Platelet, but not endothelial, P-selectin is critical for neutrophil-mediated acute postischemic renal failure

KAI SINGBARTL,*,† S. BRADLEY FORLOW,* AND KLAUS LEY*,‡,[1]

*Department of Biomedical Engineering and ‡Cardiovascular Research Center, University of Virginia, Charlottesville, Virginia, USA; and †Klinik und Poliklinik für Anästhesiologie und operative Intensivmedizin, Universitätsklinikum Münster, Münster, Germany

ABSTRACT **In a neutrophil-dependent model of acute postischemic renal failure (APRF), eliminating or blocking P-selectin reduces postischemic neutrophil infiltration and preserves kidney function. This study was designed to identify the role of platelet vs. endothelial P-selectin in APRF. Using wild-type (wt) and P-selectin-deficient (P−/−) mice, we generated chimeric mice by bone marrow transplantation. Chimeric mice exclusively expressed either platelet (Plt-P) or endothelial P-selectin (EC-P). APRF was induced by bilateral renal ischemia in situ (32 min), followed by reperfusion; 48 h after reperfusion, EC-P had significantly lower creatinine concentrations (twofold over sham) than Plt-P (eightfold over sham). Compared with wt, protection from renal failure in EC-P was similar to that observed in P−/−. Plt-P and EC-P demonstrated similar overall postischemic neutrophil infiltration as measured by renal myeloperoxidase activity. However, Plt-P showed massive neutrophil infiltration into outer and inner medulla, similar to that in wt. EC-P had only patchy, more diffuse neutrophil influx. Our study identifies platelet P-selectin as crucial for postischemic neutrophil recruitment into outer and inner medulla, which is detrimental to the development of APRF. This suggests that novel therapeutic strategies for postischemic organ failure could be aimed at neutrophil–platelet interactions.—Singbartl, K., Forlow, S. B., Ley, K. Platelet, but not endothelial, P-selectin is critical for neutrophil-mediated acute postischemic renal failure. *FASEB J.* 15, 2337–2344 (2001)**

Key Words: neutrophils · ischemia-reperfusion · acute renal failure

Postischemic organ failures of heart, brain, and kidney represent a major challenge in clinical medicine and carry a high mortality rate. Postischemic neutrophil recruitment into the kidney has recently been recognized as a key factor in the development of acute postischemic renal failure (APRF) (1–5). During reperfusion, neutrophils are thought to mediate tissue damage by two mechanisms (6). Together with red blood cells and platelets, neutrophils have been proposed to cause capillary perfusion failure or 'no-reflow', which leads to focal tissue hypoxia (7, 8). Second, exacerbated tissue injury (reflow paradox) is induced by postischemic activation of leukocytes, especially neutrophils, which then produce and release cytotoxic substances while adhering to the endothelium and infiltrating into the tissue (9). Recruitment of neutrophils into injured tissues is thought to occur in a cascade-like sequence (10, 11). Endothelial and leukocyte adhesion molecules are critically involved in this process. Capturing or tethering of freely flowing leukocytes is the first step and can result in stable rolling of these cells along the endothelium. The selectin class of adhesion molecules (L-, E-, and P-selectin) mediates leukocyte capture and rolling (12, 13). Chemokines or other chemoattractants activate integrins on slowly rolling leukocytes, leading to firm adhesion and transmigration (14). Several molecules have been implicated in the transmigration process of neutrophils across the endothelium. These include PECAM-1, ICAM-1, VE-cadherin, LFA-1, and CD47 (15–19). The blockade of certain leukocyte endothelial adhesion molecules such as β_2-integrins (20), ICAM-1 (1, 2), E-selectin (5), or P-selectin (4) significantly reduces kidney damage after ischemia/reperfusion and thus preserves kidney function.

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

Several in vivo and in vitro studies have provided evidence for P-selectin-dependent interactions between leukocytes and endothelial cells after ischemia/reperfusion (26, 27) or hypoxia/reoxygenation (28). Evidence for a role of platelet P-selectin is based on reports that neutrophils can roll on platelet P-selectin in flow chamber systems in vitro (29). Both the formation of platelet aggregates and rolling of platelets along the endothelium occur after ischemia/reperfusion (30).

[1] Correspondence: Department of Biomedical Engineering, Health Sciences Center, Box 800759, Charlottesville, VA 22908, USA. E-mail: klausley@virginia.edu

However, little is known about the interaction between neutrophils and platelets after ischemia/reperfusion and its functional relevance in postischemic organ failure. One ex vivo study showed that platelets can further aggravate neutrophil mediated dysfunction in a P-selectin-dependent model of cardiac ischemia/reperfusion (31). In a previous study (4), we showed that P-selectin is differentially expressed after ischemia/reperfusion in the kidney. In the glomeruli, P-selectin is expressed in both endothelial cells and platelets. However, in peritubular vessels, which provide blood supply to the outer medulla, only platelets stained positive for P-selectin. Postischemic neutrophil accumulation in the kidney is directed into the outer medulla (32, 33), which represents the most vulnerable region within the kidney (34). Based on these data, we hypothesized that platelets, in addition to postischemic thrombus formation, may play a role in neutrophil recruitment into the kidney after ischemia/reperfusion.

