

Plasminogen and Plasminogen Activators Protect against Renal Injury in Crescentic Glomerulonephritis

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Summary

The plasminogen/plasmin system has the potential to affect the outcome of inflammatory diseases by regulating accumulation of fibrin and other matrix proteins. In human and experimental crescentic glomerulonephritis (GN), fibrin is an important mediator of glomerular injury and renal impairment. Glomerular deposition of matrix proteins is a feature of progressive disease. To study the role of plasminogen and plasminogen activators in the development of inflammatory glomerular injury, GN was induced in mice in which the genes for these proteins had been disrupted by homologous recombination. Deficiency of plasminogen or combined deficiency of tissue type plasminogen activator (tPA) and urokinase type plasminogen activator (uPA) was associated with severe functional and histological exacerbation of glomerular injury. Deficiency of tPA, the predominant plasminogen activator expressed in glomeruli, also exacerbated disease. uPA deficiency reduced glomerular macrophage infiltration and did not significantly exacerbate disease. uPA receptor deficiency did not effect the expression of GN. These studies demonstrate that plasminogen plays an important role in protecting the glomerulus from acute inflammatory injury and that tPA is the major protective plasminogen activator.

The plasminogen/plasmin system has important functions in the dissolution of fibrin and in the breakdown of other extracellular matrix proteins (1). It has pivotal roles in clot lysis and wound healing (2, 3), but also has the potential to influence the outcome of inflammatory processes, particularly where fibrin and extracellular matrix deposition play an important role (4).

Glomerulonephritis (GN) results from immunologically mediated glomerular inflammation and is the most common cause of renal failure worldwide. Glomerular fibrin deposits are observed in severe proliferative and in crescentic forms of human GN, in which the outcome for renal function is often poor (5). These forms of GN are associated with recruitment of T cells and monocytes (6) and cross-linking of fibrin both in the glomerular tuft and within Bowman's space. Fibrin in Bowman's space is chemotactic for macrophages (7). This accumulation of macrophages and fibrin forms the histological appearance of a crescent (8, 9).

The glomerulus, being a complex filter, is particularly susceptible to injury induced by local deposition of fibrin and other matrix proteins. Defibrination studies have demonstrated that fibrin is an important mediator of glomerular injury and crescent formation in experimental GN (8, 9).

Local dysregulation of coagulation (10) and fibrinolysis (11) may contribute to glomerular fibrin deposition. Upregulation of tissue factor has been demonstrated in experimental (12) and human crescentic GN (13). Dysregulation of the plasminogen/plasmin system, with decreased glomerular fibrinolytic activity, decreased tissue type plasminogen activator (tPA) and increased plasminogen activator inhibitor-1 (PAI-1) has also been demonstrated in experimental crescentic GN (11) and in human GN (14).

The extent to which local generation of plasmin plays a protective role in preventing glomerular fibrin deposition in crescentic GN is not known. Plasmin can cleave cross-linked fibrin, directly degrade matrix proteins (1) and can activate matrix metalloproteinases (15), suggesting a protective function for plasmin in the glomerulus. Conversely, plasmin may activate latent transforming growth factor- β (16), which promotes matrix deposition (17), enhances PAI-1 production (17) and down regulates plasminogen activator production in the kidney (18).

To evaluate the plasminogen/plasmin system in immune initiated glomerular injury, proliferative GN was induced in mice with genetic deficiencies of key molecules of this system. These included mice deficient in plasminogen (*Plg*^{-/-}) (19), mice with combined deficiencies of tPA and uPA

(*tPA*^{-/-}:*uPA*^{-/-}) (2), mice with single deficiencies of either tPA (*tPA*^{-/-}) or urokinase-type plasminogen activator (*uPA*^{-/-}) (2) and mice deficient in the cell surface receptor for uPA, (*uPAR*^{-/-}) (20). Mice were compared with their appropriate genetically unmodified (or wild-type [WT]) mice in each case. GN was induced by planting an exogenous Ag, sheep anti-mouse GBM globulin, in the glomeruli of mice previously sensitized to sheep globulin (SG) which induced a rapidly progressive, proliferative GN with renal impairment in WT mice.

