P-selectin–deficient mice to study pathophysiology of sickle cell disease

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Key Points

- P-selectin–deficient SCD mice are protected from lung vaso-occlusion.
- P-selectin–deficient SCD mice will be useful in assessing the benefits of anti-P-selectin therapy in diverse complications of SCD.

Introduction

Sickle cell disease (SCD) is an autosomal-recessive genetic disorder that affects millions of people worldwide.1,2 Vaso-occlusion and hemolysis are the 2 predominant vascular events that contribute to the pathogenesis of SCD.3 Vaso-occlusion is believed to trigger acute systemic painful vaso-occlusive episode, which is the primary reason for emergency medical treatment of SCD patients.1,4 A role for P-selectin in promoting vaso-occlusion in the cremaster venules of SCD mice was first demonstrated by Kaul and Hebbel.5 Later, sickle erythrocytes were shown to adhere to P-selectin in vitro and undergo P-selectin–mediated rolling adhesion in postcapillary venules of mice in vivo, and P-selectin deletion or inhibition was shown to prevent adhesion of adoptively transferred sickle erythrocytes and vaso-occlusion in nonsickle mice in vivo.6-9 Also, platelet P-selectin–dependent neutrophil-platelet-erythrocyte heterocellular aggregates were shown to be significantly elevated in SCD patient blood.10 Epidemiological evidence suggests that painful vaso-occlusive episode in transgenic humanized SCD mice triggered precapillary pulmonary arteriole microembolism by platelet-neutrophil aggregates, which led to loss of blood flow in the lung microvasculature.11-13 Recently, we found that vaso-occlusive episode in transgenic humanized SCD mice triggered microembolism of precapillary pulmonary arterioles by platelet-neutrophil aggregates, which led to loss of blood flow in the lung microvasculature.13 Remarkably, platelet-neutrophil aggregates were attenuated, lung vaso-occlusion was prevented, and pulmonary blood flow was rescued in SCD mice following therapeutic blockade of P-selectin.13 A role for P-selectin in vaso-occlusion was further supported by a recent phase 2 study that reported a significant reduction in painful vaso-occlusive episodes among SCD patients receiving the P-selectin–blocking antibody crizanlizumab.14 Altogether, these findings suggest that SCD mice genetically deficient in P-selectin would be protected from vaso-occlusion. Such a mouse would also be useful in identifying the role of P-selectin in SCD-associated morbidities other than painful vaso-occlusive episode or acute chest syndrome.15 A role for P-selectin in systemic vaso-occlusion has been investigated using chimeric SCD mice lacking P-selectin only in the endothelium (intact in platelets)6,16 because SCD mice with global deletion of P-selectin did not exist. Here, we introduce the first SCD mice genetically lacking P-selectin in hematopoietic and nonhematopoietic compartments. Using our recently developed quantitative fluorescence intravital lung microscopy (qFILM)13,17 technique, we show that P-selectin deficiency protects SCD mice from lung vaso-occlusion.

Methods

Reagents

Violet 450 (V450) Rat anti-mouse CD49b monoclonal antibody (mAb; clone DX5) was purchased from BD Biosciences (San Jose, CA). Alexa Fluor 546 (AF546) rat anti-mouse Ly6G mAb (clone 1A8) was purchased from BioLegend (San Diego, CA). FITC-Dextran (molecular weight 70 000) was purchased from Molecular Probes (Eugene, OR). Gram-negative bacterial lipopolysaccharide (LPS) from
**Figure 1. Generation and characterization of SS-Selp\(^{-/-}\) mice.** (A) Selp\(^{-/-}\) mice were bred to Townes SS mice to generate SS-Selp\(^{-/-}\) mice. Refer to supplemental Figure 1 for the breeding strategy. (B) Genomic PCR gel image showing the presence or absence of different alleles in C57BL/6 (WT) mice, Townes AS (AS) mice, Townes SS (SS) mice, Selp\(^{-/-}\) mice, and SS-Selp\(^{-/-}\) mice. Human WT \(\beta\)-globin (WT \(\beta\)), mouse \(\beta\)-globin, mouse \(\alpha\)-globin, and mouse WT P-selectin alleles were absent in SS-Selp\(^{-/-}\) mice, but human \(\beta\), human \(\alpha\)-globin, and mouse mutant P-selectin (P-selectin \(^{-/-}\)) alleles were present in SS-Selp\(^{-/-}\) mice. (C) RT2qPCR analysis revealed a significant reduction in the mRNA levels of P-selectin in the aortas of SS-Selp\(^{-/-}\) mice compared with SS mice. Data are mean relative mRNA expression ± standard deviation (SD). (D) Western blot analysis of CD62P protein levels in platelets isolated from SS and SS-Selp\(^{-/-}\) mice. P-selectin (135 kDa) was expressed in platelets of SS mice but was absent in platelets of SS-Selp\(^{-/-}\) mice. Actin (37 kDa) was used as a loading control. Recombinant mouse P-selectin–Fc fusion protein (RP; 235 kDa; R&D Systems) was used as a positive control. Data in panels C-D are representative of 3 female SS mice and 3 female SS-Selp\(^{-/-}\) mice. Representative IHC images showing sinusoidal congestion (E) and % sirius red positive area (F) in SS and SS-Selp\(^{-/-}\) mice.
Table 1. Comparison of hematological parameters among SS, SS-Selp<sup>−/−</sup>, and Selp<sup>−/−</sup> mice

