A Role for P-Selectin in Neutrophil and Platelet Infiltration in Immune Complex Glomerulonephritis

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Abstract. P-selectin is one of the key early mediators of leukocyte adhesion in inflammatory conditions. This report examines the role of P-selectin in a neutrophil- and platelet-mediated model of glomerulonephritis (the concanavalin A [con A] model). The administration of neutralizing anti-P-selectin antibody (PB1.3) reduced the platelet influx at 10 min ($P < 0.05$) and was associated with a $60\%$ reduction in the number of oxidant-producing cells at 3 h within glomeruli. No effect on glomerular monocyte-macrophage accumulation was observed, and proteinuria was reduced by $20\%$ but did not reach significance. It is concluded that P-selectin plays an important role in mediating the neutrophil and platelet accumulation in this model and likely has a role in mediating the glomerular injury. (J Am Soc Nephrol 8: 1838–1844, 1997)

Many glomerular diseases are associated with an acute inflammatory process involving both neutrophils and platelets. Examples include diffuse proliferative lupus nephritis, cryoglobulinemic and membranoproliferative nephritis, and postinfectious nephritis (1–3). Evidence strongly suggests that the neutrophil polymorphonuclear leukocyte (PMN) contributes to glomerular injury in these conditions. First, it is known that PMN can degrade the basement membrane and injure glomerular cells in vitro via the release of proteases and oxidants (4,5). PMN-derived proteases and oxidants can also injure glomeruli and induce proteinuria in experimental animals, and antioxidants protect animals from PMN-mediated glomerular injury (6).

The role of the platelet in glomerular injury has received less attention. The observation that platelets and PMN are frequently closely associated in glomerular lesions both in animals and in man suggests a possible interaction (4,7,8). Indeed, studies in one experimental model of PMN-mediated injury showed that platelet depletion significantly reduces proteinuria by a mechanism that does not involve neutrophil localization in glomeruli and probably reflects direct intraglomerular platelet-neutrophil interaction (8).

The mechanism by which platelets can affect PMN function are diverse. In vitro studies have shown that platelets may augment PMN adherence, phagocytosis, and oxidant and protease release (9–14). Platelets may also inhibit PMN functions under certain conditions (15,16).

One major candidate mediating platelet–PMN interactions is P-selectin. P-selectin is a 140-kD transmembrane glycoprotein leukocyte adhesion molecule that is constitutively present in the alpha granules of the platelet and the Weibel-Palade bodies in the endothelial cells, and is rapidly translocated to the cell surface after stimulation of the cell with agents such as thrombin, histamine, H$_2$O$_2$, or terminal complement components (17). The selectins are crucial for early PMN attachment, before the firm adhesion mediated by the PMN $\beta_2$ integrins (CD11/CD18). Selectins allow the PMN to reversibly adhere to the endothelium, thereby allowing the PMN to roll along the vessel wall following the chemotactic gradient to the source of the inflammatory stimulus (18,19).

In addition to its role in the mediation of leukocytes to activated endothelium, P-selectin is also important for platelet–PMN adhesion. P-selectin appears to play an important role in mediating the leukocyte accumulation and fibrin deposition associated with platelet aggregates in thrombus formation (20). P-selectin-mediated adherence of platelets to PMN may also augment oxidant production by the PMN (21). This latter observation suggests that platelets and PMN could act synergistically to increase oxidant release at sites of inflammation via P-selectin.

Therefore, we examined the role of P-selectin in a platelet- and PMN-dependent model of glomerular injury (8,22). This model is induced by the infusion of concanavalin A (con A) into the renal artery of rats, followed by sheep anti-con A IgG. The con A is a lectin that binds to glomerular endothelial cell and basement membrane glycoproteins, thereby acting as a planted antigen. The infusion of anti-con A antibody then results in the formation of immune complexes on the endothelial surface, leading to complement-dependent endothelial cell injury. Platelets and PMN localize to glomeruli in this model, and by ultrastructural studies can often be shown to be in contact (22). The acute injury is associated with proteinuria
that is dependent on leukocytes, platelets, and complement (22).

