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Research Article

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Unusually Large von Willebrand Factor Multimers Increase Adhesion of Sickle Erythrocytes to Human Endothelial Cells under Controlled Flow

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Abstract

The interactions of normal erythrocytes and erythrocytes from patients having hemoglobin S hemoglobinopathies with normal human endothelial cells (EC) were investigated under flow conditions. When EC supernatant, containing 2.8–11.0 U/dl of von Willebrand factor (vWF) antigen and vWF multimeric forms larger than those present in normal plasma, was the red blood cell (RBC)–suspending medium instead of serum-free medium (SFM), the adhesion of sickle RBC, but not normal RBC, to endothelial cells was greatly increased (range of enhancement of sickle RBC adhesion, 2- to 27-fold). Adhesion of sickle RBC to endothelial cells was reduced to near serum-free levels when EC supernatant was immunologically depleted of vWF forms. Sickle RBC suspended in SFM containing 200 U/dl of purified vWF multimers of the type found in normal human plasma or 300 μg/ml human fibronectin were only slightly more adhesive to endothelial cells than sickle RBC suspended in SFM alone.

These data indicate that unusually large vWF multimers produced by endothelial cells are potent mediators of the adhesion of sickle erythrocytes to endothelial cells. Vaso-occlusive crises in sickle cell anemia may be caused, at least in part, by adhesive interactions between the abnormal surfaces of sickle RBC and the endothelium after the release of unusually large vWF multimeric forms from stimulated or damaged endothelial cells.

Introduction

The major manifestations of sickle cell anemia are periodic, localized, vaso-occlusive crises and chronic hemolytic anemia. Adhesion of sickle erythrocytes to the vascular endothelium has been proposed as one mechanism of vaso-occlusion (1–6). This investigation was undertaken to determine the role of von Willebrand factor (vWF)[1] in the adhesive interactions between sickle erythrocytes and endothelial cells.

vWF is a large, multimeric plasma protein (subunit molecular weight of 2.20×10^5 D [7]) synthesized by megakaryocytes (8) and endothelial cells (9). The circulating pool of vWF is heterogeneous with multimers ranging from 8.5×10^5 to millions of daltons (10). Large vWF multimeric forms are involved in platelet-subendothelial adhesion (11) and in shear stress–induced platelet aggregation (12). Large vWF forms bind to platelet membranes via glycoprotein Ib (13, 14) and IIb/IIIa complexes (15, 16). Arginine-glycine-aspartate amino acid sequences are involved in the binding of vWF to the IIb/IIIa complexes (17, 18).

Human endothelial cells (EC) in culture synthesize vWF multimers which are larger than the largest multimeric forms found in normal human plasma (19). These unusually large vWF forms are secreted by endothelial cells into the subendothelial matrix (20) and, under conditions of intense endothelial cell stimulation, into the plasma (21).

Human endothelial cells also produce and secrete fibronectin (22), another important cytoadhesive protein (23). It has been demonstrated that red cells of patients with sickle cell disease bind fibronectin in greater quantities than normal erythrocytes (24), and therefore fibronectin may be another important mediator of erythrocyte/endothelial cell interactions.

Microvascular occlusion in sickle cell anemia may be caused in part by capillary blockage with rigid, irreversibly sickled cells (25, 26). Deoxygenation of hemoglobin S in sickle red cells may lead to a cycle of tissue ischemia, sickling, and vaso-occlusion (26). However, recent observations (1–6) have suggested that adherence of sickle erythrocytes to the vascular endothelium may be a complementary or alternative mechanism of vaso-occlusion. Under both static (1–5) and flow (6) conditions sickle red cells adhere abnormally to cultured endothelial cells. This increase in adhesion, when compared with normal red cells, has been related to the clinical severity of vaso-occlusive events in sickle cell disease (3). Mohandas et al. (4), using a quantitative micropipette technique, demonstrated that adhesion observed in vitro could occur under the fluid forces present in the microcirculation, implying that sickle erythrocytes could also be more adhesive in vivo. We have

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1. *Abbreviations used in this paper:* EC, endothelial cell; FN, fibronectin; IRMA, immunoradiometric assay; RBC, red blood cell; SFM, serum-free medium; vWF, von Willebrand factor.

investigated the mechanism of adhesion between sickle red cells and normal human endothelial cells.

