Acute renal failure is often the result of ischemia-reperfusion (I/R) injury. Neutrophil influx is an important damaging event in I/R. Tissue-type plasminogen activator (tPA) not only is a major fibrinolytic agent but also is involved in inflammatory processes. A distinct upregulation of tPA after I/R, with de novo tPA production by proximal renal tubules, was found. For investigating the role of tPA in I/R, renal ischemia was induced in tPA−/− and wild-type (WT) mice by clamping both renal arteries for 35 min followed by reperfusion. Mice were killed 1, 5, and 10 d after reperfusion. After 1 d, tPA−/− mice displayed significantly less neutrophil influx into the interstitial area compared with WT mice. In addition, tPA−/− mice showed quicker recovery of renal function than WT mice. The protocol was repeated after injection of tPA-antisense oligonucleotides into WT mice, leading to even more explicit results: Antisense-treated mice showed less histologic damage, better renal function, and less neutrophil influx than control mice. Surprising, complement C3 concentration, levels of proinflammatory cytokines and chemokines, intercellular adhesion molecule-1 expression, and matrix metalloproteinase activity were similar in WT and tPA−/− mice. Plasmin activity levels in WT and tPA−/− kidneys were also comparable, indicating that tPA influences neutrophil influx into ischemic renal tissue independent from plasmin generation. This study shows that targeting tPA could be of therapeutic importance in treating I/R injury by diminishing neutrophil influx and preserving renal function.

Acute renal failure (ARF) is a severe and potentially life-threatening condition that is often the result of a prolonged period of renal ischemia, followed by reperfusion. Ischemia-reperfusion (I/R) injury frequently occurs in shock, sepsis, and renal artery stenosis and during renal transplantation procedures. I/R tissue damage, mainly comprising tubular necrosis, has been associated with a mortality rate of >50% throughout the past 40 yr, and treatment of this major clinical problem is still only supportive (1).

Numerous studies have shown that the inflammatory response initiated by I/R is an important determinant of tissue damage and ARF (2,3). The acute inflammatory response initiated by I/R is characterized by the induction of proinflammatory cytokines and chemokines and apparent upregulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) (3). Subsequently, the postischemic tissue is invaded by vast numbers of neutrophils that have been recognized as important contributors to actual tissue damage through the release of oxygen-derived radicals (4).

Besides their obligatory role in fibrinolysis, components of the fibrinolytic system (FS) have recently been recognized as players in the recruitment of inflammatory cells. The FS consists of two serine proteases—tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA)—that both facilitate the conversion of plasminogen into plasmin, which is the crucial protease in the fibrinolytic cascade (5). The activity of both PA is inhibited by plasminogen activator inhibitor type 1 (PAI-1) and, to a lesser extent, PAI-2 (5). Besides its contribution to fibrinolysis, tPA has been shown to play a role in various other mechanisms, such as the turnover of extracellular matrix (ECM) components through activation of matrix metalloproteinases (MMP) and immunomodulatory functions (6–8).

In the kidney, tPA is constitutively expressed by endothelial cells, glomerular cells, and epithelial cells of the distal collecting duct. Under normal conditions, no tPA expression is present in the corticomedullary tubular compartment (9). A number of studies have demonstrated the importance of tPA in a variety of glomerular diseases. tPA-deficient mice exhibited significantly increased glomerular fibrin deposition, glomerular hypercellularity, and renal failure in a model of crescentic glomerular diseases. A distinct upregulation of tPA after I/R, with de novo tPA production by proximal renal tubules, was found. For investigating the role of tPA in I/R, renal ischemia was induced in tPA−/− and wild-type (WT) mice by clamping both renal arteries for 35 min followed by reperfusion. Mice were killed 1, 5, and 10 d after reperfusion. After 1 d, tPA−/− mice displayed significantly less neutrophil influx into the interstitial area compared with WT mice. In addition, tPA−/− mice showed quicker recovery of renal function than WT mice. The protocol was repeated after injection of tPA-antisense oligonucleotides into WT mice, leading to even more explicit results: Antisense-treated mice showed less histologic damage, better renal function, and less neutrophil influx than control mice. Surprising, complement C3 concentration, levels of proinflammatory cytokines and chemokines, intercellular adhesion molecule-1 expression, and matrix metalloproteinase activity were similar in WT and tPA−/− mice. Plasmin activity levels in WT and tPA−/− kidneys were also comparable, indicating that tPA influences neutrophil influx into ischemic renal tissue independent from plasmin generation. This study shows that targeting tPA could be of therapeutic importance in treating I/R injury by diminishing neutrophil influx and preserving renal function.

