Blocking P-selectin protects from ischemia/reperfusion-induced acute renal failure

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ABSTRACT **Acute renal failure (ARF) in response to ischemia-reperfusion is thought to be associated with neutrophil infiltration. Neutrophil recruitment depends on adhesion molecules, including P-selectin. Our study sought to characterize the role of P-selectin in ischemia-reperfusion (I/R) -induced acute renal failure (ARF). In wild-type (wt) and P-selectin-deficient (P−/−) mice (both C57BL/6), ARF was induced by 32 min bilateral renal ischemia, followed by reperfusion (I/R). Wt showed a 12- and 20-fold increase in creatinine at 24 and 48 h after I/R, respectively. Similar changes were seen in blood urea nitrogen (BUN). By contrast, in P−/− creatinine and BUN increased only moderately (fourfold over sham). In wt, renal myeloperoxidase activity, indicating neutrophil infiltration, peaked after 24 h (19-fold over sham). This was significantly attenuated in P−/− (fivefold over sham). Western blot analysis revealed maximum P-selectin expression 12 h after I/R in wt. Immunostaining detected P-selectin in glomerular endothelium and in platelets adherent in glomerular and peritubular vessels. Postischemic injection of P-selectin antibody at 10 min after reperfusion, but not isotype control antibody, protected wt from ARF similar to the protection seen in P−/−. We conclude that blocking P-selectin even after onset of reperfusion protects mice from I/R-induced ARF, suggesting potential therapeutic strategies aimed at blocking P-selectin.—Singbartl, K., Green, S. A., Ley, K. Blocking P-selectin protects from ischemia/reperfusion-induced acute renal failure. *FASEB J.* 14, 48–54 (2000)**

Key Words: neutrophils · adhesion molecules · renal function · gene targeting · antibodies

ALTHOUGH THE PATHOPHYSIOLOGY of ischemia/reperfusion-induced acute renal failures is not completely understood, some key events leading to tissue injury and thereby to renal failure have been identified. In the initial phase, ischemia-induced vasoconstriction, tubular swelling, and desquamation together with endothelial cell activation and edema are found (1). Postischemic or reperfusion injury is thought to be mediated mainly by two mechanisms, which can further aggravate tissue damage: no-reflow and reflow paradox (2). No-reflow describes capillary perfusion failure resulting in focal tissue hypoxia (3). So far, red blood cells, platelets, and leukocytes have all been proposed to contribute to this phenomenon (4). Reflow paradox refers to exacerbated tissue injury due to postischemic activation of leukocytes, especially neutrophils, which then produce and secrete cytotoxic compounds while adhering to the endothelium and infiltrating into the tissue (5).

In many experimental models, neutrophils have been shown to infiltrate in postischemic kidneys (6–11). Recruitment of neutrophils into injured tissues is thought to occur by a sequence of events leading to firm endothelial adhesion and subsequently to transmigration (12, 13). Endothelial and leukocyte adhesion molecules have been proposed as mediators of this cascade-like process. Capturing or tethering of free-flowing leukocytes represents the initial step and can be converted into stable rolling of these cells along the endothelium. The selectin class of adhesion molecules (L-, P-, and E-selectin) mediates leukocyte capture and rolling (14, 15). During rolling, leukocytes are believed to get activated by chemokines or other chemoattractants leading to activation of integrins, firm adhesion, and finally to transmigration (16).

In ischemia/reperfusion-induced renal injury, only a few adhesion molecules have been studied so far (6–9, 11, 17–19). Whereas inhibiting the function of either β_2-integrins (17) or intercellular adhesion molecule 1 (ICAM-1) (6, 8, 11, 19) gave a significant reduction of kidney damage after ischemia-reperfusion (I/R), blocking L-selectin did not protect from renal failure (9). Although expression of P-selectin has been reported to be increased in response to renal ischemia-reperfusion (20), the role

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of P-selectin in the development of acute renal failure remains unknown.

