Low venular shear rates promote leukocyte-dependent recruitment of adherent platelets

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Russell, Janice, Dianne Cooper, Anitaben Tailor, Karen Y. Stokes, and D. Neil Granger. Low venular shear rates promote leukocyte-dependent recruitment of adherent platelets. Am J Physiol Gastrointest Liver Physiol 284: G123–G129, 2003. First published September 11, 2002; 10.1152/ajpgi.00303.2002—The influence of reductions in venular shear rate on platelet-endothelial (P/E) cell adhesion has not been previously addressed. The objectives of this study were to define the effects of reductions in venular shear rate on P/E cell adhesion and to determine the interdependence of P/E cell adhesion and leukocyte-endothelial (L/E) cell adhesion at low shear rates. Intravital videomicroscopy was used to quantify P/E and L/E cell adhesion in rat mesenteric venules exposed to shear rates ranging between 118 ± 9 and 835 ± 44 s⁻¹. Shear rate was altered in postcapillary venules by rapid, graded blood withdrawal, without retransfusion of shed blood. Reducing shear rate from >600 s⁻¹ to <200 s⁻¹ resulted in an eightfold increase in L/E cell adhesion, whereas P/E cell adhesion increased 18-fold. A blocking antibody directed against P-selectin blunted both the P/E and L/E cell adhesion elicited by low shear rates. Immunoneutralization of CD11/CD18 on leukocytes or rendering animals neutropenic also blocked the shear rate-dependent recruitment of both platelets and leukocytes. These findings indicate that 1) low shear rates promote P/E and L/E cell adhesion in mesenteric venules, and 2) adherent neutrophils (mediated by CD11/CD18) create a platform onto which platelets can bind to the venular wall at low shear rates. Hence, high shear rates might be expected to oppose blood cell-to-endothelial cell adhesion, whereas low shear rates should promote this cell-to-cell adhesion. This phenomenon has potentially important implications for the recruitment of circulating blood cells onto the wall of blood vessels under inflammatory conditions associated with elevated (e.g., chronic inflammatory diseases) or reduced (e.g., in postischemic tissues) blood flow (and shear rate). For example, reperfusion of ischemic tissues is generally associated with an enhanced adhesion of both leukocytes (9) and platelets (20, 21) within postcapillary venules. These adhesive interactions are generally accompanied by a reduction in venular shear rate. It appears likely, therefore, that the lower shear rates experienced by postischemic venules will contribute to the recruitment of the leukocytes and platelets during the reperfusion period.

Although the influence of low venular shear rates on leukocyte-endothelial (L/E) cell adhesion has been extensively studied (3, 4, 26), it remains unclear if and how low shear rates modulate platelet-endothelial (P/E) cell adhesion in postcapillary venules. Although some attention has been devoted to the influence of shear rate on platelet adhesion, most of this effort has been directed toward defining the role of high shear rates in promoting thrombus formation in large arteries (5, 30). Because platelets are considered to be exposed to lower shear forces compared with leukocytes due to their smaller diameter (20), one might expect that even weak receptor-ligand interactions will allow for P/E cell adhesion when venular shear rate is reduced below normal levels. In vitro studies suggest that the relative contributions of different platelet adhesion molecules [GPIIIb/IIIa, von Willebrand factor (vWF), and P-selectin]-to-P/E cell adhesion are significantly influenced by the prevailing shear rate. For example, GPIIIb/IIIa has been proposed to be a more important determinant of P/E cell adhesion at shear rates <600 s⁻¹ (a normal resting value for venules), whereas vWF plays no role at normal venular shear rates (27, 29). However, a recent in vivo study (1) of murine mesenteric venules activated with histamine...
has revealed a dominant role for vWF in mediating P/E cell adhesion at shear rates <100 s⁻¹.

