Molecular Determinants of the Prothrombogenic and Inflammatory Phenotype Assumed by the Postischemic Cerebral Microcirculation

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Background and Purpose—Circulating blood cells have been implicated in the pathogenesis of cerebral ischemia/reperfusion (I/R) injury and stroke. The objective of this study was to define the magnitude and molecular determinants of the platelet– and leukocyte–endothelial cell adhesive interactions induced by I/R in the mouse brain.

Methods—Bilateral common carotid artery occlusion was induced for 1 hour in C57BL/6 mice, followed by either 40 minutes or 4 hours of reperfusion. Fluorescent platelets were administered intravenously, and the frontal brain surface was observed with intravital fluorescence microscopy. Leukocyte–endothelial cell adhesion was monitored with the use of rhodamine-6G.

Results—Ischemia followed by 40 minutes of reperfusion resulted in the rolling (125.1±23.6/mm²) and firm adhesion (109.5±25.8/mm²) of leukocytes but not platelets in venules. However, with 4 hours of reperfusion, rolling (138.8±24.6/mm²) and firm adhesion (153.7±22.3/mm²) of platelets were detected, and this was accompanied by a more intense recruitment of rolling (374.5±54.6/mm²) and adherent (445.2±57.1/mm²) leukocytes. In mice deficient in either P-selectin (P-selectin-/-) or intercellular adhesion molecule-1 (ICAM-1-/-), the I/R-induced platelet–endothelial cell (by 80% and 60%, respectively) and leukocyte–endothelial cell (by 84% and 78%, respectively) interactions were significantly blunted compared with those of wild-type mice.

Conclusions—These findings indicate that I/R promotes the adhesion of both platelets and leukocytes in cerebral venules, with the accumulation of adherent leukocytes preceding the recruitment of platelets. Both P-selectin and ICAM-1 contribute to the inflammatory and prothrombogenic state induced by cerebral I/R. (Stroke. 2003;34:1777-1782.)

Key Words: cerebral ischemia • intercellular adhesion molecule-1 • leukocytes • platelets • selectins

Ischemic strokes in humans can be attributed largely to thrombotic or thromboembolic occlusions. Thrombolytic agents therefore offer hope for the prompt restoration of blood flow and prevention of neuronal injury after an acute ischemic stroke. Preclinical studies in a variety of vascular beds, including the brain, have revealed, however, that the sudden restoration of blood flow to an ischemic organ can initiate a cascade of events that ultimately results in an acute and potentially injurious inflammatory reaction.1,2 Ischemia/reperfusion (I/R) has been shown to elicit an inflammatory response that is characterized by leukocyte adhesion in postcapillary venules, endothelial barrier dysfunction in both capillaries and venules, and an increased production of reactive oxygen species. A rate-determining role for leukocyte–endothelial cell adhesion in reperfusion injury is supported by several reports that describe attenuated microvascular dysfunction and tissue injury after I/R in animals receiving neutralizing antibodies directed against leukocyte adhesion receptors and in mice that are genetically deficient in these adhesion receptors.1,3 In several models of ischemic stroke, antiadhesion strategies directed toward the endothelial cell glycoproteins intercellular adhesion molecule-1 (ICAM-1) or P-selectin reduce the intensity of the inflammatory response, decrease infarct volume, and enhance functional recovery after I/R.4–7 Although these preclinical studies provide some hope for the development of novel therapeutic interventions in human cerebrovascular diseases, the results of a recent multicenter acute stroke trial employing the ICAM-1 blocking antibody enlimomab showed a negative outcome.8 While mechanistic explanations for the untoward results of this trial have been provided,9 the outcome of the clinical stroke trial also raises the question of whether ICAM-1 is the most desirable target for interference with leukocyte–endothelial cell adhesion in acute stroke. This possibility is difficult to address because of limited attempts to quantitatively assess the effectiveness of ICAM-1 and...
other endothelial cell adhesion molecules (e.g., P-selectin) in mediating I/R-induced leukocyte–endothelial cell adhesion in the cerebral microvasculature.

Platelets represent another potential therapeutic target that is gaining attention in both preclinical and clinical studies of stroke. The homotypic (platelet–platelet) and heterotypic (platelet–leukocyte) aggregation of platelets can contribute to the thrombotic events that lead to ischemic stroke. Furthermore, there is mounting evidence in different vascular beds that the inflammatory changes elicited by I/R are accompanied by the recruitment of adherent platelets. The recruited platelets appear to amplify the inflammatory response to I/R and may contribute directly to the microvascular dysfunction and tissue injury. One model that was proposed to explain the prothrombogenic phenotype induced by I/R invokes a role for endothelial cell–associated P-selectin in mediating I/R-induced platelet–endothelial cell adhesion, while another model implicates ICAM-1–bound fibrinogen on endothelial cells as a ligand for glycoprotein IIb/IIIa (GpIIb/IIIa) on platelets. These models suggest that interventions directed toward ablation of I/R-induced leukocyte–endothelial cell adhesion may also be effective in blunting the corresponding recruitment of adherent platelets.

