Tissue factor deficiency and PAR-1 deficiency are protective against renal ischemia reperfusion injury

Jacob Sevastos,¹,² Sean E. Kennedy,¹,² Darren R. Davis,¹ Melissa Sam,¹,² Philip W. Peake,² John A. Charlesworth,¹,² Nigel Mackman,³,⁴ and Jonathan H. Erlich¹,²

¹Prince of Wales Clinical School, University of New South Wales (NSW), Sydney, Australia; ²Department of Nephrology, Prince of Wales Hospital, Randwick, NSW, Australia; ³Department of Immunology, Scripps Research Institute, La Jolla, CA; ⁴Department Cell Biology, Scripps Research Institute, La Jolla, CA

Ischemia/reperfusion (IR) injury is a leading cause of acute renal failure and an important contributor to allograft damage. Tissue factor (TF) is up-regulated during IR, and TF inhibition reduces renal injury. However, the underlying mechanisms by which TF contributes to injury have not been elucidated. We postulated that TF contributes to IR injury by production of coagulation proteases and subsequent signaling by protease activated receptor (PARs). We compared renal injury after 25 minutes of bilateral renal ischemia and varying periods of reperfusion in C57BL/6 mice, those expressing low levels of TF (low-TF), hirudin-treated C57BL/6, and mice lacking either PAR-1 or PAR-2. C57BL/6 mice developed severe renal failure and died within 48 hours of reperfusion. In contrast, low-TF, hirudin-treated C57BL/6, and PAR-1−/− mice were protected from renal failure and had reduced mortality, tubular injury, neutrophil accumulation, and lower levels of the chemokines KC and MIP-2. Importantly, PAR-1−/− mice had lower chemokine levels despite up-regulation of TF and fibrin deposition. In addition, treating PAR-1−/− mice with hirudin conferred no additional benefit. Somewhat surprisingly, PAR-2 deficiency did not protect from renal failure. These experiments indicate that increased TF activity after renal IR leads to increased CXC chemokine expression and subsequent neutrophil-mediated injury predominantly by thrombin-dependent PAR-1 signaling. (Blood. 2007;109:577-583)

© 2007 by The American Society of Hematology
Materials and methods

Experimental animals

Male mice aged 8 to 12 weeks and weighing 20 to 30 grams were used for all experiments. The low-TF mice (mTF−/−, hTF+), generated as previously described,24 have targeted disruption of the murine TF gene but are rescued from lethality by a minigene directing low-level (0.7% of wild type) expression of human TF. They display a typical murine distribution of TF with normal growth and a normal coagulation profile.25 PAR-1 and PAR-2 knockout mice (PAR-1−/− and PAR-2−/−) were obtained from Professor S. Coughlin, University of San Francisco. Both low-TF and PAR-1−/− mice were bred 6 generations onto the C57BL/6 background. PAR-2−/− mice were on a mixed genetic background26 and were compared with appropriate PAR-2+/+ strain controls. Preliminary experiments demonstrated that strain controls (mTF+/+ mice) for low-TF mice developed renal failure with the same kinetics and to the same degree as wild-type C57BL6 mice. Low-TF mice were therefore compared with C57BL6 wild-type (WT) mice. WT mice were obtained from the Biological Resources Centre, University of New South Wales (UNSW). Studies were approved by the UNSW Animal Ethics Committee and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Ischemia reperfusion protocol

The protocol for renal IR was modified from Zhou et al.27 Mice were anesthetized with intraperitoneal midazolam 3.2 mg/kg (warmed to 37°C) and inhaled 2% isofluorane (Abbott, Kurnell, NSW, Australia). Body fluid was maintained by subcutaneous administration of 300 μL 0.9% normal saline preoperatively. Rectal temperature was maintained at 35°C to 37°C using a thermostatically controlled operating platform. After a midline laparotomy, the renal pedicles were identified and bilaterally occluded for a period of 25 minutes using nontraumatic microaneurysm clamps (Kaiserslake, MN). Mice were on a mixed genetic background26 and were compared with appropriate PAR-2+/+ strain controls. Preliminary experiments demonstrated that strain controls (mTF+/+ mice) for low-TF mice developed renal failure with the same kinetics and to the same degree as wild-type C57BL6 mice. Low-TF mice were therefore compared with C57BL6 wild-type (WT) mice. WT mice were obtained from the Biological Resources Centre, University of New South Wales (UNSW). Studies were approved by the UNSW Animal Ethics Committee and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Plasma creatinine

Blood was collected by cardiac puncture into a heparinized syringe. Plasma creatinine was measured by the South Eastern Laboratory Services, Prince of Wales Hospital, Sydney, using an automated Jaffe method.