Materials and Methods

Experimental Model

The con A model is an immune complex model of glomerulonephritis induced by the selective renal artery perfusion of the lectin con A (22,23). Infusion of con A (150 μg) binds to glucose and mannosene residues on the glomerular endothelial cells and basement membrane glycoproteins, thereby acting as a planted antigen. Subsequent to this, anti-con A antibodies are infused and then bind to the con A, forming in situ immune complexes that are then shed from the endothelium where they localize in the subendothelial space. This model is characterized by acute complement activation, glomerular platelet and PMN infiltration, and varying degrees of fibrin deposition associated with endothelial cell injury, proteinuria, and acute renal failure (22,23). Whereas previous studies by our group used rabbit anti-con A antibodies to induce disease (22,23), in this study sheep anti-con A antibodies were used. This antibody was prepared and purified in a manner similar to that used for the preparation of the rabbit anti-con A IgG (22) and resulted in an identical histologic lesion.

Experimental Protocol

Immune complex glomerulonephritis was induced via selective right renal artery perfusion in male Sprague Dawley rats (n = 15). The treatment group received 800 μg of PB1.3 monoclonal antibody to P-selectin (n = 6), and the control group received 800 μg of an irrelevant monoclonal mouse IgG1 (n = 5). A third group of animals (n = 4) had immune complex glomerulonephritis induced without any intervention. Animals underwent survival biopsies of the perfused kidney at 10 min and 3 h, with a sacrificial biopsy at 24 h. The animals were placed in metabolic cages between 4 and 24 h with water ad libitum, and urine was collected for urine protein/creatinine ratio measurement. At sacrifice, blood was drawn for blood urea nitrogen (BUN) measurement, as well as for platelet and PMN counts. This protocol was approved by the University of Washington Animal Care Committee and conformed with guidelines of National Institutes of Health.

Renal Artery Perfusion

Male Sprague Dawley rats (Simonsen, Gilroy, CA) weighing between 200 and 300 g were anesthetized with ketamine-xylazine-acepromazine (4:2:1 vol/vol/vol) at a dose of 0.1 ml/100 g body wt intramuscularly. A midline laparotomy with a left nephrectomy was performed, and the aorta was temporarily clamped. The right renal artery was then perfused via retrograde cannulation of the superior mesenteric artery, as described previously (22). Using a perfusion pump (Sage Instrument Division, Orion Research, Inc., Cambridge, MA), the right kidney was sequentially perfused at 1 ml/min with 150 μg of con A in 1 cc phosphate-buffered saline (PBS) (Vector Laboratories, Burlingame, CA), 0.3 ml of PBS, 30 mg of polycyonal sheep anti-con A IgG (0.66 ml vol), 0.3 ml of PBS, and then 800 μg of either PB 1.3 or control monoclonal mouse IgG. After perfusion, the aorta clamps were removed and blood flow was restored. Total ischemia time was always less than 10 min. Hemostasis at the site of vessel cannulation was achieved with Gelfoam (Upjohn, Kalamazoo, MI) and gentle pressure. A biopsy of the right kidney was performed 10 min after perfusion of the anti-con A antibody, and hemostasis was provided by Gelfoam. The incision was then closed in two layers, using 4-0 silk sutures and surgical staples. The animal was injected with buprenorphine intramuscularly for pain and then placed in a warm recovery area until anesthesia wore off.

Monoclonal P-Selectin Blocking Antibody

PB1.3 (gift of Cytel Corporation, San Diego, CA) is a monoclonal IgG, antibody with neutralizing activity against rat P-selectin (24). The production and characterization of PB1.3 has been described previously, and there is no reactivity with E-selectin or L-selectin (24).

Irrelevant Murine IgG1

Mouse hybridoma IgG1, kappa (Organon Teknika Corp., West Chester, PA).

Tissue Collection and Histology

Tissue for immunostaining was fixed in methylcarnoyls, processed, and embedded. Six-μm sections were stained with the periodic acid-Schiff reagent. Indirect immunostaining was performed on methylcarnoyls fixed tissue for neutrophils (RP-3; murine monoclonal IgM antibody against rat neutrophils; gift of F. Sendo, Yamagata University School of Medicine, Yamagata, Japan) (25), macrophages (ED-1; murine monoclonal antibody against a liposomal antigen in rat macrophages; Serotec, Indianapolis, IN) (26), platelets (PL-1; murine monoclonal antibody against rat platelets; gift of W. W. Bakker, University of Groningen, Groningen, The Netherlands) (27), and P-selectin (PB 1.3) with appropriate biotinylated secondary antibodies followed by streptavidin peroxidase and colorimetric reaction.