Methods

Endothelial cell cultures. Umbilical cords were obtained from the Obstetrics Service of Jefferson Davis Hospital, Houston, TX. The umbilical veins were cannulated and rinsed with 100 ml of sterile, 37°C phosphate buffer (0.14 M NaCl, 0.0004 M KCl, 0.011 M glucose, 0.00022 M NaH_2PO_4, and 0.0081 M Na_2HPO_4). Veins were filled with 12 mg collagenase (169 U/mg; Cooper Biomedical, Inc., Malvern, PA) dissolved in 50 ml of 37°C phosphate-buffered saline (PBS; 0.0027 M KCl, 0.0015 M KH_2PO_4, 0.137 M NaCl, 0.0081 M $Na_2HPO_2 \cdot 7H_2O$, and 0.00049 M $MgCl_2$; Gibco, Grand Island, NY) and incubated for 30 min. The collagenase suspensions were collected, and the veins were rinsed with 100 ml of phosphate buffer to ensure collection of all cells. Effluent was centrifuged for 10 min at 100 *g* and the cell pellets were resuspended in complete medium (M199, Gibco; with 20% heat-inactivated fetal calf serum, HyClone Laboratories, Logan, UT; 0.10 mg/ml penicillin and streptomycin, Gibco; 0.20 mg/ml neomycin, Gibco; 0.292 mg/ml glutamine, Gibco). Cells from different cords were pooled and seeded onto 75mm × 38mm glass slides (Fisher Scientific, Springfield, NJ) pretreated with 0.5N NaOH for 2 hours to improve surface properties. Cells were grown to confluence (2–4 days) in a 37°C incubator (5% CO_2) and were then used within three days.

Endothelial cell supernatants. Endothelial cells were cultured to confluence in complete medium. Monolayers were rinsed twice in PBS (37°C), coated with 2 ml of serum-free medium (SFM; containing 5.0 μg/ml bovine insulin, Sigma Chemical Co., St. Louis, MO; 5.0 μg/ml human transferrin, Sigma Chemical Co., 0.4% human albumin, Sigma Chemical Co., 0.10 mg/ml penicillin and streptomycin; 0.20 mg/ml neomycin; 0.292 mg/ml glutamine in M199), and incubated at 37°C for 48 h. The supernatant was then removed and contained between 2.8 and 11.0 U/dl of vWF antigen (normal platelet-poor plasma contains 100 U/dl of vWF antigen). In some experiments, EC supernatant was depleted of vWF forms by incubation with rabbit anti-human vWF antibody linked to Protein-A Sepharose CL-4B (Pharmacia, Inc., Piscataway, NJ) for 2 h at room temperature. The vWF antibody did not cross-react with fibronectin. Cultures used to produce EC supernatant were not used in the red cell adhesion assays.

Purification of vWF. Human vWF multimeric forms were purified from blood bank cryosupernatant and fractionated as described previously (27).

Red cell suspensions. Blood was drawn from normal donors or patients with sickle hemoglobinopathies (HbSS, HbSC, sickle cell β-thalassemia) into sodium heparin (14.3 USP units/ml), centrifuged at 100 *g* for 10 min, and the plasma and buffy coat were removed and discarded. The red cells were washed three times in SFM and resuspended in either SFM, EC supernatant, or EC supernatant depleted of vWF. Our experiments were designed to elucidate a possible mechanism of adhesion and not to reproduce in vivo conditions. Therefore, a hematocrit of 1% was chosen to limit the amount of blood required from each donor for each experiment. For red cell adhesion assays in the presence of plasma proteins, either fibronectin (purified from human plasma; Sigma Chemical Co.) was added to SFM to produce a final concentration of 300 μg/ml or purified plasma vWF enriched in the largest multimeric forms found in the cryoprecipitate fraction of normal human plasma was added to a final concentration of 200 U/dl. Red cells suspended in SFM, EC supernatant, or EC supernatant depleted of vWF were not morphologically altered in the various suspending media when observed under phase contrast (200×) or oil immersion light microscopy (1,000×) after fixation in gluteraldehyde.

