Thrombospondin From Activated Platelets Promotes Sickle Erythrocyte Adherence to Human Microvascular Endothelium Under Physiologic Flow: A Potential Role for Platelet Activation in Sickle Cell Vaso-Occlusion

Adherence of erythrocytes to vascular endothelium likely contributes to the pathophysiology of episodic vascular occlusion in patients with sickle cell disease (SCD). In addition, coagulation activation has been reported in sickle patients during complications such as pain episodes. To test the hypothesis that platelet activation contributes to sickle erythrocyte binding, we investigated whether factors released from activated sickle platelets promote adherence of sickle erythrocytes to human microvascular endothelial cells (MEC) under flow conditions. Activated sickle platelet supernatant (APSS) promoted high levels of sickle erythrocyte adherence to MEC (55.4 ± 3.9 erythrocytes/mm²) but only moderate adherence of normal erythrocytes to MEC (14.1 ± 0.7 erythrocytes/mm²). When MEC were incubated with an antibody (OKM5) against CD36 (a thrombospondin [TSP] receptor), platelet supernatant mediated sickle erythrocyte adherence was inhibited 86%, suggesting that TSP participated in the adherence. To further define the role of TSP in adherence, additional studies using purified TSP were performed. At a concentration of 0.2 μg/mL TSP in serum-free media (SFM), sickle erythrocyte adherence to MEC was 33.9 ± 2.7 erythrocytes/mm² and sixfold greater than either sickle erythrocyte adherence in the absence of TSP or normal erythrocyte adherence in the presence of TSP. Doubling the concentration of TSP to 0.4 μg/mL proportionally increased adherence of sickle erythrocytes. Incubation of MEC with OKM5 or anti-α, monoclonal antibodies inhibited TSP-mediated sickle erythrocyte adherence more than 95%. These data suggest that activated platelet release factors, including α-granule TSP, which promote receptor-mediated sickle erythrocyte adherence to microvascular endothelium. Such factors released during in vivo platelet activation could contribute to vaso-occlusive complications by promoting erythrocyte adherence and microvascular occlusion.

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according to manufacturers' instructions. Human AB serum, endothelial cell basal media (MCDB 131), and endothelial cell growth factor were purchased from Clonetics Corp (San Diego, CA).

**Patients.** All blood samples were obtained from patients with homozygous sickle cell anemia (HbSS) at the Georgia Sickle Cell Center at Grady Memorial Hospital, Atlanta, during routine follow-up in asymptomatic periods. Phenotype was determined by cellulose acetate and citrate agar electrophoresis. β-Thalassemia was excluded by Coulter indices and quantification of HbA2 and HbF. α Gene number and β haplotypes were not performed. Patients were not receiving anticoagulant therapy and were without evidence of pregnancy, obvious infection, thromboembolic disease, or liver disease. Age-, sex-, and race-matched normal controls were recruited from Georgia Institute of Technology, and Grady Memorial Hospital. Written informed consent was obtained from all participants for collection and transfer of blood. All research was performed according to the principles of the Declaration of Helsinki. Blood was collected by venipuncture into sodium citrate (105 mmol/L, 1.9 vol/vol) or EDTA (400 mmol/L, 1:100 vol/vol) Vacutainer tubes (Becton Dickinson, Rutherford, NJ) from normal volunteers and patients with homozygous (SS) sickle cell disease.

**Endothelial cell cultures.** Human dermal MEC were isolated from neonatal foreskins as previously described. Cells were cultured to confluence in endothelial basal media (MCDB 131) supplemented with 30% (vol/vol) human AB serum; 0.029 mg/mL L-glutamine; 0.1 mg/mL penicillin, streptomycin, and amphotericin; and 0.001 mg/mL epidermal growth factor; 0.292 mg/mL L-glutamine; 0.1 mg/mL penicillin, streptomycin, and amphotericin; 0.2% human albumin; 50 μg/mL human transferrin; and 5 μg/mL bovine insulin) containing 1.6 mmol/L calcium for 37°C in a 5% CO2:95% air incubator. Medium was exchanged under sterile conditions at 48-hour intervals. For adherence assays, endothelial monolayers were subcultured onto single-well LabTek Permanox culture slides (Nunc, Inc, Naperville, IL) pretreated with 0.1% (wt/vol) porcine gelatin. Cells reached confluence within 3 to 4 days and were then used within 3 days. Confluent monolayers up to passage 6 were used in adherence assays.