P-selectin, a 140 kDa type-1 transmembrane glycoprotein, is constitutively stored in Weibel-Palade bodies of endothelial cells and in α-granules of platelets (21, 22). From there it can be released to the cell surface within a few minutes after exposure to thrombin, histamine, complement 5a, Ca^{2+}-ionophores, or adenosine diphosphate (21–24). In mice, endotoxin injection has been shown to induce P-selectin expression in liver, heart, lung, and kidney (25). P-selectin expression on the cell surface is down-regulated by internalization, degradation, and to a smaller extent by shedding into the plasma (22, 24).

This study seeks to identify the role of P-selectin in I/R-induced severe acute renal failure. To achieve this, we used gene-targeted mice with a null mutation in the P-selectin gene as well as postischemic application of function-blocking monoclonal P-selectin antibody in wild-type mice. To validate our model, we also determined the impact of neutrophils on renal function.

MATERIALS AND METHODS

Animals

Experiments were conducted in 154 adult (2–5 months old, 20–32 g body weight) C57BL/6 wild-type mice and gene-targeted mice with a null mutation in the P-selectin gene (26). Mutant mice were back-crossed into a C57BL/6 background for at least five generations and maintained in specific pathogen-free conditions at the Center for Comparative Medicine, University of Virginia.

Chemicals

Unless stated otherwise, chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Surgical procedure

Mice were anesthetized with intraperitoneal (i.p.) injections of ketamine (125 μg/g body weight, Ketalar; Parke-Davis, Morris Plains, N.J.), xylazine (12.5 μg/g body weight; Phoenix Scientific, Inc., St. Joseph, Mo.), and atropine sulfate (0.025 μg/g body weight; Elkins-Sinn, Inc., Cherry Hill, N.J.) and placed on a heating pad to maintain body temperature. Both renal pedicles were prepared using a median dorsal skin incision and bilateral paramedian opening of the retroperitoneal space. In animals undergoing I/R, both pedicles were clamped off for 32 min with hemostatic microclips. This model is known to induce severe acute renal failure in untreated wild-type mice with a 50% mortality rate at 72 h (6). Kidneys were inspected for immediate color change indicating successful clamping. After clamp removal, kidneys were checked for a change in color within 3 min to ensure reperfusion. In 19 of 154 mice, these criteria were not fulfilled, and these mice were excluded from this study. In 12 mice, kidneys showed signs of hemorrhagic infarction at the time of harvesting, indicating venous obstruction rather than arterial occlusion (27). Two mice had polycystic kidneys. These mice were also excluded. In animals subjected to sham operation, the surgical procedure was identical except that no clamps were applied. After surgery, the wounds were covered with saline-soaked gauze. Incisions were closed in two layers and animals were allowed to recover. Postoperative analgesia was provided by subcutaneous injections of buprenorphine (2 μg/g body weight) diluted with isotonic saline to provide sufficient volume substitution for 12 h. Four, 12, 24, or 48 h later mice were killed, blood samples were taken by heart puncture, and kidneys were harvested. Data from wild-type mice presented here also served as a control for identifying the role of E-selectin in ischemia/reperfusion-induced acute renal failure.

Therapeutic intervention experiments

Two separate groups of wild-type mice received 100 μg of either function-blocking monoclonal anti-mouse P-selectin antibody (RB40.34, hybridoma provided by D. Vestweber, Universität Münster, Germany; described in ref 28) or isotype-matched control antibody (PharMingen, San Diego, Calif.) i.p. 10 minutes after reperfusion. Mice were killed at 24 h after reperfusion and blood was collected.

Neutrophil depletion experiments

In two additional groups of wild-type mice, 2 μl/g body weight of either rabbit anti-mouse neutrophil serum or conventional rabbit serum (Inter-Cell Technologies Inc., Hopewell, N.J.) was injected 18 h before ischemia. Blood samples for neutrophil counts were obtained at the time of ischemia by tail bleeding. Neutrophils were counted blind using Kimura's stain. Animals were killed 24 h later, blood samples were taken, and kidneys were harvested.

Renal function

Whole blood samples were used to determine creatinine and blood urea nitrogen (BUN) concentrations (NOVA analyzer 16^{+}, NOVA Biomedical, Waltham, Mass.). Creatinine measurements were based on a three-step enzymatic assay (creatinine amidohydrolase, creatinine amidininohydrolase, and sarcosine oxidase), converting creatinine and H_2O into formaldehyde, glycine, and H_2O_2. BUN was determined by the urease method converting urea into ammonia and CO_2.

