P-Selectin–Mediated Platelet-Neutrophil Aggregate Formation Activates Neutrophils in Mouse and Human Sickle Cell Disease*

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Objective—To determine the role of platelets in stimulating mouse and human neutrophil activation and pulmonary injury in sickle cell disease (SCD).

Methods and Results—Both platelet and neutrophil activation occur in SCD, but the interdependence of these events is unknown. Platelet activation and binding to leukocytes were measured in mice and patients with SCD and in controls. Relative to controls, blood obtained from mice or patients with SCD contained significantly elevated platelet-neutrophil aggregates (PNAs). Both platelets and neutrophils found in sickle PNAs were activated. Multispectral imaging (ImageStream) and conventional flow cytometry revealed a subpopulation of activated neutrophils with multiple adhered platelets that expressed significantly more CD11b and exhibited greater oxidative activity than single neutrophils. On average, wild-type and sickle PNAs contained 1.1 and 2.6 platelets per neutrophil, respectively. Hypoxia/reoxygenation induced a further increase in PNAs in mice with SCD and additional activation of both platelets and neutrophils. The pretreatment of mice with SCD with clopidogrel or P-selectin antibody reduced the formation of PNAs and neutrophil activation and decreased lung vascular permeability.

Conclusion—Our findings suggest that platelet binding activates neutrophils and contributes to a chronic inflammatory state and pulmonary dysfunction in SCD. The inhibition of platelet activation may be useful to decrease tissue injury in SCD, particularly during the early stages of vaso-occlusive crises.

Key Words: platelet activation ■ sickle cell disease inflammation ■ neutrophil activation ■ oxidative burst ■ clopidogrel ■ P-selectin antiplatelet drugs ■ leukocytes ■ platelets ■ transgenic models

Sickled cell disease (SCD) is the most common genetic hematologic disorder in the United States. The vaso-occlusive characteristics of SCD have been viewed historically as resulting from deformed red blood cells (RBCs) that mechanically obstruct capillaries to produce tissue hypoxia.1 Present therapies for SCD are geared toward decreasing the concentration or polymerization rate of sickle hemoglobin.2 Recently, a modified paradigm has emerged suggesting that the wide spectrum of clinical manifestations of SCD results in part from recurrent episodes of disseminated microvascular ischemia-reperfusion injury.3,4 Ischemia-reperfusion injury triggers vascular inflammation, characterized by increased adhesion of leukocytes5–8 and sickle RBCs9 to vascular endothelium and activation of coagulation,10–12 blood platelets,13–20 neutrophils,7 monocytes,8,21–23 and natural killer T cells.24 Because blockade of P-selectin–mediated platelet-leukocyte aggregation is beneficial in the animal models of vascular injury,25 we reasoned that platelet-leukocyte aggregation might contribute to the vascular inflammation and tissue injury that occurs in SCD. Although increased formation of platelet-monocyte21 and platelet-RBC15 aggregates in SCD is well established, conflicting data exist regarding the occurrence of platelet-neutrophil aggregates (PNAs) in patients with SCD.18,21 The question of neutrophil activation in SCD is important because activated neutrophils play a major role in evoking vascular injury during ischemia-reperfusion by adhering to blood vessels and releasing reactive oxygen species.26 In vitro, binding of activated washed platelets to purified neutrophils results in their activation27–29, and in vivo studies demonstrate the increased formation of neutrophil-platelet aggregates as a result of inflammation.30,31

In this study, we investigated PNAs in SCD using blood obtained from NY1DD mice32,33 and patients with SCD. We found that both mice and humans with SCD have markedly increased PNAs compared with appropriate controls. Antiplatelet agents, such as clopidogrel and anti–P-selectin anti-
bodies, and platelet depletion strongly suppressed the formation of platelet-leukocyte aggregates and platelet-dependent neutrophil activation and pulmonary injury in NY1DD mice, indicating that antplatelet therapy may be helpful for limiting vascular inflammation and injury in SCD.