**Activated sickle platelet supernatant (ASPS).** Erythrocytes were separated from citrated whole blood by centrifugation at 100g for 10 minutes at 25°C. Platelet-rich plasma was separated into 2-mL aliquots until use. Platelets were recovered by centrifugation of platelet-rich plasma at 1,000g for 15 minutes at room temperature. Initially (patients S1 through S9 in Table 1), unwashed platelets were used to minimize handling and inadvertent activation. For these studies, platelets were activated by mixing the pellet with 2 mL serum-free medium (MCDB 131) supplemented with 0.292 mg/mL L-glutamine; 0.1 mg/mL penicillin, streptomycin, and amphotericin; 0.2% human albumin; 50 μg/mL human transferrin; and 5 μg/mL bovine insulin) containing 1.6 mmol/L calcium for 10 minutes at 25°C. AsPS and aggregated platelets were separated by centrifugation at 1,000g for 15 minutes. The supernatant (2 mL) was removed and used immediately in adherence assays. In subsequent experiments (patients S10 through S18 Table 1 and all patients in Figs 1-5), platelets were obtained from EDTA anticoagulated fresh blood and washed according to Dubernard and Legrand to ensure that adherence was not caused by residual plasma factors in the platelet pellet. Briefly, platelets were washed in 10 vol of modified Tyrode’s buffer (0.265 g/L CaCl2 - 2H2O, 0.214 g/L MgCl2 - 6H2O, 0.200 g/L KCl, 8.000 g/L NaCl, 0.050 g/L anhydrous NaH2PO4, 1.000 g/L D-glucose, 1.000 g/L NaHCO3), pH 6.5, containing 3.5 mg/mL human albumin, 25 μg/mL apyrase, and 100 nmol/L prostaglandin E1. Washed platelets were activated for 1 minute with 0.1 U/mL human thrombin (Sigma) in 2 mL of serum-free medium (SFM) in the presence of 0.1 mmol/L Gly-Pro-Arg-Pro, a specific inhibitor of fibrin polymerization. ASPS and aggregated platelets were separated by centrifugation at 1,000g for 15 minutes. The supernatant (2 mL) was removed and used immediately in adherence assays. ASPS produced by either method gave qualitatively similar results.

**Purified TSP.** TSP was purified from thrombin-activated normal human platelets as previously described by Santoro and Frazier.