The objectives of this study were (1) to determine the time course and magnitude of leukocyte–endothelial cell and platelet–endothelial cell adhesion in murine cerebral microvessels exposed to I/R and (2) to define the contributions of platelet–endothelial cell–associated P-selectin as well as endothelial cell–associated ICAM-1 to the I/R-induced recruitment of both platelets and leukocytes in the posts ischemic cerebral microvasculature.

Materials and Methods

Animal Preparations

Experiments were performed on male C57BL/6J (wild-type [WT]), P-selectin–deficient (P-selectin−/−), or ICAM-1–deficient (ICAM-1−/−) mice obtained from Jackson Laboratories (Bar Harbor, Me) and weighing 21 to 25 g. All mice had at least 10 backcrosses and 10 generations of inbreeding. The animals were anesthetized with an intraperitoneal injection of α-chloralose (60 mg/kg) and urethane (600 mg/kg), and lidocaine (1%) was used for local anesthesia. All mice were tracheostomized with a polyethylene catheter (PE-90; Intramedic, Clay Adams) and artificially ventilated (model 683; Harvard Rodent Ventilator) with room air. In some instances, pancuronium (0.4 mg/kg; Sigma) was administered (intravenously) to facilitate breathing. The femoral artery and vein were cannulated with a polyethylene catheter (PE-10; Intramedic, Clay Adams) to monitor mean arterial blood pressure, to sample arterial blood for blood gas analysis, and for intravenous administration of the labeled platelets and rhodamine-6G. In ischemic groups, the bilateral common carotid arteries (CCA) were occluded with 5-0 silk thread for 1 hour. The head of each mouse was fixed in a plastic frame in a sphinx position. Both frontal bones were exposed by a midline skin incision, followed by a craniectomy (2 to 3 mm) on the right and left sides. Artificial cerebrospinal fluid was used to suffuse the tissue exposed by the craniotomies. A 12-mm glass coverslip was placed over the exposed brain tissue, and the artificial cerebrospinal fluid was superfused beneath this viewing window. The aforementioned experimental procedures were reviewed and approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee.

Blood Sampling and Platelet Preparation

Mouse platelets were isolated and labeled with carboxylfluorescein diacetate succinimidyl ester (CFDASE; Molecular Probes), as previously described. Platelets were derived from WT mice for all experiments except in 1 experimental group, in which platelets were obtained from P-selectin−/− mice. In each mouse, platelets (100 × 10⁶) were infused over 5 minutes with the use of a Harvard Apparatus infusion pump, yielding approximately 10% of the total platelet count. The platelets were allowed to circulate for a period of 5 minutes before recording was performed. Previous studies, in which flow cytometry was used, have demonstrated that the platelets isolated and prepared for intravital microscopic analysis show no significant difference in the expression of P-selectin compared with platelets in whole blood. Similarly, the expression of GpIIb/IIIa is not affected by the isolation procedure (M. Ishikawa, MD, PhD, et al, unpublished data, 2002).

Intravital Fluorescence Microscopy and Video Analysis

An upright Nikon microscope equipped with a silicon-intensified target camera (C2400-08; Hamamatsu Photonics) and a mercury lamp was used to observe the cerebral microcirculation. The microscopic images were received by a charge-coupled device video camera and recorded on a video recorder (Sony) equipped with a video timer (Time-Date Generator, WJ-810; Panasonic).

Randomly selected venular segments evaluated for platelet and/or leukocyte adhesion were 30 to 40 μm in diameter and at least 100 μm in length. Platelets and leukocytes were classified as saltatory and adherent according to the duration of their immobility on the venular wall, as follows: (1) saltatory cells were adherent for >2 and <30 seconds, and (2) adherent cells were stationary for >30 seconds. Rolling, saltatory, and adherent platelets and leukocytes were expressed as the number of cells per square millimeter of venular surface, calculated from diameter and length, with the assumption of cylindrical vessel shape.