Materials and Methods

Study Design. Mice deficient in plasminogen, tPA, uPA, both tPA and uPA, and uPAR were created and characterized as previously described (2, 19, 20). Mice were 48–69 d old and clinically free of disease at induction of GN. Studies of *Plg*^{-/-}, *tPA*^{-/-}, *uPA*^{-/-}, *uPAR*^{-/-} mice and their WT mice used male mice weighing between 20 and 30 g, except *Plg*^{-/-} mice, which weighed between 10.7 and 21.3 g. *tPA*^{-/-}:*uPA*^{-/-} mice and their WT controls were female. WT female mice weighed 16.9 to 20.5 g and *tPA*^{-/-}:*uPA*^{-/-} mice weighed 13.7 to 18.8 g.

Sheep anti-mouse GBM globulin was prepared as previously described (21). Mice were sensitized with 2 mg SG in 200 μ l of Freund's Complete Adjuvant (Sigma Chemical Co., Castle Hill, Australia) injected s.c. at two sites. 5 mg of sheep anti-mouse GBM globulin was injected i.v. after 10 d to initiate glomerular disease. The dose of anti-mouse GBM globulin was reduced to 4 mg for mice weighing between 15.0 to 19.9 g and to 3 mg for mice less than 15.0 g in weight.

Animals were studied 10 days after anti-GBM globulin injection. *tPA*^{-/-} mice ($n = 10$), *uPA*^{-/-} mice ($n = 9$), and *uPAR*^{-/-} mice ($n = 8$) were matched with WT mice ($n = 9$), with equal fractions of each WT genotype. Female *tPA*^{-/-}:*uPA*^{-/-} mice ($n = 5$) were matched with female WT mice ($n = 7$). Because of severity of disease in the *Plg*^{-/-} mice ($n = 8$), they were studied 8 days after induction of disease, together with WT mice ($n = 4$). Two *Plg*^{-/-} mice, which were killed because of severe disease (at days 6 and 7), and another *Plg*^{-/-} animal that died on day 7 of disease were included in the analysis.

Assessment of Glomerular Injury. Renal tissue was fixed in Bouin's fixative, embedded in paraffin, cut in 3 μ m sections and stained with periodic acid Schiff's stain (PAS) for histological analysis. Glomerular cellularity was determined by counting cell nuclei in at least 20 glomeruli per animal and results expressed as cells per glomerular cross section (cells/gcs). The presence of PAS+ material within glomeruli was scored semiquantitatively in a minimum of 50 glomeruli per mouse as follows: 0 = no deposition of PAS+ material, 1 = up to one third of the cross-sectional area of the glomerulus staining PAS+, 2 = one third to two thirds involvement, 3 = greater than two thirds involvement. Crescent formation (defined as the presence of 3 or more layers of cells in Bowman's space) was assessed (minimum of 50 glomeruli per mouse) and results expressed as a percentage incidence.

Tissue sections (6 μ m) were stained to demonstrate macrophages using a three-layer immunoperoxidase technique, as previously described (22). The primary Ab was M1/70 (monoclonal anti-mouse Mac-1; American Type Culture Collection [ATCC], Rockville, MD). Sections of spleen provided a positive control for each animal and protein G-purified rat Ig was substituted for the primary Ab to provide a negative control. A minimum of 20 glomeruli were assessed per mouse and results expressed as cells/gcs.

Fibrin immunofluorescence was performed on sections (4 μ m) cut from frozen tissue. An IgG fraction of goat anti-mouse fibrin/fibrinogen serum (Nordic Immunological Laboratories, Drawer, CA) was conjugated to digoxigenin (Boehringer Mannheim, Castle Hill, Australia) and used at a concentration of 35 μ g/ml. Binding of the primary Ab was detected with FITC-conjugated sheep anti-digoxigenin Fab fragments (Boehringer Mannheim) at a dilution of 1 in 50. Glomerular fibrin deposition was assessed semiquantitatively (minimum of 30 glomeruli per mouse) using the scoring system described for the assessment of glomerular PAS+ material.