This study was designed to evaluate the role of platelet vs. endothelial P-selectin in postischemic neutrophil recruitment. To selectively address the role of endothelial vs. platelet P-selectin, we generated chimeric mice by bone marrow transplantation using wild-type (wt) and P-selectin deficient mice. Chimeric mice expressed P-selectin on endothelial cells, platelets, both, or neither. Bone marrow-transplanted mice then underwent bilateral renal ischemia/reperfusion and were evaluated for neutrophil infiltration, creatinine concentration, and P-selectin expression.

MATERIALS AND METHODS

Animals

We used adult (2–3 months old, 20–32 g body weight) C57BL/6 wt mice and mice with a null mutation in the P-selectin gene (35). Mutant mice were back-crossed into a C57BL/6 background for at least six generations and maintained under specific pathogen-free conditions at the Center for Comparative Medicine, University of Virginia.

Bone marrow transplantation

Bone marrow was harvested from donor mice and transplanted into recipient mice as described previously (36). Recipient mice were lethally irradiated in two doses of 600 rads, each ~4 h apart. Donor mice were killed by lethal injection of sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL). Bone marrow cells from both femurs and tibias were harvested under sterile conditions. Approximately 50 million nucleated cells were obtained from each donor mouse. Bones were flushed with RPMI (Life Technologies, Grand Island, NY) (without phenol red) containing 10% fetal calf serum (Atlanta Biologicals, Norcross, GA). Suspended bone marrow cells were washed and erythrocytes were lysed in 0.15 M NH_4Cl lysing solution. Approximately 4–5 million unfractionated bone marrow cells in 200 μl of media were injected intravenously through the tail vein of each recipient mouse. Recipient mice were housed in a barrier facility (individually ventilated cages, HEPA-filtered air, sterile bedding, autoclaved food) under pathogen-free conditions before and after bone marrow transplantation. After bone marrow transplantation, mice were maintained on autoclaved water supplemented with antibiotics (5 mM sulfamethoxazole, 0.86 mM trimethoprim; Sigma, St. Louis, MO). These conditions were maintained for 9 wk, after which mice were tail bled in order to obtain systemic and differential blood counts. Leukocytes were counted using Kimura's stain; platelet counts were obtained by ammonium oxalate staining. Pilot studies had shown that in our neutrophil-dependent model (4, 5), a neutrophil concentration of 900/μl or more was necessary to reproducibly induce severe postischemic renal failure (data not shown). Thus, only mice with a neutrophil concentration of at least 900/μl were included (n=76). Using wt and P-selectin deficient mice, we generated chimeric mice (bone marrow transplantation between mice of different genotypes, **Table 1**) and control mice (bone marrow transplantation between mice of the same genotype). Bone marrow transplantation was validated by Western blot and immunostaining (see below).

Surgical procedure

Mice were anesthetized with intraperitoneal injections of ketamine (125 μg/g body weight, Ketalar; Parke-Davis, Morris Plains, NJ), xylazine (12.5 μg/g body weight; Phoenix Scientific, St. Joseph, MO), and atropine sulfate (0.025 μg/g body weight; Elkins-Sinn, Cherry Hill, NJ) 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 wt mice, with a 50% mortality rate at 72 h (1). 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 four of 76 mice these criteria were not fulfilled, and these mice were excluded from the study. In five mice, kidneys showed signs of hemorrhagic infarction at time of harvesting, indicating venous obstruction rather than arterial occlusion (37). 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

TABLE 1. *Generation of chimeric mice and control mice by bone marrow transplantation (BMT)*

Donor mouse	Recipient mouse	P-selectin expression after BMT	Symbol
Wild-type	Wild-type	Endothelial and platelet	Wt-BMT
Wild-type	P-selectin-deficient	Platelet	Plt-P
P-selectin-deficient	Wild-type	Endothelial	EC-P
P-selectin-deficient	P-selectin-deficient	None	P−/−BMT

volume substitution for 12 h. Twenty-four and 48 h later, mice were killed, blood samples were taken by heart puncture, and kidneys were harvested.

Renal function

Serum samples were used to determine creatinine concentrations, using a commercially available enzymatic test kit (Sigma).

Myeloperoxidase activity (MPO)

MPO, indicating neutrophil infiltration into tissue, was measured in equal-sized samples of both kidneys according to our previously published protocol (4, 5). 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) hexacyltrimethylammonium 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. In triplicate, assay buffer (0.2 mg/ml *o*-dianisidine and 158 μM H_2O_2 in 50 mM KPO_4, pH 6.0) was added to supernatant at a ratio of 4:1. Changes in absorbance were recorded at 460 nm over 3.5 min. The linear part of the resulting curve was used for calculating MPO activity. 1 U of activity was defined as change in absorbance of 1.0/min at 25°C. Results were expressed as units of MPO/g of protein of supernatant as determined by bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL).

Western-blotting

Whole blood was mixed (1:2) 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 phenyl-methane-sulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Protein extracts were stored at −80°C. Samples (25 μl/lane) were run under reducing conditions on an SDS-polyacrylamide gel (4% 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 (38). A goat anti-rabbit antibody conjugated with horseradish-peroxidase served as secondary antibody (Immun-Star, Bio-Rad, Hercules, CA). Washed blots were finally impregnated with the chemiluminescence substrate and exposed to X-ray films.

Immunohistochemistry: P-selectin

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 (38). This was followed by biotinylated secondary antibody (1:250, Vector Laboratories, Burlingame, CA) in 10% goat serum to reduce background staining, and finally by avidin-biotin-peroxidase (Vector Laboratories).