Myeloperoxidase activity (MPO)

MPO, indicating neutrophil infiltration into tissue, was measured in equal-sized samples of both kidneys. This assay represents a modified combination of two previously published methods (29, 30). Briefly, samples were homogenized (1:20 w:v) in ice-cold 20 mM KPO_4 buffer (pH 7.4). After removing 17,000 × *g* supernatants (4°C, 30 min), pellets were again resuspended in ice-cold 20 mM KPO_4 buffer (pH 7.4), followed by two additional spins. Then 0.5% (w/v) hexacyltrimethylamonium bromide-10 mM EDTA in 50 mM KPO_4 (pH 6.0) was added to the remaining pellet (buffer:pellet 6:1). Suspensions were sonicated for 5 × 1 s on ice, freeze-thawed 3×, and incubated for 20 min at 4°C. After final centrifugation (17,000×*g*, 15 min, 4°C), supernatants were used to measure MPO. Assay buffer (0.2 mg/ml o-dianisidine and 158 μM H_2O_2 in 50 mM KPO_4, pH 6.0) was added in triplicate to supernatant at a ratio of 4:1. Changes in absorbance were recorded at 460 nm over 5 min. The linear part of the resulting curve was used to calculate MPO activity. 1 U of activity was defined as change in absorbance of 1.0 per minute

at 25°C. Results were expressed as units of MPO per gram of protein of supernatant as determined by bichionic acid assay (Pierce Chemical Co., Rockford, Ill.).

Western blotting

Kidneys were homogenized in ice-cold protein extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 μg/ml phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). After 10 min incubation, homogenate was centrifuged (10,000×*g*, 5 min, 4°C). Supernatant was stored at −80°C. Samples (100 μg protein per lane, bichionic acid assay) were run under reducing conditions on a sodium dodecyl sulfate-polyacrylamide gel (5% stacking gel, 7.5% separating gel). Thereafter, gels were electroblotted on nitrocellulose membranes. After blocking, blots were incubated for 2 h with a rabbit polyclonal antibody (1:5000) against a peptide corresponding to the 25 COOH-terminal amino acids of P-selectin (31). A goat anti-rabbit antibody conjugated with horseradish peroxidase served as secondary antibody. Finally, washed blots were impregnated with ECL chemiluminescence substrate and exposed to X-ray films.

Immunohistochemistry

After antigen retrieval, paraffin embedded kidney sections (5 μM) were incubated with a rabbit polyclonal antibody (1:4000) against a peptide corresponding to the 25 COOH-terminal amino acids of P-selectin (31). This was followed by biotinylated secondary antibody (1:250, Vector Laboratories Inc., Burlingame, Calif.) in 10% goat serum to reduce background staining and finally by avidin-biotin peroxidase (Vector Laboratories).

Statistics

Analysis of variance, followed by an unpaired *t* test, with Bonferroni correction when appropriate, was used for statistical analysis. Most data are shown as fold change vs. corresponding sham group. All results are given as mean ± SE for groups of six mice each. Statistical significance was set at $P < 0.05$.

RESULTS

To identify the role of neutrophils in our model, we first performed neutrophil depletion experiments. Eighteen hours before I/R, a group of wild-type mice received either anti-neutrophil serum (ANS) or control serum. At the time of ischemia, mice pretreated with ANS had significantly lower neutrophil counts than control animals (129±38/μl vs. 875±165/μl, respectively). No other differences in the differential blood count could be found. Compared to control mice, neutropenic mice showed significantly smaller increases in creatinine concentration after 24 h (**Fig. 1**). Moreover, in neutropenic mice renal MPO was only 3.0 ± 0.6 U/g protein at that time, whereas mice that had received control serum revealed renal MPO levels of 10.9 ± 3.8 U/g protein ($P<0.05$).