Serum creatinine (sCr) was measured by the alkaline picric acid method by autoanalyzer (Cobas Bio, Roche Diagnostics, Basel, Switzerland). As sCr is proportional to weight, values for *Plg*^{-/-} and *tPA*^{-/-}:*uPA*^{-/-} mice were adjusted to the mean weight of their WT group at the end of the experiment.

Measurement of Circulating Mouse Anti-Sheep Globulin Antibody. Mouse anti-sheep globulin titers were assessed by ELISA on serum collected at the end of the experiment. Microtiter plates were coated with 10 μ g/ml SG in carbonate/bicarbonate buffer (pH 9.5), washed, blocked with 1% BSA, then incubated with dilutions of mouse serum. Bound mouse Ig was detected with horseradish peroxidase conjugated rabbit anti-mouse Ig (Sigma Chemical Co.) at a dilution of 1 in 2,000 using 0.1 M 2,2'-azino-di-3-ethylbenzthiazoline sulphonate (ABTS; Boehringer Mannheim, Castle Hill, Australia) in 0.02% H₂O₂ as the substrate. The absorbance at 405 nm was read on a microtiter plate reader. Serum from each mouse was tested in serial dilutions from 1 in 100 to 1 in 126,000, with serum from six non-immunized mice used as negative controls.

Statistics. Results are expressed as the mean \pm SEM. Significances of differences were determined by the unpaired *t* test for the *Plg*^{-/-} and *tPA*^{-/-}:*uPA*^{-/-} mice and their respective WT. Multiple group comparisons (i.e., *tPA*^{-/-}, *uPA*^{-/-}, *uPAR*^{-/-} and WT mice) were analyzed by ANOVA, followed by Fisher's protected least significant differences (PLSD) test.

Results

Effects of Plasminogen and Plasminogen Activator Deficiencies on Histological Features of GN. All WT mice developed proliferative GN (Fig. 1 A) with occasional glomeruli exhibiting crescent formation. Areas of basement membrane thickening were demonstrated by silver methenamine staining (not shown) and segmental fibrin deposition was observed within the glomerular tuft by direct immunofluorescence (Fig. 1 B). Glomeruli of both *Plg*^{-/-} and *tPA*^{-/-}:*uPA*^{-/-} mice were more severely affected, with tuft necrosis and relatively hypocellular glomeruli (Fig. 1, C and E). Despite this relative hypocellularity, there were more macrophages within glomeruli and significantly increased numbers of glomeruli exhibiting crescent formation compared to their respective WT controls (Table 1). Crescents in the *Plg*^{-/-} and *tPA*^{-/-}:*uPA*^{-/-} animals were larger than those found in WT mice and contained both cellular elements (Fig. 1, C and E, arrowed), and fibrin (Fig. 1, D and F, arrowed). Deposition of PAS+ material within glomeruli was significantly increased (Table 1) indicating more extra cellular matrix deposition in nephritic *Plg*^{-/-} and *tPA*^{-/-}:*uPA*^{-/-} mice, compared to WT animals.

