Leukocyte dependence of platelet adhesion in postcapillary venules

Dianne Cooper, Janice Russell, Keith D. Chitman, Matthew C. Williams, Robert E. Wolf, and D. Neil Granger. Leukocyte dependence of platelet adhesion in postcapillary venules. Am J Physiol Heart Circ Physiol 286: H1895–H1900, 2004. First published January 8, 2004; 10.1152/ajpheart.01000.2003.—Reperfusion of ischemic tissues results in development of a proinflammatory, prothrombogenic phenotype, culminating in the recruitment of leukocytes and platelets within postcapillary venules. Recent studies have indicated an interdependence of platelet and leukocyte adhesion, suggesting that heterotypic blood cell interactions may account for postischemic platelet recruitment. The objectives of this study were to 1) determine whether ischemia-reperfusion (I/R)-induced platelet recruitment is leukocyte dependent and 2) quantify the contributions of leukocytes and endothelial cells in this platelet recruitment. Intravital microscopy was used to monitor the recruitment of fluorescently labeled platelets in postcapillary venules of the small intestine after 45-min ischemia and 4-h reperfusion. To assess the leukocyte dependence of platelet adhesion, platelets from wild-type mice were infused into mice deficient in neutrophils and/or lymphocytes and mice deficient in key leukocyte adhesion molecules (CD18 and ICAM-1). These antileukocyte strategies resulted in significantly reduced platelet recruitment. Simultaneous visualization of platelets and leukocytes enabled quantification of leukocyte-dependent and endothelium-dependent platelet adhesion. It was observed that in wild-type animals 74% of I/R-induced platelet adhesion was a result of platelet-leukocyte interactions. Although the majority of adherent platelets were associated with leukocytes, <50% of adherent leukocytes were platelet bearing, suggesting that not all adherent leukocytes support platelet adhesion. These results are consistent with leukocytes playing a major role in supporting I/R-induced platelet adhesion.

Ischemia; neutrophil; cell adhesion molecules

Reperfusion of ischemic tissues alters the microvasculature in a manner consistent with an acute inflammatory and prothrombogenic response. Intravital microscopic analyses of the interactions among leukocytes, platelets, and the vessel wall of postischemic venules have revealed an initial rapid recruitment of leukocytes followed by a slower, more gradual adherence of postischemic venules (8, 9). Although the majority of adherent platelets were associated with leukocytes, <50% of adherent leukocytes were platelet bearing, suggesting that not all adherent leukocytes support platelet adhesion. These results are consistent with leukocytes playing a major role in supporting I/R-induced platelet adhesion.

P-selectin and ICAM-1 (14, 15). The observation that some adhesion molecules participate in the recruitment of both leukocytes and platelets has raised the possibility of a codependence of leukocyte adhesion on platelets and platelet adhesion on leukocytes.

Increased interactions between leukocytes and platelets occur in the circulation of patients suffering from a variety of ischemic disorders, including myocardial infarction (16) and stroke (6). Immuneutralization studies have identified platelet-associated P-selectin and leukocyte-associated CD18 and P-selectin glycoprotein ligand (PSGL)-1 as key mediators of these interactions (1, 7, 26). Platelet-leukocyte aggregates have been observed by intravital and electron microscopy after I/R (9, 14) that were also shown to be dependent on platelet P-selectin expression (14). Such heterotypic interactions between blood cells result in reciprocal activation of the involved cell types and may lead to increased interactions with the vessel wall. Recent studies have identified an interdependence of platelet and leukocyte adhesion after I/R and during low venular shear rates (20, 21, 24). Reduced platelet adhesion, through either administration of antiplatelet serum or immuneutralization of platelet-associated adhesion molecules (GPIIb/IIIa, P-selectin), results in decreased leukocyte emigration in the postischemic small intestine (21). Similarly, platelet-associated P-selectin, rather than the endothelial form, mediates neutrophil-dependent postischemic renal failure (24). Although platelets play a role in mediating leukocyte recruitment, the converse may also occur, as observed after large reductions in venular shear rates (20). Antileukocyte strategies such as immuneutralization of the leukocyte adhesion molecule CD11/CD18 or rendering animals neutropenic with antineutrophil serum (ANS) appear to block shear rate-dependent recruitment of both leukocytes and platelets.