In addition to the adhesive interactions that platelets and leukocytes can establish with vascular endothelium, they can also bind to one another. These heterotypic interactions are possible, because leukocytes express receptors [e.g., P-selectin glycoprotein ligand-1 (PSGL-1)] for adhesive ligands on platelets (e.g., P-selectin) and vice versa (15). Indeed, flow chamber experiments have revealed that circulating leukocytes can roll on and adhere to immobilized platelets (17), and there is evidence that circulating platelets will bind to adherent leukocytes (11). Furthermore, these studies indicate that the ability of one population of immobilized blood cells (e.g., platelets) to bind other moving blood cells (e.g., leukocytes) is more pronounced at low (<100 s⁻¹) shear rates (7). These findings raise the possibility that the recruitment of adherent platelets and/or leukocytes into postcapillary venules exposed to low shear rates may involve both a direct adhesive interaction between circulating cells and endothelial cells as well as an indirect interaction mediated by already adherent blood cells.

Major objectives of the present study were to: 1) define the dependence of P/E and L/E cell adhesion on venular shear rate, 2) determine whether shear rates induced P/E cell adhesion affects (or is affected by) the corresponding recruitment of adherent leukocytes (L/E cell adhesion), and 3) estimate the contribution of reduced shear rate to the P/E and L/E cell adhesion seen in venules exposed to ischemia-reperfusion (I/R). These objectives were addressed by applying the technique of intravital videomicroscopy to rat mesenteric venules exposed to graded reductions in shear rate or to an I/R insult.

MATERIALS AND METHODS

Surgical procedure. Male Sprague-Dawley rats (160–260 g) were fasted 18–24 h before the experiment. The animals were anesthetized by intraperitoneal injection of 120 mg/kg thiobutabarbital (Inactin). A tracheotomy was performed to facilitate breathing during the experiment. The right carotid artery was cannulated for blood withdrawal and blood pressure measurement. The jugular vein was cannulated for administration of platelets and monoclonal antibodies. A midline abdominal incision was made to allow for exteriorization of the small bowel and associated mesentery (18).

Blood sampling and platelet preparation. Approximately 0.9 ml of blood was collected via the carotid artery from the experimental animal and anticoagulated with 0.1 ml acitrate dextrose buffer (blood volume was replaced with 2.0 ml of bicarbonate-buffered saline). Platelet-rich plasma was obtained and centrifuged at 600 g. The platelet pellet was resuspended in PBS and fluorescence labeled by incubation with 90 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 min. The fluorescently labeled platelet solution was then centrifuged, resuspended in 500 μl of PBS (pH 7.4), and protected from light. With the aid of a hemocytometer, blood cell counts yielded 6 × 10¹⁰ % leukocytes in the platelet suspension (6).

Flow cytometry. To determine whether the platelet isolation procedure influenced platelet activation status, flow cytometry was used to compare P-selectin expression on both isolated washed platelets and platelets in whole blood. Blood was collected via the carotid artery into acid citrate dextrose buffer at a ratio of 1:10. Platelets were isolated as described above for analysis of washed platelets. Before staining with antibodies, isolated washed platelets were suspended at a concentration of 1 × 10⁶ cells/ml in FACS buffer (2% FCS in PBS), and whole blood was diluted 1:8 in FACS buffer. Both preparations were divided into nonstimulated and thrombin-stimulated samples. Before thrombin stimulation, Gly-Pro-Arg-Pro (GPRP; 0.8 mM final concentration; Sigma, St. Louis, MO) was added to whole blood to prevent fibrin polymerization and platelet aggregation. Human thrombin was then added to both isolated washed platelets and GPRP-treated blood at a final concentration of 1 U/ml. An anti-P-selectin monoclonal antibody RMP-1 provided by Dr. D. C. Anderson (Pharmacia and Upjohn, Kalamazoo, MI) and the F(ab')₂ fragment of the anti-GPIII/IIa monoclonal antibody 7E3 (Centocor, Malvern, PA) were conjugated with FITC as previously described (12). Nonstimulated and thrombin-stimulated samples were incubated with either the FITC-conjugated anti-GPIII/IIa antibody for identification of platelet populations, or FITC-conjugated anti-P-selectin antibody to assess platelet activation, for 20 min at room temperature. Cells were then washed twice with FACS buffer and analyzed on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA).