### Table 1. ASPS Promotes Sickle Erythrocyte Adherence to MEC

<table>
<thead>
<tr>
<th>Patient</th>
<th>SFM</th>
<th>ASPS</th>
<th>PPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>2.1 ± 0.4</td>
<td>18.4 ± 1.6</td>
<td>324 ± 23</td>
</tr>
<tr>
<td>S2</td>
<td>0.7 ± 0.3</td>
<td>29.6 ± 1.7</td>
<td>114 ± 1.4</td>
</tr>
<tr>
<td>S3</td>
<td>3.5 ± 0.6</td>
<td>94.8 ± 8.3</td>
<td>64.7 ± 6.3</td>
</tr>
<tr>
<td>S4</td>
<td>4.3 ± 0.9</td>
<td>82.7 ± 7.2</td>
<td>30.5 ± 5.4</td>
</tr>
<tr>
<td>S5</td>
<td>1.6 ± 0.6</td>
<td>30.7 ± 2.7</td>
<td>771 ± 59</td>
</tr>
<tr>
<td>S6</td>
<td>3.5 ± 1.0</td>
<td>29.2 ± 3.1</td>
<td>48.6 ± 4.7</td>
</tr>
<tr>
<td>S7</td>
<td>4.4 ± 0.7</td>
<td>70.5 ± 6.1</td>
<td>294 ± 17</td>
</tr>
<tr>
<td>S8</td>
<td>4.4 ± 0.7</td>
<td>520 ± 19</td>
<td>183 ± 3.5</td>
</tr>
<tr>
<td>S9</td>
<td>2.1 ± 0.5</td>
<td>170 ± 1.9</td>
<td>244 ± 34</td>
</tr>
<tr>
<td>S10</td>
<td>4.9 ± 0.6</td>
<td>22.3 ± 1.8</td>
<td>84.3 ± 6.2</td>
</tr>
<tr>
<td>S11</td>
<td>0.4 ± 0.2</td>
<td>12.2 ± 1.5</td>
<td>160 ± 10</td>
</tr>
<tr>
<td>S12</td>
<td>3.0 ± 0.6</td>
<td>25.2 ± 2.4</td>
<td>72.5 ± 5.6</td>
</tr>
<tr>
<td>S13</td>
<td>4.4 ± 0.7</td>
<td>47.5 ± 3.4</td>
<td>69 ± 1.4*</td>
</tr>
<tr>
<td>S14</td>
<td>5.6 ± 0.6</td>
<td>76.9 ± 5.2</td>
<td>93 ± 1.3*</td>
</tr>
<tr>
<td>S15</td>
<td>1.8 ± 0.5</td>
<td>164 ± 7.1</td>
<td>41 ± 0.9*</td>
</tr>
<tr>
<td>S16</td>
<td>3.1 ± 0.8</td>
<td>36.9 ± 2.3</td>
<td>40 ± 0.9*</td>
</tr>
<tr>
<td>S17</td>
<td>4.9 ± 0.7</td>
<td>65.7 ± 3.3</td>
<td>34 ± 1.0*</td>
</tr>
<tr>
<td>S18</td>
<td>2.2 ± 0.6</td>
<td>9.3 ± 1.2</td>
<td>10.6 ± 1.8*</td>
</tr>
</tbody>
</table>

Adhesion of washed sickle erythrocytes suspended in SFM, ASPS, or 30% autologous platelet-poor sickle plasma (PPP) in SFM to human MEC was quantified at 1.0 dynes/cm². Sodium citrate anticoagulated blood was used for patients S1 through S9 and EDTA anticoagulated blood was used for patients S10 through S18. Similar data were obtained for citrate and EDTA anticoagulated blood. ASPS > SFM, P < .001 for each experiment. PPP > SFM, P < .001.

* P = not significant.
THROMBOSPONDIN PROMOTES SICKLE CELL ADHERENCE

Briefly, washed platelets were suspended in 20 mM Tris-HCl containing 150 mM NaCl and 100 mM L CaCl2 at pH 7.6, also containing 5 mM glucose per unit of platelets. Cells were activated with 0.5 U/mL thrombin for 1 to 2 minutes at 37°C. Thrombin was inhibited by 2 U/mL hirudin and 2 mM L phenylmethylsulfonyl fluoride (PMSF). Aggregated platelets were removed by centrifugation at 200 g for 5 minutes at 4°C. An additional centrifugation of the supernatant at 20,000 g for 30 minutes ensured removal of all particulate matter. The supernatant was then made 1 mM L in CaCl2. TSP was purified from the supernatant by heparin-Sepharose affinity chromatography (Pharmacia-LKB Biotechnology, Uppsula, Sweden) and eluted stepwise with 250 to 600 mM NaCl. TSP-rich fractions were pooled and applied to a BioGel A-0.5 m column (BioRad Laboratories, Richmond, CA) to remove low molecular weight contaminants. TSP concentrations were determined by the method of Lowry et al. or by its absorbance at 280 nm.

