and NAPlr deposition in the glomeruli of APSGN patients (11), in clear contrast to the similarity of plasmin-like activity and NAPlr deposition in the present study. We suspect that direct complement activation by NAPlr is mediated predominantly in the circulation rather than in situ.

From these findings, we propose the following APSGN induction mechanism. Infection of the throat or skin with GAS...
induces the release of cytoplasmic NAPiR into the circulation. Circulating NAPiR binds to the renal glomeruli on the mesangial matrix and GBM, probably through its adhesive character (12). Bound NAPiR then traps plasmin and maintains its activity, which may induce glomerular damage in situ by degrading the GBM by itself or by activating pro-MMP. Plasmin activity may also mediate inflammation by activating and accumulating monocytes and neutrophils in situ (36,37). Such glomerular damage may induce urinary abnormalities during the latent period of the disease. Finally, the developed antibody forms immune complexes that can readily pass through the altered GBM and thereafter accumulate in the subepithelial space as humps. This final step of immune complex deposition, accompanied by the activation of complement and immune cell accumulation, leads to the full-blown and overt disease state.

In summary, we identified upregulated glomerular plasmin-like activity in relation to NAPiR deposition in human APSGN. Instead of immunohistochemical detection, we used an in situ zymography method and identified functional proteolytic activity within glomeruli in situ. Our results suggest the important role of plasmin in the development of APSGN and strongly support the idea that NAPiR isolated from GAS is a nephritogenic agent for APSGN.

Acknowledgments

We are grateful to our colleagues Satoko Kiyono and Mami Morisugi for expert secretarial assistance and to Tatsuyo Harasawa, Central Research Laboratory, National Defense Medical College, for excellent technical assistance.

References

13. Winram SB, Lottenberg R: The plasmin-binding protein Plr of group A streptococci is identified as glyceraldehyde-3-phosphate dehydrogenase. Microbiology 142[Suppl]: 2311–2320, 1996