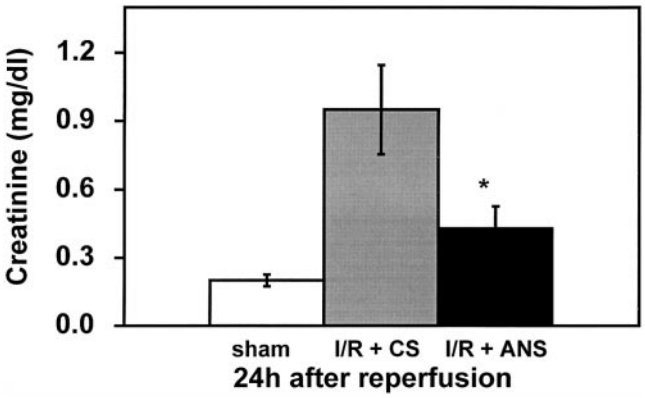

Figure 1. Role of systemic neutrophil concentration at the time of ischemia on postischemic kidney function as measured by creatinine concentration. Wild-type mice were injected with either rabbit anti-mouse neutrophil serum (black column, ANS) or preimmune rabbit serum (gray column, CS) as control 18 h before bilateral ischemia. Creatinine was measured 24 h after. The white column gives the results from untreated, sham-operated wild-type mice (sham). *$P < 0.05$ vs. control group.

In wild-type mice, 32 min bilateral renal pedicle clamping, followed by reperfusion, led to dramatically elevated creatinine concentrations after 24 (13-fold over sham) and 48 h (19-fold over sham) (**Fig. 2*a***). A significant increase in BUN (1) concentrations (Fig. 2*b*) could also be seen after 24 (10-fold over sham) and 48 h (10-fold over sham). Concomitant with this drastic impairment of renal function, untreated wild-type mice revealed a continuous increase in MPO, reaching 17.9 ± 3.6 U/g protein at 24 h after reperfusion (19-fold increase above sham control, Fig. 2*c*). Thereafter, MPO declined, reaching 10.3 ± 3.5 U/g protein (ninefold above sham) 48 h after reperfusion. Creatinine and BUN concentrations trailed MPO by ~24 h, reaching a maximum at 48 h after reperfusion.

In contrast to the dramatic rise in both creatinine and BUN concentrations seen in wild-type mice, P-selectin-deficient mice had significantly smaller elevations in creatinine (Fig. 2*a*) and BUN (Fig. 2*b*) concentrations after 24 and 48 h of reperfusion. In P-selectin-deficient mice, renal MPO was only 4.5-fold elevated above corresponding sham values 24 h after I/R (Fig. 2*c*). This indicates a significant reduction of neutrophil influx by ~85% compared to wild-type mice.

Protein extracts from kidneys of representative sham-operated and I/R wild-type mice were analyzed for P-selectin expression using Western blot (**Fig. 3*a***). P-selectin was highly up-regulated soon after reperfusion, peaked at 12 h, and declined thereafter. In sham-operated animals, a similar but far less intense expression pattern was found. Western blot analysis of kidney samples from P-selectin-deficient mice confirmed that no P-selectin was expressed (Fig. 3*b*) in these mice.