Methods

Human Subjects
Peripheral venous blood samples were obtained from consenting adult patients with SCD (hemoglobin S homozygotes) and age- and race-matched healthy control subjects (normal hemoglobin A) during a routine health examination at the Adult Hemoglobinopathy Clinic at Washington University, St Louis, Mo. All patients were at steady state (ie, they reported no more than typical pain at phlebotomy). The human protocol was approved by the institutional review boards of Washington University and the University of Virginia, Charlottesville.

Mice
Transgenic NY1DD mice with a C57BL/6 genetic background were used as a model for SCD.33 NY1DD mice are deficient in mouse β-globin and express a fused human α-ββ′-globin transgene. These mice have a normal hematocrit at baseline but exhibit multiple organ damage and leukocytosis.32 Hypoxia/reoxygenation (H/R) evokes hemolysis associated with the development of increased inflammation. Congenic C57BL/6 female mice, aged 8 to 12 weeks, were used as wild-type (WT) sex- and age-matched controls (Jackson Laboratory, Bar Harbor, Me). Experimental procedures were approved by the University of Virginia Animal Care and Use Committee.

Assessment of Platelet Activation in Whole Blood
Attempts to prepare platelets from NY1DD mice were complicated by the existence of platelet-platelet and platelet-leukocyte aggregates. Thus, platelet function was assessed in whole blood. Details about mouse blood collection and assessment of platelet activation in whole blood are described in the supplemental Methods (available online at http://atvb.ahajournals.org).

Platelet-Leukocyte Aggregates in Whole Blood
For mouse studies, heparin-anticoagulated blood was incubated with rat anti–mouse CD16/CD32 antibody to block the FcγRIIa receptor. A 4-color flow cytometry assay was developed to analyze simultaneous platelet binding to neutrophils and monocyes in whole blood. Rat anti–mouse CD41-PE or rat anti–mouse platelet glycoprotein (GP) IX–fluorescein isothiocyanate (FITC) antibodies were used to label platelets. The combinations of rat anti–mouse CD11b–activated protein C, CD45-PerCP-Cy5.5, and glucocorticoid receptor (GR)–1–FITC antibodies and isotype controls (all from BD Biosciences Franklin Lakes, NJ) were used for labeling leukocytes. Erythrocytes were lysed with high-yield lysing solution (Caltag Labs). Neutrophils and monocyes were distinguished from other cells by their size and granularity and by anti–CD11b–activated protein C and anti–GR-1–FITC antibody binding. Within the CD11b/CD45 double-positive gate, PNA formation was calculated from CD41-PE/GR1–FITC double-positive events and platelet-monocyte aggregates were calculated from CD41-PE–positive and GR-1–FITC–negative events. The activation of platelets bound to leukocytes was assessed by counting CD62P–positive events in the CD45-PerCP-Cy5.5/GPIX–FITC double-positive gate.

Human blood samples were collected by venipuncture into tubes (Vacutainer-ACD) and prepared for flow cytometry as previously described. PNAS in these samples were identified as CD14-negative and CD41+/CD16+ events and platelet-monocyte aggregates as CD16-negative and CD41+/CD14+ events in the CD11b+/CD45+ leukocyte gate. The activation of platelets bound to neutrophils was determined by counting CD62P+ events on PNAS. Fluorescence intensity was measured with a color analyzer (CyAn ADP LX 9 Color Analyzer; DakoCytomation, Glostrup, Denmark), and data analysis was performed with computer software (FlowJo). Details about antibodies used in human studies are given in the supplemental Methods.

Multispectral Imaging Flow Cytometry
PNAs were imaged using multispectral imaging flow cytometry (ImageStream flow cytometer; Amnis Corp, Seattle, Wash). At least 5000 cells were collected from each sample, and data were analyzed using image analysis software (IDEAS; Amnis Corp). Platelets were labeled using an anti–CD41-PE antibody. Neutrophil images were identified as GR-1–FITC+/high (H) scatter and CD11b+ events. Neutrophil-bound platelets were identified using a GR-1 membrane mask and the small spot intensity feature of IDEAS software. The number of platelets bound per neutrophil was determined using the spot count and peak intensity algorithms of IDEAS software and confirmed by examination of images positive for CD41 and GR-1.