| Parameter          | Normal range | SS, mean ± SD | SS-Selp<sup>−/−</sup>, mean ± SD | Selp<sup>−/−</sup>, mean ± SD | P       |
|--------------------|--------------|---------------|---------------------------------|-------------------------------|---------|
| WBCs, ×10<sup>9</sup>/L | 1.8-10.7     | 29.4 ± 13     | 46.9 ± 5                       | 4.5 ± 1                       | .01<sup>†</sup>; <.011 |
| Neutrophils, ×10<sup>9</sup>/L | 0.1-2.4   | 6.7 ± 4       | 23.3 ± 7                       | .08 ± 0.7                     | .01<sup>†</sup>; <.011 |
| Lymphocytes, ×10<sup>9</sup>/L | 0.9-9.3   | 21.3 ± 8      | 19.8 ± 4                       | 3.3 ± 1                       | .70; <.01 |
| Monocytes, ×10<sup>9</sup>/L | 0.0-0.4   | 1.3 ± 1       | 3.2 ± 1                       | 0.4 ± 0.1                     | .04<sup>†</sup>; .02† |
| Platelets, ×10<sup>12</sup>/L | 592-2972 | 306.3 ± 81    | 458.8 ± 104                    | 888.5 ± 140                   | .05; <.01 |
| Hemoglobin, g/dL  | 11.0-15.1   | 6.9 ± 1       | 7.3 ± 1                       | 11.6 ± 0.4                    | .61; <.01 |
| Hematocrit, %     | 35.1-45.4   | 26.9 ± 5      | 25.8 ± 5                       | 46.7 ± 2                      | .72; <.01 |

Data are reported for 4 male and 3 female SS mice, 4 female SS-Selp<sup>−/−</sup> mice, and 2 male and 2 female Selp<sup>−/−</sup> mice. WBCs, white blood cells.

*Significant (P < .05) difference between SS mice and SS-Selp<sup>−/−</sup> mice, Student t test with unequal variances.

†Significant (P < .05) difference between SS-Selp<sup>−/−</sup> mice and Selp<sup>−/−</sup> mice, Student t test with unequal variances.

Escherichia coli 0111:B4 was from Sigma-Aldrich (St. Louis, MO). Recombinant murine P-selectin (CD62P) Fc chimera was purchased from R&D Systems (Minneapolis, MN). DC Protein Assay Reagent A, B, and S were purchased from Bio-Rad (Hercules, CA). Bolt LDS sample buffer (4X), Bolt MES SDS Running Buffer (20X), Bolt transfer buffer (20X), Bolt 4% to 12% Bis-Tris plus MOPS (20X), Bolt 4% to 12% Bis-Tris plus MOPS Running Buffer (20X), Bolt LDS sample buffer (4X), Bolt 4% to 12% Bis-Tris plus MOPS Running Buffer (20X), Bolt M-Per Mammalian Protein Extraction Reagent, and SuperSignal West Pico Chemiluminescent Substrate were purchased from Thermo Fisher Scientific (Rockford, IL).

