Endothelial cell P-selectin mediates a proinflammatory and prothrombogenic phenotype in cerebral venules of sickle cell transgenic mice

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Wood, Katherine C., Robert P. Hebbel, and D. Neil Granger. Endothelial cell P-selectin mediates a proinflammatory and prothrombogenic phenotype in cerebral venules of sickle cell transgenic mice. Am J Physiol Heart Circ Physiol 286: H1608–H1614, 2004. First published January 15, 2004; 10.1152/ajpheart.01056.2003.—Whereas the adhesion of leukocytes and erythrocytes to vascular endothelium has been implicated in the vasculopathic processes associated with sickle cell disease, the role of platelet–venule wall interactions in this process remains undefined. The objectives of this study were to: 1) determine whether the adhesion of platelets and leukocytes in cerebral venules differs between sickle cell transgenic (βS) mice and their wild-type (WT) counterparts (C57Bl/6) under both resting and posthypoxic conditions, and 2) define the contributions of P-selectin to these adhesion processes. Animals were anesthetized, and platelet and leukocyte interactions with endothelial cells of cerebral postcapillary venules were monitored and quantified using intravital microscopy in WT, βS, and chimeric mice produced by transplanting bone marrow from WT or βS mice into WT or P-selectin-deficient (P-sel−/−) mice. Platelet and leukocyte adhesion to endothelial cells in both unstimulated and posthypoxic βS mice were significantly elevated over WT levels. Chimeric mice involving bone marrow transfer from βS mice to P-sel−/− mice exhibited a profound attenuation of both platelet and leukocyte adhesion compared with βS bone marrow transfer to WT mice. These findings indicate that βS mice assume both an inflammatory and prothrombogenic phenotype, with endothelial cell P-selectin playing a major role in mediating these microvascular responses.

platelet; leukocyte; brain; bone marrow transplant

SICKLE CELL DISEASE (SCD) is a painful and life-threatening condition that involves multiple organ pathology, including acute chest syndrome and stroke. Studies on the pathogenesis of SCD have revealed that the vasculature is a principal target of the disease (3, 14) and that the affected tissues assume an inflammatory phenotype (7, 12, 21). Enhanced cytokine production (8, 23), oxidative stress (18, 26, 28), altered endothelium-dependent vasorelaxation (19), and increased endothelial cell adhesion molecule expression (9, 32, 33) are manifestations of the inflammatory state that occurs during SCD. Recent studies using the sickle transgenic mouse model have revealed increased adhesive interactions between circulating erythrocytes and leukocytes with the walls of postcapillary venules, which may impede microvascular perfusion and promote vasoocclusive crises. These adhesion responses appear to involve both direct adhesive interactions of the circulating blood cells with venular endothelium and indirect interactions that involve the binding of erythrocytes to leukocytes already adherent to venular endothelium (40). A role for P-selectin in mediating leukocyte and erythrocyte interactions in mice with SCD has been demonstrated using both blocking monoclonal antibodies (18) and P-selectin knockout mice (40). There is a growing body of evidence that implicates platelets in the vasculopathy of both small and large blood vessels observed in SCD. Platelets from SCD patients exhibit an abnormal phenotype marked by surface-mobilized, activation-dependent antigens and microparticle morphology (37, 46). Increased circulating levels of platelet products (thrombospondin, IL-1) have been noted in SCD patients and have been implicated in the abnormal adhesion of SCD erythrocytes (2, 4, 34). It has been demonstrated that antibodies directed against the thrombospondin receptor (reticulocyte CD36) dramatically inhibit platelet supernatant-mediated adhesion of SCD erythrocytes to monolayers of cultured endothelial cells (4, 34). Furthermore, studies of large vessel cerebral arterial disease in SCD stroke patients have revealed hyperplastic arterial vessels with overlying thrombi (10). Despite evidence that SCD patients are more prone to thrombus formation (16, 25, 43, 45) and its consequences (e.g., stroke), there has been no reported effort to determine whether circulating SCD platelets, like erythrocytes and leukocytes, exhibit enhanced adhesive interactions within the microvasculature. This possibility appears tenable inasmuch as P-selectin, which mediates the adhesion of erythrocytes and leukocytes in venules of SCD mice (18, 40), has been implicated in the recruitment of adherent platelets in murine venules exposed to inflammatory conditions such as ischemia-reperfusion (24) and hypercholesterolemia (36). Most intravital microscopic studies of the microvascular responses to SCD have focused on skeletal muscle circulation (cremaster) despite limited evidence implicating muscle involvement in this disease. The organ systems most profoundly affected by SCD include the lung, brain, kidney, spleen, and long bones. An important clinical consequence of the cerebral vascular response to SCD is stroke. Brain involvement in SCD is not uncommon, especially in children. Indeed, the prevalence of stroke in the SCD population is reported to range between 6 and 30% (20, 30, 31). Although the cerebral circulation is known to be a major target organ in the pathogenesis of SCD, there has been no documented attempt to examine the brain microvascular responses to SCD. In this study, the cerebral microcirculation was examined in wild-type (WT) and sickle cell transgenic (βS) mice to