H2O2-producing cells were identified as described previously (28), using a method that detects H2O2 produced by leukocytes with endogenous peroxidase activity (29). Frozen sections were incubated for 20 min at 37°C in Tris-buffered solution, pH 7.0, with 0.5 mg/ml 3,3-diaminobenzidine (DAB; Sigma, St. Louis, MO). In the presence of H2O2, the endogenous peroxidase activity will generate a colorimetric reaction from the DAB.

Immunofluorescence Staining

Sheep IgG was demonstrated on snap-frozen tissue, using FITC-conjugated rabbit anti-sheep IgG (Organon Teknika) (8), con A was detected by use of biotinylated goat anti-con A antibody, followed by FITC-conjugated streptavidin (Amersham Life Science, Arlington Heights, IL). C3 was detected with FITC-conjugated goat anti-rat C3 (Organon Teknika).

Quantitative Studies

The PMN-, H2O2-, and ED-1-stained cells were enumerated in a blinded manner in a minimum of 20 glomeruli per biopsy, using glomeruli only if they represented an equatorial cross-section. The platelet influx was graded semiquantitatively per glomerulus with the following scale: rare or no platelets, grade 0; few platelets (aggregates in 2 or 3 capillary loops), grade 1; moderate platelet influx (involving up to 25% glomerular capillaries), grade 2; intense platelet influx (involving up to 50% of capillaries), grade 3; and maximal platelet influx (greater than 50% capillary involvement), grade 4. Each glomerulus was given a numeric score. The scores were totaled for the entire biopsy and divided by the number of glomeruli counted in that biopsy for a mean score for each biopsy.

Biochemical Assays

Urine was collected and analyzed for protein excretion, using the sulfosalicylic acid method. Urine creatinine was measured using the...
Statistical Analyses

All values are mean ± SEM. Comparisons between the groups were done by t test. Analysis of the oxidant cells was performed using a single-tailed test based on our a priori hypothesis that P-selectin would mediate the oxidant production by the infiltrating leukocytes. The platelet influx data were analyzed nonparametrically using the Median test.

Results

Experimental Model

Glomerulonephritis was induced by the selective renal artery perfusion of con A and anti-con A IgG. As reported previously (22,23), this results in an acute complement-dependent glomerular endothelial cell injury. At 10 mm, a marked platelet influx into glomeruli can be documented (Figure 1), with only minimal leukocyte influx at this time. By 3 h, a prominent accumulation of glomerular leukocytes is observed, the majority of which are PMN (Figure 2). Many of the PMN, as well as monocytes, are producing oxidants such as H₂O₂ (Figure 3). At 24 h, the platelet and PMN influx has largely subsided, although the glomerulus still exhibits hypercellularity primarily because of a macrophage (ED-1 + cell) infiltration (Figure 4). There is also endothelial cell swelling, with a varying amount of intracapillary fibrin deposition that rarely occluded individual capillary loops.

Expression of P-Selectin

In normal rat glomeruli, P-selectin is absent (data not shown). In contrast, P-selectin was heavily expressed by infiltrating platelets in the con A model, especially at the 10-min time point. At 3 h, the P-selectin was expressed primarily on platelets but also to varying degrees on the glomerular endothelium, and even rarely on the PMN itself (Figure 5a). Furthermore, in animals perfused with the murine anti-P-selectin antibody (PB 1.3), the anti-P-selectin antibody could be localized 10 min after perfusion to the infiltrating platelets and less to the endothelium by directly applying biotinylated goat anti-mouse IgG antibody on the tissue, followed by peroxidase-streptavidin and the DAB colorimetric reaction (Figure 5b). No mouse IgG could be detected on platelets in animals with nephritis perfused with control mouse IgG (Figure 5c).

Effect of P-Selectin Blockade

Glomerular Platelet Influx. The mean glomerular platelet influx score was determined 10 min after infusion of the anti-con A antibodies, because this is the time of maximal platelet infiltration (22). There was a marked reduction in glomerular platelet accumulation in the PB1.3-treated group versus control rats (P < 0.05), as shown in Figure 6.

Glomerular PMN Infiltration. Very few PMN were present in the anti-P-selectin or control groups at 10 min (data not shown). However, at 3 h, when the maximal neutrophil
influx is expected (22), there was a significant reduction in PMN in the PB1.3-treated group (7.2 ± 1.5 RP-3-positive cells per glomerulus versus 2.8 ± 1.0 in the control versus PB1.3-treated group (P < 0.05) (Figure 7).

