Red blood cell (RBC) adhesion to the vascular endothelium is increased in several pathologic conditions, including sickle cell disease and malaria. However, RBC interactions with components of the subendothelial matrix are not well-characterized. Under in vitro flow conditions of 1 dyne/cm², washed RBCs bound to the purified adhesive molecules thrombospondin (TSP) and laminin. Sickle RBCs had the greatest adhesion of all tested RBCs. The adhesion of sickle RBCs to immobilized TSP was inhibited by the anionic polysaccharides high molecular weight (MW) dextran sulfate and chondroitin sulfate A, but not other anionic polysaccharides of similar structure and/or charge density. These data were consistent with the RBC adhesive molecule being a sulfated glycolipid. Therefore, TSP-binding lipids from normal and sickle RBCs were isolated and characterized. The TSP-binding lipid was purified by alkaline methanolysis, anion exchange chromatography, and preparative thin layer chromatography (TLC). A homogeneous band on TLC was identified using a specific overlay TSP-binding assay. TSP binding to the purified lipid was stable to base and neuraminidase treatment, labile to acid treatment, and was inhibited by high MW dextran sulfate, similar to that seen with intact RBCs binding to immobilized TSP under conditions of flow. In addition, soluble laminin bound to the purified RBC lipid. This acidic TSP- and laminin-binding lipid(s) isolated from both sickle and normal RBC membranes may contribute to erythrocyte interactions with the subendothelial matrix, thereby participating in the pathogenesis of vaso-occlusive disease.

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From the Departments of Pediatrics and Pathology, Medical College of Wisconsin, Milwaukee; Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee.

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Address reprint requests to Cheryl A. Hillery, MD, Blood Research Institute, The Blood Center of Southeastern Wisconsin, PO Box 2178, Milwaukee, WI 53201-2178.

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sought to identify specific purified extracellular matrix adhe-
sive proteins that preferentially bind RBCs under conditions of
controlled flow. Because the enhanced binding of RBCs
to the vascular endothelium has been described in sickle cell
disease, sickle RBCs served as a pathologic model in this
investigation. Both normal and sickle RBCs were found to
to have increased adhesion to the purified extracellular matrix
proteins TSP and laminin. In addition, the adhesion of RBCs
to TSP and laminin was inhibited by anionic polysaccha-
drides. Because sulfated glycolipids are known to bind to
TSP and laminin, and this interaction is inhibited by anionic
polysaccharides, we hypothesized that a RBC membrane lip-
id(s) contributes to the adhesive properties of RBCs.
Therefore, we isolated and characterized an acidic lipid(s)
from normal and sickle erythrocytes that binds the adhesive pro-
teins TSP and laminin.

MATERIALS AND METHODS

Reagents. Chondroitin sulfates A and C were purchased from
Calbiochem (San Diego, CA). Heparan sulfate (Bovine intestinal
mucosa), keratan sulfate (Bovine cornea), dermatan sulfate (Porcine
skin), dextran sulfates (average MW 500 kD and MW 5 kD), fu-
coidan (Fucus vesiculosus), gangliosides (Type II, Bovine brain,
15% N-acetyl-neuraminic acid), cholesterol 3-sulfate, M199 tissue
culture medium, azide A, and neuraminidase were obtained from
Sigma (St Louis, MO). Sepharose 4B, heparin-Sepharose CL-6B, and
DEAE-Sepharose 6B were obtained from Pharmacia (Piscataway,
NJ). Human laminin, human fibronectin, human vitronectin, anti-
human laminin monoclonal antibody (MoAb) 4E10 and the en-
terchitoning system (GIBCO).

Immune complexes were detected using the ELISA amplification
system. Both normal and sickle RBCs were found to
inhibit a 5 to 10 minute rinse period, the number of adherent RBCs per
unit surface area were counted by direct microscopic visualization
of the adhesive surface in a previously calibrated grid of known area
in 4 to 6 random areas near the center of the flow surface. For
inhibition experiments, the inhibitor was added to the red cell sus-
pension for 30 minutes at 37°C before the initiation of the flow
experiment.