Generation of P-selectin–deficient SCD mice

Male and female (~12- to 16-week-old) Townes SCD mice [SS mice; homozygous for Hba<sup>tm1[HBA]Tow</sup>, homozygous for Hbb<sup>tm2[HBG1,HBB*]Tow</sup>] and nonsickle control mice [AS mice; homozygous for Hba<sup>tm1[HBA]Tow</sup>, compound heterozygous for Hba<sup>tm1[HBA]Tow</sup> and Hbb<sup>tm2[HBG1,HBB*]Tow</sup>, compound heterozygous for Hba<sup>tm1[HBA]Tow</sup> and Hbb<sup>tm2[HBG1,HBB*]Tow</sup>, compound heterozygous for Hba<sup>tm1[HBA]Tow</sup> and Hbb<sup>tm2[HBG1,HBB*]Tow</sup>, compound heterozygous for Hba<sup>tm1[HBA]Tow</sup> and Hbb<sup>tm2[HBG1,HBB*]Tow</sup>, compound heterozygous for Hba<sup>tm1[HBA]Tow</sup> and Hbb<sup>tm2[HBG1,HBB*]Tow</sup> and Hbb<sup>tm2[HBG1,HBB*]Tow</sup> and Hbb<sup>tm2[HBG1,HBB*]Tow</sup>] were used in this study. Townes SS mice have human α-sickle and β-sickle globin (β<sup>s</sup>) genes knocked into the locus where mouse α and β genes were knocked out. Townes AS mice are sickle trait mice and, thus, do not develop SCD. Townes SS and AS mice have been used previously as SCD and control nonsickle mice, respectively.

Townes SS mice were bred and genotyped in-house. Breeding pairs of P-selectin–deficient (Sel<sup>p−/−</sup>) mice (B6.129S7-Selp<sup>tm1Bay/J</sup>; stock number 002289) were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house.21 Townes SS mice were bred to Selp<sup>−/−</sup> mice to generate P-selectin–deficient SS (SS-Selp<sup>−/−</sup>) mice using the breeding strategy described in supplemental Figure 1. Mice used in experiments were 12 to 16 weeks old and between the second and sixth generation. Mice were euthanized as per the guidelines of the American Veterinary Medical Association and the Department of Laboratory Animal Research at the University of Pittsburgh. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited pathogen-free animal facility at the University of Pittsburgh. The study was approved by Animal Care and Use Committee of the University of Pittsburgh.

Genotyping of SS-Selp<sup>−/−</sup> mice

DNA was isolated from crude mouse tail lysate, and polymerase chain reactions (PCR) were conducted on a thermocycler (Applied Biosystems, Foster City, CA) using a combination of protocols provided by The Jackson Laboratory for genotyping Townes SS and Selp<sup>−/−</sup> mice. The PCR products were used in 1.5% agarose gel electrophoresis, followed by visualization under UV light to determine the amplicon size. The following primers were used.

α-chain. The following primers were used for α-chain: human α reverse (5′-TCC TGC AGG GTG AGG AAG GAA GG-3′); mouse α reverse (5′-CCC CAAG GGC ACT CCA GGA AAG ACT-3′); common (5′-TCT ATC GAT CAC ATG TAG GAC TAG-3′).

β-chain. The following primers were used for β-chain: human β<sup>A</sup> reverse (5′-GTT TAG CCA GGA ACC GTT TCA G-3′); human β<sup>S</sup> reverse (5′-AAT TCT GGG TTA TCG GCA AG-3′); mouse β reverse (5′-ATG TCA GAA GCA AAT GTG AGG AGC A-3′); common (5′-TGG AGT AAC GAT GAC TAG GAA GG-3′).

P-selectin. The following primers were used for P-selectin: mouse Selp<sup>−/−</sup> forward (5′-CTG AAT GAA CGT CAG GAC GA-3′); mouse Selp<sup>−/−</sup> reverse (5′-ATA CT TCT CGG CAG GAG CA-3′); mouse wild type Selp forward (5′-TTG GAA ATC AGA AGG AAG TGG-3′); mouse wild type Selp reverse (5′-AGA GTT ACT CTT GAT GTA GAT CTC C-3′).