Intravital microscopy. Rats were placed in a supine position on an adjustable acrylic microscope stage, and the mesentery was prepared for microscopic observation as described previously (18). Briefly, the mesentery was draped over a nonfluorescent coverslip that allowed for observation of a 2-cm² segment of tissue. The exposed bowel wall was covered with Saran Wrap (Dow Chemical), and then the mesentery was superposed with bicarbonate-buffered saline (37°C, pH 7.4) that was bubbled with a mixture of 5% CO₂–95% N₂.

An upright multipurpose microscope system (Zeiss, Thornwood, NY) with a 40× water immersion objective lens (Achromplan 40×/0.75 W) was used to observe the mesenteric microcirculation. The mesentery was either transilluminated (with a 12-V, 100-W direct current-stabilized light source) for visualization of adherent leukocytes or epi-illuminated (HBO 50W mercury lamp) for visualization of the fluorescently labeled platelets. CFSE (excitation: 490 nm, emission: 518 nm) was viewed with a reflector slide equipped with an excitation filter of 450–490 nm, a dichroic mirror of 510 nm, and a barrier filter of 515–565 nm. The fluorescent microscopic images were received by a charge-coupled device (CCD) video camera and optimized by a CCD camera control (Dage MTI, Michigan City, IN) attached to an intensifier with a controller (Dage MTI). The transilluminated images were received by a chromachip camera (model JE3362; Javelin) and viewed on a monitor. The images were then recorded on a video recorder (JVC, Elmwood, NJ) for off-line evaluation. A video time/date generator (Panasonic, Secaucus, NJ) projected the time, date, and stopwatch function on the monitor.

Single, unbranched venules with diameters ranging between 25 and 35 μm and a length of >150 μm were selected for study. Venular diameter (Dv) was measured either on- or off-line using a video caliper (Microcirculation Research Institute, Texas A & M University, College Station, TX). Red blood cell centerline velocity (V RBC) was measured in venules using an optical Doppler velocimeter (Microcirculation Research Institute). Venular blood flow was calculated from the product of mean red blood cell velocity (Vmean = V RBC/1.6) and microvascular cross-sectional area, assuming cylindrical
Wall shear rate (WSR) was calculated on the basis of the Newtonian definition: 

$$\text{WSR} = \frac{\pi \rho V_{\text{mean}} D_v}{11000}$$

The numbers of adherent leukocytes and platelets were determined off-line during playback of recorded images. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period of ≥30 s (18). Adherent platelets were defined as those cells remaining stationary for a period of ≥2 s. Adherent leukocytes and platelets were expressed as the number of cells per square millimeter of venular surface, calculated from $D_v$ and length, assuming cylindrical vessel shape (6).

Experimental protocols. After a 30-min stabilization period, fluorescently labeled platelets ($4 \times 10^8$) were infused over a 5-min period and allowed to circulate for 5 min before baseline measurement. Criteria for acceptance of a vessel preparation were shear rates $>500$ s$^{-1}$ and L/E cell adhesion of $\leq 5/100$-μm length. To study the relationship between L/E or P/E cell adhesion and shear rate, venular blood flow was altered from baseline by graded reductions in blood volume without retransfusion of shed blood (no reperfusion), thereby exposing mesenteric venules to one normal and three reduced shear rates, each for a period of 5–10 min. This protocol was selected to achieve the following desired shear rates expressed relative to baseline: 100 (baseline), 50, 37.5, and 20%. In some experiments, rats were pretreated with either a blocking MAb directed against P-selectin (2 mg/kg RMP-1) (31) or CD18 (2 mg/kg WT3) (2) or antineutrophil serum (ANS; 1.0 ml/kg; Accurate Chemicals and Scientific, Westbury, NY) (13). The MAbs RMP-1 and WT3 were administered 5 min before the baseline measurements of shear rate and P/E and L/E cell adhesion. Rats receiving ANS were given 1 ml/kg ip 3 h before the induction of anesthesia. The mesenteric area was scanned for 3–5 venules/shear rate and each was recorded for a period of 1.5 min.