Lipid preparation. Washed RBC ghosts (100 to 200 mL) were
prepared by hypotonic lysis (7.5 mmol/L NaH2PO4, 1 mmol/L
EDTA, pH 7.5) of washed RBCs as previously described.24 Contami-
nating leukocytes and platelets are resistant to lysis and were
removed by discarding the small thick white pellet that is underneath
the fluffy red cell ghosts after each centrifugation. Washed RBC
ghosts were extracted (Ghosts: H2O:MeOH:CHCl3, 1:3:10.5, vol/
vol/vol/vol) for 30 minutes at 22°C with stirring.25 After drying in
a rotary evaporator, the crude lipid extract was treated with alkaline
methanolysis (0.2 mol/L NaOH in MeOH, 37°C, 30 minutes) to
degrade ester linked phospholipids, then neutralized with 0.9 volume
of 0.2 mol/L HCl in MeOH and dried to a syrup.26 The extract was
desalted by dialysis against distilled H2O, lyophilized, and resus-
pended in CHCl3:MeOH:H2O (30:60:8). The extract was then frac-
tioned by anion exchange chromatography on a DEAE-Sepharose
6B column equilibrated with CHCl3:MeOH:H2O (30:60:8). Neutral
lipids, sphingomyelin, and cholesterol do not bind to the column
and are recovered in the flow through. The acidic lipids were eluted
with stepwise increasing concentrations of NH4HCO3 (0.01 mol/L
to 0.5 mol/L) in MeOH. Lipids were resolved by thin layer chroma-
tography (TLC) on HPTLC aluminum silica gel 60 sheets (E. Merck)
with CHCl3:MeOH:2% CaC12 (60:35:7). Following air drying, the
plate was sprayed with phosphate buffered saline containing 1% BSA and
incubated at 4°C overnight. After blocking again with Binding buffer,
bound protein was quantitated

Overlay binding assay of TSP to lipids separated by TLC. Lipid
was applied to HPTLC aluminum silica gel 60 sheets and resolved
with CHCl3:MeOH:2% CaCl2 (35:7). Following air drying, the
plates were treated with 0.015% methacrylate in hexane to minimize
lipid diffusion. After evaporation of the solvent, the plate
was sprayed with phosphate buffered saline (PBS), blocked with
Binding buffer for 45 minutes at 22°C, and immersed in a solution
of TSP in Binding buffer (0.5 µg/mL) at 4°C overnight. After wash-
ing with PBS, bound TSP was detected using anti-TSP MoAb C6.7

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ERYTHROCYTE ADHESION TO THROMBOSPONDIN AND LAMININ

Proteins. RBCs, 10 for AA-RBCs, and 4 for Hi Retic) or laminin (LM, N = 20 for RBCs, healthy controls (AA-RBCs) and patients with high reticulocyte counts (25 to 80% reticulocytes), did not show an increased adhesion to thrombospondin or laminin compared to AA-RBCs. In addition, SS-RBCs to the adhesive ligands TSP and laminin was more than 75% of SS-RBC adhesion to immobilized TSP. However, chondroitin sulfate C, which differs from chondroitin sulfate A only in the position of sulfation of N-acetyl-

The glycosaminoglycans keratan sulfate and heparan sulfate, which possess charge densities similar to the chondroitin sulfates,37 caused only mild to moderate inhibition. In addition, fucoidan and heparin, which have higher charge densities than the chondroitin sulfates,37 were only moderately inhibitory, even at high concentrations. This suggests that the inhibition is not solely caused by charge interactions. When the anionic polysaccharide, dextran sulfate was added to the sickle RBCs, the HMW form (MW 500 kD) caused followed by horseradish peroxidase conjugated rabbit antimouse IgG. Bound TSP was visualized by chemiluminescence using the ECL detection system (Amersham, Arlington Heights, IL).

RESULTS

Adhesion of erythrocytes to purified extracellular matrix proteins. To determine specific adhesive proteins that bind RBCs, the normal hemoglobin AA red blood cells (AA-RBCs) and hemoglobin SS red blood cells (SS-RBCs) to several purified extracellular matrix proteins was tested under the conditions of controlled flow at a wall shear stress of 1 dyne/cm2. These forces are similar to forces found in the postcapillary venule, a proposed site of vaso-occlusion in malaria1 and sickle cell disease.3-6.7 To examine whether either of these RBC membrane components were involved in mediating cytoadhesion to immobilized TSP, OKM-5, a murine anti-CD36 MoAb that blocks TSP binding to CD36,33 and the TSP peptide CSVTCG, which blocks the interaction of TSP with CD36,36 were tested for their ability to inhibit SS-RBC adhesion to immobilized TSP. Both OKM-5 and CSVTCG failed to inhibit the binding of SS-RBCs to immobilized TSP under conditions of controlled flow (Fig 2).