Histopathology

Histological damage of the kidneys was determined by a semiquantitative assessment of tubular injury. Kidneys were fixed in 4% paraformaldehyde for 24 hours and then embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin (H&E). Tubular injury was identified by the presence of any flattened tubular cells, loss of brush border, and cast formation. Ten high-powered fields (hpf’s; × 400 magnification) from the outer medulla and corticomedullary junction were examined from each animal to determine the percentage of tubules showing evidence of injury and scored according to the following scale: 0, no injury; 1, less than 10%; 2, 10% to 25%; 3, 26% to 75%; 4, greater than 75%.28 The 10 scores were averaged to give the tubular injury score for each specimen.

Infiltrating neutrophils (polymorphonuclear leukocytes) were quantified by counting polymorphonuclear cells (PMNs) from 10 consecutive hpf’s along the outer medulla and corticomedullary junction. The sum of the 10 counts was ascribed as the neutrophil score, measured as PMN per hpf for each specimen. A single investigator, blinded to the mouse genotype and surgical procedure, performed all scoring.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was assayed as a measure of neutrophil activity in kidneys using a modified protocol.29 A quarter of each kidney was homogenized in 1 mL of 50 mM potassium phosphate buffer (pH 6.0) and centrifuged at 15 600g at 4°C for 30 minutes. The pellet was resuspended in 1 mL 5 mM potassium phosphate and recentrifuged under the same conditions. This process was repeated. After washing, the pellets were resuspended in 1 mL extraction buffer (50 mM potassium phosphate buffer [pH 6.0] containing 0.5% hexadecyltrimethylammoniumbromide), followed by 3 rounds of freeze-thawing. The suspension was incubated at 4°C for 20 minutes then centrifuged at 15 600g at 4°C for 15 minutes. The supernatant (100 μL) was mixed with 100 μL reaction buffer (50 mM potassium phosphate buffer [pH 6.0] containing 0.6 mg/mL O-dianisidine dihydrochloride and 0.03% hydrogen peroxide). Absorbance was measured at 450 nm after 5 minutes of incubation. After normalization for protein concentration, the MPO content was expressed as units of MPO activity per milligram of protein. Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol.

Relative reverse transcriptase polymerase chain reaction (RT-PCR)

Expression of mRNA was evaluated by relative RT-PCR with an 18S ribosomal RNA internal standard. Kidney tissue was harvested into RNAlater (Ambion, Austin, TX) and stored at −80°C. RNA was extracted using TRIZOL (Invitrogen, Mt Waverley, VIC, Australia), and cDNA was transcribed with the RevertAid cDNA synthesis kit (MBI Fermentas, Hanover, MD) using random hexamer primers. Transcribed cDNA was amplified in a 20-μL reaction containing 1.5 to 2.5 mM MgCl2, 150 μM each dNTP, 1.0 μM each primer (KC: 5′-CATGCGCCTGGAGTACCTC-3′, 5′-TCTCTGTTCTCTCTGTCAGA-3′, MIP-2: 5′-TGGCGGCT-CTCAAGTCTGCTG3′, 5′-AAATTTTGTGACGGCCTTGA3′; TF: 5′-AGAAACACTTGTGATCA-3′, 5′-GTGGTCTGAGCCTCTCTCCGA-3′), 18S RNA internal standard primers, and competimers (Ambion) and 0.4 U DNA polymerase. Initial experiments were performed for each PCR to determine the exponential phase of the reaction. Amplification consisted of a denaturation period of 90 seconds at 94°C followed by 30 sequential cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. RT-PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide, and band intensities for both the target gene and 18S were quantified using Kodak Digital Science 1D software (Kodak, New Haven, CT). Results are expressed as the ratio of the target gene band intensity to the 18S internal standard band intensity.