Collectively, the available literature suggests that heterotypic blood cell interactions may account for a significantly larger fraction of the accumulation of platelets on the venular wall after I/R than the platelet recruitment resulting from direct adhesion to the venular endothelium. This possibility has not been addressed in a systematic, quantitative manner. Hence, the overall study objective was to determine whether platelet adhesion in postischemic intestinal microvasculature was a result of platelet-leukocyte and/or platelet-endothelial cell interactions. This objective was achieved by using two experimental strategies, i.e., by 1) determining the effect of various antileukocyte strategies on platelet recruitment, using mice deficient in specific leukocyte populations (neutrophils and lymphocytes), and 2) simultaneously monitoring the accumu...
lation of leukocytes and platelets with the vessel wall to determine whether platelets were interacting directly with leukocytes or with venular endothelium.

**MATERIALS AND METHODS**

*Animals.* Wild-type (C57BL/6J), severe combined immunodeficiency (SCID) (C57BL/6-J-Pkdcscid/SzJ), CD18-deficient (C57BL/6J-Igbs2tm1Bay), and ICAM-1-deficient (C57BL/6J-Icam1tm1Bay) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Male mice (8–12 wk old) were used.

*Surgical procedure.* Animals were anesthetized with ketamine hydrochloride (150 mg/kg ip) and xylazine (7.5 mg/kg ip). The right carotid artery was cannulated for blood pressure measurement. The right jugular vein was cannulated for platelet infusion. A midline laparotomy was performed, the animal was placed in a supine position, and a loop of small bowel was exteriorized and superfused with warm bicarbonate-buffered saline.

*Blood sampling and platelet preparation.* Approximately 0.9 ml of blood was harvested via the carotid artery into a polypropylene tube containing 0.1 ml of acid-citrate-dextrose buffer (Sigma, St. Louis, MO) and centrifuged at 120 g for 10 min. Platelet-rich plasma was obtained by two sequential centrifugations (120 g for 8 min and 120 g for 3 min). Platelets were pelleted at 550 g for 10 min and resuspended in PBS (pH 7.4). Platelets were incubated for 10 min (room temperature) with the fluorochrome carboxyfluorescein diacetate succinimidyl ester (CFSE, final concentration 90 μM; Molecular Probes, Eugene, OR). The fluorescently labeled platelet solution was centrifuged, resuspended in 500 μl of PBS, and protected from light. The platelet suspension contained <0.01% leukocytes. This isolation procedure does not induce P-selectin expression (3). Platelets were derived from wild-type mice for all experiments, with the exception of one group, which used CD18-deficient platelets. Platelets (100 × 10^9) were infused over 5 min with a Harvard Apparatus (South Natick, MA) infusion pump, yielding 12.51 mm^2 (155.68–92.96 mm^2) per animal with a pseudoshear rate of 30 s within a 200-μm vessel segment, assuming a cylindrical vessel shape (13).

In additional experiments, both leukocyte and platelet interactions with the venular wall were examined simultaneously within a 100-μm vessel segment with a ×20 objective. Rhodamine 6G (0.02%) was administered intravenously for leukocyte visualization. Platelets (isolated and analyzed as described in Blood sampling and platelet preparation) were further distinguished as associated with adherent leukocytes or interacting with the vessel wall. Leukocytes remaining stationary for ≥30 s were considered firmly adherent.