To determine whether sulfated glycolipids were responsible for the adhesion of RBCs to TSP or laminin, we performed SS-RBC adhesion studies using a number of anionic polysaccharides as potential inhibitors. As shown in Fig 3, the glycosaminoglycan chondroitin sulfate A inhibited more than 75% of SS-RBC adhesion to immobilized TSP. On the other hand, chondroitin sulfate C, which differs from chondroitin sulfate A in the substitution of glucuronic acid with iduronic acid, also had less inhibitory activity compared to chondroitin sulfate A. The glycosaminoglycans keratan sulfate and heparan sulfate, which possess charge densities similar to the chondroitin sulfates,37 caused only mild to moderate inhibition. In addition, fucoidan and heparin, which have higher charge densities than the chondroitin sulfates,37 were only moderately inhibitory, even at high concentrations. This suggests that the inhibition is not solely caused by charge interactions.

Fig 1. Increased adhesion of sickle RBCs to thrombospondin and laminin. Washed RBCs from patients with sickle cell disease (SS-RBCs), healthy controls (AA-RBCs) and patients with high reticulocyte counts (25 to 80% reticulocytes) but no hemoglobinopathy (High Retic) were perfused through flow chambers previously coated (2 pg/cm2) with BSA (BSA, N = 32 for SS-RBCs and 9 for AA-RBCs), vWF (vWF, N = 3 for SS-RBCs and AA-RBCs), TSP (TSP, N = 49 for SS-RBCs, 10 for AA-RBCs, and 4 for Hi Retic) or laminin (LM, N = 20 for SS-RBCs, 10 for AA-RBCs, and 2 for Hi Retic) at a wall shear stress of 1 dyne/cm2 as described in Materials and Methods. After completion of the rinse period, adherent RBCs per unit area were counted by direct microscopic visualization. Adherent RBCs/mm2 are depicted as the mean ± SE.

Fig 2. Sickle cell adhesion to immobilized TSP is not inhibited by CD36 blocking antibodies or peptides. Washed SS-RBCs were incubated with control buffer (None, N = 6), anti-CD36 blocking MoAb OKM-5 (OKM-5 5 µg/mL, N = 6), control isotype specific MoAb MBC 35.5 (Ctrl, 5 µg/mL, N = 4), blocking peptide CSVTCG (CSVT, 200 µmol/L, N = 6) and control scrambled peptide SGTCVC (Scr, 200 µmol/L, N = 6) for 60 minutes at 22°C before the flow adhesion assay. Treated RBCs were perfused through flow chambers as described in Fig 1.
Fig 3. Sickle cell adhesion to TSP is inhibited by chondroitin sulfate A (CSA) and high molecular weight (MW) dextran sulfate (DS). Washed SS-RBCs were incubated with control buffer (None, N = 10) or 500 μg/mL of indicated anionic polysaccharide for 30 minutes at 37°C before the flow adhesion assay. Treated RBCs were perfused through flow chambers coated with TSP as described in Fig 1. CSA (N = 10); CS-C, chondroitin sulfate C (N = 4); DrS, dermatan sulfate (N = 5); KS, keratan sulfate (N = 3); HS, heparan sulfate (N = 5); Fuc, fucoidan (N = 7); Hep, heparin (N = 4); DS-Hi, dextran sulfate, MW 500 kD (N = 12); and DS-Lo, dextran sulfate, MW 5 kD (N = 3).

Fig 4. Dose-response curve of HMW DS and CSA for SS-RBC binding to TSP (A and B) or laminin (C). SS-RBCs were incubated with DS MW 500 kD ([A], N = 3, [C], N = 3 for all points except 500 μg/mL where N = 10) or CSA ([B] N = 3) at the indicated concentration for 30 minutes at 37°C before the flow adhesion assay. Treated RBCs were perfused through flow chambers coated with immobilized TSP (A and B) or immobilized laminin (C) as described in Fig 1.