Enzyme-linked immunosorbent assay

The CXC chemokines KC and MIP-2 were quantified in the kidney by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Frozen tissue was homogenized in an extraction buffer containing protease inhibitor mix (Roche Diagnostics, Mannheim, Germany). Chemokines were expressed as picograms per milliliter per milligram of total protein. Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol.

Measurement of tissue factor activity

Functional TF activity was measured by a 1-stage prothrombin assay.24 Half of a frozen kidney was homogenized in 15 mM octyl-β-D-glucopyranoside
To further assess the functional role of thrombin, WT and low-TF mice were subjected to 25 minutes of bilateral renal ischemia followed by up to 48 hours of reperfusion. TF activity was measured in seconds using a Start 4 automatic coagulation analyser (Diagnostica Stago, Asnieres Cedex, France). TF activity was expressed as arbitrary units per milligram of total protein, by reference to a standard curve of rabbit thromboplastin (Sigma, Castle Hill, NSW, Australia).

**Fibrin immunofluorescence**

Fibrin deposition was assessed as previously described. Briefly, kidneys were snap frozen in liquid nitrogen. Frozen sections were fixed in acetone for 1 to 2 minutes before blocking with 1% bovine serum albumin (BSA) and 1% horse serum in PBS for 1 hour at room temperature and then incubating at room temperature for a further hour with 1:100 sheep anti-rabbit fibrinogen, crossreactive to mouse fibrin (gift from Dr P. Tipping, Centre for Inflammatory Diseases, Monash University, Melbourne, VIC, Australia). Bound antibody was detected with a FITC-conjugated donkey anti-goat IgG (1:100 in 1% BSA in PBS; Jackson ImmunoResearch, West Grove, PA), and the section was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The area of FITC staining was averaged over 10 consecutive hpf’s and quantified in square micrometers using SlideBook 4.0 software (Intelligent Imaging, Denver, CO).

**Statistical analysis**

Statistical analysis was performed using JMP statistical software package for Macintosh version 5 (SAS, Cary, NC). Normally distributed data were expressed as the mean ± standard error of the mean (SEM), and groups were compared using a 2-tailed Student's t test. Nonparametric data from histological scoring were compared using the Mann-Whitney U test. Survival was compared using Kaplan-Meier methods. A P value below .05 was considered significant.

**Results**

**TF–thrombin–PAR-1 signaling contributes to renal failure and mortality following renal IR injury**

Our hypothesis was that the TF-coagulation protease-PAR signaling system would contribute to renal IR injury. To test this hypothesis, WT (C57BL/6), low-TF, PAR-1−/−, and PAR-2−/− mice were subjected to 25 minutes of bilateral renal ischemia followed by up to 48 hours of reperfusion. To further assess the functional role of thrombin, WT and PAR-1−/− mice were treated with the specific thrombin inhibitor hirudin and then subjected to renal IR injury. Mice subjected to sham surgery were used as controls.

WT mice developed severe renal failure after IR with elevated plasma creatinine compared with sham-operated mice at 2 hours (60.8 ± 2.4 μM versus 19.5 ± 2.7 μM), 5 hours (89.8 ± 2.6 μM versus 21.8 ± 2.6 μM), and 24 hours (203.1 ± 12.0 μM versus 22.9 ± 2.1 μM) after reperfusion (P < .001 at each time point). By 48 hours all WT mice had died (Figure 1). The mean plasma creatinine of sham-operated WT mice did not differ from control mice or sham-operated mice of other genotypes or treatments (data not shown), and there were no deaths by 48 hours.

A functional role for TF and PAR-1 in IR was demonstrated, because both low-TF and PAR-1−/− mice had reduced mortality (P < .001 compared with WT mice undergoing renal IR) (Figure 1) and less serum creatinine at 5 and 24 hours (Figure 2). The functional importance of thrombin was confirmed, because hirudin treatment gave similar protection to renal function as PAR-1 deficiency or reduced TF levels (Figures 1-2). Hirudin treatment of WT mice significantly reduced the mortality of injured mice at 48 hours but trended to be less effective than PAR-1 deficiency or low-TF. There was, however, no statistically significant difference in mortality between low-TF, hirudin-treated WT, and PAR-1−/− mice. PAR-2 deficiency afforded no protection from renal failure compared with controls at 24 hours of reperfusion (Figure 2C). These data indicated that PAR-1 activation appeared to be the predominant mechanism by which thrombin provided proinflammatory signaling, because hirudin treatment of PAR-1−/− mice gave no additional benefit over PAR-1 deficiency (data not shown).