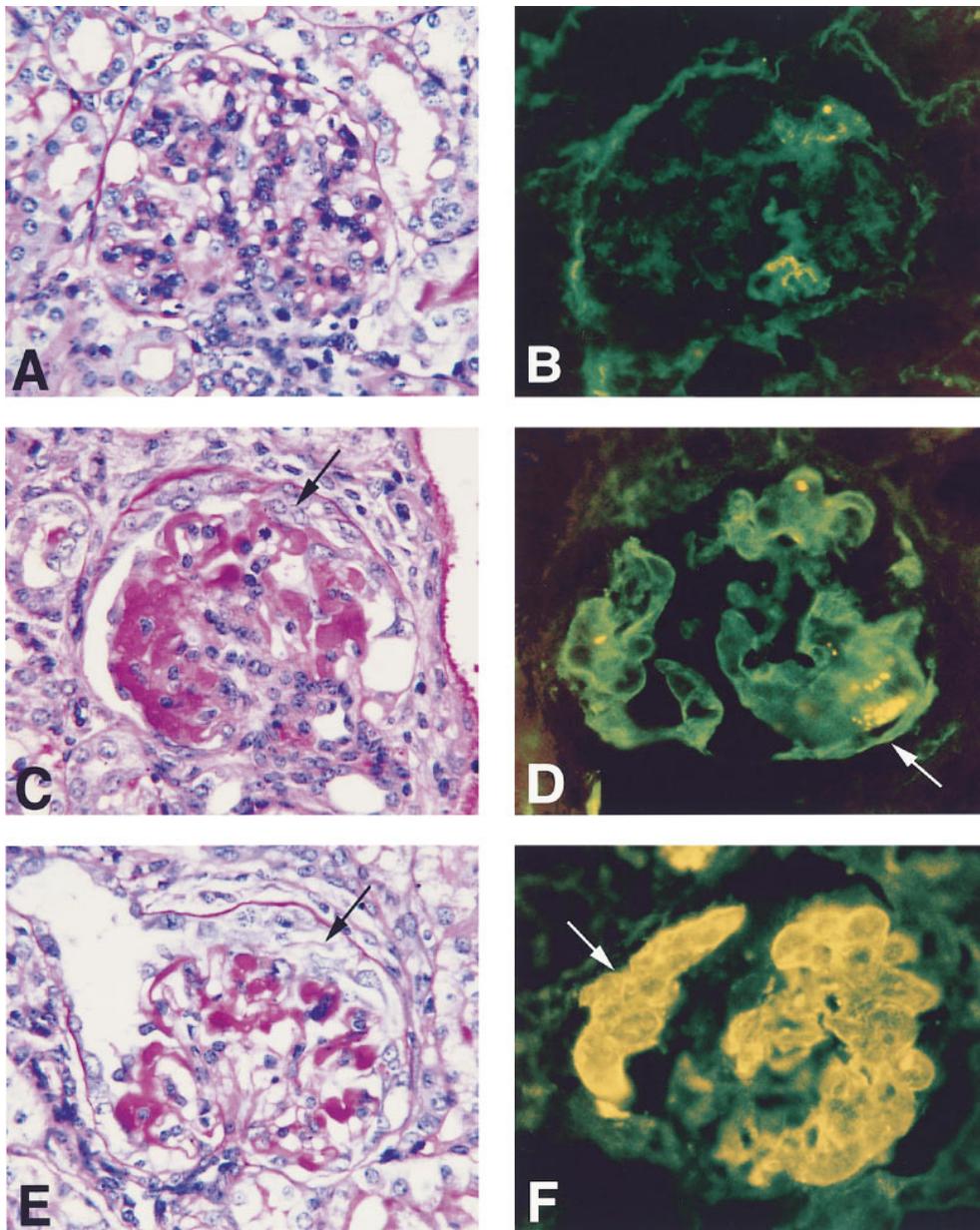


Figure 1. Photomicrographs of representative glomeruli from WT mice (A and B, *Plg*-deficient mice (C and D) and combined *tPA*- and *uPA*-deficient mice (E and F) with GN demonstrating light microscopic appearances (PAS stain, magnification $\times 350$) and fibrin deposition (immunofluorescence, magnification $\times 400$). WT mice with GN at day 10 showed proliferative GN (A) and segmental fibrin deposition within the glomerular tuft (B). *Plg*-deficient mice at day 8 developed hypocellular glomeruli with extensive deposition of PAS+ material in the glomerular tuft and cellular crescent formation (C, arrowed) and extensive fibrin deposition within the glomerular tuft and in Bowman's space (D, arrowed). Mice with combined *tPA*, *uPA* deficiency at day 10 showed similar histological features to *Plg*-deficient mice (E) and similar extensive glomerular fibrin deposition (F).

Of mice deficient in a single plasminogen activator or uPAR, only *tPA*^{-/-} mice exhibited histologically more severe disease with increased glomerular hypercellularity and increased numbers of glomerular macrophages. Deposition of PAS+ material was increased compared to WT mice (Table 1) but did not reach the extent of the *Plg*^{-/-} or *tPA*^{-/-}:*uPA*^{-/-} mice. Mice deficient in uPA had fewer macrophages infiltrating glomeruli compared to WT mice, but no other differences in the histological appearances or quantitative indices of renal injury (Table 1). Nephritic *uPAR*^{-/-} mice showed similar histological appearances to WT mice (Table 1).