Isolation and characterization of a RBC lipid that binds TSP and laminin. Because these data were consistent with RBC membrane sulfated glycolipids mediating binding to TSP and laminin, TSP-binding lipids from normal RBCs and sickle RBCs were isolated and characterized. Lipids were extracted from washed red cell ghosts from either normal healthy controls or patients with sickle cell disease. Following dialysis against distilled H2O, the alkaline-treated lipid extracts were fractionated by anion exchange chromatography. As shown in Fig 5, TSP binding lipids eluted coincident with or just after peak sulfated glycolipid fractions as determined by parallel azure A analysis.

To further characterize the biochemical properties of RBC-derived TSP-binding lipids, we performed TLC analysis of the peak TSP-binding lipid fractions from the DEAE-Sepharose column. As shown in Fig 6A, three major bands were resolved by TLC: Band 1, Rf = 0.44, Band 2, Rf = 0.4, and Band 3, Rf = 0.3. TSP bound to only Band 2, which runs with similar mobility as purified bovine brain sulfatide. Further purification by preparative TLC yielded a homogeneous lipid with strong TSP binding properties (Fig 6B, a marked inhibition of the adhesion of sickle RBCs to immobilized TSP. However, the lower molecular weight dextran sulfate (MW 5 kD) did not significantly inhibit adhesion of SS-RBCs to immobilized TSP, even at high concentrations. A dose-response curve for increasing concentrations of high MW dextran sulfate revealed a 50% inhibitory concentration of approximately 0.1 μg/mL (Fig 4A). The dose-response curve for chondroitin sulfate A revealed a 50% inhibitory concentration that was approximately 10-fold higher, in the 5 to 10 μg/mL range (Fig 4B). Thus, the enhanced adhesion of sickle RBCs to immobilized TSP was inhibited, in a dose-dependent manner, by the anionic polysaccharides chondroitin sulfate A and HMW dextran sulfate to a greater degree than other anionic polysaccharides with similar structure and/or charge density.

Because sulfated glycolipids also bind to laminin, the effect of anionic polysaccharides on the adhesion of sickle RBCs to immobilized laminin was tested. The addition of 500 μg/mL HMW dextran sulfate inhibited the adhesion of sickle RBCs to immobilized laminin by 60 ± 5% (mean ± SE, N = 10). The dose response curve with increasing amounts of HMW dextran sulfate revealed a 50% inhibitory concentration that was approximately 50 μg/mL (Fig 4C). This 50% inhibitory concentration for HMW dextran sulfate is approximately 50-fold higher than that required for the same level of inhibition for the adhesion of sickle RBCs to TSP.
ERYTHROCYTE ADHESION TO THROMBOSPONDIN AND LAMININ

A

AA lipid

- TSP Binding

- Sulfatide content

Optical Density

1.2

1.0

0.8

0.6

0.4

0.2

0.0

N1 N2 N3 N4 N5

Fractions from DEAE-Sepharose column

B

SS lipid

- TSP Binding

- Sulfatide content

Optical Density

1.2

1.0

0.8

0.6

0.4

0.2

0.0

N1 N2 N3 N4 N5

Fig 5. TSP binding and sulfatide content of lipid fractions from DEAE-Sepharose column. Following alkaline methanolysis and desalting by dialysis, lipid extracts from AA-RBCs (A) AA lipid or SS-RBCs (B) SS lipid were fractionated by anion exchange chromatography on DEAE-Sepharose. Acidic lipids were eluted with increasing concentrations of NH4HCO3 in MeOH: 0.01 mol/L (N1), 0.02 mol/L (N2), 0.05 mol/L (N3), 0.1 mol/L (N4), and 0.5 mol/L (N5). The sulfated glycolipid content of each fraction was quantitated by azure A analysis (∆, Sulfatide content). TSP binding to immobilized lipid was detected by a solid phase binding assay as described in Materials and Methods (∗, TSP Binding). In both AA-RBC and SS-RBC lipid preparations, TSP-binding lipid fractions were recovered coincident with or just after peak sulfated glycolipid fractions (azure A analysis). The results are depicted as the mean ± SE of N = 5 AA-RBC and N = 5 SS-RBC preparations.