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Materials and Methods

Animals. WT C57BL/6 mice, CD45.1 congenic B6.SJL-PtprcPepc/P Boy mice (which express CD45.1), and B6.129S7-SleptmBbay (P-selectin/f^{-/-}) mice on a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME). The β^s mice and their negative controls (C57BL/6) were provided by Dr. Robert P. Hebbel, Dept. of Medicine, University of Minnesota. The β^s mice, and heterozygous for knockout of murine P-selectin on the activity or viability of isolated platelets as assessed by flow cytometry (36), were used to isolate and label donor platelets from WT and β^s mice. Platelets were isolated from whole blood obtained via the carotid artery of donor mice. The blood was collected in a sterile 50-ml centrifuge tube (Clay Adams; Parsippany, NJ) that was previously exposed by a longitudinal midline skin incision. A craniectomy was made by using a craniotome, and a craniocutaneous defect was created by using a microsurgical technique. The brain was then exposed and the skull was fixed on the skull with ethyl cyanoacrylate (Elmer’s Products; Columbus, OH), and a covered craniocutaneous defect was exposed by a longitudinal midline skin incision. The brain was then exposed and the skull was fixed.
Intravital fluorescence microscopy. Platelets were visualized, as previously described (6), with a Nikon Xanaphot upright microscope assisted by a silicon intensified target camera (C-2400, Hamamatsu Photonics). Cerebral venules were epifluorescently illuminated, observed through a 20× long distance objective lens (Leitz Weiztler), and recorded on videotape using a videocassette recorder (BRS601MU, JVC). A video time/date generator projected the time, date, and stopwatch function on the monitor (diagonal 50.6 cm, PVM-203, Sony Trinitron). CFSE (excitation: 490 nm, emission: 518 nm) and acridine orange (excitation: 500 nm, emission: 526 nm) visualization required a filter block with an excitation filter (B-2A, Nikon) for 450–490 nm, a dichroic mirror for 510 nm, and a barrier filter of 520 nm. The cerebral surface was scanned for five nonoverlapping venular segments per animal, and each was recorded for 1 min. Platelet and leukocyte adhesion were quantified separately within cerebral venules.

Video analysis. Leukocyte and platelet interactions with the endothelium were determined by offline analysis of videotaped images as previously described (5). Nonoverlapping venular segments of 300 μm length with diameters ranging between 20 and 80 μm (51.1 mean diameter) were chosen for quantification of adherent cells. Platelets and leukocytes were considered adherent if they arrested on the vascular endothelium ≥2 s. Platelet adherence was expressed as the number of cells per square millimeter of venular surface, calculated from diameter and length, assuming cylindrical vessel shape (24).

Statistical analysis. All values are reported as means ± SE. ANOVA with the Fisher’s post hoc test was used to compare groups, with statistical significance set at P < 0.05.