Tissue-Type Plasminogen Activator Modulates Inflammatory Responses and Renal Function in Ischemia Reperfusion Injury

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Acute renal failure is often the result of ischemia-reperfusion (I/R) injury. Neutrophil influx is an important damaging event in I/R. Tissue-type plasminogen activator (tPA) not only is a major fibrinolytic agent but also is involved in inflammatory processes. A distinct upregulation of tPA after I/R, with de novo tPA production by proximal renal tubules, was found. For investigating the role of tPA in I/R, renal ischemia was induced in tPA−/− and wild-type (WT) mice by clamping both renal arteries for 35 min followed by reperfusion. Mice were killed 1, 5, and 10 d after reperfusion. After 1 d, tPA−/− mice displayed significantly less neutrophil influx into the interstitial area compared with WT mice. In addition, tPA−/− mice showed quicker recovery of renal function than WT mice. The protocol was repeated after injection of tPA-antisense oligonucleotides into WT mice, leading to even more explicit results: Antisense-treated mice showed less histologic damage, better renal function, and less neutrophil influx than control mice. Surprising, complement C3 concentration, levels of proinflammatory cytokines and chemokines, intercellular adhesion molecule-1 expression, and matrix metalloproteinase activity were similar in WT and tPA−/− mice. Plasmin activity levels in WT and tPA−/− kidneys were also comparable, indicating that tPA influences neutrophil influx into ischemic renal tissue independent from plasmin generation. This study shows that targeting tPA could be of therapeutic importance in treating I/R injury by diminishing neutrophil influx and preserving renal function.

ulonephritis (10). In addition, treatment with recombinant tPA notably decreased matrix accumulation in mesangioproliferative glomerulonephritis in rats (11). These studies indicate a protective role of tPA in the glomerular compartment.

Recently, a possible deleterious role of tPA within the tubulointerstitial compartment of the kidney has been recognized. Yang et al. (12) showed that tPA−/− mice displayed considerably less fibrosis in a model of obstructive nephropathy, as a result of reduction of MMP-9–induced tubular basement membrane disruption. In addition, our study demonstrates that tPA-gene deletion in mice leads to significantly less inflammation and quicker recovery of renal function after I/R injury.

Materials and Methods

**Mice and Experimental Protocol**

**tPA−/− mice,** constructed on a C57Bl/6 background (13), were bred in our animal facility. C57Bl/6 mice (Charles River, Maastricht, The Netherlands) served as a wild-type (WT) control.

Renal ischemia was induced by clamping both renal arteries for 35 min in 8- to 10- wk-old male mice under general anesthesia (0.07 ml/10 g mouse of fentanyl citrate fluanisone midazolam mixture, containing 1.25 mg/ml midazolam [Roche, Mijdrecht, The Netherlands], 0.08 mg/ml fentanyl citrate, and 2.5 mg/ml fluanisone [Janssen Pharmaceuticals, Beerse, Belgium]). After removal of the clamps, the abdomen was closed. Mice (n = 8 per group) were killed 1, 5, and 10 d after surgery. Plasma was collected and kidneys were partly fixed in formalin and partly snap-frozen. Sham-operated mice underwent the same procedure without clamping of the arteries and were killed 1 d after surgery. All experimental procedures were approved by the Animal Care and Use Committee of the University of Amsterdam, The Netherlands.