Experimental Protocols

After a ventral midline cervical incision was made, the bilateral CCA were exposed and ligated with 5-0 silk sutures under the microscope. After 1-hour occlusion of the bilateral CCA, the ligatures were removed. Cranial windows at bilateral frontal bones were then established for observation of platelet- and leukocyte–vessel wall interactions. Platelets were infused, and their interactions were recorded in 5 to 6 randomly selected venular segments. Leukocyte–vessel wall interactions were then monitored and recorded. The latter was achieved by intravenous administration of 50 μL of 0.02% rhodamine-6G (Sigma Chemical), followed by a continuous infusion (2 mL/h) of the fluorochrome at the same concentration for 5 to 10 minutes. WT mice were assigned to 1 of 5 experimental groups: (1) control (no ischemia and no exposure of bilateral CCA); (2) 1-hour ischemia (bilateral CCA occlusion) and 40 minutes of reperfusion; (3) 1-hour ischemia and 4 hours of reperfusion; (4) 1 hour and 40 minutes between sham operation (exposure but no ligation of bilateral CCA) and platelet monitoring; and (5) 5 ligatures between sham operation and platelet monitoring. P-selectin−/− and ICAM-1−/− mice were exposed to the 1-hour ischemia and 4-hour reperfusion protocol (group 3 described above) before platelet–vessel wall interactions were recorded. CFDASE-labeled WT platelets, ie, platelets isolated from WT mice, were infused into and monitored in P-selectin−/− and ICAM-1−/− mice. In another experimental group, CFDASE-labeled P-selectin−/− platelets were monitored in WT mice.

Statistical Analysis

Data were analyzed by ANOVA and Scheffé’s post hoc test. Some data were analyzed by paired Student’s t test. The data are reported as mean±SE. Statistical significance was set at P<0.05.
Results

Vessel Diameter and Physiological Variables
No significant differences in venular diameter (33.4±1.5 μm) (obtained after platelet adhesion measurements) as well as blood pH (7.37±0.02), Po₂ (105±7 mm Hg), and Pco₂ (34±3 mm Hg) were detected between animal groups. In all bilateral CCA occlusion groups, baseline blood pressure did not differ from the sham control value of 88±2 mm Hg. No differences in blood pressure were noted between groups during the ischemia and reperfusion periods.

Platelet–Endothelial Cell Interactions in WT Mice
In WT mice, platelet adhesion in pial venules was unaffected by 60 minutes of ischemia followed by 40 minutes of reperfusion. However, large and highly significant increases in platelet rolling, saltation, and adherence were noted in mice subjected to 60 minutes of ischemia and 4 hours of reperfusion (Figures 1A and 2). The I/R-induced platelet adhesion was detected only in venules, with no such interactions noted in arterioles of different sizes (15 to 80 μm).

Leukocyte–Endothelial Cell Interactions in WT Mice
Bilateral CCA occlusion followed by 40 minutes of reperfusion resulted in significant increases in leukocyte rolling, saltation, and adherence. Four hours of reperfusion was associated with significant further increases in all 3 forms of leukocyte–vessel wall interaction (Figures 1B and 3). Leukocytes did not roll or adhere in arterioles at any time during these experiments.

Platelet–Vessel Wall Interactions in P-Selectin<sup>−/−</sup> and ICAM-1<sup>−/−</sup> Mice
When WT platelets were monitored in postischemic venules of P-selectin<sup>−/−</sup> mice, there was a dramatic reduction in adhesion compared with adhesion values obtained when WT platelets were administered to WT mice, which implicates endothelial cell–associated P-selectin in the I/R-induced adhesion response. A similar attenuation of platelet adhesion was noted when P-selectin<sup>−/−</sup> platelets were monitored in postischemic venules of WT mice, which suggests that platelet-associated P-selectin also plays a role in this model. When WT platelets were monitored in ICAM-1<sup>−/−</sup> mice, all of the I/R-induced platelet adhesion responses were significantly attenuated compared with responses seen when WT platelets were administered to WT mice (Figure 4A).

Leukocyte–Vessel Wall Interactions in P-Selectin<sup>−/−</sup> and ICAM-1<sup>−/−</sup> Mice
The I/R-induced recruitment of rolling leukocytes was significantly attenuated in P-selectin<sup>−/−</sup> mice compared with the rolling responses noted in WT mice exposed to cerebral I/R. However, ICAM-1<sup>−/−</sup> mice exhibited a leukocyte rolling response to I/R that was similar to that observed in WT mice. The enhanced salutation and adherence of leukocytes normally seen in WT mice exposed to cerebral I/R were significantly blunted in both P-selectin<sup>−/−</sup> and ICAM-1<sup>−/−</sup> mice (Figure 4B).