Adhesion assay. Confluent endothelial monolayers on glass slides made up the base of a modified Richardson flow chamber (Fig. 1) held in place by an applied vacuum. The chamber, with slide of endothelial cells in place, was mounted on the stage of an inverted, phase-contrast microscope (Nikon). Medium and red cell suspensions were kept at 37°C in an adjacent water bath and the chamber was maintained at 37°C by an air curtain incubator.

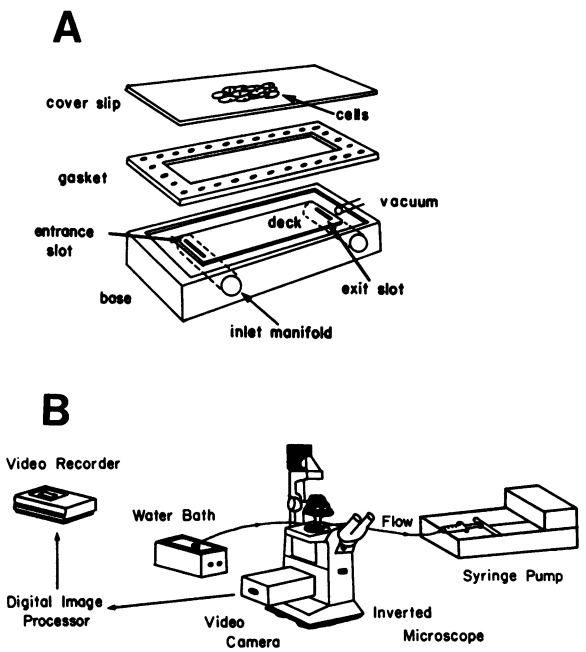

Figure 1. Adhesion assay apparatus. (*A*) An EC monolayer on a glass slide forms the base of the flow chamber and is held in place by a vacuum applied at the vacuum port. The depth of the parallel-plate flow chamber is determined by the thickness of the gasket, and for the experiments reported is 104 μ. (*B*) The chamber, with EC monolayer in position, is inverted and mounted on the microscope stage. Media and red cell suspensions are kept in an adjacent 37°C water bath and flow over the EC monolayer at a rate of 0.0764 ml/min (wall shear stress, 1.0 dyn/cm^2) by use of the syringe pump. The EC monolayer is kept at 37°C by an air curtain incubator mounted on the microscope (not shown). A video camera and video cassette recorder are mounted on the microscope to facilitate red cell counting and to record experiments for later analysis.

The endothelial cell monolayer was rinsed for 5 min with SFM at a constant flow rate of 0.0764 ml/min generated by a Harvard syringe pump producing a wall shear stress of 1.0 dyn/cm^2 (a shear stress typically found in the venules [28]). The red cell suspension was then passed over the endothelial cell monolayer for 10 min followed by a 20-min rinse with SFM to remove nonadherent red cells. Even at 1% hematocrit, the endothelial cell monolayer was entirely covered with red cells for the entire 10 min of perfusion, and thus many of the cells were able to interact with the endothelial surface. Therefore, the adhesion values observed were an accurate representation of the adhesive subfraction of each donor's erythrocytes. No static incubation of red cells on the endothelial cell monolayer occurred before rinsing began. The number of adherent cells was counted in 24 random fields ranging over the entire slide.

vWF antigen level. vWF antigen was quantified by solid-phase immunoradiometric assay (IRMA) using rabbit anti-human vWF antibody and rabbit [^{125}I]-anti-human vWF antibody as previously described (29).

vWF multimer patterns. vWF multimers were separated by sodium dodecyl sulfate agarose gel electrophoresis, overlaid with [^{125}I]-anti-human vWF IgG and analyzed by autoradiography using 1% agarose and a continuous buffer system (30, 31).

Fibronectin antigen level. Fibronectin antigen was quantified by

Laurell 'rocket' immunoelectrophoresis as previously described (32).