Immunohistochemistry: neutrophils

Paraffin embedded kidney sections (5 μm) were incubated (1:4000) with a rat anti-mouse monoclonal antibody (clone 7/4; Serotec, Raleigh, NC) against a polymorphic 40 kDa antigen expressed by neutrophils (39). This was followed by a biotinylated secondary antibody (1:250, Vector Laboratories) in 10% goat serum to reduce background staining, and finally by avidin-biotin-peroxidase (Vector Laboratories). Neutrophils were then located and counted in representative kidney sections (n=5).

Statistics

For statistical analysis, one-way ANOVA, Dunnett's test, Student-Newman-Keuls test, and paired t test were used. All results are given as mean ± SE. Statistical significance was set at $P < 0.05$.

RESULTS

Bone marrow transplantation

Using our previously described bone marrow transplantation protocol (36), we generated chimeric and control mice (Table 1). Successful bone marrow transplantation was confirmed by Western blot analysis of whole blood and immunostaining on paraffin-embedded kidney sections. Lethally irradiated wt mice that had received bone marrow from P-selectin-deficient mice exclusively expressed endothelial P-selectin (EC-P mice). Lethally irradiated P-selectin-deficient mice injected with bone marrow from wt mice expressed only platelet P-selectin (Plt-P mice). Wild-type mice receiving bone marrow from wt mice (Wt-BMT) and P-selectin-deficient mice obtaining bone marrow from P-selectin-deficient mice (P−/−BMT) served as positive and negative controls, respectively. Only whole blood from Wt-BMT and Plt-P mice, but not from EC-P or P−/−BMT, stained positive for P-selectin in Western blots using whole blood protein extracts (**Fig. 1*a***). Moreover, immunostaining on kidney sections shows that in Wt→Wt mice P-selectin expression was found in both platelets and endothelium (Fig. 1*b*). However, in Plt-P mice, P-selectin expression was restricted to platelets (Fig. 1*c*) whereas in EC-P it was seen only in endothelial cells (Fig. 1*d*). Leukocyte and platelet counts were not significantly different between the groups of mice (**Table 2**).

APRF

After a 32-min ischemia, the creatinine concentration reached its maximum at 48 h after reperfusion (1, 4, 5). Here Wt-BMT mice revealed the highest creatinine concentration 48 h after ischemia/reperfusion, which was ~11-fold higher than in corresponding sham-operated animals (2.96±0.96 mg/dl vs. 0.22±0.02 mg/dl) (**Fig. 2**). By contrast, in P−/−BMT mice the creatinine concentration was significantly lower and only twofold over that of matching sham-operated mice (0.61±0.19 mg/dl vs. 0.27±0.03 mg/dl). This corresponded to an ~80% protection from APRF in P−/−BMT mice, similar to the protection observed in

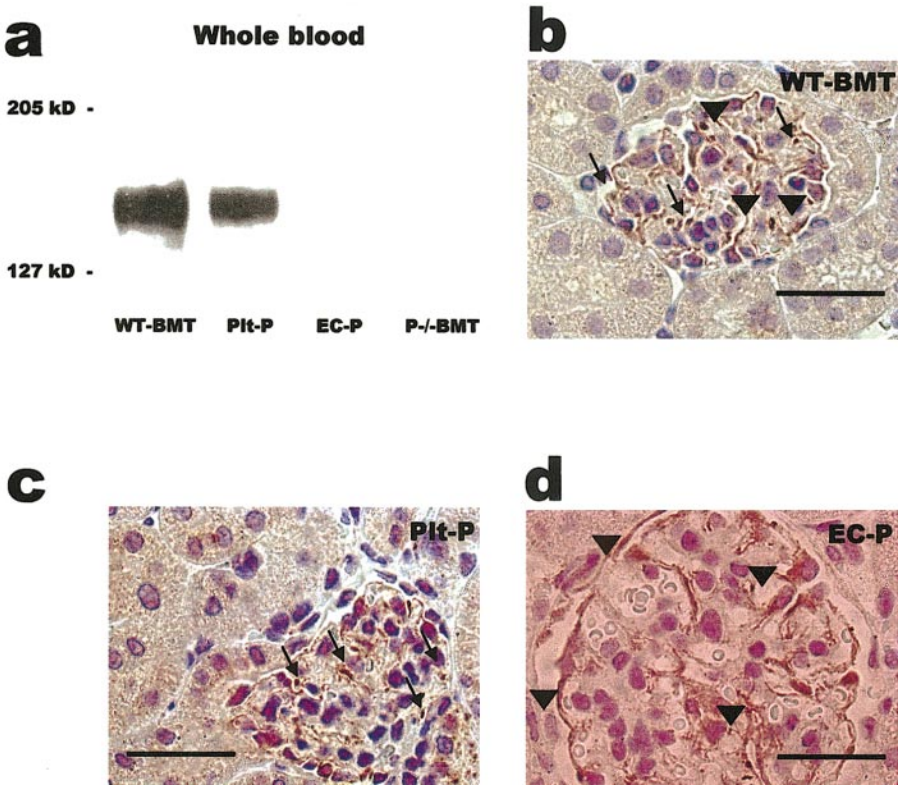

Figure 1. Confirmation of successful bone marrow transplantation. *a)* Western blot analysis for P-selectin in whole blood from all groups of mice studied. Only blood samples from Wt-BMT and Plt-P mice, but not those from EC-P and P−/−BMT mice, showed a positive signal for P-selectin. *b–d*) Immunostaining for P-selectin on paraffin-embedded kidney sections (5 μm) from all groups of mice studied (24 h after I/R). *b)* Kidney sections from Wt-BMT mice expressed P-selectin in platelets (arrows) and endothelium (arrowheads). *c)* In Plt-P mice, P-selectin expression was limited to platelets (arrows). *d)* By contrast, P-selectin expression in EC-P mice could be observed only in endothelial cells (arrowheads). Hematoxylin counterstain. Bar = 40 μm.