RBC suspensions. Fresh erythrocytes separated from plasma were washed three times in Delbecco’s phosphate-buffered saline (DPBS; pH 7.4). 100 mM MgCl2, 6-H2O, 0.200 mM KCl, 0.200 mM L anhydrous K2HPO4, 8.000 mM L NaCl, 1.150 mM L anhydrous NaH2PO4, Sigma, St Louis, MO) without calcium chloride supplemented with 0.2% (w/vol) human albumin, 5.0 µg/mL human transferrin, and 5.0 µg/mL bovine insulin. For adherence assays, washed erythrocytes were suspended to 1% hematocrit in SFM ASPS, SFM containing 30% autologous plasma, SFM containing 0.2 µg/mL TSP, SFM containing 0.4 µg/mL TSP, SFM containing 0.1 U/mL thrombin, or SFM containing 1.0 U/mL thrombin.

Erythrocyte adherence assay. Confluent MEC monolayers were incubated with 5 µg/mL anti-CD36 monoclonal antibody (MoAb) OKM5 (generously donated by Dr Patricia Rao, Ortho Diagnostic, Raritan, NJ), 10 µg/mL monoclonal anti-a, antibody 3F12, which has affinity for the a chain of the a5b3 vitronectin receptor; or 50 µg/mL of the peptide Arg-Gly-Asp-Ser in endothelial cell culture media for 1 hour at 37°C before an adherence assay. Antimajor histocompatibility complex (MHC) Class I antibody (W6/32) at 10 µg/mL was used as a negative control antibody.

Statistical methods. The mean ± SEM adherent erythrocytes/mm² was calculated for individual experiments and a combined mean ± SEM was determined using a pooled variance as an estimate of per field of view) ranging over the entire endothelial cell monolayer and results were normalized to adherent erythrocytes per square millimeter of endothelium (erythrocytes/mm²).

In an attempt to inhibit sickle erythrocyte adherence, MEC monolayers were incubated with 5 µg/mL anti-CD36 monoclonal antibody (MoAb) OKM5 (generously donated by Dr Patricia Rao, Ortho Diagnostic, Raritan, NJ), 10 µg/mL monoclonal anti-a, antibody 3F12, which has affinity for the a chain of the a5b3 vitronectin receptor, or 50 µg/mL of the peptide Arg-Gly-Asp-Ser in endothelial cell culture media for 1 hour at 37°C before an adherence assay. Antimajor histocompatibility complex (MHC) Class I antibody (W6/32) at 10 µg/mL was used as a negative control antibody.

Statistical methods. The mean ± SEM adherent erythrocytes/mm² was calculated for individual experiments and a combined mean ± SEM was determined using a pooled variance as an estimate of
the population variance for each set of experiments. A single-classification analysis of variance using a fixed-effects model was used to distinguish between treatment effects and random error within individual experiments. Nonparametric methods were used to compare population results because sickle adherence values are not normally distributed using the chi-squared goodness of fit test. To compare population results from paired observations, the Wilcoxon two-sample test was used.

RESULTS

At a shear stress of 1.0 dyne/cm², ASPS-mediated adherence of sickle erythrocytes to microvascular endothelium ranged from 9.3 ± 1.2 to 520 ± 19 erythrocytes/mm² (mean ± SEM) for 18 sickle patients studied (Table 1). This was significantly greater (P < .001 for each experiment) than the adherence recorded when sickle cells were suspended in SFM alone (0.4 ± 0.2 to 5.5 ± 0.6 erythrocytes/mm²). For the same patients, adherence of sickle erythrocytes suspended in 30% autologous plasma ranged from 3.4 ± 1.0 to 771 ± 59 erythrocytes/mm² (Table 1). Similar results were obtained for citrate and EDTA anticoagulated blood. Adherence in plasma did not correlate with adherence in ASPS. ASPS promoted fourfold higher adherence of sickle erythrocytes (23.1 ± 1.9 to 147 ± 6.7 erythrocytes/mm²) as compared with the moderate levels of adherence (6.9 ± 1.0 to 20.2 ± 1.4 erythrocytes/mm²) observed with normal erythrocytes from antigen-matched donors (Fig 1).