Real-time reverse-transcriptase quantitative PCR for P-selectin

Aortas were isolated from 3 SS-Selp<sup>−/−</sup> mice and 3 SS mice. Total RNA isolation was performed using Invitrogen TRizol Reagent (Invitrogen, Carlsbad, CA), as per the vendor’s instructions. Reverse transcription was performed to synthesize complementary DNA using a High-Capacity cDNA Reserve Transcription Kit (Applied Biosystems). Real-time amplification of complementary DNA was
Figure 2. Genetic deletion of P-selectin attenuates pulmonary vaso-occlusion in SCD mice. (A) SS and SS-Selp$^{-/-}$ mice were challenged IV with 0.1 μg/kg LPS, and qFILM was used 2 to 2.5 hours later to visualize the pulmonary microcirculation. (B) IV LPS (0.1 μg/kg) triggered occlusion of pulmonary arteriolar bottlenecks (large dotted ovals) in SS mice by large aggregates of neutrophils (red) and platelets (green). The same FOV is shown over 3 time points. A neutrophil (red; small dashed circles) flows toward the occlusion but cannot pass through it and is forced to flow toward another open vessel to the side of the occlusion. Supplemental Video 1 shows the complete time series for the FOV in panel B. (C) In contrast to SS mice, the majority of FOVs in SS-Selp$^{-/-}$ mice were free of pulmonary vaso-occlusions. The same FOV is shown over 3 time points. A neutrophil (red; small dashed circles) is seen trafficking up the pulmonary arteriole that has no aggregates present. Supplemental Video 2 shows the complete time series for the FOV in panel C. The pulmonary microcirculation was labeled with FITC-Dextran and pseudo-colored purple. Neutrophils and platelets were labeled by IV administration of AF546-conjugated anti-Ly6G mAb and V450-conjugated anti-CD49b mAb, respectively. Neutrophils are shown in red, and platelets are pseudo-colored green. The arrows denote the direction of blood flow, and the asterisks (*) denote alveoli. Scale bars, 20 μm. The diameters of the vessels are 34 μm in panels B-C, respectively. (D-F) The neutrophil-platelet aggregates blocking pulmonary arterioles were quantified as described in Methods. After IV LPS administration, SS-Selp$^{-/-}$ mice had a significantly decreased average number of pulmonary vaso-occlusions per FOV (D), percentage of FOVs with pulmonary vaso-occlusions (E), and large pulmonary vaso-occlusions (area > 1000 μm$^2$) (F) compared with SS mice. The average number of pulmonary vaso-occlusions per FOV and large pulmonary vaso-occlusions...
conducted using PowerUp SYBR Green Master Mix on a StepOnePlus Real-Time PCR system (Applied Biosystems). β-Actin was used as a housekeeping gene. The following primers were used: mouse P-selectin forward primer, 5'-TCCAGGAAGCTCTGACCTGCTTG-3'; mouse P-selectin reverse primer, 5'-GCAGCGATTAGAAGACTCCGTAT-3'; β-actin forward primer, 5'-AGGGCCAGTCTACATCTAC-3'; and β-actin reverse primer, 5'-AGGAGGGCTGGAGGAGGCC-3'.

Western blot analysis for P-selectin

Blood was drawn from the inferior vena cava of SS and SS-Selp−/− mice into trisodium citrate and supplemented with an equal volume of washing buffer (10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM glucose, and 1 mM pyruvate; pH 7.4) and centrifuged at 100g (22°C) for 10 minutes. The supernatant was treated with 0.5 µM prostaglandin I2 and centrifuged at 1100g (22°C) for 15 minutes. The platelet pellet was resuspended in washing buffer containing 0.5 µM prostaglandin I2 and centrifuged at 1100g (22°C) for 15 minutes. The platelet pellet was resuspended in ice-cold radioimmunoprecipitation assay buffer, supplemented with protease and phosphatase inhibitor cocktail, incubated for 5 minutes on ice, and centrifuged at 3000g (4°C) for 15 minutes to remove cell debris. Lysate (supernatant) was snap-frozen in liquid nitrogen and stored at −80°C. Protein concentrations in platelet lysates were measured using a Bio-Rad DC Protein Assay. Total protein (50 µg) was separated and blotted using a Mini Gel System and Mini Blot Module (both from Invitrogen), as described elsewhere.22

Histology and IHC

Immunohistochemistry (IHC) of paraffin-embedded liver tissue sections was performed as described elsewhere.23,24 Tissue sections (4 µm) were stained with hematoxylin and eosin (H&E) and Sirius Red. An Olympus Provis microscope was used to capture images, and Nikon NIS-Elements software was used for image analysis. Quantification of Sirius Red staining was done using ImageJ software.