*Experimental protocols.* All platelet recipients were fasted for 24 h. In the I/R groups, the superior mesenteric artery was clamped for 45 min. Sham-treated animals underwent identical manipulation except for the ischemia. Platelet adhesion was measured after 4-h reperfusion. For experiments in which both leukocyte and platelet adhesion were quantified, platelets were infused as described in Blood sampling and platelet preparation, followed by infusion of rhodamine 6G.

*Clinical results.* Neutropenic SCID (SCID - ANS) recipient mice after 45-min ischemia and 4-h reperfusion in postcapillary venules of the small intestine. Minimal platelet adhesion occurred in sham-treated animals (19.4 ± 17.69 mm^-2); however, this number increased significantly (155.68 ± 92.96 mm^-2) in wild-type mice after I/R. No significant difference between pseudoshear rates in sham-treated animals (575 ± 35 s^-1) and those undergoing I/R (518 ± 42 s^-1) was evident, indicating that the increased platelet adhesion did not result from decreased shear stress. The role of leukocytes in mediating this I/R-induced platelet adhesion was revealed by using ANS-treated wild-type mice and SCID mice. There was a significant decrease in the total number of adherent platelets in both SCID (72.24 ± 14.71 mm^-2) and neutropenic (81.92 ± 12.51 mm^-2) mice. Depletion of both leukocyte subsets (SCID mice treated with ANS)

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**RESULTS**

I/R causes leukocyte-dependent recruitment of platelets in postcapillary venules. Previous studies demonstrated a significant increase in platelet-endothelial cell adhesion both immediately and hours after I/R (3, 14, 17). Figure 1 summarizes total platelet adhesion (because leukocytes were not fluorescently labeled in these experiments, platelet adhesion could not be classified as being to either adherent leukocytes or the endothelium) after 45-min ischemia and 4-h reperfusion in postcapillary venules of the small intestine. Minimal platelet adhesion occurred in sham-treated animals (19.4 ± 17.69 mm^-2); however, this number increased significantly (155.68 ± 92.96 mm^-2) in wild-type mice after I/R. No significant difference between pseudoshear rates in sham-treated animals (575 ± 35 s^-1) and those undergoing I/R (518 ± 42 s^-1) was evident, indicating that the increased platelet adhesion did not result from decreased shear stress. The role of leukocytes in mediating this I/R-induced platelet adhesion was revealed by using ANS-treated wild-type mice and SCID mice. There was a significant decrease in the total number of adherent platelets in both SCID (72.24 ± 14.71 mm^-2) and neutropenic (81.92 ± 12.51 mm^-2) mice. Depletion of both leukocyte subsets (SCID mice treated with ANS).
did not cause a further decrease in platelet adhesion compared with neutropenic or SCID mice (61.32 ± 10.37 mm\(^{-2}\)). Differential blood counts obtained for animals treated with ANS revealed a significant decrease in the number of neutrophils (95% reduction) after treatment. ANS treatment also resulted in a significant decrease in the number of circulating lymphocytes and monocytes (45% and 70% reductions, respectively) and platelets (15% reduction). Neutrophil counts in SCID mice were not significantly different from those of wild-type mice (950 ± 157 and 985 ± 806 μl\(^{-1}\), respectively).

The observation that platelet adhesion was decreased in animals lacking circulating leukocyte populations raised the possibility that some of the observed platelet adhesion may occur as a result of platelets binding already adherent leukocytes. Thus experiments were performed with animals deficient in the leukocyte adhesion molecules CD18 or ICAM-1 and in a group of animals treated with a CD18-blocking MAb. Blocking I/R-induced leukocyte-endothelial cell adhesion also significantly decreased platelet recruitment (Fig. 2). Reductions in platelet adhesion were comparable between the CD18 (68.39 ± 12.73 mm\(^{-2}\)) and ICAM-1 (65.28 ± 15.87 mm\(^{-2}\))-deficient animals, with a further reduction in animals treated with the CD18-blocking MAb (45.14 ± 7.5 mm\(^{-2}\)). Platelet adhesion in animals treated with the isotype control Ab was not significantly different from that of untreated animals (164 ± 19 mm\(^{-2}\)). Because CD18 has been identified on platelets as well as leukocytes (19), one group of wild-type animals received CD18-deficient platelets. Surprisingly, platelet adhesion was also significantly decreased in this group (93.79 ± 20.32 mm\(^{-2}\)), albeit to a lesser extent than in the other groups.