RESULTS

Comparisons of arterial blood pressure (79.8 ± 3.1 vs. 76.9 ± 3.1 mmHg), venular diameter (52.2 ± 2.7 vs. 53.1 ± 2.6 μm), and venular pseudoshear rate (400.4 ± 40.5 vs. 305.4 ± 29.7 s⁻¹) between unstimulated WT and β⁵ mice revealed no statistically significant differences. Figure 1 illustrates that the number of adherent leukocytes in the cerebral venules of unstimulated β⁵ mice (60 ± 11 cells/mm²) was approximately twice the adhesion detected in their unstimulated WT counterparts (34 ± 5 cells/mm², P = 0.047). This finding is consistent with previous reports of a twofold increase in leukocyte adhesion in cremasteric postcapillary venules of β⁵ mice.

Major objectives of this study were to determine whether larger numbers of adherent platelets are observed in cerebral venules of β⁵ mice (compared with WT) and to define the contribution of platelets versus endothelial cells to any platelet-endothelial cell interactions that are elicited in venules of β⁵ mice. When platelets isolated from β⁵ donors were monitored in venules of β⁵ recipients (WT → WT), significant platelet adhesion (654 ± 240 cells/mm²) was noted, and the intensity of adhesion was significantly greater than that observed when WT platelets were monitored in WT recipients (WT → WT; 147 ± 35 cells/mm², P = 0.03) (Fig. 2). When platelets isolated from WT donors were infused into β⁵ recipients (WT → β⁵), platelet adhesion (454 ± 141 cells/mm²) was similar to that noted in β⁵ controls (WT → β⁵). However, when platelets isolated from β⁵ donors were infused into WT recipients (β⁵ → WT), platelet adhesion (101 ± 50 cells/mm²) was similar to that noted in WT controls (WT → WT; 147 ± 35 cells/mm²), i.e., minimal platelet adhesion was observed. These experiments revealed that platelet adhesion in unstimulated β⁵ mice is significantly increased compared with WT mice, and this thrombogenic response is endothelial cell dependent.

Another objective of the study was to determine whether 2 h of whole body hypoxia (10% O₂-0.05% CO₂, balance nitrogen) followed by 4 h reoxygenation (room air) alters the adhesion of leukocytes and platelets in the cerebral venules of β⁵ and WT mice (Figs. 3 and 4). WT mice challenged with H/R exhibited leukocyte (27 ± 6 vs. 34 ± 5 cells/mm²) and platelet (124 ±...
44 vs. 147 ± 35 cells/mm²) adhesion responses that are similar to those seen in normoxic WT mice. The response of leukocyte adhesion to H/R in controls is consistent with published reports that address H/R-induced responses in cremasteric venules of SCD mice. When β⁵ mice were exposed to H/R, platelet adhesion was unaffected compared with their normoxic, unstimulated counterparts (571 ± 171 vs. 654 ± 240 cells/mm²). Although leukocyte adhesion was enhanced by H/R in β⁵ mice, this response was not statistically significant (86 ± 18 vs. 59 ± 10 cells/mm²).

The role of endothelial cell P-selectin in mediating the leukocyte and platelet adhesion responses to H/R in β⁵ mice was addressed in the bone marrow of chimeric mice (Figs. 5 and 6). Transfer of marrow from β⁵ mice into WT mice (β⁵/WT) yielded significantly higher H/R-induced leukocyte

Fig. 3. Hypoxia-reoxygenation (H/R)-induced leukocyte adhesion in β⁵ mice. Effects of H/R on leukocyte adhesion in cerebral postcapillary venules of WT and β⁵ mice are shown. Mean responses of leukocytes adherent for ≥2 s. 15 to 16 animals were studied in each group. *Significant differences, P < 0.05 (one-way ANOVA and Fisher’s test).

Fig. 4. H/R-stimulated platelet adhesion is similar to baseline adhesion in β⁵ mice. The effects of H/R on platelet adhesion in cerebral postcapillary venules of WT and β⁵ mice are shown. Mean responses of platelets adherent for ≥2 s. Six to 8 animals were studied in each group. *Significant differences, P < 0.05 (one-way ANOVA and Fisher’s test).