Discussion
Despite a growing recognition of the potential importance of leukocyte–endothelial cell adhesion in experimental stroke models, there have been very few attempts to monitor and quantify leukocyte–endothelial cell adhesion in the cerebral microvasculature after I/R, and only 1 report has addressed the role of a single adhesion molecule, CD11/CD18, in mediating the I/R-induced leukocyte–endothelial cell adhesion. Previous efforts to study leukocyte–endothelial cell adhesion in the postischemic brain have employed porcine, gerbil, and rat models. The present report summarizes an initial effort to study I/R-induced adhesion of both leukocytes and platelets in the cerebral microvasculature of mice.

There are several advantages to studying I/R-induced leukocyte–endothelial cell adhesion in mouse brain. Bilateral CCA occlusion, which does not yield reproducible forebrain ischemia in the rat because the anterior and posterior circu-
lations are connected via the circle of Willis, is a simple and highly reproducible model of global cerebral ischemia in C57BL/6 mice.\textsuperscript{22–26} In C57BL/6 mice, the posterior communicating arteries are hypoplastic, allowing bilateral CCA occlusion to produce consistent ischemia and tissue injury.\textsuperscript{22–26} The mouse also affords the opportunity to visualize the postischemic cerebral microcirculation under fluorescence microscopy without the need to cut the dura matter, which is very thin and relatively avascular in mice. Finally, C57BL/6 mice represent the most commonly used background strain for developing knockout and transgenic mice, which have proven to be particularly useful for studying molecular mechanisms of I/R injury in other vascular beds.\textsuperscript{27}

The findings of this study support published reports that conclude that the postischemic microvasculature of the brain assumes an inflammatory phenotype that is characterized by the recruitment of rolling, saltatory, and firmly adherent leukocytes.\textsuperscript{18–21} As noted in several other vascular beds, I/R-induced leukocyte–endothelial cell interactions occur exclusively in the postcapillary segment (venules) of the microcirculation,\textsuperscript{1} which is likely explained by the preferential expression of leukocyte adhesion receptors (e.g., P-selectin, ICAM-1) in venules.\textsuperscript{28}

Several reports describe an increased expression of ICAM-1, P-selectin, and other endothelial cell adhesion molecules in the postischemic cerebral microvasculature. Similarly, increased circulating levels of soluble ICAM-1 and P-selectin have been detected in the serum of stroke patients,\textsuperscript{29,30} which is consistent with the view that a portion of these cell adhesion molecules are shed from the vessel wall after endothelial cell activation.\textsuperscript{29,30} The results of the present study indicate that the increased endothelial expression of ICAM-1 and P-selectin is of quantitative significance in mediating the leukocyte–endothelial cell adhesive interactions elicited by cerebral I/R. P-selectin\textsuperscript{−/−} mice subjected to cerebral I/R exhibited a profound reduction in leukocyte adhesion. Although ICAM-1\textsuperscript{−/−} mice did not exhibit an attenuated I/R-induced leukocyte rolling, deficiency of this

**Figure 3.** Changes in leukocyte rolling (A), saltation (B), and adherence (C) observed in pial venules after 60 minutes of ischemia followed by either 40 minutes or 4 hours of reperfusion. Six to 8 animals were studied in each group. *P<0.05 relative to corresponding sham and control values.

**Figure 4.** Role of P-selectin and ICAM-1 in I/R-induced rolling, saltation, and adhesion of platelets (A) and leukocytes (B). Six to 8 animals were studied in each group. WT P-selectin\textsuperscript{−/−} and ICAM-1\textsuperscript{−/−} mice were exposed to 1 hour of cerebral ischemia followed by 4 hours of reperfusion. WT mice received CFDASE-labeled platelets from either WT (WT→WT) or P-selectin\textsuperscript{−/−} (P-sel\textsuperscript{−/−}→WT) donor mice. P-selectin\textsuperscript{−/−} (WT→P-sel\textsuperscript{−/−}) and ICAM-1\textsuperscript{−/−} (WT→ICAM-1\textsuperscript{−/−}) mice also received CFDASE-labeled platelets from WT mice. *P<0.05 relative to sham group; #P<0.05 relative to WT (WT→WT) in platelets and to WT in leukocytes.
endothelial cell adhesion molecule was associated with substantial reductions in the number of saltatory and adherent leukocytes at 4 hours after reperfusion. Collectively, our results from the cell adhesion molecule–deficient mice are consistent with the multistep model of leukocyte recruitment that invokes a role for P-selectin as a mediator of leukocyte rolling and ICAM-1 as a mediator of firm adhesion. Since leukocyte rolling is a prerequisite for firm adhesion, it is not unexpected that P-selectin deficiency would reduce all forms of leukocyte–endothelial cell adhesion, while ICAM-1 deficiency would affect stationary adhesion but not leukocyte rolling.