Experiments were also performed to determine whether the thrombin (used to activate platelets) present in ASPS contributed to sickle cell adherence. Adherence of sickle cells suspended in SFM containing 0.1 U/mL human thrombin (equivalent to the thrombin concentration in ASPS) was 2.9 ± 0.3 erythrocytes/mm² and was not significantly different than adherence of sickle erythrocytes suspended in SFM alone (3.3 ± 0.3 erythrocytes/mm²) for six sickle patients. In two experiments testing the effect of thrombin alone at 1.0 U/mL (a thrombin concentration 10 times that present in ASPS) adherence was 1.5 ± 2.5 and 2.4 ± 2.5 erythrocytes/mm² (mean ± SD), respectively, in SFM and 10.6 ± 5.9 and 1.6 ± 1.8 erythrocytes/mm², respectively, in SFM containing 1.0 U/mL thrombin. Thus, even in the presence of 1.0 U/mL thrombin, the sickle erythrocyte adherence was not significantly elevated as compared with adherence in SFM. More importantly, the adherence recorded in SFM containing thrombin alone was much less than that observed when sickle erythrocytes were suspended in ASPS.

Preincubation of microvascular endothelium with monoclonal anti-CD36 (a TSP receptor) antibody (OKM5) inhibited ASPS-mediated sickle erythrocyte adherence by 86% (Fig 2). Anti-MHC Class I antibody (W6/32) did not inhibit ASPS-mediated sickle erythrocyte adherence (data not shown). These observations suggest that TSP released from platelet α-granules may be involved in sickle cell adherence to microvascular endothelium.

To directly test the role of TSP in promoting adherence of sickle erythrocytes to vascular endothelium, we examined the ability of purified TSP to promote sickle erythrocyte adherence to MEC. As shown in Fig 3, addition of 0.2 μg/mL TSP to SFM significantly elevated sickle erythrocyte adherence to MEC. In these experiments, adherence ranged from 8.1 ± 0.8 to 83.9 ± 6.9 erythrocytes/mm² and averaged 33.9 ± 2.7 erythrocytes/mm². TSP at 0.2 μg/mL also promoted moderate levels of normal erythrocyte adherence (1.2 ± 0.5 to 9.4 ± 1.2 erythrocytes/mm²) (Fig 3), which was significant for only 4 (P < .05) of the 6 normal donors studied. When the TSP concentration was doubled from 0.2 to 0.4 μg/mL, a corresponding increase in adherence was observed for the four patients studied (Fig 4). Preincubation of MEC with either monoclonal anti-CD36 antibody (OKM5) or monoclonal anti-α, antibody (3F12) inhibited TSP-mediated adherence (at 0.2 μg/mL) of sickle erythrocytes by 99% (range: 89% to 100%) and 96% (range: 87% to 100%), respectively (Fig 5). Similarly, preincubation of microvascular endothelium with the tetrapeptide Arg-Gly-Asp-Ser inhibited 97% of the TSP-mediated adherence for two patients studied (data...
 attachment of sickle erythrocytes to the vascular endothelium, temper in sickle patients during asymptomatic periods and vaso-

sickling.
delay erythrocyte capillary transit allowing for significant sickling.
microvascular endothelium at 200 ng/mL TSP and this increases proportionately as the TSP concentration is increased to 400 ng/mL (Fig 4). Studies of TSP levels in plasma and amounts released in ASPS from samples obtained during pain episodes and steady-state may provide further insights into the relationship of TSP to complications; however, local levels of TSP in the microvasculature may be the more important determinants of complications.

Our previous data indicate that a significant fraction of autologous plasma-mediated sickle cell adhesion can be attributed to differences in patient RBCs. These differences, although not defined, appear to be related to the variability in erythrocyte binding of adherence factors. It is possible that in the present study, RBCs from different patients bind variable amounts of TSP and thus adhere to endothelium to a varying degree. In addition, it is likely that endothelial cells in culture may have variable expression of receptors that may contribute to variability in erythrocyte binding between experiments. Further studies will be necessary to define the contribution of erythrocyte, platelet, plasma, endothelial cell, and possibly leukocyte factors in the variation in binding between individuals over time. Once these are well characterized, careful studies of variation during complications may help define the role and mechanisms of endothelial adherence in complications.