Effect of Plasminogen and Plasminogen Activator Deficiencies on Glomerular Fibrin Deposition in GN. In contrast to the segmental fibrin deposition observed only within the glom-

erular tuft in WT mice (Fig. 1 B), *Plg*^{-/-} and *tPA*^{-/-}:*uPA*^{-/-} mice developed extensive glomerular fibrin deposition within the glomerular tuft and in Bowman's space (Fig. 1, D and F). Semi-quantitative scoring of the extent of glomerular fibrin deposition confirmed that these differences were statistically significant compared to their WT controls (Fig. 2). Mice deficient in *tPA* also showed significant increases in glomerular fibrin deposition over WT (Fig. 3), though the extent of glomerular fibrin deposition did not reach that of the *Plg*^{-/-} or the *tPA*^{-/-}:*uPA*^{-/-} mice. There were no significant changes in glomerular fibrin deposition in nephritic *uPA*^{-/-} and *uPAR*^{-/-} mice compared to WT mice.

Effect of Plasminogen and Plasminogen Activator Deficiencies on Renal Function in GN. The importance of the increased

Table 1. Histological Features of GN in Mice

Group	Glomerular crescent formation	PAS+ Material (0-3+)	Glomerular Hypercellularity	Glomerular Macrophages
	% glomeruli		cells/gcs	cells/gcs
WT (Plg)	4.8 ± 1.9	0.79 ± 0.08	66.3 ± 1.2	1.47 ± 0.17
<i>Plg</i> ^{-/-}	18.1 ± 3.1*	1.94 ± 0.27*	54.0 ± 1.9 [‡]	4.85 ± 1.03*
WT (tPA, uPA)	2.9 ± 1.2	0.64 ± 0.09	58.6 ± 2.8	1.38 ± 0.05
<i>tPA</i> ^{-/-} : <i>uPA</i> ^{-/-}	15.4 ± 2.9 [§]	1.55 ± 0.24 [§]	45.3 ± 4.4*	3.92 ± 0.52 [‡]
WT	3.9 ± 1.9	0.75 ± 0.20	60.1 ± 1.2	1.46 ± 0.14
<i>tPA</i> ^{-/-}	16.1 ± 8.0	1.27 ± 0.05 [¶]	69.7 ± 1.6 [¶]	2.22 ± 0.28 [‡]
<i>uPA</i> ^{-/-}	6.1 ± 1.3	0.80 ± 0.07	62.4 ± 2.2	0.82 ± 0.08*
<i>uPAR</i> ^{-/-}	9.6 ± 3.5	0.87 ± 0.06	64.8 ± 2.3	1.31 ± 0.18

Comparison of histological features of GN in plasminogen (*Plg*^{-/-}), combined plasminogen activator-deficient (*tPA*^{-/-}:*uPA*^{-/-}), tPA (*tPA*^{-/-}), uPA (*uPA*^{-/-}) and uPA receptor (*uPAR*^{-/-})-deficient mice and their appropriate WT controls. Results are expressed as the mean ± SEM.

*Results significantly different to WT at the $P < 0.05$ level; [‡] $P < 0.01$ level; [§] $P < 0.005$ level; [¶] $P < 0.001$ level (Fisher's PLSD test).

glomerular deposition of fibrin and other matrix proteins induced by deficiency of plasminogen activation was demonstrated by the increased impairment of renal function observed in plasminogen or plasminogen activator-deficient mice. *Plg*^{-/-} and *tPA*^{-/-}:*uPA*^{-/-} mice developed more severe renal failure, measured by sCr compared to their appropriate WT groups (Fig. 2). Mice deficient in tPA alone also developed more renal impairment compared to their WT control. *uPA*^{-/-} mice showed a trend towards more severe renal impairment (sCr $P = 0.08$ compared to WT). The severity of renal impairment of nephritic *uPAR*^{-/-} mice was unchanged from that of WT mice with GN (Fig. 3).