P-selectin-deficient mice (4). Thus, bone marrow transplantation does not interfere with our model of neutrophil-dependent APRF.

Mice that expressed endothelial but not EC-P had only an ~ twofold increase in creatinine concentration 48 h after ischemia/reperfusion (0.58±0.34 mg/dl vs. 0.25±0.02 mg/dl), which was significantly smaller than that in Wt-BMT mice (Fig. 2). The protection from APRF seen in EC-P mice equaled that found in P−/−BMT mice. This rise was significantly lower than that observed in mice in which P-selectin expression was restricted to only platelets (Plt-P); here, the creatinine concentration increased ~ eightfold over sham after I/R (2.13±0.46 mg/dl vs. 0.28±0.02 mg/dl) (Fig. 2).

Postischemic neutrophil recruitment

We have previously shown (4, 5) that in wt mice, renal myeloperoxidase activity reaches its maximum at 24 h after ischemia/reperfusion. After I/R, Plt-P mice and EC-P mice had similar, statistically significant increases in absolute as well as relative renal myeloperoxidase activities vs. sham controls (**Fig. 3*a***). MPO activity was elevated ~ sixfold over matching sham-operated animals (Fig. 3*b*), indicating a comparable overall neutrophil infiltration.

Several studies (1, 33, 40) have shown that in wt mice postischemic neutrophil infiltration into the kidney is concentrated in the outer medulla, which represents the most vulnerable region in the kidney (34). To a lesser extent, neutrophils also infiltrate into the inner medulla and cortical region. Using immunostaining on paraffin-embedded kidney sections with a monoclonal antibody against a neutrophil antigen, we localized postischemic neutrophil infiltration into kidneys from Plt-P mice and EC-P mice. In Plt-P mice, we found a massive, highly concentrated influx of neutrophils into the outer medulla (**Fig. 4*a***) and some infiltration into the inner medulla (Fig. 4*c*). In the outer medulla, both intra- and extravascular neutrophils were found (Fig. 4*a*), whereas in the inner medulla neutrophils were seen mainly inside the peritubular vessels (Fig. 4*c*). By contrast, EC-P mice demonstrated a different pattern of postischemic neutrophil recruitment (Fig. 4*b, d*). Here the influx of neutrophils into the outer medulla was

TABLE 2. *Total and differential leukocyte counts and platelet counts (cells/μl) from chimeric and control mice*

	Wt-BMT	Plt-P	EC-P	P−/−BMT
Total WBC	12,000 ± 300	14,000 ± 2,000	11,000 ± 2,000	14,000 ± 900
PMN	1,400 ± 200	1,900 ± 300	1,200 ± 100	1,500 ± 100
MNC	11,000 ± 2,600	11,000 ± 1,900	9,600 ± 2,100	12,000 ± 900
Plts	850,000 ± 64,000	600,000 ± 110,000	570,000 ± 130,000	590,000 ± 60,000
Mice	11	9	9	12

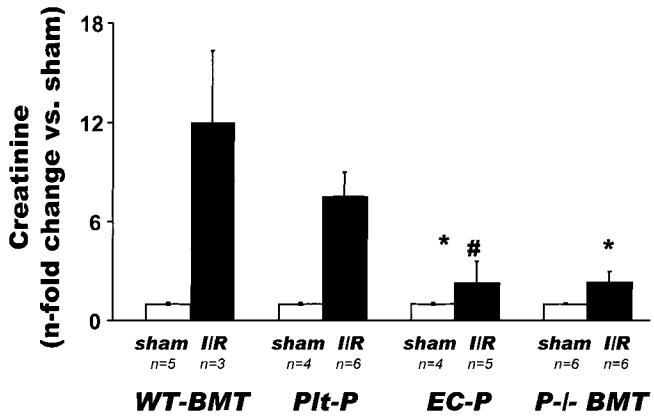

Figure 2. Renal function as measured by serum creatinine concentration in Wt-BMT, Plt-P, EC-P, and P−/−BMT mice at 48 h after 32 min bilateral renal ischemia and subsequent reperfusion. *$P < 0.05$ vs. Wt→Wt. #$P < 0.05$ vs. Plt-P.

drastically reduced (Fig. 4*b*) and almost completely abolished in the inner medulla (Fig. 4*d*). Instead, more neutrophils were found in sections of the renal cortex of EC-P mice (Fig. 4*f*) than in Plt-P mice (Fig. 4*e*). Thus, the overall neutrophil infiltration in EC-P mice was similar in extent, but more diffuse than in Plt-P mice.

DISCUSSION

Our data show that development of acute renal failure after postischemic renal injury requires P-selectin on platelets but not on endothelial cells. We had previously localized P-selectin positive platelets in capillaries of the outer medulla (4). However, the dominant role of platelet P-selectin was surprising, because after ischemia/reperfusion P-selectin is also expressed on glomerular endothelial cells (4). Even though P-selectin −/− mice reconstituted with wt bone marrow (Plt-P) and wt mice reconstituted with P-selectin−/− bone marrow (EC-P) demonstrated equal total neutrophil recruitment into the kidney, only EC-P were protected from renal failure. In these mice, neutrophil accumulation was redirected from a highly concentrated infiltrate in the outer medulla to a more diffuse pattern. The dissociation between global neutrophil recruitment and outcome suggests that neutrophils recruited into certain vulnerable postischemic regions mediate renal damage at a clinically relevant level. These findings also call into question the value of global measures of neutrophil infiltration without histological studies to localize the infiltrating cells.

