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

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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 fibrin(ogen) 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 metalloproteinase 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.

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 NAPiR 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.

Table 1. Characteristics of APSGN patients

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*PSGN, acute poststreptococcal glomerulonephritis.

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 East 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 U/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 amiloride; 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 Lumi- naVision 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.
In Vitro Zymography for Plasmin Activity

Results

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

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 NAPIr-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 NAPIr deposition as assessed by IF staining of serial sections (data not shown). The activity was completely abolished by the addition of aprotinin or α2-antiplasmin (α2-AP).

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

The similar distribution of glomerular plasmin-like activity and NAPIr 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 \textit{in situ} zymography truly represented that of plasmin(ogen). The glomerular staining pattern of \textit{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 \textit{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.

\textbf{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 \textit{in vitro} and contribute to the development of APSGN \textit{in vivo} (11). Poon-King et al. (27) reported the \textit{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
upregulated glomerular plasmin activity in APSGN or in any type of human glomerulonephritis in situ.

Here, we showed prominent intraglomerular plasmin-like activity in NAPr-positive APSGN patients by in situ zymography with a plasmin-sensitive synthetic substrate. We selected Tos-Lys-NE as the substrate for this assay because the C-terminal end of lysine is most vulnerable to plasmin activity (24), and this substrate is applicable to histologic analysis (26). D-Val-Leu-Lys-p-nitroanilide (25), and Tos-Gly-Pro-Lys-p-nitroanilide (19,21) are also commercially available synthetic peptides for plasmin assay, but these peptides are not applicable to histologic analysis. Because of the extremely high sensitivity of naphthyl ester, the histologic localization obtained with the present method was sharp and hence could be used for serial section assays or for double staining. However, the definitive identification of the proteolytic activity detected by this method as plasmin in vivo is difficult. To the best of our knowledge, no substrate is completely specific for plasmin. Tos-Lys-NE, D-Val-Leu-Lys-p-nitroanilide (25), and Tos-Gly-Pro-Lys-p-nitroanilide (19,21) have high affinity for and are sensitive to plasmin, but they are also sensitive to other proteases. We attempted to specify the enzyme with the use of several inhibitors. The addition of EDTA, E-64, tPA antibody, or amiloride did not suppress the activity; thus, we could tell that the protease was not an MMP, a cysteine protease, tPA, or uPA but could not exactly specify that it was plasmin. E-64 was used to assess the contribution of cysteine proteases because SPEB, another potent nephritogenic antigen, is a cysteine protease that is deposited in the glomeruli of APSGN patients (9). Our results indicate that cysteine proteases did not contribute to the activity assayed. Aprotinin is a widely used plasmin inhibitor and did suppress the activity in vivo, but it potentially inhibits other proteases such as trypsin or chymotrypsin. α2-AP inhibited plasmin activity in vitro (Figure 1), but it did not inhibit the glomerular plasmin-like activity in vivo in the present study (Figure 2E). However, this result was in keeping with our concept that deposited NAPr can trap and maintain plasmin activity in vivo, because receptor-bound plasmin should be protected from inactivation by α2-AP, according to the results of Boyle et al. (16).

ε-Aminocaproic acid and tranexamic acid are lysine analogs that are also popularly used as plasmin inhibitors. However, neither sufficiently inhibited plasmin activity in vitro in our assay system (data not shown), probably because of the extremely high affinity of the substrate for plasmin that we used. Thus, we did not identify a specific inhibitor of in vivo activity. However, the identical distribution of proteolytic activity with the plasmin receptor (NAPr); the relation of the proteolytic activity with plasminogen deposition; complete inhibition of the activity by aprotinin; and exclusion of MMP, cysteine proteases, tPA, and uPA strongly suggest that it represents plasmin. Nonetheless, we used the term “plasmin-like” instead of “plasmin.”

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 fibrinogen deposition and NAPr deposition or plasmin-like activity is not surprising, because plasmin, which is suspected to be bound by and co-localized with NAPr, should have the ability to degrade fibrinogen, thereby decreasing the fibrinogen 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)olyis 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). NAPr is a potential candidate for the nephritogenic antigen because it is highly antigenic (12). However, this study clearly showed that NAPr 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 NAP1r deposition in the glomeruli of APSGN patients (11), in clear contrast to the similarity of plasmin-like activity and NAP1r deposition in the present study. We suspect that direct complement activation by NAP1r 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 NAPlR into the circulation. Circulating NAPlR binds to the renal glomeruli on the mesangial matrix and GBM, probably through its adhesive character (12). Bound NAPlR 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 NAPlR 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 NAPlR isolated from GAS is a nephritogenic agent for APSGN.

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References