**IR injury results in increased TF mRNA and functional activity**

Having demonstrated a functional role for TF–thrombin–PAR-1 signaling in renal failure and mortality, we examined the molecular and cellular events that were likely contributors to renal injury. First, we examined the effect of IR injury on TF mRNA expression in WT (C57BL/6) mice. WT mice treated with hirudin, and PAR-1−/− mice.

In all cases, TF mRNA was increased at 2 hours and remained elevated throughout the 24-hour period of study (Figure 3A,C,E). Sham surgery had no effect on renal TF mRNA expression at any time point in any of the mice (Figure 3A,C,E). This result was the same for the other genotypes (data not shown). Low-TF mice do not express mouse TF mRNA. TF functional activity in WT and PAR-1−/− mice was increased in a time-dependent manner (Figure 3B,D,F). TF activity was reduced in hirudin-treated mice, which is likely because of residual levels of hirudin being present in renal tissue extracts that inhibit thrombin activity in the one-stage prothrombin assay used to measure renal TF activity. As expected renal TF activity from low-TF mice was very low (0.24 ± 0.1 au/mg protein at 24 hours of reperfusion).

Both PAR-1 deficiency and hirudin treatment were associated with renoprotection despite up-regulation of TF mRNA. This suggests that TF contributes to injury via thrombin generation and the activation of PAR-1.

**Reduced TF expression or inhibition of thrombin–PAR-1 signaling results in reduced renal injury, neutrophil infiltration, and MPO activity**

To further assess the effect of IR injury on renal tissue, tubular infiltration was assessed by a histological scoring system, and neutrophil infiltration was assessed by both counting of PMNs on tissue sections and by tissue MPO activity 24 hours after ischemia. Renal architecture in all
The dilution of standard was designated 100 units. Data are expressed as mean ± SEM. Renal TF activity was very low from sham-operated mice was normal (Figure 4A). WT mice displayed significant injury 24 hours after ischemia with tubular cast formation, loss of brush border, and tubular necrosis (Figure 4B). Low-TF, hirudin-treated WT mice, and PAR-1−/− mice 24 hours after ischemia showed qualitatively similar histological changes to each other with relatively much less severe injury compared with WT mice (Figure 4B-E). Tubular injury was significantly less in low-TF, hirudin-treated WT mice, and PAR-1−/− mice, compared with WT mice (mean tubular score: low-TF, 2.58 ± 0.10, hirudin-treated WT, 2.60 ± 0.04, and PAR-1−/−, 2.61 ± 0.03 versus WT 3.76 ± 0.00; P < .001 for each pair; Figure 4F). Renal neutrophil infiltration and MPO activity were significantly reduced in low-TF, PAR-1−/−, and hirudin-treated WT mice compared with WT mice undergoing renal IR injury (Figure 5).

Reduced PAR-1 signaling results in reduced CXC chemokine response

We then examined the effect of reduced PAR-1 signaling on renal chemokine expression. Analysis of the expression of various inflammatory mediators by RNase protection assay indicated that KC and MIP-2 were strongly up-regulated (data not shown). Subsequent RT-PCR and ELISA confirmed that KC and MIP-2 mRNA and protein were both increased following renal IR injury (Figure 6), with up-regulation of mRNA by 2 hours and protein by 5 hours, both of which persisted for 24 hours. Sham-operated mice showed no significant change in renal chemokine expression. Low-TF mice, hirudin-treated WT, and PAR-1−/− mice showed similar degrees of reduction of chemokine mRNA and protein expression (Figure 6).

Fibrin deposition does not correlate with renoprotection

Inhibition of TF or thrombin will reduce the generation of fibrin. To analyze fibrin deposition in this model, levels of fibrin in kidney specimens of WT (n = 12), low-TF (n = 8), hirudin-treated WT mice (n = 12), and PAR-1−/− mice (n = 12), obtained after 24 hours of reperfusion, were assessed by immunohistochemical staining and compared with sham-operated and control mice. Significant levels of fibrin deposition were not detected in control mice and sham-operated mice (Figure 7). In contrast, fibrin staining was detected in the WT and PAR-1−/− mice after reperfusion (Figure 7). Low levels of fibrin were also detected in injured low-TF mice and hirudin-treated mice. These results indicated that levels of fibrin deposition did not correlate with markers of renal injury.
Discussion

This study provides evidence that TF contributes to renal IR via thrombin-dependent activation of PAR-1. Disruption of the key stages of this pathway led to decreased renal injury, lower mortality, and reduced expression of proinflammatory chemokines.