Platelet-leukocyte adhesion predominates over platelet-endothelium adhesion after I/R. The second major objective of this study was to determine whether platelet adhesion was a result of platelet-endothelial cell or platelet-leukocyte interactions. Leukocytes and platelets were visualized simultaneously to determine whether platelets were adhering directly to the vessel wall or to adherent leukocytes. The numbers of adherent platelets per square millimeter observed in the experiments in Figs. 3 and 4 are more than double the numbers observed in Figs. 1 and 2. As indicated above, experiments in which platelets and leukocytes were observed simultaneously were carried out under a higher magnification than when observing platelets alone, thus allowing adherent platelets and leukocytes to be distinguished. Because of the higher magnification used, adhesion was assessed over the shorter vessel length of 100 μm, resulting in an increased number of adherent platelets when converted to number of platelets per square millimeter. Figure 3 demonstrates that in wild-type mice 26% (125.69 ± 41 mm\(^{-2}\)) of platelet adhesion after I/R results from direct platelet-endothelial cell interactions, with the remaining 74%...
of platelets binding to adherent leukocytes. Similar levels of platelet-endothelial cell adhesion were observed in ICAM-1-deficient animals (106.62 ± 11.8 mm⁻²) or mice treated with a CD18-blocking MAb (107.5 ± 24.61 mm⁻²), indicating that this platelet-endothelial cell adhesion is independent of leukocyte adhesion.

When leukocyte adhesion was assessed (Fig. 4), sham-treated animals demonstrated low leukocyte adhesion within venules (13.55 ± 6.89 mm⁻²). This was significantly elevated after I/R (444.56 ± 61.66 mm⁻²). Leukocyte adhesion was significantly decreased in ICAM-1-deficient mice (146.03 ± 53.29 mm⁻²) or after immunoneutralization of CD18 (129.78 ± 32.61 mm⁻²). Platelet-bearing leukocytes were diminished to a greater extent in the ICAM-deficient animals (29.11 ± 7.64 mm⁻²) compared with animals treated with the CD18-blocking MAb (82.27 ± 25.14 mm⁻²), although this was not statistically significant.

Platelet-endothelium and platelet-leukocyte adhesion are largely P-selectin dependent. Both in vivo and in vitro studies have identified platelet-attached P-selectin as a key adhesion molecule mediating platelet-leukocyte interactions, probably through its interactions with PSGL-1 (1, 14). To address this possibility in our model, a group of animals were treated with a P-selectin-blocking MAb 5 min before the onset of reperfusion. This significantly decreased platelet recruitment to sham treatment levels (29.34 ± 6.9 mm⁻²), implicating P-selectin as the principal adhesion molecule mediating I/R-induced platelet recruitment. This is consistent with an earlier study showing that platelet recruitment after I/R is P-selectin dependent, with platelet-associated P-selectin largely mediating this process (3).

DISCUSSION

Inflammatory cell recruitment and activation play an important role in the pathogenesis of various diseases (e.g., atherosclerosis and I/R injury). The inflammatory phenotype that is assumed by the vasculature of diseased tissues is accompanied by a prothrombogenic phenotype, characterized by platelet adhesion in postcapillary venules (14, 17). Adhesion of leukocytes and platelets in venules have been viewed as largely independent processes linked by expression of adhesion molecules on activated endothelium. However, there is mounting evidence for the recruitment of blood cells on the vessel wall either through a direct interaction with endothelial cells or indirectly by binding already adherent blood cells (20, 21). The present study determined the relative contributions of these direct and indirect adhesion mechanisms for the accumulation of platelets in postcapillary venules after I/R.