Hydrogen Peroxide Production by Neutrophils
We developed a new 5-color flow cytometry assay to assess oxidative burst simultaneously in PNAS and single neutrophils in whole blood. The respiratory burst was monitored by dihydrodichromamine (DHR) 123, which produces fluorescent rhodamine123 after oxidation by hydrogen peroxide. Heparinized whole blood, 0.5 mL, from WT or sickle mice treated for 3 days with clopidogrel or vehicle and then subjected to H/R was incubated for 30 minutes with a cocktail of DHR 123 and appropriate fluorescent antibodies at 37°C and then transferred to ice. DHR 123 fluorescence was identified in both CD45+/CD11b+/GR1–/CD41+ and CD45+/CD11b+/GR1–/CD41– regions and analyzed with a color analyzer (CyAn ADP LX9).

Hypoxia/Reoxygenation and Drug Treatment of Mice
Mice were placed in a hypoxia chamber (8% oxygen plus 92% nitrogen; Coyer Laboratory Products, Ann Arbor, Mich) for 3 hours and then reoxygenated for 3 hours in ambient air. The number of platelet-leukocyte aggregates did not change during hypoxia (data not shown) but increased to a maximum 3 hours after reoxygenation. Some mice received carotid artery injections of 100 μg of blocking anti–P-selectin antibody (RB40.34; BD Biosciences PharmMingen) or control IgG just before the reoxygenation period. In other experiments, vehicle or clopidogrel, 30 mg/kg per day, was administrated to mice by oral gavage for 3 days before H/R.

Platelet Depletion Effects on Pulmonary Neutrophil Accumulation and Vascular Permeability
Platelets were depleted by a single intravenous injection of anti–GPibα antibody, 2 μg/g (Emfret Analytics, Wurzburg, Germany).44 After 24 hours, circulating platelets were reduced in sickle mice by 85% (from a mean±SD of 950±40×10⁶/μL to a mean±SD of 142±8×10⁶/μL). Subsequently, mouse lungs were harvested and neutrophils/lungs were counted by flow cytometry, as previously described.44 Pulmonary vascular permeability was assessed by Evans blue dye extravasation, as described in the supplemental Methods.

Representative data shown in the Figures are typical of 3 or more replicate experiments. Where applicable, results are given as the mean±SD. WT and NY1DD mice, or black healthy controls and patients with SCD, were compared using the t test.

Results

Activation of α₁β₂β₃ Integrin and Increased Membrane Expression of CD40L and P-Selectin on Sickle Platelets
There are several mouse models of SCD.35 One frequently used model is the Berkeley sickle mouse, which expresses exclusively human sickle hemoglobin and develops severe SCD.36,37 However, these mice exhibit marked thrombocyto-
Increased P-selectin on platelet-leukocyte aggregates in sickle mice. Platelets and leukocytes were recognized by their forward and side scatter characteristics. Platelet singlets were further identified with anti–GPIX-FITC. Platelet-leukocyte complexes were identified by anti–CD45-PerCP-Cy5.5 and anti–GPIX-FITC positive fluorescence. P-selectin expression was assessed using anti–CD62P-PE. A and B, Representative histograms showing P-selectin expression on platelet singlets (A) and platelet-leukocyte heteroaggregates (B) (n=5). Data are expressed as mean±SD.

Increased Platelet-Leukocyte Aggregation in SCD

The percentage of platelets found in PNAs or platelet-monocyte aggregates in mouse peripheral blood was assessed by 4-color flow cytometry. At baseline, approximately 2.5-fold more sickle than WT neutrophils and 1.7-fold more sickle than WT monocytes had adhered CD41+ platelets (Figure 3A and B). Neutrophils aggregated with platelets were more activated in sickle than WT blood, based on higher expression of CD11b (mean fluorescence intensity, 459±39 versus 233±50; P<0.001; n=10). We also detected eosinophils-platelet aggregates that were also significantly increased in sickle compared with WT mice (supplemental Figure II). Once formed, platelet-leukocyte aggregates were stable and did not change in number for up to 4 hours after the addition of heparin or anti–P-selectin–blocking antibody, RB40.34 to blood (data not shown).