We demonstrated that TF plays a prominent role in IR-related inflammation and tissue injury by showing that mice with low levels of TF were protected from renal injury. These mice had a significant survival advantage compared with WT mice and a striking reduction in renal PMN accumulation after IR. We have documented up-regulation of the TF gene in renal tissue 2 hours after ischemia and a progressive increase in functional activity up to 24 hours after ischemia. Frank et al.30 also showed up-regulation of TF mRNA and activity in a similar murine model of renal IR. After prolonged renal ischemia and contralateral nephrectomy in a rat model, TF was up-regulated in the kidney with increased TF staining in glomeruli, blood vessels, and stimulated monocytes.14 In a separate study, this group also demonstrated a survival advantage and reduction in histological injury in rats treated with TFPI. The same group, using a less severe model, has subsequently shown a survival advantage and protection from tubular necrosis by the use of TF antisense oligonucleotides.15 The mechanism underlying TF-mediated injury was not explored, although it was postulated that necrosis was caused by microcirculatory incompetence and microthrombus formation. Our data, however, suggest

Figure 4. Effect of IR on renal tubular injury. (A–E) Hematoxylin and eosin–stained tissue sections of (A) WT mice undergoing sham surgery followed by 24 hours of reperfusion and of (B) WT, (C) low-TF, (D) C57BL/6 hirudin-treated, and (E) PAR-1/– mice undergoing 25 minutes of renal ischemia followed by 24 hours of reperfusion. In panel B, tubular cast formation is indicated by *. (F) Tubular injury scores of WT (n = 16), low-TF (n = 7), and WT (n = 7) mice treated with hirudin and PAR-1/– (n = 16) mice undergoing 25 minutes of bilateral ischemia followed by 24 hours of reperfusion or WT mice undergoing sham surgery. IR injury resulted in significant tubular injury in WT mice compared with sham-operated animals (**P < .001). Low-TF, WT mice treated with hirudin and PAR-1/– mice had significantly reduced tubular injury compared with WT (**P < .001). Images were captured using an Olympus BX51 microscope equipped with a uPlanFl 40 ×0.75 numerical aperture (NA) air objective (Olympus, North Ryde, Australia) connected to a Retiga EXi digital camera, and captured with Q capture for Apple Macintosh (QImaging, Burnaby, BC, Canada). Data are expressed as mean ± SEM.

Figure 5. Effect of IR injury on renal polymorphonuclear (PMN) cell infiltration. PMN infiltration was assessed by (A) measuring renal myeloperoxidase activity and (B) counting PMN cells on H&E-stained tissue sections of WT (n = 16), low-TF (n = 7), WT mice treated with hirudin (n = 7) and PAR-1/– (n = 16) mice undergoing 25 minutes of bilateral ischemia followed by 24 hours of reperfusion or WT mice undergoing sham surgery. IR mice undergoing IR had significantly increased MPO and PMN counts compared with sham-operated mice (**P < .001). Low-TF, WT mice treated with hirudin and PAR-1/– mice had significantly reduced renal PMN accumulation compared with WT (**P < .001). Data are expressed as mean ± SEM.