Several studies have shown that neutrophil infiltration into the postischemic kidney is important for the development of renal failure (1, 41–44). However, none of these studies investigated the role of platelets for neutrophil recruitment. Our study is the first to specifically show a role for platelet-induced neutrophil recruitment in postischemic renal injury. Moreover, it is the first in vivo study to demonstrate the relevance of platelet-induced neutrophil recruitment for the development of any postischemic organ failure. So far, only one ex vivo (31) study has shown a role for platelets in postischemic organ dysfunction. Lefer et al. (31) showed that postischemic cardiac reperfusion with platelets and neutrophils resulted in a far greater myocardial dysfunction than reperfusion with either cell type alone. Dual-cell reperfusion also leads to a much higher postischemic neutrophil infiltration than reperfusion with neutrophils alone. Adding either a sialyl Lewis^x-oligosaccharide or a recombinant soluble P-selectin ligand to the reperfusion solution drastically reduced both cardiodepressant effects and neutrophil infiltration. Kogaki et al. (45) examined neutrophil adhesion after hypoxia-reoxygenation in a static endothelial cell/cardiomyocyte coculture system in vitro. The coincubation with platelets resulted in significantly higher neutrophil adhesion and transmigration than the incubation with endothelial cells/cardiomyocytes alone. This increase was inhibited by a monoclonal anti-P-selectin antibody, suggesting a critical role for P-selectin in platelet-mediated neutrophil adhesion in this model.

After ischemia/reperfusion, neutrophils accumulate in the outer medulla (32, 33), which has long been known to undergo extensive postischemic damage (46). These findings suggest an association between neutrophil infiltration in this area and subsequent

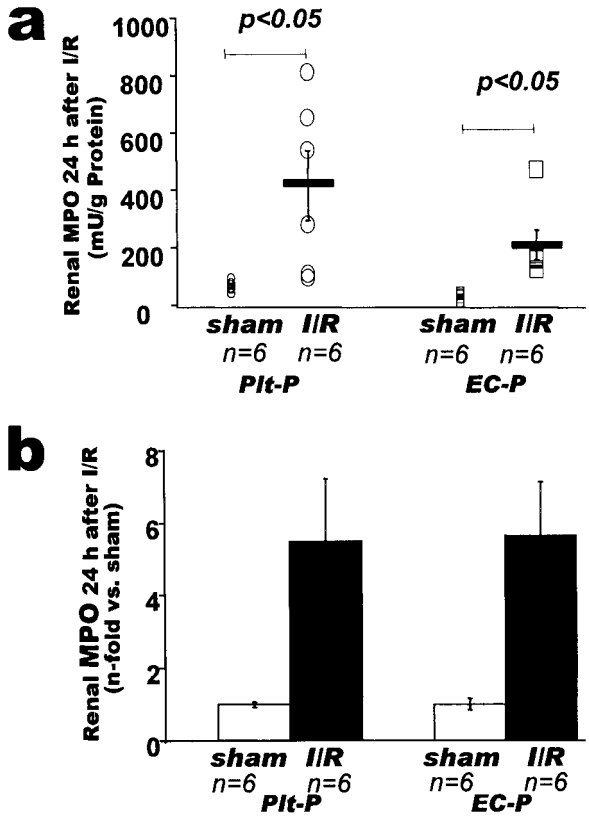

Figure 3. Renal myeloperoxidase activity, indicating neutrophil infiltration, in Plt-P and EC-P mice at 24 h after 32 min bilateral renal ischemia and after reperfusion. *a)* Absolute renal myeloperoxidase activities, mean ± SE. MPO in sham-operated controls showed a small but significant difference. *b)* Relative renal myeloperoxidase activities (fold increase±SE).