A novel and potentially important observation in the present study is that venular endothelial cells in the postischemic brain assume a prothrombogenic phenotype that is characterized by the presence of rolling, saltatory, and adherent platelets in pial venules. It is noteworthy that the appearance of a prothrombogenic phenotype, which is not seen until 4 hours after reperfusion, lags behind the inflammatory phenotype, which is manifested as early as 40 minutes after reperfusion. Platelet–vessel wall interactions have been described in other tissues exposed to I/R.11,12 In mouse intestine, platelet adhesion is noted in both arterioles and venules as early as 5 minutes after reperfusion.10 In rat retina, however, the recruitment of rolling and adherent platelets in venules (but not arterioles) is not seen until 4 hours after reperfusion and reaches a peak at 12 hours after reperfusion.11 Our findings and published work on the retina suggest that platelet recruitment in venules of neuronal tissue either involves a transcription-dependent mechanism, requires sufficient time for accumulation of specific mediators of platelet adhesion, or both.

Our experiments also provide interesting information concerning the adhesion glycoproteins that may account for the prothrombogenic phenotype assumed by postischemic cerebral venules. Since the rolling, saltation, and adherence of platelets induced by I/R were largely abolished in WT mice receiving P-selectin−/− platelets as well as in P-selectin−/− mice receiving WT platelets, it appears that P-selectin expression on both the venular wall and platelet is necessary for the platelet adhesion. The finding that platelet-associated P-selectin contributes to I/R-induced platelet adhesion in pial venules is inconsistent with data derived from other vascular beds in which endothelial cell–associated P-selectin, but not platelet P-selectin, has been implicated as a mediator of I/R-induced platelet recruitment.10 In these studies it was proposed that platelet-associated P-selectin glycoprotein ligand-1 (PSGL-1) may serve as the ligand for platelet binding to endothelial cell P-selectin.10,11 Our findings also implicate ICAM-1 in I/R-induced platelet adhesion in pial venules. In a published report on platelet adhesion in postischemic mouse intestine,12 it was shown that ICAM-1−/− mice exhibited an attenuation of platelet adhesion of a magnitude comparable to that seen in our brain experiments. This observation, coupled with additional data related to fibrinogen deposition in the postischemic intestinal vascularity of WT and ICAM-1−/− mice, led to the proposal that ICAM-1−bound fibrinogen on endothelial cells serves as a ligand for GpIIb/IIIa on platelets. The protective effects of ICAM-1 deficiency against cerebral I/R-induced platelet recruitment noted in our study may result from a comparable mechanism. However, another molecular model that could explain the responses noted for both leukocyte and platelet adhesion in the postischemic brain of ICAM-1−/− and P-selectin−/− mice involves the rolling and adhesion of platelets onto leukocytes that are already adherent to venular endothelium. Leukocytes utilize endothelial P-selectin as well as ICAM-1 to roll and firmly adhere, respectively, to venular endothelium. The adherent leukocytes, which constitutively express PSGL-1, then create a platform onto which platelets can bind using P-selectin. This model would explain why P-selectin expressed on both platelets and endothelial cells is required for I/R-induced platelet adhesion. It would also explain why either ICAM-1 or P-selectin deficiency reduces infarct volume in experimental stroke models14,15 and blunts the recruitment of both platelets and leukocytes.

In conclusion, the results of this study provide evidence that I/R leads to the induction of an inflammatory and prothrombogenic state in the cerebral microvasculature. Both ICAM-1, expressed on endothelial cells, and P-selectin, expressed on endothelial cells as well as platelets, contribute to the recruitment of leukocytes and platelets. The I/R-induced platelet accumulation may serve to amplify the inflammatory response and render the brain more vulnerable to microthrombosis and consequently amplify the ischemic insult. Unlike antithrombotic agents such as heparin and tissue plasminogen activator, blockade of P-selectin can interfere with platelet adhesion without predisposing the tissues to bleeding.31 Consequently, P-selectin may prove to be a more effective therapeutic target for stroke than either CD11/CD18 or ICAM-1. The therapeutic potential of antithrombotic strategies in stroke is supported by a recent report that describes a significant reduction in infarct volume in mouse brain after middle cerebral artery occlusion with the antithrombotic agent CD39/ectopyrase.32

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