Because the number of PNAs in blood from children with SCD was previously reported to be similar to controls,18 we studied platelet-neutrophil interactions in adults with SCD using 5-color flow cytometry. The percentage of neutrophils and monocytes found aggregated with platelets in blood obtained from patients with SCD versus controls was 2.5- and 2.1-fold higher, respectively (Figure 3C). Human neutrophils in PNAs were more activated in patients with SCD than in control subjects based on the expression of CD11b (mean fluorescence intensity, 311±80 versus 210±57; P<0.05; n=7). The number of P-selectin–positive platelets in the blood of patients with SCD was not different from that of controls. However, the surface expression of P-selectin given as mean fluorescence intensity on PNAs was significantly greater in patients with SCD than in controls: 63.2±13.3 versus 24.8±2.0 (P<0.05; n=7). In sum, as in mice, patients with SCD had increased numbers of PNAs, and aggregation resulted in activation of both platelets and neutrophils.

Fluorescence Imaging Analysis of PNAs

We used fluorescence imaging (ImageStream) to confirm the existence of PNAs and to determine the number of platelets

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**Figure 1.** Increased activated α6β3 integrin and CD40L on platelets in SCD mouse blood. A, Activation of the α6β3 integrin in platelets at rest and after stimulation with 1 μmol/L ADP was measured by binding of JON/A-PE antibody. After stimulation, blood samples were diluted and immediately analyzed by flow cytometry. The percentages of the total platelet population activated (JON/A+) in resting and ADP-stimulated WT and SCD blood are plotted as mean±SD (n=5) for all groups. B, The expression of platelet CD40L in resting and convulxin-stimulated blood was determined from binding of anti–CD40L-PE antibody. Platelets were identified by forward light scatter and standard saline citrate characteristics and by GPIX-FITC binding. The percentages of the total platelet population that are CD40L+ in blood from WT mice and mice with SCD are plotted as mean±SD from 3 to 4 independent experiments.

**Figure 2.** Increased P-selectin on platelet-leukocyte aggregates in sickle mice. Platelets and leukocytes were recognized by their forward and side scatter characteristics. Platelet singlets were further identified with anti–GPIX-FITC. Platelet-leukocyte complexes were identified by anti–CD45-PerCP-Cy5.5 and anti–GPIX-FITC positive fluorescence. P-selectin expression was assessed using anti–CD62P-PE. A and B, Representative histograms showing P-selectin expression on platelet singlets (A) and platelet-leukocyte heteroaggregates (B) (n=5). Data are expressed as mean±SD.

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penia,38 which does not parallel the increase in platelet number usually noted in human SCD at baseline.39 Therefore, we focused our studies on the NY1DD mouse model of SCD that develops moderate SCD.32,33 Like patients with SCD, NY1DD mice exhibit a proinflammatory phenotype with leukocytosis and increased platelet number.24 Because platelet activation is well established in human SCD,10,20 we first determined if it is replicated in NY1DD mice by using the rat mAb directed against mouse α6β3 (JON/A) antibody that recognizes the activated conformation of the murine fibrinogen receptor, α6β3.40 The percentage of JON/A-positive platelets was significantly greater in sickle (6.4%) compared with WT (3%) mouse blood (P<0.01) and was increased significantly more in SCD than in WT mice after stimulation for 10 seconds with ADP (Figure 1A). Surface expression of CD40L was used as an additional marker of platelet activation. Compared with WT mice, unstimulated sickle blood contained more CD40L+ platelets (P<0.001) (Figure 1B). Stimulation of heparinized blood for 15 seconds with the strong platelet agonist convulxin, 50 nmol/L, induced a rapid increase in CD40L in WT platelets and significantly greater activation in sickle platelets. Convulxin triggered a 2.3±0.3- and a 3.5±0.2-fold increase in surface expression of CD40L in WT and SCD mice, respectively, based on the geometric mean of fluorescence intensity.