Fig. 5. Endothelial cell-associated P-selectin mediates H/R-stimulated leukocyte adhesion in β⁵ chimeras. Effects of H/R (2 h hypoxia, 4 h reoxygenation) on leukocyte-endothelial (L/E) adhesion in cerebral postcapillary venules of WT control chimeras (WT/WT), β⁵ chimeras (β⁵/WT), and β⁵/P-selectin⁻/⁻ chimeras (β⁵/P-selectin⁻/⁻) are shown. Mean responses of leukocytes adherent for ≥2 s. Five to 7 animals were studied in each group. *Significant difference versus WT/WT; #significant difference versus β⁵/WT, P < 0.05 (one-way ANOVA and Fisher’s test).

Fig. 6. Endothelial cell-associated P-selectin mediates H/R-stimulated platelet adhesion in β⁵ chimeras. Effects of H/R (2 h hypoxia, 4 h reoxygenation) on platelet-endothelial cell (P/E) adhesion in postcapillary cerebral venules of WT control chimeras (WT/WT), β⁵ chimeras (β⁵/WT), and β⁵/P-selectin⁻/⁻ chimeras (β⁵/P-selectin⁻/⁻) are shown. Mean responses of WT platelets adherent for ≥2 s. Five animals were studied in each group. *Significant difference versus WT/WT; #significant difference versus β⁵/WT, P < 0.05 (one-way ANOVA and Fisher’s test).
(67 ± 12 vs. 24 ± 10 cells/mm², P = 0.01) and platelet (914 ± 198 vs. 175 ± 79 cells/mm², P = 0.002) adhesion responses compared with WT mice receiving bone marrow from WT mice (WT/WT). However, the transfer of bone marrow from β⁸ mice into P-selectin⁻/⁻ mice (β⁸/P-selectin⁻/⁻) yielded leucocyte (30 ± 7 vs. 24 ± 10 cells/mm²) and platelet (284 ± 53 vs. 175 ± 79 cells/mm²) adhesion responses to H/R that were not different from those noted in WT/WT chimeras and were significantly reduced compared with leucocyte (67 ± 12 cells/mm², P = 0.03) and platelet (914 ± 198 cells/mm², P = 0.004) adhesion observed in β⁸/WT chimeras. These observations are consistent with the transfer of both the inflammatory and prothrombogenic phenotypes into WT mice following transplantation of bone marrow derived from β⁸ mice. In addition, the blunted adhesion responses of platelets and leucocytes in β⁸/P-selectin⁻/⁻ chimeric mice strongly implicates endothelial cell-associated P-selectin in these adhesion responses.

**DISCUSSION**

There is a growing body of evidence indicating that acute and chronic inflammatory conditions are associated with microvascular dysfunction, which is characterized by endothelial cells that assume both an inflammatory and prothrombogenic phenotype. For example, the recruitment of rolling and adherent leucocytes in postcapillary venules exposed to ischemia and reperfusion is accompanied by an intense accumulation of rolling and firmly adherent platelets (24). Colocalization of adherent platelets and leucocytes has been observed in response to transient retinal ischemia (17, 27, 39), as well as in venules of experimental animals with chronic arterial hypertension (1, 13, 22, 35). The results of the present study indicate that sickle cell disease is another pathological condition that produces both an inflammatory and prothrombogenic state in the microcirculation.

The results of our study demonstrate that SCD affects the cerebral microcirculation by 1) promoting the adhesion of platelets and leucocytes to venular endothelium, 2) inducing platelet and leucocyte adhesion responses that are P-selectin dependent, and 3) eliciting platelet and leucocyte responses that result primarily from changes in the function/adhesivity of endothelial cells, rather than circulating blood cells. The endothelial P-selectin-dependent inflammatory and prothrombogenic phenotype that we have observed in the cerebral microcirculation of β⁸ mice may explain, at least in part, the greater vulnerability of the brain to ischemic strokes in patients with SCD. This appears tenable in view of several published reports that implicate the recruitment of platelets and leucocytes in the brain injury and tissue dysfunction that is associated with strokes (11, 41, 42).