Figure 6. IR injury induces both renal KC and MIP-2 mRNA and protein. Mice underwent renal ischemia followed by 5 to 24 hours of reperfusion or sham surgery. (A,C) KC and MIP-2 mRNA was assessed by RT-PCR and levels expressed relative to 18S ribosomal RNA. (B,D) KC and MIP-2 protein was assessed by ELISA, and levels were expressed as picogram per milliliter (samples were loaded equally for total protein). WT mice undergoing IR had significantly elevated KC and MIP-2 mRNA and protein compared with sham-operated WT mice (**P < .001). Low-TF WT mice treated with hirudin and PAR-1/– mice had significantly reduced KC and MIP-2 mRNA and protein compared with WT mice (**P < .001). Data are expressed as mean ± SEM.
However, because hirudin treatment may also reduce factor Xa levels, was protective against renal IR without altering the expression of TF. Further protection was obtained by treating PAR-1 pathways (eg, PAR-4) also appears to be minimal in renal IR because no IR. The contribution of thrombin-regulated signaling by non–PAR-1 may not be the major mediator of TF-related IR injury. Similarly, Frank et al found that defibrinogenated mice were not protected from lethality in renal IR. In vitro studies have shown that TF-VIIa-FXa complex activates PAR-2.39,20 The absence of any benefit or more severe renal injury in PAR-2–deficient mice suggests PAR-2 signaling was not a significant pathway in this model. In contrast, activation of PAR-2 in mice contributes to mortality following administration of LPS,27 induces acute lung injury,22 and is pivotal in mediating chronic joint inflammation following adjuvant arthritis.23

Regulation of renal hemodynamics is a further potential mechanism by which PARs may contribute to renal function following renal IRI. In the isolated perfused rat kidney PAR-1 activation results in vasoconstriction.30 Hence, in our model at least some of the benefit of PAR-1 deficiency or reduced PAR-1 signaling may derive from improved glomerular hemodynamics.

Fibrin, like thrombin, may potentially induce chemokine signaling.36 Further, some studies suggest that fibrin is important in macrophage accumulation in inflammatory diseases such as glomerulonephritis and cardiac IR.40,41 In the latter case, fibrinopeptide B binding to VE-cadherin was shown to be the important mechanism regulating monocyte trans-endothelial cell migration.41 However, Frank et al found that defibrinogenated mice were not protected from lethality in renal IR injury. A potential explanation of this discrepancy is that residual levels of fibrinogen in that model were sufficient to form fibrin and the fibrinopeptide B breakdown product, thereby promoting leukocyte trans-endothelial cell migration. Alternatively, there may be differences in mechanisms of inflammation between cardiac and renal IIR models. However, our finding of similar levels of interstitial fibrin in the WT and PAR-1−/− mice after 24 hours of reperfusion, suggests that any contribution of fibrin to renal IR is downstream of the TF–thrombin–PAR-1 axis. It is possible that PAR-1–dependent chemokine production may be required for fibrin to express a proinflammatory role in renal reperfusion injury. As with cardiac reperfusion, it is possible that binding of the β chain of fibrin to VE-cadherin may also be important for leukocyte accumulation in renal IR; however, thrombin-mediated PAR-1 signaling and chemokine expression are critical upstream events.

In conclusion, we have shown that deficiency of TF or lack of PAR-1 signaling is protective in murine renal IR. The predominant mechanism appears to be by reduced CXC chemokine expression and consequent neutrophil infiltration. Inhibition of PAR-1 may therefore provide an important new strategy for reducing reperfusion injury.

In the current study, TF deficiency was associated with reduced up-regulation of MIP-2 and KC after IR. Inhibiting these chemokines with neutralizing antibodies has been shown to protect mice from renal IR via reduced neutrophil infiltration.3 TF may regulate chemokine expression and consequent neutrophil recruitment directly via TF cytoplasmic tail-dependent NF-κB activation11 or in combination with FVIIa by a protease-activated receptor mechanism (reviewed by Rao and Pendurthi32). However, we found that WT mice treated with hirudin had a similar degree of protection from renal IR injury as low-TF mice, despite up-regulation of TF mRNA. This suggests that a major pathway for TF proinflammatory signaling in IR injury is by the activation of the coagulation cascade and thrombin production. Therefore, our results suggest that direct cell signaling by TF alone or in complex with VIIa may not be the major mediator of TF-related IR injury. Similarly, Frank et al found that treatment with the factor Xa inhibitor, fondaparinux, was protective against renal IR without altering the expression of TF. However, because hirudin treatment may also reduce factor Xa levels, we cannot rule out a role of Xa activation of PAR-1 in the injury.33,34