P-selectin on activated platelets is a major mediator of adhesion to leukocytes.41 In unstimressed mice, the percentage of P-selectin–positive platelets was low (<1%) in both WT and sickle mice (Figure 2A). The percentage of P-selectin–positive platelets increased in response to convulxin to 34% in WT mice and to 50% in SCD mice (supplemental Figure Ia). In contrast, platelets that aggregated with leukocytes were partially P-selectin positive: 5.8±0.3% in WT mice and 16.0±1.8% in SCD mice (Figure 2B). Convulxin treatment increased the percentage of P-selectin–positive platelets in platelet-leukocyte aggregates to 44% in WT mice and to 71% in sickle mice (P<0.005) (supplemental Figure Ib).
attached to each neutrophil (Figure 4). The percentage of neutrophils with adherent platelets was significantly higher in mice with SCD than in WT mice (15.3±2.4% versus 5.8±0.9%, respectively). These numbers varied with age, sex, and handling of mice; therefore, it was important to use age- and sex-matched mice. In this experiment, the average number of adherent platelets per neutrophil-platelet aggregate was approximately 2.4-fold as high in mice with SCD compared with WT mice (Figure 4C and D). This was confirmed by measuring the mean fluorescence intensity of anti-CD41 on adhered platelets, indicative of the number of platelets per neutrophil, which was 2.3-fold higher in sickle mice compared with WT mice. The expression of CD11b was 1.7-fold higher in neutrophils aggregated with platelets compared with single neutrophils in sickle mice. There was no significant difference in CD11b expression between platelet-free and platelet-positive neutrophils in WT mice. In SCD, 40% of PNAs included more than 2 (up to 6) platelets, whereas in WT mice, only 20% of aggregates had more than 1 adhered platelet.

Hypoxia/Reoxygenation Enhances P-Selectin–Dependent Formation of PNAs

To investigate whether H/R has an effect on the formation of PNAs, WT and SCD mice were placed in a hypoxia chamber and exposed to 8% oxygen and 92% nitrogen for 3 hours, followed by 3 hours of reoxygenation in ambient air. Flow cytometric analysis revealed that H/R elicited a significant increase in PNAs in blood from sickle mice (Figure 5A). The injection of anti–P-selectin antibody just before reoxygenation led to a significant reduction in PNAs after H/R. Treatment of mice with SCD with clopidogrel or platelet depletion also resulted in significant suppression of CD41⁺ neutrophils both at baseline and after subsequent H/R in sickle mice (Figure 5A). Platelet binding to neutrophils has been reported to increase neutrophil adhesion to endothelium and may be responsible for endothelial activation.45,46 To test the hypothesis that activated platelets contribute to endothelial dysfunction in SCD, platelets were depleted and pulmonary microvascular permeability was measured with Evans blue dye. Platelet depletion was associated with a decreased number of pulmonary neutrophils, from 1.71±0.18×10⁵ per lung to 0.32±0.11×10⁵ per lung (n=10, \( P<0.05 \)) and with decreased pulmonary vascular leakage (Figure 5B) in NY1DD mice. These findings implicate platelet activation and exposure of platelet P-selectin as functionally important events mediating PNA formation, which may contribute to vaso-occlusive crisis in SCD.

Adherent Platelets Promote Neutrophil Oxidative Activity

We investigated whether platelet binding to neutrophils influences neutrophil activation in mice with SCD by measuring oxidative burst and expression of CD11b adhesive receptors using 5-color flow cytometry. The oxidative burst that results in production of hydrogen peroxide was detected from the intracellular oxidation of a fluorescent dye (ie, DHR 123) (Figure 6). In mice with SCD, CD41⁺ PNAs (Figure 6A) produced substantially more hydrogen peroxide (ie, more oxidative burst) (Figure 6B) and had significantly higher expression of CD11b (Figure 6C) than CD41-free single neutrophils (Figure 6A), suggesting that platelet binding activates neutrophils in vivo.