Oligodeoxynucleotide (ODN)-treated C57Bl/6 mice received 24 h before surgery and during the operation an intraperitoneal injection of tPA antisense (GTATCTATGTCAACAGGCT) or nonsense (ACTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTACTAGTAC

**Histology and Immunohistochemistry**

Four-micrometer paraffin sections were stained with periodic acid-Schiff after diastase. Tubular injury, characterized by necrosis, dilation, cast deposition, and loss of brush border, was graded to the extent of corticomedullary region involvement in 10 randomly chosen, nonoverlapping fields (×100 magnification), on a scale from 0 to 5, as follows: 0, absent; 1, 0 to 10%; 2, 10 to 25%; 3, 25 to 50%; 4, 50 to 75%; and 5, 75 to 100% (17). Total values were expressed as tubular injury scores.

Immunostainings were performed in a standard manner. Primary antibodies used were anti-mouse Ly6-G (Pharmingen, Erembodegem, Belgium), F4/80 (Serotec, Oxford, UK), anti-active caspase 3 (Cell Signaling Technology, Beverly, MA), aquaporin-4 (Chemicon, Hampshire, UK), anti-mouse fibrinogen (Ilex; Accurate Chemical & Scientific, Westbury, NY), and anti-mouse ICAM-1 (R&D Systems, Minneapolis, MN). For detection of FITC-labeled ODN, sections were stained using rabbit anti-FITC (DAKO, Glostrup, Denmark).

After incubation with the secondary (horseradish peroxidase-labeled) antibodies (DAKO), horseradish peroxidase was visualized using 3,3-diamino-benzidine tetrachloride (Sigma, St. Louis, MO). Neutrophils were counted in eight randomly chosen fields (×400 magnification). The amount of fibrin and ICAM-1 staining was measured in eight randomly chosen fields (×200 magnification) using digital image analysis (Image pro-plus; Mediacybernetics, Dortmund, Germany).

**Determination of Renal Function**

Plasma urea and creatinine were determined by routinely used clinical diagnostic urease and creatinase assays.

**In Vitro Migration Assay**

Chemotaxis system plates (Neuroprobe Inc., Gaithersburg, MD) were filled with HAM-F12 medium (Life Technologies, Breda, The Netherlands) that contained various concentrations of formyl-methionyl-leucyl-phenylalanine (Sigma), recombinant murine tPA (Molecular Innovations Inc., Southfield, MI), or medium alone. Wells were covered with a 5-μm pore size filter on which 50 μl of mouse whole blood was applied. After incubation for 45 min at 37°C, a Ly6G staining was performed on the bottom side of the filter and the number of transmigrated neutrophils was counted.

**ELISA**

Concentrations of tPA, IL-1β, IL-6, keratinocyte-derived chemokine (KC), macrophage inflammatory protein-2 (MIP-2) and myeloperoxidase (MPO) were measured in renal homogenates by specific ELISA according to the manufacturers’ instructions (tPA: Molecular Innovations; MPO: HyCult Biotechnology, Uden, Netherlands; others: R&D Systems). Complement C3 was measured by ELISA, using anti-mouse C3 (ICN Biomedicals, Irvine, CA) as capture antibody and biotinylated anti-mouse C3c (Nordic, Tilburg, The Netherlands) as detection antibody.

**Plasmin Activity**

Plasmin activity in kidney homogenates was determined following the method of Arza et al. (18) using the chromogenic substrate S-2403 (Chromogenix, Milan, Italy). Kidney homogenates were allowed to

After hybridization, slides were washed, and bound alkaline phosphatase activity was visualized with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate, toluidine salt (Roche). A number of slides were immunostained for aquaporin-4, a specific epithelial marker for proximal tubules in mice (16), after the ISH was completed.
incubate with the substrate for 10, 30, or 45 min at 37°C, after which the extinction at 405 nm was measured. Measurements were corrected for the protein concentrations of the samples.

**Gelatin Zymography for MMP**

*In situ* fluorescence zymography was performed on unfixed frozen sections as described previously (19) using DQ-gelatin as a substrate (EnzChek; Molecular Probes, Eugene, OR). Gelatin zymography was performed as described previously (20) using similar amounts of sonicated tissue, loaded onto a 10% polyacrylamide gel that contained 0.025% gelatin (Bloom 225; Sigma). Bands were analyzed by densitometry using National Institutes of Health Image software.