Hirudin, a specific inhibitor of thrombin, will inhibit all protease-dependent actions of thrombin, including procoagulant effects, PAR signaling, and activation of other molecules (eg, protein C). We therefore used mice deficient in the main cellular receptor of thrombin, PAR-1, to further elucidate the pathway of TF-dependent IR injury. We found similar degrees of renal protection in PAR-1−/− mice to low-TF and hirudin-treated WT mice. PAR-1−/− mice were also found to have lower CXC chemokine expression and less neutrophil infiltration than WT mice after IR, suggesting a proinflammatory role for PAR-1 in renal IR. The contribution of thrombin-regulated signaling by non–PAR-1 pathways (eg, PAR-4) also appears to be minimal in renal IR because no further protection was obtained by treating PAR-1−/− mice with hirudin. Matrix metalloproteinase 1 (MMP-1) is another activator of PAR-1.35 In addition, thrombin has been shown to induce MMP-1 expression.36 However, the fact that hirudin treatment, low-TF, and PAR-1 deficiency all gave a similar degree of protection of renal function suggests that, if MMP-1 does have a major role in the injury, it is downstream of the TF thrombin pathway.

In vitro studies have shown that TF-VIIa-FXa complex activates PAR-2,19,20 Thrombin may induce TF expression in a variety of cells. However, TF up-regulation in this model appeared not to be thrombin dependent because both hirudin-treated and PAR-1−/− mice displayed equivalent TF mRNA expression.

Thrombin, a specific inhibitor of thrombin, will inhibit all protease-activated receptor mechanisms (reviewed by Rao and Pendurthi32). However, we found that WT mice treated with hirudin had a similar degree of protection from renal IR injury as low-TF mice, despite up-regulation of TF mRNA. This suggests that a major pathway for TF proinflammatory signaling in IR injury is by the activation of the coagulation cascade and thrombin production. Therefore, our results suggest that direct cell signaling by TF alone or in complex with VIIa may not be the major mediator of TF-related IR injury. Similarly, Frank et al found that treatment with the factor Xa inhibitor, fondaparinux, was protective against renal IR without altering the expression of TF. However, because hirudin treatment may also reduce factor Xa levels, we cannot rule out a role of Xa activation of PAR-1 in the injury.33,34

Hirudin, a specific inhibitor of thrombin, will inhibit all protease-dependent actions of thrombin, including procoagulant effects, PAR signaling, and activation of other molecules (eg, protein C). We therefore used mice deficient in the main cellular receptor of thrombin, PAR-1, to further elucidate the pathway of TF-dependent IR injury. We found similar degrees of renal protection in PAR-1−/− mice to low-TF and hirudin-treated WT mice. PAR-1−/− mice were also found to have lower CXC chemokine expression and less neutrophil infiltration than WT mice after IR, suggesting a proinflammatory role for PAR-1 in renal IR. The contribution of thrombin-regulated signaling by non–PAR-1 pathways (eg, PAR-4) also appears to be minimal in renal IR because no further protection was obtained by treating PAR-1−/− mice with hirudin. Matrix metalloproteinase 1 (MMP-1) is another activator of PAR-1.35 In addition, thrombin has been shown to induce MMP-1 expression.36 However, the fact that hirudin treatment, low-TF, and PAR-1 deficiency all gave a similar degree of protection of renal function suggests that, if MMP-1 does have a major role in the injury, it is downstream of the TF thrombin pathway.
Acknowledgment

This work was supported by postgraduate medical research scholarships from the Australian Kidney Foundation and the National Health and Research Council of Australia.

D.R.D. holds a National Health and Medical Research Council Peter Doherty Fellowship (ID no. 222974).

Authorship

Contribution: J.S. performed the majority of experiments and participated from the Australian Kidney Foundation and the National Health and Medical Research Council. M.N. provided key reagents and assisted with manuscript preparation; N.M. provided key reagents and assisted with manuscript preparation; D.R.D. performed some experiments and participated in data analysis; P.W.P. provided experimental input and assisted with manuscript preparation; J.A.C. provided critical comment and assisted with manuscript preparation; N.M. provided key reagents and assisted with manuscript preparation; and J.H.E. led the project and was responsible for experimental design, analysis, and manuscript preparation.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Jonathan Erlich, Department of Nephrology, Prince of Wales Hospital, Barker St, Randwick, NSW 2031, Australia; e-mail: j.erlich@unsw.edu.au.

References

Tissue factor deficiency and PAR-1 deficiency are protective against renal ischemia reperfusion injury

Jacob Sevastos, Sean E. Kennedy, Darren R. Davis, Melissa Sam, Philip W. Peake, John A. Charlesworth, Nigel Mackman and Jonathan H. Erlich