Two strategies were used to address the importance of leukocyte adhesion to platelet recruitment in postischemic venules: 1) examining the platelet adhesion response in leukocyte (lymphocytes and/or neutrophils)-depleted mice and in mice deficient in key leukocyte adhesion molecules (CD18 or ICAM-1) and 2) assessing the magnitude of platelet and leukocyte colocalization in venules of wild-type mice with and without CD18-blocking MAb and ICAM-1-deficient mice. Together, the results strongly suggest that leukocytes play a major role in supporting platelet adhesion after I/R, with 74% of adherent platelets associated with adherent leukocytes and the remaining 26% of platelet adhesion representing a direct interaction with venular endothelium.

Previous studies indicated that several leukocyte populations are recruited into postischemic venules, including neutrophils and T lymphocytes (23). Neutrophil adhesion occurs minutes after reperfusion, with several hours required for T cell recruitment (23). To address the contribution of these two leukocyte populations to platelet adhesion, we studied both SCID and neutropenic mice after gut I/R. Platelet adhesion was comparably decreased in both groups (by 54% and 47%, respectively). Differential blood counts revealed that neutrophils were not the only blood cell affected by ANS treatment. Lymphocyte counts were also significantly decreased in these animals, making it difficult to determine the exact role played by lymphocytes/neutrophils in platelet recruitment. Data from SCID mice (which had normal neutrophil counts) suggest that lymphocytes are required for platelet adhesion. Nonetheless, it remains unclear whether platelets are binding directly to adherent lymphocytes or whether lymphocytes participate by modulating the recruitment of neutrophil recruitment as previously shown in SCID mice exhibiting an attenuated recruitment of neutrophils into the postischemic intestine (23). The lack of an additive effect in both the ANS-treated mice (in which neutrophils are decreased by 95% and lymphocytes by 45%) and in ANS-treated SCID mice (which lack both cell populations) suggests that the lymphocyte and neutrophil responses to I/R may be series-coupled, rather than parallel-coupled, events. There are several lines of evidence indicating that neutrophils are more likely than lymphocytes to support platelet adhesion in venules of the postischemic intestine, including the significant decrease in platelet recruitment that is observed in ICAM-1-deficient mice (discussed below), the observation by others of platelet-neutrophil aggregates in the postischemic small bowel (14), and in vitro studies that show a greater propensity for platelet-neutrophil rather than platelet-lymphocyte aggregation in N-formylmethionyl-leucyl-phenylalanine-stimulated whole blood (12).

Many studies implicate CD18-ICAM-1 interactions in the leukocyte adhesion observed in venules of postischemic tissues (5, 9). Our leukocyte adhesion results are consistent with the importance of β₂-integrins and ICAM-1 in this model. However, a novel aspect of our work is the revelation that CD18/ICAM-1-mediated leukocyte adhesion is a quantitatively important mechanism for platelet adhesion in postischemic microvessels. The reduction in platelet recruitment in animals deficient in key leukocyte adhesion molecules suggests that leukocytes must adhere to the vessel wall to mediate platelet recruitment. There is evidence in the literature to suggest that such leukocyte-dependent platelet recruitment is not an I/R-specific event. Recent studies suggest that a similar mechanism may exist in LPS-treated mice (2) and during hemorrhage-induced reductions in venular shear rate (20). Platelet recruitment in these models was found to be dependent on the presence of circulating leukocytes and/or leukocyte adhesion. Differences between these models are apparent, however, as a role for CD18/ICAM-1 was not apparent in endotoxicemic mice, because neither CD18-deficient mice nor mice treated with the same CD18-blocking MAb used here displayed a reduction in LPS-induced platelet adhesion (2). With similar strategies, LPS-induced leukocyte adhesion was also found to be independent of CD18.
A surprising finding in the present study was the significant decrease in platelet adhesion observed when CD18-deficient platelets were infused into wild-type mice. However, CD18 has been identified on both human and murine platelets (19) and a functional role for platelet-associated $\beta_2$ integrins interacting with endothelial ICAM-1 has been described in pulmonary fibrosis (18). It is possible that platelet-associated CD18 is responsible for some of the platelet adhesion observed in our study through its interactions with ICAM-1 on leukocytes and/or endothelial cells.