In a separate group of animals, we evaluated the effects of ischemia (45 min) and reperfusion (60 min) (I/R) on P/E and L/E cell adhesion and shear rate in mesenteric venules. In the I/R group, the superior mesenteric artery was ligated with a snare created from polyethylene tubing. Reperfusion was induced by gently removing the arterial ligature. Sham animals were subjected to an identical protocol without actually securing the ligature. L/E cell adhesion and shear rate were observed for 30 min before the induction of ischemia (actual or simulated) to ensure acceptable baseline conditions. P/E and L/E cell adhesion and shear rate were measured 60 min after actual or simulated reperfusion.

![Fig. 1](image1.png)

Fig. 1. Dependence of leukocyte- and platelet-endothelial cell adhesion on shear rate in rat mesenteric venules. Absolute measured values (A) and predicted binding density for labeled and unlabeled platelets (B) are shown. *P < 0.05 relative to shear rate >600 s$^{-1}$.

![Fig. 2](image2.png)

Fig. 2. Responses of shear rate-dependent platelet (A) and leukocyte (B) recruitment to P-selectin (P-sel) blockade. *P < 0.05 relative to shear rate >600 s$^{-1}$; †P < 0.05 relative to untreated.
Experimental procedures described above were reviewed and approved by the Louisiana State University Health Sciences Center-Shreveport Institutional Animal Care and Use Committee and performed according to the criteria outlined in Guide for the Care and Use of Laboratory Animals, [DHEW Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

**Statistics.** Data were analyzed using standard statistical analysis, i.e., one-way ANOVA and Fisher's post hoc test. All values are reported as means ± SE from 5–9 rats, and statistical significance was set at P < 0.05.

**RESULTS**

Flow cytometric analysis of platelets isolated for intravital microscopy revealed no significant differences in either percentage of P-selectin-positive platelets (3.8±1.1 vs. 3.4±0.9%) or the mean fluorescence intensity of P-selectin expression (31.4±0.7 vs. 32.9±1.4) on unstimulated washed platelets vs. platelets in whole blood. On stimulation with thrombin, the percentage of P-selectin-positive platelets increased significantly in both washed platelets and whole blood (35.2±6.5 and 73.3±6.3%, respectively).

Figure 1 summarizes results of the studies designed to assess the dependence of L/E and P/E cell adhesion in rat mesenteric venules on shear rate. The blood cell adhesion data were analyzed for four ranges of venular shear rate, 835±44, 467±19, 319±9, and 118±9 s\(^{-1}\) induced by brief, graded reductions in blood volume. Data reveal that graded reductions in venular shear rate are associated with recruitment of increasing numbers of adherent leukocytes and platelets. Figure 1A presents the actual recorded data and illustrates that reducing shear rate appears to exert a more profound influence on the recruitment of leukocytes compared with platelets. Figure 1B platelet data are corrected for the fact that only 5% of the total circulating platelets in our experiments were fluorescently labeled. Assuming that the fluorescent and nonfluorescent platelets behaved identically in these experiments, then a much larger number of adherent platelets are predicted at lower shear rates, greatly exceeding the density of adherent leukocytes.

Effects of pretreatment with a P-selectin blocking MAb on the recruitment of adherent platelets (Fig. 1A) and leukocytes (Fig. 1B) at low shear rates are summarized in Fig. 2. P-selectin blockade significantly attenuated the recruitment of both platelets and leukocytes at low shear rates. The observation that P-selectin immunoneutralization reduced the adhesion of both populations of circulating cells raised the possibility that the shear rate-dependent recruitment of platelets may be dependent on P-selectin-mediated L/E cell adhesion rather than P-selectin on the platelets themselves. Therefore, additional experiments were performed to determine whether leukocyte-directed interventions (CD18 immunoneutralization or ANS) altered the recruitment of platelets induced by low shear...
rates. Figure 3B illustrates that either blocking L/E cell adhesion with a CD18-specific MAb or rendering the rats neutropenic with ANS profoundly reduced the L/E cell adhesion normally seen at low shear rates. In Fig. 3A, effects of these interventions on shear rate-dependent recruitment of platelets are shown. Both CD18 immunoneutralization and neutropenia resulted in highly significant ($P < 0.01$) reductions in platelet recruitment at low shear rates.