Band 2). Similar findings were obtained from both normal and sickle RBC lipid preparations. Further biochemical analysis of the purified TSP-binding lipid (Band 2) revealed that TSP binding was stable to base treatment that degrades phosphoglycerolipids, but spares most sulfated glycolipids and gangliosides (Fig 7). TSP binding to the purified lipid was also stable to neuraminidase treatment that destroys sialic acid residues in gangliosides. However, TSP binding was labile to solvolysis with acid that selectively desulfates most sulfated glycolipids, but spares most gangliosides [10]. These properties were similar to the sulfatide control (Fig 7). To determine if TSP binding to the purified lipid was primarily because of charge interactions, TSP binding to other acidic or similarly charged lipids was tested. As shown in Fig 7, TSP did not bind to either purified gangliosides or cholesterol 3-sulfate, which have a similar charge as sulfatides.

To determine if the adhesive protein laminin also bound to the purified RBC lipid, soluble laminin was incubated with the immobilized purified lipid in the solid-phase binding assay. As shown in Fig 8, soluble laminin bound to the purified RBC lipid. In addition, laminin did not bind to the acidic lipids, gangliosides, or to cholesterol 3-sulfate. Therefore, the binding of laminin to the purified RBC lipid was not solely because of charge interactions.

To further characterize the purified lipid, the effect of anionic polysaccharides on TSP binding was tested. TSP binding to purified RBC lipid was inhibited the most by HMW dextran sulfate, similar to previous findings for the inhibition of intact RBCs to immobilized TSP under conditions of flow (Fig 9). The two gangliosides with the highest charge density, fucoidan and heparin, caused approximately 60% inhibition of TSP binding to the purified lipid, suggesting that a charge effect may be important in this more purified system.

DISCUSSION

The enhanced adhesion of erythrocytes to purified, immobilized TSP and laminin under conditions of flow (1 dyne/cm²) has not previously been reported. The level of adhesion was further increased in erythrocytes obtained from patients with sickle cell disease but not from patients with high reticulocyte counts but no hemoglobinopathy. This suggests that the increased adhesion of sickle RBCs was not simply because of the increased numbers of reticulocytes found in...
sickle cell disease. Sugihara et al. have reported enrichment of sickle reticulocyte adhesion to immobilized, purified TSP under static conditions, but did not study total RBC adhesion nor flow conditions. In contrast, the adhesion of RBCs to other purified adhesive extracellular matrix proteins was minimal, including vWF, fibronectin, and vitronectin. The high level of adhesion of RBCs to purified TSP and laminin suggests that binding of erythrocytes to exposed TSP or laminin in the vasculature may play an important role in the evolution of vascular pathology.

The enhanced adhesion of erythrocytes to TSP is in agreement with previous reports that soluble TSP, but not vWF, vitronectin, fibrinogen, or fibronectin, increased the adhesion of sickle RBCs to cultured endothelial cells in vitro. This suggests that soluble TSP can participate in the binding of sickle RBCs to intact vascular endothelium. In addition, there is evidence that TSP mediates the adhesion of Plasmodium falciparum-infected RBCs to vascular endothelium in vitro, probably via the expression of parasitic proteins on the infected erythrocyte membrane. In several studies, the supernatant derived from stimulated endothelial cell cultures (rich in HMW vWF) increased the adhesion of SS-RBCs to endothelial cells. In addition, the treatment of ex vivo mesocoeum with desmopressin, known to increase circulating levels of vWF, increased the adhesion of SS-RBCs. The effect was blocked by anti-vWF antibodies, supporting a role for vWF in SS-RBC adhesion. However, the data in this study do not support a role for erythrocyte adhesion to immobilized purified vWF or endothelial cell supernatant rich in HMW vWF. Because the binding characteristics of adhesive ligands can vary depending on the shear force and whether the ligand is in solution phase versus the solid phase, the above discrepancies may be due to the fact that the previous studies entailed soluble vWF and intact endothelium whereas this system used purified immobilized vWF.