Assessment of Systemic Immune Responses. In addition to the activity of the coagulation and fibrinolytic systems, the extent of immune response to the disease initiating antigen is important in determining the severity of injury in this model of GN. Although deficiencies of the plasminogen/plasmin system are not known to modulate immune responses, the specific immune response to the nephritogenic Ag (SG) was quantified in the various groups of mice with GN by measurement of circulating levels of mouse anti-sheep globulin antibodies. Ab titers, measured by ELISA at dilutions of sera from 1 in 100 to 1 in 102,400, were not significantly different for any genetically deficient mice compared with their WT control group, except in *Plg*^{-/-} mice that showed slightly lower absorbance values at the two highest antibody dilutions. These minor differences are unlikely to significantly modulate the expression of GN and could not contribute to the more severe disease seen in these *Plg*^{-/-} mice.

Discussion

These studies demonstrate that generation of plasmin plays a critical role in protecting glomerular structure and

function in rapidly progressive GN. A similar degree of severe glomerular damage was observed with plasminogen deficiency and combined deficiency of tPA and uPA, suggesting that these two plasminogen activators contribute the major plasminogen activating activity in GN. This supports the earlier observation that tPA and uPA are the only two physiologically significant plasminogen activators in mice (19). Mice deficient in tPA alone also developed more severe renal injury than WT mice, but not to the extent observed in combined PA or the *Plg*-deficient mice. More severe GN did not develop in *uPA*^{-/-} or *uPAR*^{-/-} mice.

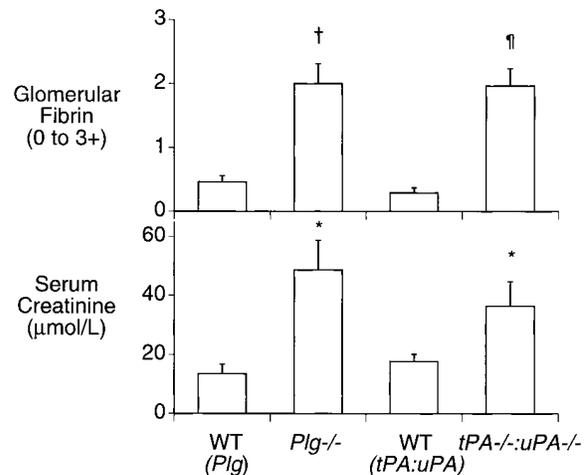


Figure 2. Glomerular fibrin deposition and renal function (sCr) in *Plg*^{-/-}, WT (*Plg*), *tPA*^{-/-}:*uPA*^{-/-} and WT (*tPA*:*uPA*) mice (* indicates $P < 0.05$, [‡] $P < 0.01$, [†] $P < 0.001$, compared to appropriate WT control by unpaired *t* test).

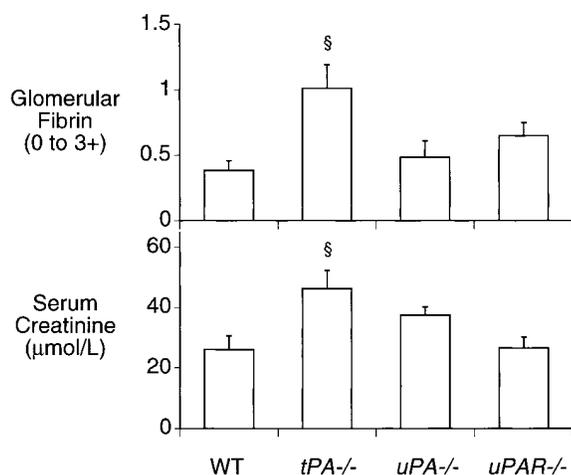


Figure 3. Glomerular fibrin deposition and renal function (sCr) in *tPA*^{-/-}, *uPA*^{-/-}, *uPAR*^{-/-}, and WT mice. [§]*P* < 0.005 compared to WT control by Fisher's PLSD test.

In the initial characterization of mice with combined deficiencies of tPA and uPA, minor spontaneous renal fibrin deposition was reported in a minority of animals beyond the age of 2 to 3 mo (2). However, in this study disease was induced in *tPA*^{-/-}·*uPA*^{-/-} mice that were younger than 2 mo old to avoid any possibility that spontaneous glomerular fibrin deposition may significantly influence the results. In the original description of *Plg*^{-/-} mice, dilated renal pelvises had been noted in four mice, without fibrin deposition (19). None of the *Plg*^{-/-} mice in the current study had macroscopic or microscopic evidence of pelvicalyceal dilatation or obstruction.