qFILM

Recently, qFILM has been used by our group to study platelet-neutrophil aggregate-mediated pulmonary vaso-occlusion in transgenic humanized SCD mice.13 In the current study, qFILM was used to assess pulmonary vaso-occlusion in SS and SS-Selp−/− mice following IV challenge with saline or LPS. The qFILM experimental setup has been described in detail previously.13,17,22 Briefly, SS and SS-Selp−/− mice were injected IV with saline or 0.1 µg/kg LPS via the tail vein. Approximately 2 to 2.5 hours later, mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine HCl (Henry Schein Animal Health, Dublin, OH) and 20 mg/kg xylazine (LOYD Laboratories, Shenandoah, IA). A cannula was inserted into the right carotid artery, and a tracheotomy was performed to facilitate mechanical ventilation with 95% O2 and supply maintenance anesthesia (1%-2% isoflurane). The left lung was surgically exposed, and a small portion of the lung was immobilized against a coverslip using a vacuum enabled micro-machined device, as described elsewhere.13,17,22

Statistical analysis

Means were compared between groups using the unpaired Student t test. Percentages were compared using fourfold table analysis with χ2 statistics.25,26 Unless otherwise stated, error bars represent standard error (SE). P < .05 was considered significant.

Results and discussion

Townes SS male mice were bred to Selp−/− female mice (Figure 1A), using the breeding steps described in supplemental Figure 1, to generate SS-Selp−/− mice. Identical to SS mice, SS-Selp−/− mice lacked murine α- and β-globin genes but expressed human α and β5 globin genes (representative gel shown in Figure 1B). SS-Selp−/− mice also lacked the P-selectin gene expressed in WT mice, but they expressed the mutant P-selectin gene expressed in Selp−/− mice (Figure 1B). P-selectin is stored preformed in Weibel-Palade bodies and α-granules of endothelial cells and platelets, respectively.27,29 Additionally, P-selectin is known to be transcriptionally upregulated in inflamed endothelial cells30,31 and chronically expressed on endothelium in SCD mice in vivo.32 Therefore, P-selectin protein levels were detected in isolated mouse platelets, whereas P-selectin messenger RNA (mRNA) levels were assessed in harvested mouse aortas. Real-time reverse-transcriptase quantitative PCR (RT2qPCR) of aortic
tissue revealed significantly reduced P-selectin mRNA levels in aortas of SS-\textit{Selp}^{−/−} mice compared with SS mice (Figure 1C). Western blots confirmed that P-selectin protein was absent in platelets isolated from SS-\textit{Selp}^{−/−} mice but was present in normal SS mice platelets (Figure 1D). Because SS-\textit{Selp}^{−/−} females were unable to breed, the mice colony was maintained by breeding SS-\textit{Selp}^{−/−} males to littermate female mice heterozygous for murine β and human β\textsuperscript{S} globin genes. P-selectin deficiency also led to significantly elevated counts of circulating neutrophils and monocytes in SS-\textit{Selp}^{−/−} mice compared with SS mice (Table 1), most likely as a result of the lack of P-selectin–dependent rolling along the vascular endothelium, leading to impaired recruitment of these cells to sites of inflammation or bone marrow. However, the hemoglobin and hematocrit values in SS-\textit{Selp}^{−/−} mice were comparable to those in SS mice; these values were below the normal range, suggestive of hemolytic anemia (Table 1). Next, the histology of isolated liver sections was assessed using the approach described previously\textsuperscript{23,24} H&E staining (Figure 1E; normal range, suggestive of hemolytic anemia (Table 1). Next, blood-brain barrier permeability in SCD mice.\textsuperscript{38} Regardless of the difference in P-selectin expression between murine and human endothelial cells, SS-\textit{Selp}^{−/−} mice would be useful in future studies to assess the role of P-selectin and the benefits of anti–P-selectin therapy in diverse pathologies of SCD.

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### Authorship
Contribution: M.F.B. performed qFILM studies and analyses; E.T. generated SS-\textit{Selp}^{−/−} mice and performed genotyping and western blots; S.G. contributed to mice genotyping; T.B. isolated platelets from mice; E.T. and T.P.-S. performed RTqPCR experiments; S.C.W. was involved in qFILM studies; T.P.-S. and S.P.M. performed histology and IHC of liver sections; and P.S. designed the experiment, supervised the project, and wrote the manuscript with contributions from all coauthors.

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