**Glomerular Oxidant-Producing Cells.** The number of oxidant-producing cells was determined at 3 h, the point of maximal leukocyte influx. There was a greater than 50% reduction in oxidant-producing cells in the PB1.3-treated group (3.8 ± 1.2 H₂O₂-positive cells per glomerulus versus 8.3 ± 2.2 H₂O₂-positive cells per glomerulus, PB1.3 versus control (P < 0.05) (Figures 2 and 8).

**Glomerular Macrophage Influx.** In this model, there is a progressive mononuclear cell infiltration over the 24-h time course. This was first observed in the 3-h biopsy, with 3.9 ± 0.4 ED-1-positive cells per glomerulus in the PB1.3-treated group compared with 3.9 ± 0.4 in the control group. The 24-h time point showed 6.3 ± 1.0 ED-1-positive cells per glomerulus in the PB1.3-treated group and 7.5 ± 0.7 in the control group (P = NS, Table 1).

**Circulating PMN and Platelet Counts.** Both the control and PB1.3 groups had a thrombocytosis and equivalent WBC counts at 24 h at the time of sacrifice, and there was no significant difference between groups (Table 1).

**Functional Data.** Serum BUN measurements at 24 h were not different between groups (Table 1). The urine protein/creatinine ratios tended to be lower in PB1.3-treated animals, but did not reach statistical significance (1.98 ± 0.4 versus 2.5 ± 0.3, P = NS) (Table 1).

**Immunofluorescence Staining.** Frozen sections of biopsies at 3 h demonstrated equivalent (4+) staining of sheep IgG and con A in a linear pattern along the capillary loops. Immunofluorescence staining for C3 also demonstrated 3+ to 4+ staining in the same distribution in both groups.

**Discussion**

In this study, we examined the role of P-selectin in a model of glomerular disease characterized by endothelial cell injury, with immune deposits localizing primarily to the subendothelial space. With this model, we had previously demonstrated that the injury was mediated by both platelets and PMN and suggested that the platelets and PMN could be acting synergistically to mediate the glomerular damage (8). We hypothesized that P-selectin was a critical mediator of the platelet–PMN interaction and that by blocking this interaction we could alter the localization of PMN and the platelet-mediated stim-
Figure 6. Mean platelet influx score. The histogram shows the mean platelet score comparing control animals with anti-P-selectin-treated (PB1.3) animals from the 10-min time point in con A glomerulonephritis (2.2 ± 0.2 versus 1.1 ± 0.25; *p < 0.05).

Figure 7. Glomerular PMN infiltration. The bar histogram compares RP-3+ cells per glomerulus in control versus anti-P-selectin (PB1.3)-treated animals at 3 h in con A glomerulonephritis (7.2 ± 1.5 versus 2.8 ± 1.0; *p < 0.05).

Figure 8. Glomerular oxidant-producing cells. Comparison of the number of hydrogen peroxide-producing leukocytes per glomerulus in control versus anti-P-selectin (PB1.3)-treated animals at 3 h in the con A model (8.3 ± 2.2 versus 3.8 ± 1.2; *p < 0.05).

To study the role of P-selectin in the con A model, we administered either neutralizing anti-P-selectin antibody or control antibody to rats at the end of the con A/anti-con A renal artery perfusion, because platelets are activated almost immediately in this model and we wanted to maximize antibody delivery. Because we perfused the blocking P-selectin antibody after the induction of the model, it obviated concerns that the antibody would alter the binding of the con A or the anti-con A antibody to the glomerular endothelium. Indeed, the immunofluorescence staining for con A, sheep IgG, and C3 was equivalent in both the treated and control animals.

The antibody used to block P-selectin has been well characterized, is specific for P-selectin, and has been shown to block P-selectin in the rat in previous in vitro studies (24). The dose of the antibody selected was based on in vivo studies in which P-selectin was blocked in a model of lung injury (24).

An important observation of this study was that administration of anti-P-selectin antibody did not alter circulating platelet or PMN counts. We were initially concerned that the anti-P-selectin antibody might act to platelet-deplete animals after the induction of glomerulonephritis, because P-selectin is expressed in the platelet surface after activation. However, platelet counts were similar in treatment and control groups. In addition, no effect on circulating PMN counts was observed, which contrasts with the P-selectin knockout mouse, in which a twofold compensatory increase in circulating PMN number is present (30,31).