Recently, two groups have proposed that CD36 on the surface of sickle reticulocytes plays a role in TSP mediated adhesion of SS-RBCs to endothelium. However, in the present study, both the blocking MoAb OKM5 directed against CD36, and the blocking TSP peptide, CSVTCG, failed to inhibit the adhesion of SS-RBCs to immobilized TSP under conditions of flow. This difference in results
could be because of the fact that the other studies were primarily evaluating RBC adhesion to endothelial cells via soluble TSP, whereas our study involved direct RBC binding to immobilized TSP. However, Sugihara et al. did report that both OKM-5 and CSVTCG inhibited the adhesion of sickle reticulocytes to immobilized TSP under static conditions. However, this discrepancy in results may be caused by the fact that this group limited their data to the reticulocyte subpopulation of RBCs, known to contain CD36, or to the static conditions of their assay system. Because CD36 is primarily found on the reticulocyte subpopulation of RBCs, the lack of adhesion of high reticulocyte controls to TSP further argues against the hypothesis that CD36 mediates the enhanced adhesion of sickle RBCs to immobilized TSP at a shear stress of 1 dyne/cm². However, the possibility that reticulocytes in certain disease states, such as sickle cell disease, have an altered or abnormally exposed form of CD36 has not been ruled out by this study. Overall, these data suggest a limited role for CD36 as the etiology of the enhanced adhesion of sickle RBCs to immobilized TSP under the conditions of this study. However, our studies have not ruled out the possibility that CD36 may play a role in erythrocyte binding to soluble TSP. TSP bound to endothelial cells, the reticulocyte subpopulation of RBCs binding to immobilized TSP, or RBC binding that may occur under other types of shear forces.

The data in this study provide evidence that an acidic RBC membrane glycolipid(s) likely participates in erythrocyte adhesion to TSP and laminin. Sickle RBC binding to immobilized TSP was inhibited by HMW dextran sulfate and chondroitin sulfate A whereas sickle RBC binding to immobilized laminin was partially inhibited by HMW dextran sulfate. These findings are similar to previous reports that the binding of TSP and laminin to purified sulfatide is differentially inhibited by anionic polysaccharides. In agreement, an acidic RBC lipid(s) that binds TSP and laminin was identified and purified from both normal and sickle RBC membranes. This lipid had biochemical properties consistent with a sulfated glycolipid, including stability to base and neuraminidase treatment, acid lability, and detection by azure A analysis. In addition, TSP binding to the immobilized purified lipid was inhibited by HMW dextran sulfate, similar to previous findings for the inhibition of intact sickle RBCs binding to immobilized TSP under the conditions of flow. However, chondroitin sulfate A did not preferentially inhibit the binding of TSP to the immobilized lipid. This may be explained by alterations in conformation of either TSP, because it is soluble instead of being immobilized in this adhesion assay, or the lipid, because it is now purified and no longer in the native RBC lipid bilayer. Alternatively, the purification process may have altered some of the properties of this lipid, such as by inadvertent oxidation or loss of associated membrane components.

The TSP- and laminin-binding lipid purified in this study was found in preparations from both normal and sickle erythrocytes. This implies that this lipid is normally present on RBCs, but may be modified or abnormally exposed on the sickle erythrocyte surface. The sickle red cell membrane has increased levels of oxidative damage that could alter the results of this lipid. In addition, in sickle cell disease the typical lipid bilayer is disrupted with loss of the normal phospholipid asymmetry. This disruption of the lipid membrane bilayer could expose or alter the conformation of native RBC membrane lipids and proteins, thereby modulating adhesive properties of these membrane components. We propose that the TSP- and laminin-binding RBC lipid purified in this study contributes to the low level binding of normal RBCs to TSP and laminin detected and that this lipid is abnormally exposed or modified on sickle RBCs, thereby participating in the enhanced adhesion of sickle RBCs to TSP and laminin.

In this study, we have shown that erythrocytes have increased adhesion to the purified adhesive molecules TSP and laminin under conditions of controlled flow and that an acidic lipid(s) with biochemical properties consistent with a sulfated glycolipid on the RBC surface likely contributes to this adhesion. One model for erythrocyte adhesion to vascular endothelium is that a multivalent adhesive ligand, such as TSP or laminin, binds to sulfated glycolipids on the RBC surface and links the RBC to the subendothelial matrix or another adhesive molecule on the vascular endothelium. In this way, sulfated glycolipids may be important adhesive molecules that are functionally relevant to RBC adhesion. The enhanced adhesive properties of sickle RBCs may be caused by lipids naturally present in the red cell membrane that have been biochemically altered or abnormally exposed. Further characterization of this lipid(s) will be required to determine whether this lipid(s) is abnormally exposed or modified on RBCs in pathologic states, thereby participating in the enhanced adhesion of RBCs to adhesive proteins and potentially in the pathogenesis of vaso-occlusive diseases.

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Increased adhesion of erythrocytes to components of the extracellular matrix: isolation and characterization of a red blood cell lipid that binds thrombospondin and laminin

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