**Statistical Analyses**

Data were analyzed with an unpaired *t* test or Mann-Whitney *U* test when appropriate. *P* < 0.05 was regarded as statistically significant.

**Results**

**tPA is Upregulated during Renal Ischemia**

To investigate local tPA production upon I/R, we measured tPA protein levels by specific ELISA in kidney homogenates from I/R and sham-operated mice. Postischemic kidneys showed significantly higher levels of tPA in comparison with sham-operated kidneys (*T* = 24 h after operation; Figure 1).

To reveal the cellular source of tPA, we performed ISH on ischemic and sham kidneys. Sham kidneys showed tPA mRNA in podocytes (Figure 2A), endothelial cells (Figure 2B), and epithelial cells of the distal collecting ducts (data not shown). Postischemic kidneys showed a positive signal at the same sites and in damaged proximal tubules as well, which points to tubular tPA production upon ischemia-induced injury (Figure 2, C through F). Aquaporin-4 staining identified tPA mRNA-positive cells as proximal tubular epithelial cells (TEC; Figure 2, G and H). The observed upregulation of renal tPA expression strongly suggests a pathophysiologic role for tPA during I/R.

**tPA−/− Mice Show a Reduction of Ischemia-Induced Inflammation and a Quicker Recovery of Renal Function**

To investigate the role of tPA during renal I/R injury, we submitted WT and tPA−/− mice to 35 min of renal ischemia, followed by reperfusion. On day 1, no difference in renal function was observed between WT and tPA−/− mice. However, the tPA−/− mice showed a quicker recovery of renal function (Figure 3). Despite better renal function at day 5 in tPA−/− mice, the degree of renal damage was similar in both groups at all time points (Figure 4A). Tubular cell apoptosis as assessed by counting active caspase-3–positive cells was similar in both groups (data not shown). As expected, tPA−/− mice tended to display more and larger interstitial fibrin deposits at day 1, although the observed difference did not reach statistical significance (Figure 4B).

Striking, tPA−/− mice showed a significantly lower number of infiltrating neutrophils into the renal interstitium on day 1, when compared with WT mice (Figure 4C). The difference in neutrophil influx was confirmed by MPO-ELISA. WT mice
Figure 3. Renal function. Plasma urea and creatinine levels of wild-type (WT; □) and tPA−/− (●) mice. Both types of mice show a severe impairment of renal function at day 1 and show total recovery after 10 d. tPA−/− mice, however, tend to show quicker recovery of renal function at day 5. Data expressed as mean ± SEM; **P < 0.01; ***P < 0.001.

Figure 4. Histologic characteristics. (A) Periodic acid-Schiff after diastase staining of postischemic kidneys from WT and tPA−/− mice (representative microphotographs and semiquantitative scoring of tubular injury (T = 24 h after ischemia-reperfusion [I/R])). No differences in TEC damage between WT (□) and tPA−/− mice (●). Data expressed as mean total score ± SEM. (B) Fibrin staining (positive staining in brown). Although tPA−/− mice seem to display more and larger interstitial fibrin deposits than WT mice at day 1, there is no statistically significant difference. (□, WT; ●, tPA−/−; data expressed as mean percentage of slide surface ± SEM). (C) Representative microphotographs of Ly6G staining at T = 24 h (positive staining in brown). tPA−/− mice (●) show significantly less neutrophil influx than WT mice (□) at day 1, data expressed as mean number of granulocytes per hpf ± SEM. Magnification, ×40 in A and B; ×20 in C.
displayed significantly higher levels of MPO in kidney homogenates than tPA−/− mice (day 1: 225 ± 81 [WT] versus 79 ± 17 [tPA−/−], P < 0.05; day 5: 34 ± 3 [WT] versus 21 ± 4 [tPA−/−], P < 0.05; on day 10, MPO levels were undetectable). The number of infiltrating macrophages showed no differences between both genotypes (data not shown).

tPA-Antisense Treatment Reduced Renal Dysfunction, Tubular Damage, and Granulocyte Influx