The interaction between CD36 and TSP is somewhat controversial. In some reports, TSP binding to the CD36 receptor is observed, and the MoAb OKM5 abolishes the binding of TSP to the surface of stimulated platelets. Other data indicate that OKM5 may not bind TSP. More recent studies with purified human TSP and CD36 have shown that these proteins bind to each other. The data of Lawler and Hynes, on the other hand, indicate that under some conditions the vitronectin receptor (a, b3 integrin) and not CD36, functions as the TSP receptor. One possible explanation of some of these observations is that CD36 and a, b3 are closely associated on the endothelial cell surface and antibody binding to one receptor causes steric inhibition of binding to the other. The present data do not directly address which receptor binds TSP. However, our data clearly indicate that incubation of endothelial cells with an antibody against either of the proposed TSP receptors quantitatively inhibits sickle erythrocyte binding mediated by TSP and ASPS. Furthermore, incubation with an antibody to an epitope not thought to be involved in adhesion (anti-MHC Class I) does not inhibit ASPS or TSP-mediated sickle RBC adhesion.

The mechanism of TSP enhancement of sickle erythrocyte binding to MEC is uncertain. TSP may serve as a molecular bridge between receptors on the endothelial and erythrocyte membranes. Alternatively, TSP binding to the endothelial cell could induce conformational changes in the cell surface receptors that potentiate membrane interactions between sickle erythrocytes and endothelial cells. CD36 is known to be expressed early in erythroid development and has recently been identified on normal erythrocytes. Although
the level of CD36 expression on normal RBCs is low (approximately 50 surface molecules per RBC), it is sufficient to play a role in biologically important cell-cell adhesion events such as the CD36-dependent rosetting of normal erythrocytes around Plasmodium falciparum-infected erythrocytes. The higher levels of platelet supernatant and TSP-mediated adhesion of sickle erythrocytes over normal erythrocytes observed here could reflect higher CD36 expression on the generally younger erythrocytes in sickle blood as well as conformational changes in CD36 on sickle cells elicited by membrane damage.

The present data suggest a role for acute-phase reactants in sickle cell pain episodes. Possibly, platelet activation promotes sickle erythrocyte adherence and vaso-occlusion. This hypothesis would suggest that aspirin or antiplatelet therapy should ameliorate or modulate the frequency and/or severity of pain episodes. Several investigators have reported that use of prophylactic anticoagulant therapy in SCD was associated with clinical improvement, although in some studies the benefits appeared minimal. However, these data do not rule out a role for platelet activation in pain events because the small number of patients studied, the relative infrequency of pain events in the study populations, and the low compliance were all cited as factors that made demonstration of an intervention effect difficult. In light of the present data that define a potential role for platelet activation in vaso-occlusion via erythrocyte adherence, additional, larger prospective studies may show the efficacy of anticoagulant therapy in eliminating or reducing sickle cell vaso-occlusive episodes.

In summary, the present data suggest that activated platelets release factors, including α-granule TSP, which promote receptor-mediated sickle erythrocyte adherence to microvascular endothelium. Such factors released during platelet activation could contribute to vaso-occlusive complications by promoting erythrocyte adherence and microvascular occlusion. Clearly, additional studies will further ascertain the role of intravascular coagulation and vaso-occlusion via this mechanism. These studies should include investigations of receptors on sickle erythrocytes, modulation of binding to microvascular endothelium, and evaluation of TSP levels in patients during vaso-occlusive complications and asymptomatic periods. Such studies will further elucidate the role of intravascular coagulation in the initiation of vascular occlusion in SCD and lead to potential therapies targeting this mechanism.

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Thrombospondin from activated platelets promotes sickle erythrocyte adherence to human microvascular endothelium under physiologic flow: a potential role for platelet activation in sickle cell vaso-occlusion

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