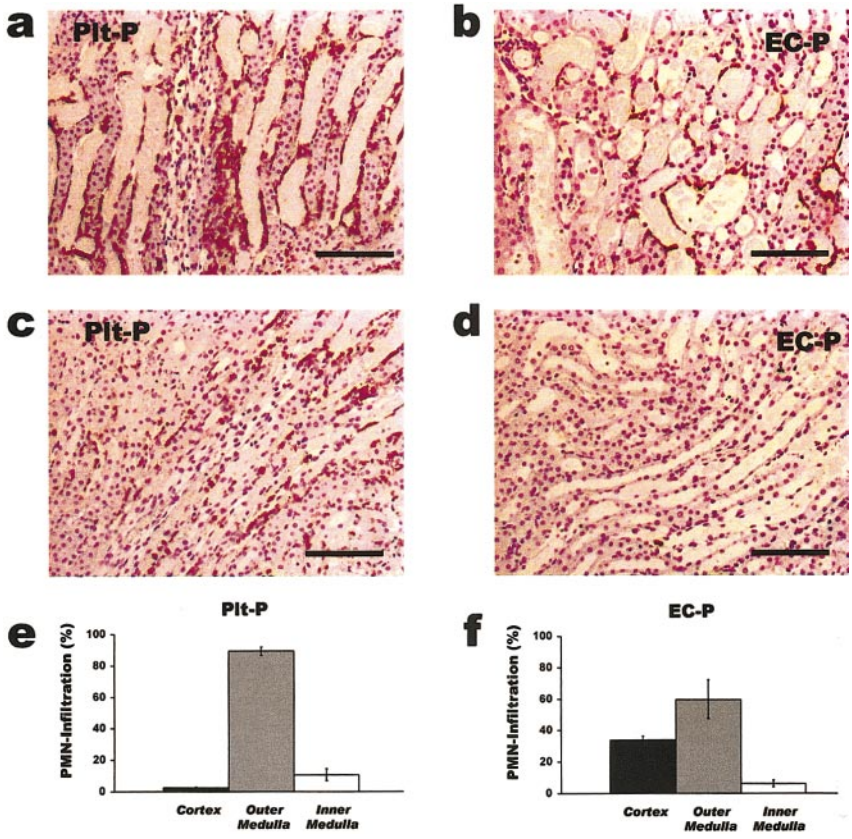

Figure 4. Immunostaining for neutrophils in paraffin-embedded kidney (5 μm) sections 24 h after renal ischemia/reperfusion. *a)* In Plt-P mice, there was a massive neutrophil infiltration into the outer medulla and some into the inner medulla (*c*). By contrast, in EC-P mice the influx of neutrophils into the outer medulla was drastically reduced (*b*) and that into the inner medulla almost completely abolished (*d*). Hematoxylin counterstain. Bar = 100 μm. *e, f)* Counting and locating neutrophils in representative kidney sections (n=5) support these findings. Data are given as percent of total neutrophils counted.

renal damage. Our present data provide a potential mechanistic link between platelet-mediated neutrophil infiltration into the outer medulla and acute renal failure. In mice lacking platelet P-selectin, neutrophil recruitment into the outer medulla was reduced, and these mice were protected from APRF. Although we show here that neutrophil recruitment to the outer medulla requires platelet P-selectin, the mechanism of recruitment remains unknown. It is possible that platelets may adhere to endothelial cells and subsequently cause secondary attachment of neutrophils. This has been shown in vitro (47–49) and in vivo, especially after arterial injury by angioplasty (48–50). However, platelets could also adhere to circulating neutrophils after ischemia/reperfusion, which could then become trapped in narrow peritubular capillaries. The latter possibility is supported by a recent intravital microscopy study (30). In a mouse model, Massberg et al. (30) showed that after intestinal ischemia/reperfusion, platelet–endothelial cell-interactions were almost completely blocked by eliminating endothelial P-selectin, but platelet–leukocyte interactions were platelet P-selectin dependent (30). Since the development of APRF in our model depends on platelet but not on endothelial P-selectin, it appears more likely that circulating platelets and neutrophils form aggregates, which then adhere or become trapped in the renal microcirculation.

In addition to P-selectin (4), blockade or elimination of E-selectin (5), β_2-integrins (20), or ICAM-1 (1, 2) has been shown to reduce kidney damage after ischemia and reperfusion. By contrast, L-selectin blockade had no effect (51). Our study not only adds to this list of protective interventions in postischemic renal failure, it also provides evidence that selective elimination of platelet P-selectin is protective. This novel finding suggests that targeted therapy aimed at platelets or, more specifically, platelet–leukocyte interaction may be beneficial in patient management to prevent postischemic renal failure. Importantly, such interventions may impair host defense or coagulation much less than globally blocking leukocyte adhesion molecules. FJ

We thank Dr. Samuel A. Green, University of Virginia, for providing polyclonal anti-P-selectin antibody for this study and Dr. Daniel C. Bullard, University of Alabama, Birmingham, for providing breeder pairs, from which the colony of P-selectin deficient mice was established. This work was supported by National Institutes of Health grant HL-54136 to K.L.

REFERENCES

1. Kelly, K. J., Williams, W. W. J., Colvin, R. B., Meehan, S. M., Springer, T. A., Gutierrez-Ramos, J. C., and Bonventre, J. V. (1996) Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *J. Clin. Invest.* **97,** 1056–1063
2. Rabb, H., Mendiola, C. C., Saba, S. R., Dietz, J. R., Smith, C. W., Bonventre, J. V., and Ramirez, G. (1995) Antibodies to ICAM-1 protect kidneys in severe ischemic reperfusion injury. *Biochem. Biophys. Res. Commun.* **211,** 67–73
3. Linas, S. L., Whittenburg, D., Parsons, P. E., and Repine, J. E. (1995) Ischemia increases neutrophil retention and worsens acute renal failure: role of oxygen metabolites and ICAM 1. *Kidney Int.* **48,** 1584–1591