To analyze whether tPA-antisense treatment could influence clinical outcome, as well as to rule out possible adaptive mechanisms that might occur in knockout animals, we repeated the I/R protocol after administration of tPA antisense ODN or nonsense ODN to WT mice. It is interesting that antisense-treated mice displayed significantly better renal function, associated with less renal tubular damage when compared with nonsense-treated mice (Figure 5, A through C). In accordance, antisense-treated mice showed an extremely low number of infiltrating neutrophils in comparison with nonsense-treated mice (Figure 5D). For evaluating in vivo targeting of ODN to the kidney, FITC-labeled ODN were injected intraperitoneally and detected by anti-FITC immunostaining. Intrapitoneally administered ODN were predominantly targeted to renal proximal TEC (shown in Figure 6A) and to a far lesser extent to lung, liver, and spleen (data not shown). On 1 d after I/R, antisense-treated mice showed a significantly reduced tPA activity in kidney homogenates compared with nonsense-treated mice, as determined by casein zymography (Figure 6B). uPA activity was unaffected.

These findings confirm the observations in tPA−/− mice, thus adding weight to the conclusion that tPA is crucially involved in the migration of neutrophils into the renal interstitium during I/R. To clarify the mechanism at play, we performed the following experiments.

In Vitro Migration Assay

The observed difference in neutrophil influx was not due to an a priori low number of circulating neutrophils in the knockout mice: Peripheral blood smears from both mouse strains were counted and revealed similar numbers of circulating neutrophils (data not shown). To dissect whether the observed difference in neutrophil influx was a result of intrinsic defects of tPA−/− neutrophils, we performed an in vitro migration assay using whole blood. Neutrophils of WT and tPA−/− mice showed a similar migratory potential toward a gradient of the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (Figure 7A).

To investigate whether tPA has a direct chemotactic effect on neutrophils, we performed an in vitro migration assay using recombinant murine tPA (r-tPA). As shown in Figure 7B, r-tPA did not exert chemotactic activity.

Proinflammatory Cytokines and Chemokines

Proinflammatory cytokines and chemokines were measured by ELISA in kidney homogenates. With regard to IL-6, KC, and MIP-2, no differences were found between WT and tPA−/− mice on day 1 after I/R (data not shown). IL-1β concentration was higher in WT than in tPA−/− mice, namely 14.1 ± 0.5 (WT) versus 12.5 ± 1.0 (tPA−/−) mean pg/mg protein ± SEM (P < 0.05). TNF-α levels were too low to be detected in this assay (data not shown).

In Figure 5, tPA-antisense treatment confirms the observations in tPA−/− mice. Antisense-treated mice ( ) show lower plasma urea levels (A), lower plasma creatinine levels (B), less tubular damage (C), and fewer infiltrating neutrophils (D) than nonsense-treated mice ( ) at day 1 after 1/R. Data expressed as mean ± SEM; *P = 0.05; **P < 0.05; ***P < 0.005; ****P < 0.001.
ICAM-1 Expression Is Similar in WT and tPA−/− Mice

Because ICAM-1 is one of the most important adhesion molecules that enable neutrophil influx during I/R and upregulation of ICAM-1 by tPA has been described (21), ICAM-1 immunostainings were performed. Peritubular endothelial cells stained positive for ICAM-1. Quantitative assessment of ICAM-1 staining showed similar expression in WT and tPA−/− mice (data not shown).

Plasmin Activity and Complement C3 Levels Are Similar in WT and tPA−/− Mice

Because tPA has the ability to generate plasmin, which subsequently can activate the complement system (22), plasmin activity and complement C3 levels were determined in kidney homogenates (T = 24 h after I/R). Plasmin activity and complement levels were similar in WT and tPA−/− kidneys (Figure 8). These data indicate that the plasminogen-plasmin-complement axis cannot account for the observed differences in neutrophil mobilization.