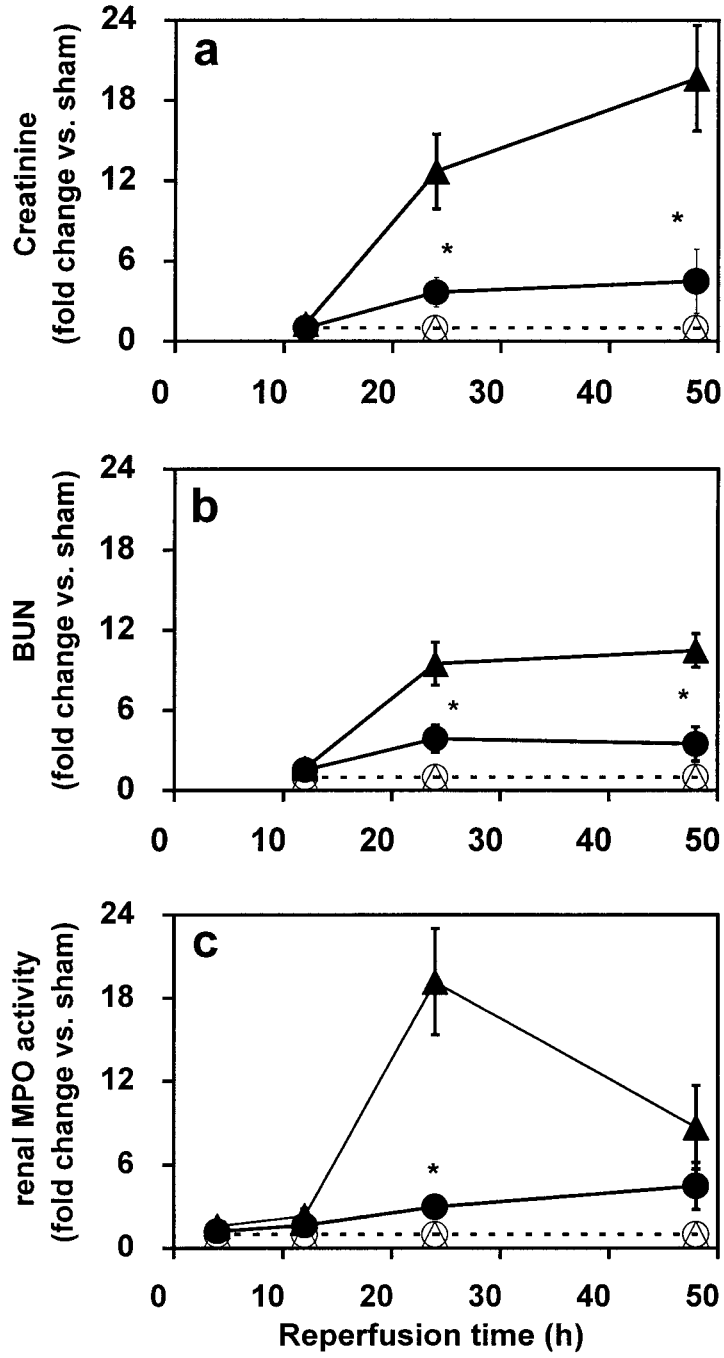

Figure 2. Renal function and neutrophil accumulation as measured by myeloperoxidase activity after 32 min bilateral renal ischemia in wt (I/R: filled triangles; sham: open triangles and P-selectin-deficient mice (I/R: filled circles; sham: open circles). Creatinine (*a*), BUN (*b*), and MPO (*c*) were measured at 4 (MPO only), 12, 24, and 48 h. $*P < 0.05$ vs. wild-type mice.

Immunostaining localized P-selectin expression at 12 h after ischemia-reperfusion to glomeruli, peritubular vessels, and to a lesser extent to arteries and veins. In glomeruli, P-selectin expression was found in platelet as well as in endothelial cells (**Fig. 4***a*), whereas in peritubular vessels only platelets stained positive for P-selectin (Fig. 4*b*). Sham-operated animals also revealed P-selectin expression (Fig. 3), but this was considerably smaller and could only be found in glomeruli (data not shown).

To identify any therapeutic benefit from blocking P-selectin, another group of wild-type mice that had undergone 32 min bilateral ischemia, received either function blocking monoclonal anti-P-selectin antibody (RB40.34) or isotype- matched control antibody (IgG_1) i.p. 10 minutes after clamp removal. This treatment resulted in a significant attenuation of creatinine increase at 24 h after reperfusion, similar to that seen in mice gene deficient for P-selectin (**Fig. 5**).

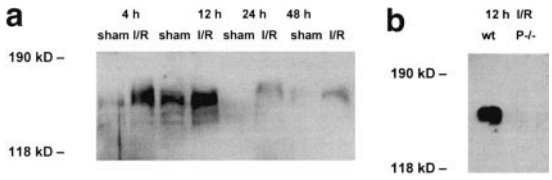

Figure 3. Renal P-selectin expression after 32 min bilateral ischemia. Western blot analysis of protein extracts from representative wild-type kidney samples (*a*). Kidney specimen from a representative P-selectin-deficient mouse (P−/−) harvested 12 h after I/R, i.e., at the maximum of P-selectin expression in wild-type mice, showed no P-selectin expression (*b*).

DISCUSSION

Our study shows that P-selectin is necessary to produce severe acute renal failure in response to ischemia-reperfusion. A causative role of P-selectin is further supported by our observation that neutrophil infiltration into postischemic kidneys is dramatically reduced in P-selectin-deficient mice. Even postischemic blockade of P-selectin can dramatically preserve renal function at clinically relevant levels.