Figure 4 summarizes the changes in L/E cell adhesion, P/E cell adhesion, and shear rate in mesenteric venules exposed to I/R. In sham rats experiencing the same protocol as the I/R group but without superior mesenteric artery ligation, 140 ± 40 adherent leukocytes/mm$^2$ and 7 ± 7 adherent platelets/mm$^2$ were detected with shear rates of 728 ± 71 s$^{-1}$. In venules exposed to I/R, the number of adherent leukocytes and platelets increased to 687 ± 104 and 272 ± 82 cells/mm$^2$, respectively. The venular shear rate at 60 min after reperfusion was reduced to 324 ± 45 s$^{-1}$.

On the basis of the effects of brief reductions in venular shear rate on L/E and P/E cell adhesion predicted in Fig. 1A and the shear rate values detected in venules exposed to I/R (Fig. 4B), we estimated the proportion of recruited leukocytes and platelets induced by this I/R insult that can be attributed to shear rate dependent and independent mechanisms (Fig. 5). This analysis revealed that ~95% of the I/R-induced leukocyte recruitment may be due to the corresponding lower shear rate, whereas the reduced shear rate may account for ~65% of the I/R-induced platelet recruitment.

**DISCUSSION**

I/R results in the recruitment of adherent leukocytes and platelets in the microcirculation (9, 20). These blood cells have been implicated in the microvascular dysfunction (9, 20), tissue necrosis (8, 9), and apoptosis (28) that commonly occur in postischemic tissues. Recognition that blood cells contribute to the end organ damage elicited by I/R and other pathological conditions has generated much interest in defining the factors that regulate blood cell-endothelial cell adhesion in microvessels. Some of the major factors known to modulate blood cell-endothelial cell interactions include adhesion molecules (e.g., P-selectin) expressed on endothelial cells and/or blood cells, products of endothelial cell (e.g., nitric oxide) and blood cell (e.g., superoxide) activation, and hydrodynamic dispersal forces (e.g., shear rate) generated by the movement of blood in the microcirculation (10). Although all of these factors have been implicated in the recruitment of adherent platelets and leukocytes in postischemic tissues, relatively little is known about how the reduced venular shear rates that accompany reperfusion affect P/E cell adhesion and whether leukocytes influence the recruitment of platelets at low venular shear rates. These issues were addressed in the present study.

Findings of our study indicate that low shear rates (without reperfusion) promote the recruitment of adherent platelets and leukocytes in postcapillary venules of the rat mesentery. The shear rate-dependent recruitment of adherent leukocytes noted in this study is both qualitatively and quantitatively similar to that previously reported for rat (18) and cat (3, 4, 26) mesentery. A novel finding of the present report is that low shear rates also elicit the adhesion of platelets in postcapillary venules, with the largest increment in platelet recruitment seen when shear rate is reduced <300 s$^{-1}$. The number of fluorescently labeled platelets detected at the lowest shear rates (~412 cells/mm$^2$ venule) is more than twice the density of adherent platelets measured in mouse intestinal venules after intraperitoneal administration of 0.5 mg/kg of *Escherichia coli* lipopolysaccharide (6), a potent inducer of P/E cell adhesion (14).
Although it appears that low shear rates result in the recruitment of more adherent leukocytes than platelets (Fig. 1A), because only ~5% of the platelets were fluorescent, one would predict that a far larger number of platelets are actually adherent to the venular wall at low shear rates (Fig. 1B). This suggests that approximately eight times more platelets than leukocytes bind the venular wall at the lowest shear rates studied. Difference in magnitude of platelet vs. leukocyte recruitment cannot be explained by cell size, because in vitro evaluation of the attachment rates of different-sized PSGL-1-coated particles to immobilized P-selectin under flow conditions have revealed a more profound influence of lowering shear rate on the attachment of larger particles (>10 μm) compared with 5-μm particles (24). An alternative explanation is that expression of a receptor for platelet attachment on the vessel wall is increased when shear rate is reduced.