Discussion

Circulating blood leukocytes in SCD, particularly neutrophils,7,45,46 display an activated phenotype that may predispose them to endothelial adhesion and amplification of vascular inflammation and vaso-occlusion. In this study, we sought to determine if platelet activation that occurs in SCD15,16,47 actively contributes to SCD-associated neutrophil activation. To our knowledge, our data reveal for the first time an increased formation of PNAs in blood obtained from patients with SCD and from NY1DD sickle mice. In both sickle patients and NY1DD sickle mice, the most activated neutrophils were those with adhered platelets, as assessed by oxidative burst and increased expression of CD11b adhesive receptors (Figure 3). In sickle mice, the formation of PNA was exacerbated after hypoxia/reoxygenation, suggesting that platelet activation and adhesion to neutrophils might occur as
a result of H/R during vaso-occlusive crisis. Because the most activated neutrophils were those with adhered platelets, we hypothesized that it may be possible to reduce neutrophil activation in SCD by inhibiting platelet activation. Indeed, the treatment of mice with SCD with the antiplatelet drug clopidogrel, which blocks P2Y12 ADP receptors, or with anti–P-selectin antibody significantly reduced the number of PNAs and decreased overall neutrophil activation. The depletion of platelets with anti–GPIb antibody also reduced the number of PNAs and decreased pulmonary neutrophil infiltration and vascular permeability in sickle lung. This novel link between platelet and neutrophil activation in SCD provides a new perspective into SCD pathological features and treatment.

In vitro evidence indicates that platelet binding to leukocytes likely changes both platelet and leukocyte activation. Thrombotic processes associated with enhanced platelet activation and the formation of platelet-leukocyte aggregates have been observed in several chronic inflammatory diseases. Despite the fact that activation of platelets, monocytes, neutrophils, and eosinophils has been observed in human SCD, the only previously reported increases in heteroaggregates that contain platelets are those formed between platelets and monocytes or platelets and sickle RBCs. The analysis of platelet function in SCD is complicated by the fact that severe chronic inflammation may result in platelet depletion, margination, and desensitization. Moreover, previous studies have not attempted to measure in vivo the ratio of platelets bound per neutrophil or the effect of platelet binding on the oxidative activity of individual neutrophils. In a study of severe SCD in children with nocturnal hypoxia, the fraction of PNAs was not significantly changed compared with that of controls. The discrepancy with our findings could be partly because of the difference in the patient age and duration of the disease or different techniques of blood sample preparation and analysis. We used 5-color flow cytometry of cell markers with confirmation by imaging (ImageStream) analysis to define PNAs in ACD-anticoagulated blood, whereas Inwald et al used specific light scatter characteristics of neutrophils and CD61 platelets in citrate-anticoagulated blood. In NY1DD mice with mild SCD, we were able to consistently demon-
strate that platelet binding to neutrophils has a significant impact on the overall activation of neutrophils, which likely promotes adhesion to endothelial cells and induction of pulmonary vascular injury in mice with SCD.5,46,50 This is in agreement with previous studies showing that ligation of the P-selectin glycoprotein ligand 1 on neutrophils by P-selectin Ig chimera leads to production of reactive oxygen intermediates and increases neutrophil expression of cell surface CD11b.55 The analysis of platelet-leukocyte aggregates revealed more platelets bound to neutrophils in SCD: on average, approximately 2.6 platelets per neutrophil in PNAs in SCD versus approximately 1.1 platelets per neutrophil in WT mice. Thus, the binding of multiple platelets associated with increased neutrophil adhesive and oxidative activities may represent a newly recognized mechanism contributing to neutrophil activation in peripheral blood in vivo.

Several investigators have reported that only activated platelets expressing surface P-selectin bind to leukocytes.41 To test the hypothesis that P-selectin is required for neutrophil activation in SCD, we studied the effects of the antplatelet drug clopidogrel (3 days), injection of 100 μg of anti–P-selectin antibody, or platelet depletion with anti–GPIbβ antibody prevented the formation of platelet-neutrophil aggregates in mice with SCD. B, Platelet depletion with anti–GPIbβ antibody (24 hours) reduced a pulmonary vascular leak measured with Evans blue dye. Data are given as mean±SD (n=3). **P<0.005 and ***P<0.001.