4. Singbartl, K., Green, S. A., and Ley, K. (2000) Blocking P-selectin protects from ischemia/reperfusion-induced acute renal failure. *FASEB J.* **14,** 48–54
5. Singbartl, K., and Ley, K. (2000) Protection from ischemia-reperfusion induced severe acute renal failure by blocking E-selectin. *Crit. Care Med.* **28,** 2507–2514
6. Müller, J. M., Vollmar, B., and Menger, M. D. (1997) Pentoxifylline reduces venular leukocyte adherence ('reflow paradox') but not microvascular 'no reflow' in hepatic ischemia/reperfusion. *J. Surg. Res.* **71,** 1–6
7. Rabb, H., and Postler, G. (1998) Leucocyte adhesion molecules in ischaemic renal injury: kidney specific paradigms? *Clin. Exp. Pharmacol. Physiol.* **25,** 286–291
8. Mason, J., Joeris, B., Welsch, J., and Kriz, W. (1989) Vascular congestion in ischemic renal failure: the role of cell swelling. *Miner. Electrolyte Metab.* **15,** 114–124
9. Menger, M. D., and Vollmar, B. (1994) In vivo analysis of microvascular reperfusion injury in striated muscle and skin. *Microsurgery* **15,** 383–389
10. Springer, T. A. (1995) Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu. Rev. Physiol.* **57,** 827–872
11. Butcher, E. C. (1991) Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* **67,** 1033–1036
12. Kansas, G. S. (1996) Selectins and their ligands: current concepts and controversies. *Blood* **88,** 3259–3287
13. Vestweber, D., and Blanks, J. E. (1999) Mechanisms that regulate the function of the selectins and their ligands. *Physiol. Rev.* **79,** 181–213
14. Jung, U., Norman, K. E., Scharffetter-Kochanek, K., Beaudet, A. L., and Ley, K. (1998) Transit time of leukocytes rolling through venules controls cytokine-induced inflammatory cell recruitment in vivo. *J. Clin. Invest.* **102,** 1526–1533
15. Muller, W. A. (1995) The role of PECAM-1 (CD31) in leukocyte emigration: studies in vitro and in vivo. *J. Leukoc. Biol.* **57,** 523–528
16. Bullard, D. C., Qin, L., Lorenzo, I., Quinlin, W. M., Doyle, N. A., Bosse, R., Vestweber, D., Doerschuk, C. M., and Beaudet, A. L. (1995) P-selectin/ICAM-1 double mutant mice: acute emigration of neutrophils into the peritoneum is completely absent but is normal into pulmonary alveoli. *J. Clin. Invest.* **95,** 1782–1788
17. Gotsch, U., Borges, E., Bosse, R., Boggemeyer, E., Simon, M., Mossmann, H., and Vestweber, D. (1997) VE-cadherin antibody accelerates neutrophil recruitment in vivo. *J. Cell Sci.* **110,** 583–588
18. Lu, H., Smith, C. W., Perrard, J., Bullard, D., Tang, L., Shappell, S. B., Entman, M. L., Beaudet, A. L., and Ballantyne, C. M. (1997) LFA-1 is sufficient in mediating neutrophil emigration in Mac-1-deficient mice. *J. Clin. Invest.* **99,** 1340–1350
19. Cooper, D., Lindberg, F. P., Gamble, J. R., Brown, E. J., and Vadas, M. A. (1995) Transendothelial migration of neutrophils involves integrin-associated protein (CD47). *Proc. Natl. Acad. Sci. USA* **92,** 3978–3982
20. Rabb, H., Mendiola, C. C., Dietz, J., Saba, S. R., Issekutz, T. B., Abanilla, F., Bonventre, J. V., and Ramirez, G. (1994) Role of CD11a and CD11b in ischemic acute renal failure in rats. *Am. J. Physiol.* **267,** F1052–F1058
21. Geng, J. G., Bevilacqua, M. P., Moore, K. L., McIntyre, T. M., Prescott, S. M., Kim, J. M., Bliss, G. A., Zimmerman, G. A., and McEver, R. P. (1990) Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature (London)* **343,** 757–760
22. McEver, R. P., Beckstead, J. H., Moore, K. L., Marshall-Carlson, L., and Bainton, D. F. (1989) GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J. Clin. Invest.* **84,** 92–99
23. Berman, C. L., Yeo, E. L., Wencel-Drake, J. D., Furie, B. C., Ginsberg, M. H., and Furie, B. (1986) A platelet alpha granule membrane protein that is associated with the plasma membrane after activation. Characterization and subcellular localization of platelet activation-dependent granule-external membrane protein. *J. Clin. Invest.* **78,** 130–137
24. Foreman, K. E., Vaporciyan, A. A., Bonish, B. K., Jones, M. L., Johnson, K. J., Glovsky, M. M., Eddy, S. M., and Ward, P. A. (1994) C5a-induced expression of P-selectin in endothelial cells. *J. Clin. Invest.* **94,** 1147–1155
25. Sanders, W. E., Wilson, R. W., Ballantyne, C. M., and Beaudet, A. L. (1992) Molecular cloning and analysis of in vivo expression of murine P-selectin. *Blood* **80,** 795–800
26. Sawaya, D. E. J., Zibari, G. B., Minardi, A., Bilton, B., Burney, D., Granger, D. N., Mcdonald, J. C., and Brown, M. (1999) P-selectin contributes to the initial recruitment of rolling and adherent leukocytes in hepatic venules after ischemia/reperfusion. *Shock* **12,** 227–232
27. Kanwar, S., Smith, C. W., and Kubes, P. (1998) An absolute requirement for P-selectin in ischemia/reperfusion-induced leukocyte recruitment in cremaster muscle. *Microcirculation* **5,** 281–287
28. Ichikawa, H., Flores, S., Kvietys, P. R., Wolf, R. E., Yoshikawa, T., Granger, D. N., and Aw, T. Y. (1997) Molecular mechanisms of anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. *Circ. Res.* **81,** 922–931
29. Diacovo, T. G., Roth, S. J., Buccola, J. M., Bainton, D. F., and Springer, T. A. (1996) Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood* **88,** 146–157
30. Massberg, S., Enders, G., Leiderer, R., Eisenmenger, S., Vestweber, D., Krombach, F., and Messmer, K. (1998) Platelet-endothelial cell interactions during ischemia/reperfusion: the role of P-selectin. *Blood* **92,** 507–515
31. Lefer, A. M., Campbell, B., Scalia, R., and Lefer, D. J. (1998) Synergism between platelets and neutrophils in provoking cardiac dysfunction after ischemia and reperfusion: role of selectins. *Circulation* **98,** 1322–1328
32. Willinger, C. C., Schramek, H., Pfaller, K., and Pfaller, W. (1992) Tissue distribution of neutrophils in postischemic acute renal failure. *Virchows Arch. B.* **62,** 237–243
33. Lindner, J., Song, J., Xu, F., Klibanov, A., Singbartl, K., Ley, K., and Kaul, S. (2000) Noninvasive ultrasound imaging of inflammation using microbubbles targeted to activated leukocytes. *Circulation* **102,** 2745–2750
34. Brezis, M., and Rosen, S. (1995) Hypoxia of the renal medulla—its implications for disease. *N. Engl. J. Med.* **332,** 647–655
35. Bullard, D.C., Qin, L., Lorenzo, I., Quinlin, W. M., Doyle, N. A., Bosse, R., Vestweber, D., Doerschuk, C. M., and Beaudet, A. L. (1995) P-selectin/ICAM-1 double mutant mice: acute emigration of neutrophils into the peritoneum is completely absent but is normal into pulmonary alveoli. *J. Clin. Invest.* **95,** 1782–1788
36. Jung, U., and Ley, K. (1999) Mice lacking two or all three selectins demonstrate overlapping and distinct functions for each selectin. *J. Immunol.* **162**, 6755–6762
37. Racusen, L. C., Trpkov, K., and Solez, K. (1995) Pathology of acute renal failure. In *Acute Renal Failure in the Critically Ill* (Bellomo, R., and Ronco, C., eds) pp. 1–27, Springer Verlag, Berlin
38. Green, S. A., Setiadi, H., McEver, R. P., and Kelly, R. B. (1994) The cytoplasmic domain of P-selectin contains a sorting determinant that mediates rapid degradation in lysosomes. *J. Cell Biol.* **124,** 435–448
39. Hirsch, S., and Gordon, S. (1983) Polymorphic expression of a neutrophil differentiation antigen revealed by monoclonal antibody 7/4. *Immunogen* **18,** 229–239
40. Chiao, H., Kohda, Y., McLeroy, P., Craig, L., Housini, I., and Star, R. A. (1997) Alpha-melanocyte-stimulating hormone protects against renal injury after ischemia in mice and rats. *J. Clin. Invest.* **99,** 1165–1172
41. Thornton, M. A., Winn, R., Alpers, C. E., and Zager, R. A. (1989) An evaluation of the neutrophil as a mediator of in vivo renal ischemic-reperfusion injury. *Am. J. Pathol.* **135,** 509–515
42. Hellberg, P. O., Kallskog, O. T., Ojteg, G., and Wolgast, M. (1990) Peritubular capillary permeability and intravascular RBC aggregation after ischemia: effects of neutrophils. *Am. J. Physiol.* **258,** F1018–F1025
43. Hellberg, P. O., and Kallskog, T. O. (1989) Neutrophil-mediated post-ischemic tubular leakage in the rat kidney. *Kidney Int.* **36,** 555–561
44. Paller, M. S. (1989) Effect of neutrophil depletion on ischemic renal injury in the rat. *J. Lab. Clin. Med.* **113,** 379–386