Previous work from this laboratory has shown the colocalization of platelets and PMN in this model within the glomerular capillary loops (8). In this study, we demonstrated that this was associated with P-selectin expression by the platelets and, to a lesser extent, by the glomerular endothelial cells. Interestingly, in some sections, P-selectin-positive PMN were identified, which may reflect tightly adherent platelets or bound soluble P-selectin. We were also able to demonstrate that mouse anti-P-selectin antibody bound to the activated platelets
within the glomeruli, as documented by identifying mouse IgG on platelets in the tissue sections of the treated rats but not the control animals with nephritis.

A major finding was that blocking P-selectin also reduced the platelet influx into the glomerulus. This was accomplished without a change in the circulating platelet count. Platelet activation is common in inflammatory and exudative glomerulonephritis, and platelets or platelet-derived materials have often been identified in glomeruli of renal biopsies (32). The observation that the platelet influx could be reduced by blocking P-selectin suggests a novel way to reduce the platelet infiltration and lessen their contribution of vasoactive, mitogenic, and chemotactic substances to the glomerular milieu (32,33).

A second finding in this study was that the inhibition of P-selectin resulted in less PMN infiltration at 3 h. This may have occurred via one of several mechanisms, including: blocking the release of chemotactic factors generated by a PMN–platelet interaction (32); blocking endothelial cell P-selectin (34); or as a consequence of the decrement in the platelet influx. Tipping et al. also reported that P-selectin blockade reduced the PMN influx in a mouse model of anti-glomerular basement membrane nephritis (34), in which it was postulated to be due to an effect on endothelial cell P-selectin expression. This may also be the mechanism in this model, because in previous studies we did not observe any effect of platelet depletion on the PMN influx (22).

We also examined the effect of inhibition of P-selectin on the number of H$_2$O$_2$-producing leukocytes within the glomerulus. The assay used to detect the H$_2$O$_2$-producing cells will detect H$_2$O$_2$ production only in leukocytes with endogenous peroxidase activity, such as PMN and monocytes. The observation that the number of oxidant-producing cells present in control animals with nephritis was greater than the number of PMN present suggests that other cells, in addition to PMN (such as monocytes), were producing H$_2$O$_2$ in the lesions. Of interest, however, was the finding that inhibition of P-selectin resulted in a significant reduction in oxidant-producing cells. This likely reflects the inhibition of the PMN infiltration because the reduction in H$_2$O$_2$-producing cells (4.5/glom) corresponded to the reduction in PMN (4.4/glom). However, a separate effect on the P-selectin-mediated platelet-mediated augmentation of oxidant production by PMN (21) cannot be excluded.

Despite the inhibition of PMN and platelet infiltration, no significant reduction in proteinuria was observed. This may be because the monocyte–macrophage infiltration was not altered. Indeed, in a previous study in which neutrophil depletion resulted in a reduction in proteinuria, the method used to deplete PMN (anti-PMN sera and irradiation) also depleted all leukocyte populations (22).

Previous studies on P-selectin in glomerulonephritis have yielded conflicting results. Tipping et al. showed in a murine model of nephrotoxic nephritis that P-selectin inhibition with a polyclonal antibody resulted in a decrease in the neutrophil influx and decreased proteinuria (34). In contrast, Mayadas et al. studied a similar model in P-selectin knockout mice and found a worsening of the disease in the absence of P-selectin (30). The proposed mechanism for the latter finding is based on the work of Brady and colleagues, in which the platelet–PMN interaction mediated by P-selectin allows for the transcellular biosynthesis of certain eicosanoids that may have anti-inflammatory action, specifically lipoxin A$_4$ (35,36). Papayianni et al. have provided compelling evidence for the transcellular synthesis of lipoxin A$_4$ mediated by P-selectin (15) in a model similar to the one reported here. However, this latter model results in mild and transient glomerular endothelial cell injury, and the PMN influx is relatively minor compared with the current model. Thus, P-selectin may have both beneficial and detrimental functions in glomerulonephritis, and additional studies are necessary to determine whether modulation of P-selectin will be of benefit in the treatment of glomerular disease.

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