Several studies have shown neutrophil infiltration into the kidney after ischemia-reperfusion (6–11). However, the effect of neutrophils on renal function after ischemia-reperfusion remains unsettled. In some studies preischemic neutrophil depletion provided protection from renal failure (6, 32–34), whereas other studies could not demonstrate a protective role of neutrophil depletion (18, 35). In addition to variations in model design, different levels of neutrophil depletion achieved may have contributed to these conflicting results. We achieved a significant reduction in neutrophil counts of ~85% at the time of ischemia and show that neutrophils play a crucial role in this model of acute renal failure. This demonstrates that our model is suitable to study the role of adhesion molecules such as P-selectin, relevant to neutrophil infiltration in the postischemic kidney.

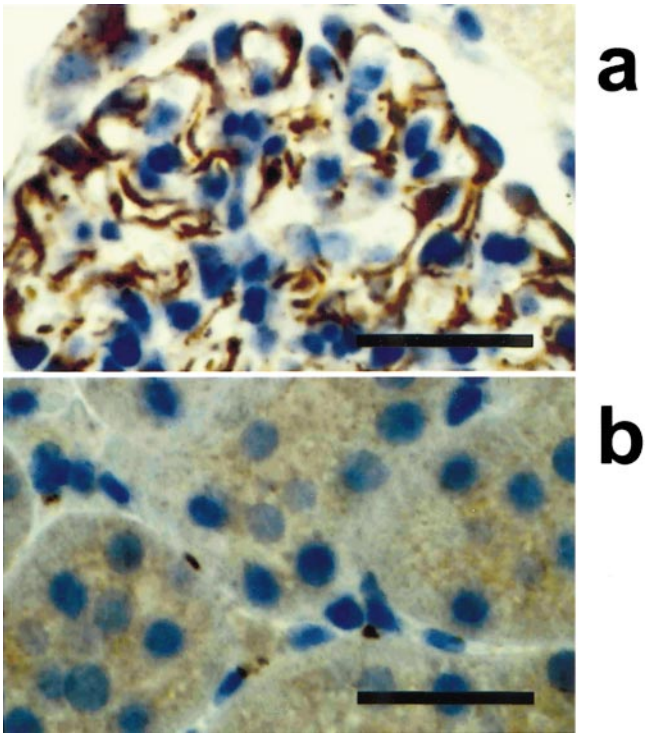

Figure 4. Localization of P-selectin expression in kidneys after 32 min bilateral renal ischemia. 12 h after ischemia and reperfusion, P-selectin expression was seen in glomeruli (*a*) and in peritubular vessels (*b*). In glomeruli both endothelium and platelets (arrows) stained positive for P-selectin. By contrast, in peritubular vessels P-selectin was only found in platelets (arrows). Hematoxylin counterstain. Bar= 40 μM.

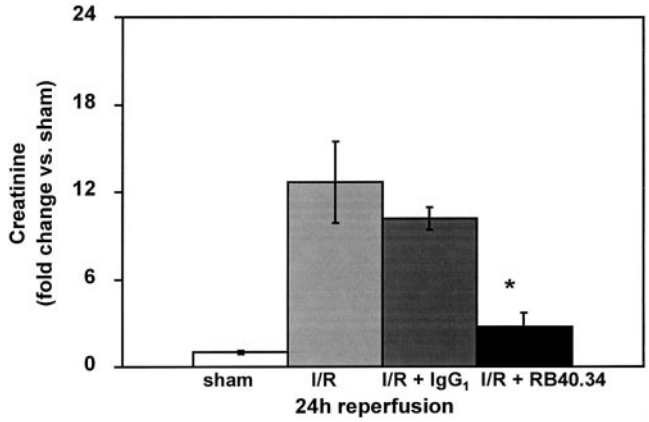

Figure 5. Effect of postischemic P-selectin blockade on renal function after I/R. Wild-type-mice were injected with either function-blocking monoclonal anti-P-selectin antibody (RB40.34, dark gray column) or isotype-matched control antibody (IgG_1, black column) 10 min after 32 min bilateral renal ischemia. Blood samples were taken at 24 h after reperfusion for determining creatinine concentrations. Results from untreated wild-type mice either sham operated or after ischemia-reperfusion are given by the white and light gray columns, respectively. Data are given as mean ± SE for groups of six mice each. $*P < 0.05$ vs. wild-type mice treated with control antibody.