Our observation that leucocytes adhere in the cerebral microcirculation of β⁸ mice is consistent with published reports from other laboratories describing enhanced baseline and H/R- or tumor necrosis factor-α-stimulated leucocyte adhesion in cremasteric venules of SCD mice (18, 40). Our data show an approximate twofold increase in baseline leucocyte adhesion in cerebral venules of β⁸ mice (Fig. 1) that is not significantly enhanced by 2 h hypoxia followed by 4 h reoxygenation (Fig. 3). Kaul and Hebbel (18) reported a doubling of leucocyte adhesion in cremasteric venules of β⁸ mice. Similarly, Turhan and associates (40) noted that baseline and tumor necrosis factor-α-stimulated leucocyte rolling and adhesion in postcapillary and collecting venules of the cremaster muscle of sickle cell transgenic mice are nearly twice those observed in WT controls. The quantitatively similar leucocyte adhesion responses of the brain and cremasteric circulations to experimental SCD raises the possibility that this inflammatory phenotype is exhibited in a variety of different vascular beds.

Previous intravital microscopic studies of leucocyte adhesion in cremasteric venules of mice with experimental SCD have demonstrated significant attenuation of adhesion following administration of a P-selectin blocking monoclonal antibody (18). These observations, coupled to reports of elevated expression of P-selectin on circulating endothelial cells isolated from patients with SCD (33) and recent data that describe an increased expression of P-selectin in different regional vascular beds of β⁸ mice (44), implicate endothelial cell P-selectin as a key mediator of the leucocyte recruitment associated with experimental SCD. The results of the present study support and significantly extend these observations. We observed that bone marrow transfer from β⁸ mice into P-selectin⁻/⁻ mice yields a leucocyte adhesion response that is comparable to that observed in WT mice receiving bone marrow from other WT mice but is significantly lower than the adhesion response seen when bone marrow is transferred from β⁸ mice into WT mice. Our findings indicate that P-selectin expressed by cerebral venular endothelium is critical for the recruitment of adherent leucocytes in β⁸ chimeric mice. An important and novel observation in the present study is that endothelial cell-associated P-selectin also mediates platelet recruitment observed in cerebral venules of β⁸ mice. Therefore, our observations suggest that therapeutic application of P-selectin-directed interventions may be useful in blunting both the inflammatory and prothrombogenic responses that are elicited in the cerebral circulation by SCD.

In addition to demonstrating that SCD cerebral venules exhibit enhanced platelet interactions with the vascular wall, our studies also provide novel insight into the relative contributions of endothelial cell versus platelet activation in SCD prothrombogenic responses. Our data show an approximate fourfold increase in baseline platelet adhesion in cerebral venules of β⁸ mice (Fig. 2) that is not significantly enhanced by 2 h hypoxia followed by 4 h reoxygenation (Fig. 4). Moreover, we demonstrate that β⁸ platelets adhere comparably to WT platelets in WT mice, whereas the adhesion of WT platelets is comparable to β⁸ platelets in β⁸ mice (Fig. 2). These observations indicate that β⁸ mice exhibit a prothrombogenic phenotype that largely reflects changes in the adhesivity of the venular wall rather than circulating platelets. This possibility is supported by our findings in the β⁸ chimeric mice that were created to determine whether the elevated platelet adhesion noted in the cerebral microvasculature of β⁸ mice, similar to leucocyte adhesion, is mediated by vascular P-selectin. Complete inhibition of platelet adhesion was noted in chimeric mice wherein the vessel wall (recipient mice) was deficient in P-selectin while circulating platelets (from β⁸ marrow donor) retained the capacity to express P-selectin (Fig. 6). Whereas endothelial cells clearly play a major role in promoting the adhesion of platelets as well as leucocytes in β⁸ chimeric mice,
the exact nature of this endothelial cell-dependent response remains unclear. One possible explanation relates to reports describing an oxidative stress in the vasculature of SCD mice (3, 26). This oxidative stress may contribute to a P-selectin involved, proadhesive environment on the endothelial cell surface that facilitates the capture of both platelets and leukocytes, as previously demonstrated in other experimental models (6, 15).

GRANTS

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