Once we established the dependence of platelet adhesion on leukocyte-endothelial cell adhesion, simultaneous visualization of platelets and leukocytes was used to determine whether this dependence resulted from platelets binding to adherent leukocytes. The majority of platelet adhesion (74%) resulted from interactions with adherent leukocytes (Fig. 3). Although the majority of adherent platelets were associated with leukocytes, Fig. 4 shows that <50% of adherent leukocytes were platelet bearing, suggesting that not all adherent leukocytes support platelet adhesion. As expected, fewer platelet-bearing leukocytes were observed in ICAM-1-deficient mice and mice treated with the CD18-blocking MAb. Surprisingly, the percentage of platelet-bearing leukocytes was lower in ICAM-1-deficient animals than after CD18 immunoneutralization. An explanation for this difference is not clear, but it may reflect a role for ICAM-1-fibrinogen-mediated interaction with a platelet ligand such as GPIIb/IIIa. Such interactions have been observed between endothelium-derived ICAM-1 and fibrinogen after I/R (15). Our findings may also reflect different abilities of adherent lymphocytes and neutrophils to support platelet adhesion. Because it was previously shown that lymphocyte recruitment in the postischemic intestine is ICAM-1 independent (23), lymphocytes should still adhere in ICAM-1-deficient mice. Hence, the reduction in platelet-bearing leukocytes observed in these mutants may reflect a diminished capacity of lymphocytes (which also express ICAM-1) to support platelet adhesion.

Figure 5 proposes a model that may explain the dependence of platelet adhesion on leukocyte-endothelial cell adhesion. Hours after the onset of reperfusion, neutrophils and lymphocytes are recruited to the vessel wall through interactions between leukocyte-associated PSGL-1, CD18, and $\alpha_\beta$-integrins, with P-selectin, ICAM-1, and mucosal addressin cell adhesion molecule-1 expressed on endothelial cells. Platelets adhere to the vessel wall, primarily through an association with adherent leukocytes, with a minority of platelets interacting directly with the vessel wall. The dramatic reduction in platelet adhesion observed in animals treated with an antibody blocking P-selectin suggests that platelet-associated P-selectin has a role in both leukocyte-dependent and -independent platelet recruitment. This model also explains why a reduction in leukocyte adhesion within postischemic venules significantly blunts platelet recruitment.

An interesting and potentially important revelation of our colocalization experiments is that only 40–45% of adherent leukocytes in postischemic venules are platelet bearing. An explanation for the fact that <50% of adherent leukocytes sustain platelet adhesion is not readily available from our results. It is conceivable that this pattern of platelet adhesion may reflect differences in the level of activation between those adherent leukocytes that sustain platelet adhesion and those that do not. This possibility is supported by a recent study demonstrating significantly diminished platelet adhesion in intestinal venules of endotoxin (LPS)-challenged p47phox-knockout mice (a component of superoxide-producing neutrophilic NADPH oxidase) compared with LPS-treated wild-type mice (2). Superoxide promotes platelet-endothelium adhesion in culture (22), and neutrophil-derived superoxide has been shown to activate platelets in vitro (12). Furthermore, the extent of platelet-neutrophil aggregate formation in vitro is dependent on the level of both neutrophil and platelet activation (1). Hence, adherent, activated leukocytes that are producing superoxide may exhibit an elevated adhesivity to platelets whereas those adherent leukocytes not producing superoxide may not. The exact mechanisms that account for this propensity for only a fraction of adherent leukocytes to sustain platelet adhesion warrant further investigation.
REFERENCES