Activity and Localization of MMP

Numerous studies have shown that tPA can convert pro-MMP to active MMP, which have the ability to degrade ECM components and as a result clear the way for migrating neutrophils (8). MMP localization and activity were evaluated by in vitro migration assay with recombinant murine tPA (r-tPA) as chemoattractant, at concentrations of 1 and 10 ng/ml. There is no significant difference in the number of transmigrated neutrophils in comparison with the number of neutrophils that migrate toward medium alone.
situ fluorescence gelatin zymography on frozen sections of kidneys (T = 24 h after I/R) and gelatin gel zymography of kidney homogenates. WT and tPA− /− kidneys showed comparable gelatinolytic activity, which was localized predominantly in damaged proximal tubules (Figure 9). Mean surface as well as the intensity of the fluorescence signal was similar in both mouse strains (surface: 35.3 ± 1.8 [WT] versus 33.2 ± 2.4 [tPA− /− ] mean percentage fluorescence signal of slide surface ± SEM, P = 0.8; intensity: 38.9 ± 3.7 [WT] versus 42.5 ± 3.2 [tPA− /− ] mean fluorescence intensity units ± SEM, P = 0.4).

Subsequently, gelatin gel zymography was performed to identify the active MMP in the investigated kidneys. Although MMP-9 activity showed large variability among the different samples, active MMP-2 and MMP-9 were present in similar quantities (MMP-9: 30.6 ± 9.0 [WT] versus 49.5 ± 14.9 [tPA− /− ], P = 0.3; MMP-2: 3.5 ± 1.3 [WT] versus 3.2 ± 1.1 [tPA− /− ], P = 0.8, mean AU ± SEM).

**Discussion**

This study shows distinctive upregulation of tPA expression in damaged renal tubules during I/R. Furthermore, we show that tPA deletion or targeting in mice leads to less inflammation and quicker functional recovery, indicating a deleterious role of tPA during I/R.

Although postischemic upregulation of tPA expression has been reported comprehensively in brain ischemia (23,24) and in lung I/R (25), we describe for the first time an upregulation of endogenous tPA in ischemic kidneys at the protein level and an enhanced expression of tPA mRNA in damaged proximal TEC. This upregulation of renal tPA expression strongly suggests a pathophysiologic role for tPA during I/R. We demonstrate that tPA deletion or inhibition with antisense ODN results in a powerful anti-inflammatory effect during I/R injury.

The tPA− /− mice in our study displayed the same degree of tubular damage as the WT mice but less neutrophil influx. It is widely accepted that tissue damage in this model is related to the influx of neutrophils (26). Apparently, the strikingly low numbers of neutrophils in the interstitial space did not result in less tissue damage in the tPA− /− mice. One possible explanation for this discrepancy could be that tPA− /− neutrophils display a more damaging behavior, e.g., the production of more harmful reactive oxygen species than WT neutrophils. Indeed, in vitro studies have shown that tPA can diminish neutrophil superoxide production (27,28). The antisense-treated mice, however, showed a clear correlation between the extent of inflammation and the degree of tubular damage, corresponding with better renal function when compared with the nonsense-treated mice. This could be explained by direct targeting of the
ODN to the proximal tubules after intraperitoneal administration, resulting in a difference in neutrophil migration without affecting neutrophil reactive oxygen species production. Fibrin deposits have been reported comprehensively in a wide variety of renal diseases, such as crescentic glomerulonephritis (10), mesangio proliferative glomerulonephritis (11), and murine autoimmune glomerulonephritis (29), and in renal I/R as well (30). Although tPA−/− mice seemed to show more fibrin deposits in the renal interstitial areas compared with WT mice after I/R, there was no statistically significant difference. In a model of brain ischemia, tPA−/− mice displayed more fibrin deposits than WT mice (31). The large abundance of tPA throughout the kidney probably compensates partly for the lack of tPA. This may well account for the observation that plasmin activity and fibrin deposits were not statistically different in WT and tPA−/− mice in our study. These results indicate that modulation of the inflammatory response by tPA in renal I/R takes place in a plasmin-independent manner.

The complement system is crucially involved in the inflammatory response to renal I/R (26,32) and can be activated by tPA (33). Indeed, C3−/− mice show reduced neutrophil influx and as a result are protected from renal I/R injury (32). We found no difference in complement C3 levels between WT and tPA−/− mice, indicating that the observed differences in neutrophil influx are not mediated by the complement system. The most probable explanation for this result is that tPA has shown to activate complement via the plasminogen-plasmin axis (33), and WT and knockout mice displayed the same plasmin activity in our system. One factor from the FS, PAI-1, has proved to act in vitro as a chemotactic factor for macrophages (34). In a similar assay using r-tPA, we could not find a direct chemotactic effect of tPA on neutrophils.