There was a significantly increased propensity to form crescents in glomeruli of mice with plasminogen or combined plasminogen activator deficiency. Crescents are the result of accumulation of fibrin and macrophages, together with proliferation of glomerular epithelial cells in Bowman's space. They are indicative of severe glomerular injury and a poor outcome for renal function. Fibrin has previously been shown to be an important chemotactic stimulus for accumulation of macrophages in Bowman's space and crescent formation (7). The enhanced crescent formation and macrophage infiltration in *Plg*^{-/-} and *tPA*^{-/-}·*uPA*^{-/-} mice is consistent with the observation that enhanced glomerular fibrin accumulation occurring in the absence of local plasmin generation provides a greater stimulus for inflammatory cell recruitment and crescent formation. The more intense inflammatory reaction resulted in necrosis of the glomerular tuft and hypocellular glomeruli.

tPA is the predominant PA within mouse (23), rabbit (11) and human (24, 25) glomeruli and is produced mainly by glomerular endothelial cells. The major site of uPA production within the murine kidney is the tubular epithelium (23), although human glomerular epithelial cells in culture also produce uPA (24, 26) and uPA immunofunctional activity has been demonstrated in rabbit (11) and human glo-

meruli (25). uPA has been proposed to have a role in maintenance of tubular patency (23).

Mice deficient in tPA alone showed significantly increased renal failure, glomerular hypercellularity, glomerular macrophage numbers, glomerular fibrin deposition and deposition of PAS+ material within glomeruli. They also demonstrated a trend towards increased crescent formation. Glomerular capillary thrombosis was minimal in nephritic *tPA*^{-/-} mice and their glomeruli were hypercellular. However, the severity of injury in these mice was not sufficient to cause necrosis of the glomerular tuft.

Mice deficient in uPA alone did not show significant differences in histological indices of disease from WT mice, but did show a trend towards increased renal impairment. The significance of this result is unclear, but the extensive expression of uPA in renal tubular epithelial cells of normal mice suggests that tubular dysfunction may have contributed to any increased renal impairment in *uPA*^{-/-} mice. The demonstration of significantly reduced glomerular macrophage infiltration in *uPA*^{-/-} mice supports the hypothesis that uPA assists migration of macrophages to sites of inflammatory injury.

The increased renal impairment, fibrin deposition and other histological indices of disease in the *tPA*^{-/-} mice suggest a more important role for tPA in protecting glomeruli against inflammatory injury, but do not exclude a role for uPA. However, the more severe glomerular injury in mice deficient in both plasminogen activators implies that the effects of uPA deficiency in glomerular inflammation become evident in the absence of tPA.

Deficiency of the uPA receptor did not significantly modify the histological or functional manifestations of GN. The observation that glomeruli of *uPAR*^{-/-} mice contained similar numbers of macrophages to WT mice suggests that unlike uPA, *uPAR* is not important in macrophage infiltration into nephritic glomeruli. In normal mice, *uPAR* mRNA is expressed only at low levels in the kidney, in contrast to the abundant renal expression of uPA mRNA (27). Sites of uPA expression in the kidney do not correspond well to its receptor (27), suggesting that in the kidney uPA can act independently of *uPAR*. Although a prominent role for uPA and *uPAR* could not be demonstrated in this short term model of renal injury, this does not preclude a significant role for these proteins in chronic renal injury, where prolonged accumulation of matrix proteins leads to renal scarring and glomerulosclerosis.

In summary, these studies demonstrate an important protective role for plasminogen and plasminogen activators in acute inflammatory renal injury associated with crescentic GN. They are consistent with previous observations that tPA and uPA appear to be the major plasminogen activators in vivo but also suggest that the major protective role in acute inflammatory glomerular injury is attributable to tPA. uPA facilitates recruitment of macrophages into nephritic glomeruli. The uPA receptor does not appear to play any significant role in protecting the glomerulus in GN. Therapies aimed at enhancing or replacing fibrinolytic activity in crescentic GN may be of benefit in human disease.

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