Because P-selectin has been previously implicated in the recruitment of both leukocytes and platelets in postischemic mesenteric venules (16, 20), we evaluated the contribution of this adhesion molecule to the P/E and L/E cell adhesion observed at low venular shear rates. Immunoneutralization of P-selectin with a ratspecific MAb RMP-1 (31) resulted in a 50–60% reduction in L/E and P/E cell adhesion at the lowest shear rate studied, suggesting that this adhesion molecule contributes to the shear rate-dependent recruitment of both cell populations. Inasmuch as venular endothelial cells can capture leukocytes using endothelial P-selectin-leukocyte-PSGL-1 interactions and platelets can bind to leukocytes via P-selectin (platelet)-PSGL-1 (leukocyte) interactions, the effectiveness of the P-selectin MAb in reducing the recruitment of both platelets and leukocytes at low shear rates may reflect a linkage between the two recruitment processes. To address this possibility, we examined whether depletion of circulating neutrophils with ANS or immunoneutralization of the β2-integrin CD11/CD18, which has previously been shown to mediate shear-rate-dependent L/E cell adhesion (3, 26), affect the recruitment of platelets at low shear rates. Rendering rats neutropenic with ANS virtually abolished L/E cell adhesion at low shear rates, indicating that neutrophils are the dominant leukocyte participants in this process. Because ANS treatment (which did not alter the number of circulating platelets) also abolished the recruitment of platelets at low shear rates, it appears that platelets are recruited into venules at low shear rates through a leukocyte-dependent mechanism. Immunoneutralization of CD11/CD18, an adhesion molecule not known to directly mediate P/E cell adhesion, was also effective in reducing (but not abolishing) the recruitment of both leukocytes and platelets, which is consistent with leukocyte-dependent platelet recruitment.

A model that can be proposed to explain the dependence of platelet recruitment on L/E cell adhesion is shown in Fig. 6. At low shear rates, leukocytes utilize endothelial P-selectin as well as CD11/CD18 to bind to venular endothelium. Adherent leukocytes, which constitutively express PSGL-1, then create a platform onto which platelets can bind using P-selectin. Such a model would explain why P-selectin immunoneutralization attenuates the adhesion of both platelets and leukocytes when shear rate is low. It would also explain the ability of CD11/CD18 immunoneutralization to blunt the P/E cell adhesion response and for neutrophil depletion to completely abolish it.

Several experimental models, including mesentery, cremaster muscle, and retina, have revealed that venular shear rate is significantly reduced after reperfusion of ischemic tissue (22, 23, 25). Because reperfusion is also associated with a pronounced P/E and L/E cell adhesion, the question arises as to what proportion of the reperfusion-induced L/E and P/E cell adhesion can be attributed to the reduced shear rate. Results of the present study suggest that in the mesentery ~95 and 65% of the L/E and P/E cell adhesion, respectively, may be due to the low shear rate (~300 s⁻¹) that venules are exposed to during reperfusion. Our previous work on cat mesentery indicates that, whereas reduced shear rates induced by low flow ischemia are indeed associated with the significant recruitment of adherent leukocytes (10), the magnitude of the L/E cell adhesion is not as intense as that seen after reperfusion. This difference likely results from the production and release of inflammatory mediators during the early reperfusion period and suggests that one cannot attribute the exaggerated adhesion response simply to a reduced venular shear rate. Nonetheless, our findings suggest that increasing shear rate during the reperfusion period may be an effective strategy for preventing or attenuating the proinflammatory and prothrombotic genotypes induced by I/R.

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