IL-1β, IL-6, KC, and MIP-2 have been shown to play a pivotal role in I/R-related inflammation (2,3). In our study, the anti-inflammatory effect of tPA deletion does not result from differences in these chemokines and cytokines. The statistically significant lower IL-1β concentration in the kidneys of tPA−/− mice is probably not significant in a biologic sense, as the subtle difference in IL-1β concentration is highly unlikely to account for the dramatic difference in neutrophil influx. An earlier publication on collagen-induced arthritis in mice showed that tPA−/− mice experienced more severe joint disease, associated with significantly increased levels of IL-1β in synovial fluid (35). Furthermore, tPA−/− mice showed more fibrin deposits and more infiltrating inflammatory cells in the phalangeal joints, compared with WT mice. Another study also reported more severe arthritic disease, associated with more fibrin deposits in tPA−/− mice after injection of methylated BSA into the knee joint (36). Combined with our results, these studies illustrate that tPA can exert both pro- and anti-inflammatory effects, depending on the disease model and the involved organ. Because fibrin is of critical importance in the pathogenesis of arthritis (37), the exacerbating effect of tPA deficiency probably is a direct result of decreased fibrinolytic activity, as is the case in crescentic glomerulonephritis (10).

Other key factors in the inflammatory response to I/R are endothelial adhesion molecules, of which P-selectin, E-selectin, and ICAM-1 are the most important (3,26,38). Indeed, blocking these molecules has proved to protect against I/R injury (39–41). In addition, ICAM-1−/− deficient mice are protected against renal I/R (42). P-selectin and E-selectin show a quick upregulation after I/R with return to control levels after 12 to 24 h (43). Indeed, no noteworthy staining of E-selectin was observed after 24 h in our study (data not shown). However, ICAM-1 expression has been shown to reach its maximum after 24 h (44). Endothelial ICAM-1 expression was observed in our study, without noticeable differences between WT and tPA−/− mice. The influence of tPA on ICAM-1 expression has been studied extensively in the ischemic brain. Surprisingly, r-tPA can cause both up- and downregulation of ICAM-1 expression, depending on the time point of administration (21). In several other studies, tPA has proved to influence the interaction between neutrophils and ICAM-1, with varying effects. In vitro experiments have demonstrated that tPA can enhance neutrophil–ICAM-1 adhesion (7) but in other circumstances can decrease neutrophil adhesion and transmigration as well (45). Therefore, the exact effects of tPA on the interaction between neutrophils and ICAM-1 remain unclear.

In cerebral I/R, tPA has been shown to activate MMP-9, leading to partial destruction of the blood-brain barrier, which subsequently results in cerebral edema and brain tissue damage. By now, there is extensive evidence that tPA initiates this harmful cascade through interaction with the LDL receptor–related protein (LRP) (46–48). In the kidney, LRP-positive but otherwise unspecified cells have been recognized in a model of obstructive nephropathy (49). In this study, we could not detect LRP-expressing cells in renal tissue (data not shown), suggesting that the observed immunomodulatory effects of tPA in renal I/R are not accomplished via this receptor. In renal disease, MMP-2 and MMP-9 have been implicated in a variety of inflammatory and remodeling kidney diseases (50). Especially in obstructive nephropathy, tPA−/− mice are partly protected against the development of fibrosis as a result of diminished MMP-9 induction, leading to less basement membrane disruption in comparison with WT mice (12). We did not find distinct differences in MMP-9 and MMP-2 activity between WT and tPA−/− mice that might contribute to differences in basement membrane destruction. This suggests that involvement of MMP is of lesser impact in I/R than in obstructive nephropathy, where extensive ECM turnover takes place.

Taken together, this study shows de novo tPA expression in renal tubules during I/R. Elimination of tPA by antisense treatment diminishes neutrophil influx and results in preservation of renal function during I/R injury, indicating that inhibition of tPA could be of great therapeutic importance in the treatment of ischemic ARF.

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