In our experiments, we found a sharp increase in total, i.e., surface and intracellular P-selectin expression as early as 4 h after reperfusion, indicating ischemia/reperfusion-induced biosynthesis. P-selectin expression was maximal at 12 h after reperfusion. In a 30 min model of murine renal ischemia, followed by reperfusion, Zizzi et al. (20) showed that P-selectin surface expression in perfused vessels, as measured by injection of radio-labeled P-selectin antibody, peaked within 5 h of reperfusion and declined thereafter. However, postischemic kidneys are known to have a disturbed regional perfusion pattern, which can last for up to 48 h (36). Blood flow in the outer medulla is reduced and blood flow in the inner medulla is increased (36). Thus, the observed difference in P-selectin expression may be explained at least partially by P-selectin expressed on cell surfaces in blood vessels that were not perfused.

We found that sham operation could also induce

considerable P-selectin expression, which was restricted to glomeruli. By contrast, after ischemia-reperfusion, P-selectin was found in glomeruli and peritubular vessels. In glomeruli, both endothelial and platelet P-selectin were detected whereas in peritubular vessels, only platelets stained positive for P-selectin. In a recent biopsy study of human kidney transplants, P-selectin expression was also found in platelets in both glomeruli and peritubular vessels (37). Activated and adherent platelets have been shown to support leukocyte rolling, adhesion, and transmigration via P-selectin and β_2-integrins in a way similar to that of activated endothelium (38).

Development of acute renal failure in our model is strongly neutrophil dependent; blocking P-selectin dramatically reduced renal myeloperoxidase activity, indicating attenuated neutrophil infiltration. Therefore, we suggest that inhibition of P-selectin dependent neutrophil recruitment may be the mechanism responsible for the protection seen in P-selectin-deficient mice and in wild-type mice after antibody treatment. As the peritubular vessels provide blood supply to the outer medulla representing the most ischemia/hypoxia-sensitive zone of the kidney (39), neutrophils adherent to either endothelium or platelets could damage the surrounding tissue by two mechanism. First, adherent neutrophils can impair or obstruct blood flow in small vessels leading to local tissue hypoxia in downstream regions (no-reflow phenomenon). Second, while sticking to and later infiltrating through the endothelium, neutrophils can release cytotoxic substances, destroying neighboring cells and tissue matrix (reflow-paradox). Both mechanisms have been postulated in ischemia/reperfusion-induced acute renal failure (3).

Blocking P-selectin to protect from ischemia/reperfusion-induced organ failure has been shown in the heart (40, 41), brain (42, 43), and liver (44). In a rat model of cold renal ischemia-reperfusion, Takada et al. (45) showed a protective effect of soluble P-selectin glycoprotein ligand 1 (PSGL-1), suggesting a role for P-selectin under these circumstances. However, PSGL-1 may bind to and block E- and L-selectin in addition to P-selectin (14).

Our study is the first showing a clear role for P-selectin in ischemia/reperfusion-induced acute renal failure. Similar results have been obtained for ICAM-1 (6) and CD11a/CD11b (17). It is not possible to compare our results with that of blocking CD11a/CD11b (17), as the study protocol differs from ours with respect to animals (uninephrectomized rats) and duration of ischemia. ICAM-1-deficient mice showing decreased renal myeloperoxidase levels were protected against I/R-induced renal failure (6) in the same model of renal ischemia presented here. Since no data from sham-operated, ICAM-1-deficient mice were reported in that study, the efficiency of blocking P-selectin cannot rigorously be compared with that of blocking ICAM-1. However, in ICAM-1-deficient mice, absolute creatinine concentrations at 24 and 48 h were reduced by 60 and 90%, respectively.

In conclusion, in our neutrophil-dependent model of severe ischemia/reperfusion-induced acute renal failure, blocking P-selectin reduces neutrophil infiltration into postischemic kidneys and protects from the development of renal failure, even after the onset of reperfusion. FJ

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