Figure 5. Antiplatelet treatments suppress H/R-induced formation of platelet-neutrophil aggregates, neutrophil activation, and lung vascular permeability in mice with SCD. A, WT mice and mice with SCD were subjected to 3-hour hypoxia (8% oxygen plus 92% nitrogen), followed by 3-hour reoxygenation (H/R). Prior oral treatment with clopidogrel (3 days), injection of 100 μg of anti–P-selectin antibody, or platelet depletion with anti–GPIbβ antibody prevented the formation of platelet-neutrophil aggregates in mice with SCD. B, Platelet depletion with anti–GPIbβ antibody (24 hours) reduced a pulmonary vascular leak measured with Evans blue dye. Data are given as mean±SD (n=3). **P<0.005 and ***P<0.001.

Figure 6. Neutrophils with attached platelets (CD41+) have increased oxidative burst and CD11b expression in sickle mice. A, Gating strategy for evaluation of oxidative burst in platelet-positive or platelet-free neutrophils. B and C, Quantitative analysis in neutrophils of oxidative burst (B) and CD11b expression (C). Data are given as mean±SD (n=5). *P<0.05 and ***P<0.001.

in neutrophil-platelet aggregates in sickle mice at baseline and after H/R. The injection of P-selectin–blocking antibody to H/R-treated sickle mice before reoxygenation almost completely prevented the formation of new PNAs, suggesting that H/R leads to P-selectin–dependent platelet-neutrophil binding. The inability of anti–P-selectin to dissociate preexisting PNAs in SCD is consistent with the idea that P-selectin bound to P-selectin glycoprotein ligand 1 is not accessible to anti–P-selectin antibody or that the initial platelet adhesion to neutrophils occurs via surface P-selectin; however, stabilization of aggregates requires additional adhesive interactions, such as binding of neutrophil CD11b to platelet GPIb.56 These observations provide strong evidence that platelets play a role in enhancing neutrophil activation in vivo in SCD. The physiological significance of increased formation of platelet-leukocyte aggregates in peripheral blood in inflammatory diseases is unknown. Previous studies57–59 of P-selectin–positive platelet-leukocyte aggregates indicate that leukocytes with attached platelets tether and roll on endothelial cells with higher avidity than single leukocytes, thereby exacerbating endothelial inflammation.

Recently, we showed that NY1DD mice display baseline pulmonary injury.34 In the current study, we found that platelet depletion reduced the number of circulating PNAs, significantly suppressed neutrophil migration to the lung, and reduced lung vascular permeability, suggesting that platelets contribute to acute lung injury in SCD.43 Activated platelets with surface expression of P-selectin are essential for neutrophil recruitment into kidney in acute posts ischemic renal failure40 and for leukocyte extravasation into airways after allergen challenge.61 Thus, targeting platelet activation and...
membrane expression of P-selectin may be a useful strategy to reduce lung injury in SCD, particularly at the onset of vaso-occlusive crisis. In previous studies, long-term anti-platelet drug therapy (ticlopidin or low-dose aspirin) and anticoagulant treatment (low-dose heparin or warfarin) have not been effective in the prophylaxis of acute vaso-occlusive events in human SCD. However, these studies have not evaluated dose ranging nor measured the levels of platelet activation or the extent of vascular damage before and after treatment. Thus, it is not known if these treatments were sufficiently aggressive to reduce platelet activation and platelet-leukocyte aggregation.

Our findings suggest that, in addition to their procoagulant role, platelets may contribute directly to ongoing vascular inflammation in SCD by activating neutrophils. Consequently, therapies targeting platelet function and platelet interactions with other blood cells may help to control inflammation. The contribution of platelet activation to SCD pathophysiological features should be further investigated using newly available antiplatelet agents with the evaluation of biomarkers, including P-selectin expression and platelet-leukocyte aggregate formation. The fact that H/R induces an increase in platelet-leukocyte interaction, which decreases to baseline levels after the administration of clopidogrel or anti-P-selectin antibody, suggests that platelet-leukocyte cross-talk contributes to vaso-occlusive events in SCD. Further studies will be required to revisit antiplatelet therapy for SCD, with emphasis on inhibiting P-selectin-mediated platelet-leukocyte interactions.

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Disclosures
None.