45. Kogaki, S., Sawa, Y., Sano, T., Matsushita, T., Ohata, T., Kurotobi, S., Tojo, S. J., Matsuda, H., and Okada, S. (1999) Selectin on activated platelets enhances neutrophil endothelial adherence in myocardial reperfusion injury. *Cardiovasc. Res.* **43,** 968–973
46. Venkatachalam, M. A., Bernard, D. B., Donohoe, J. F., and Levinsky, N. G. (1978) Ischemic damage and repair in the rat proximal tubule: differences among the S1. *S* **2,** and S3 segments. *Kidney Int.* **14**, 31–49
47. Yeo, E. L., Sheppard, J. A., and Feuerstein, I. A. (1994) Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model). *Blood* **83,** 2498–2507
48. Merhi, Y., Provost, P., Chauvet, P., Theoret, J. F., Phillips, M. L., and Latour, J. G. (1999) Selectin blockade reduces neutrophil interaction with platelets at the site of deep arterial injury by angioplasty in pigs. *Arterioscler. Thromb. Vasc. Biol.* **19,** 372–377
49. Merhi, Y., Provost, P., Guidoin, R., and Latour, J. G. (1997) Importance of platelets in neutrophil adhesion and vasoconstriction after deep carotid arterial injury by angioplasty in pigs. *Arterioscler. Thromb. Vasc. Biol.* **17,** 1185–1191
50. Merhi, Y., Lacoste, L. L., and Lam, J. Y. (1994) Neutrophil implications in platelet deposition and vasoconstriction after deep arterial injury by angioplasty in pigs. *Circulation* **90,** 997–1002
51. Rabb, H., Ramirez, G., Saba, S. R., Reynolds, D., Xu, J., Flavell, R., and Antonia, S. (1996) Renal ischemic-reperfusion injury in L-selectin-deficient mice. *Am. J. Physiol.* **271,** F408–F413

Received for publication April 19, 2001.
Revised for publication July 13, 2001.