References
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Supplement Material

Methods

Assessment of platelet activation in whole blood

Mouse blood was collected from the retro-orbital plexus and mixed with heparin (20 U/mL). In some experiments, platelets in whole blood were activated with 1 μM ADP or 50 nM convulxin and then fixed with 1% (w/v) paraformaldehyde (PFA) before the addition of fluorescent antibodies. Platelet activation was assessed by 2-color flow cytometry. Platelets in whole blood were identified by characteristic light scattering and membrane expression of the platelet specific glycoprotein IX (GPIX) detected with rat anti-mouse GPIX antibody (Emfret Analytics, Wurzburg, Germany). Surface exposure of platelet activation antigens was measured with PE-labeled rat anti-mouse CD62P (Emfret Analytics) or rat anti-mouse CD40L (BD Biosciences PharMingen, San Jose, CA). The activation of αIIbβ3 integrin was assessed by binding of PE-labeled JON/A antibody. Appropriate rat IgGs were used to determine non-specific binding. Blood samples were incubated with the antibodies for 30 min at 20°C and analyzed with a FACSCalibur (Becton Dickinson, San Jose, CA). Data analysis utilized FlowJo Software (Tree Star Inc, Ashland, OR).

Platelet-eosinophil aggregates

To detect platelet-leukocyte aggregates, blood was stained to detect leukocytes, monocytes, eosinophils, neutrophils, and platelets, respectively, anti–CD45-PerCP-Cy5.5, anti–mouse Siglec-F-PE (BD Biosciences PharMingen), anti–CD11b-APC, anti-GR1-PE-Cy7 and anti-GPIX-FITC antibodies. Blood samples were subjected to flow cytometry analysis and data collected on a CYAN flow cytometer.
**Lung vascular permeability**

Pulmonary vascular permeability was evaluated by measurement of Evans blue dye extravasation (EBD, 30 mg/kg body weight, 200 µl) injected intravenously into anesthetized mice 30 min prior to lung removal. The chest was opened, the inferior vena cava transected, and the pulmonary vasculature flushed with 10 ml of saline via the right ventricle to remove excess intravascular dye. The lung lobes were removed, weighed, homogenized, and incubated in 100 % formamide at 37°C for 24 hours to extract EBD. The concentration of dye extracted was analyzed by spectrophotometry. Correction of optical densities ($E$) for contaminating heme pigments was performed using the equation:

$$E_{620}^{\text{corrected}} = E_{620} - (1.426 \times E_{740} + 0.03)$$

Results are presented as µg EBD per g of lung tissue.

**Human antibodies**

Monoclonal mouse antibodies (MAbs) against human antigens were purchased from the following sources: Pacific Blue-conjugated CD45 (HI30), PerCP-Cy5.5-CD45 (HI30), FITC-CD14 (61D3), PE-CD16 (eBioCB160), PE-CD11b (ICRF44), PE-Cy7-CD11b (ICRF44) and appropriate IgG1 and IgG2a controls were from eBiosciences (San Diego, CA). APC-conjugated CD41 and FITC-conjugated CD62P were obtained from BD Biosciences Pharmingen (San Jose, CA).
Supplemental Figure I. Rapid increase in expression of P-selectin on platelet-leukocyte aggregates after activation with convulxin. Blood samples were activated with 50 nM convulxin for 30 s on orbital shaker. To stop reaction the Lyse/Fix buffer was added (BD) and samples were washed. After incubation with antibodies, samples were analyzed by flow cytometry. a) P-selectin exposure on single platelets b) P-selectin exposure on platelet-leukocyte aggregates.
Supplemental Figure II. Platelet-eosinophil aggregates are increased in SCD. A distinctive population of blood CD45+ leukocytes with a high content of granules (SSC High) that is CD11b+ and GR-1 dim (dashed arrow) was confirmed to be ~98% eosinophils based on expression of Siglec-F (CD33rSiglec), a marker present on mature circulating mouse eosinophils. Platelet (CD41+)-eosinophil aggregates are more abundant in sickle (29%) than in WT (9%) mice as shown by whole blood flow cytometry.