Statistics. The data for each donor were analyzed using an F-test (33), which distinguishes between the field-to-field variance and the treatment variance (EC supernatant vs. SFM, EC supernatant vs. vWF-depleted EC supernatant, etc.).

Results

The EC supernatants contained relatively small amounts of vWF antigen (2.8–11.0 U/dl), but a major component of the multimers were unusually large forms not found in normal pooled plasma (Fig. 2). The endothelial cell supernatants also contained small amounts of fibronectin (< 20 μg/ml). This fibronectin was present in the supernatants even after they were depleted of vWF (data not shown).

The adhesion of normal red cells ranged from 2.52 to 4.08 RBC/mm^2 in SFM and increased slightly to 4.17–5.81 RBC/mm^2 in EC supernatant (Table I). The adhesion of sickle red cells suspended in SFM to endothelial cells was 3.04–10.24 RBC/mm^2, and increased greatly to 12.76–132.12 RBC/mm^2 when the sickle red cells were suspended in EC supernatant (average 11-fold increase; range 2- to 27-fold for eight experiments with six different patients, Table I). When the EC supernatant was immunologically depleted of vWF multimers, the adhesion of sickle red cells decreased to nearly the SFM control level (3.13–9.81 RBC/mm^2). The antigen level in the vWF-depleted EC supernates was < 1.5 U/dl for the four patients studied (column 3, Table I). Fig. 3 shows an endothelial cell monolayer before and after the 10-min perfusion of sickle erythrocytes in EC supernatant.

When fibronectin (FN) or purified plasma vWF were added to a suspension of sickle red cells (HbSS or HbSC) in SFM (300 μg/ml FN or 200 U/dl of the largest vWF multimers found in cryoprecipitate), the extent of adhesion to the endothelial monolayers increased only modestly (Table II). The enhancement with either FN or vWF was 1.5- to 2.5-fold, much less than the enhancement of sickle erythrocyte adhesion to endothelial cells in the presence of endothelial cell supernatant.

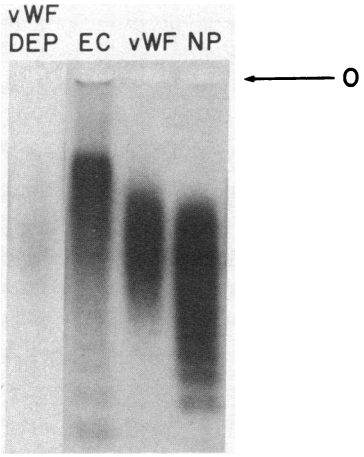

Figure 2. vWF multimer patterns of test samples. vWF multimers were separated by sodium dodecyl sulfate-1% agarose gel electrophoresis. *vWF,* sample of vWF multimers purified from the cryoprecipitate fraction of normal plasma, including largest multimeric forms found in normal plasma. *EC,* sample of endothelial cell supernatant containing unusually large vWF multimers not found in *NP. vWF DEP,* EC supernatant preparation immunologically depleted of vWF forms. *NP,* sample of normal pooled, platelet-poor plasma, shown for comparison.

Table I. Adhesion of Sickle and Normal Red Blood Cells to Endothelial Cells

	Adhesion in SFM	Adhesion in EC SUP	Adhesion in vWF DEP-EC SUP
Normal red cells (HbAA)			
Donor 1	2.52±3.19	5.73±6.41 ($P < 0.01$)	
Donor 2	4.08±4.16	4.17±4.71 (P = NS)	
Donor 3	3.47±4.96	5.03±4.07 (P = NS)*	
Donor 4	2.69±3.38	5.81±7.28 ($P < 0.01$)	
Sickle red cells (HbSS)			
Patient 1	5.73±4.91	19.10±11.38§	—
Patient 2	10.24±6.37	68.92±37.37§	—
Patient 3	3.23±3.06‡	35.21±15.70§	—
Patient 4	3.04±4.19‡	83.33±56.86§	—
Patient 5	5.90±5.22	35.94±24.43§	3.13±5.40¶
Patient 6	4.95±4.84	30.96±17.01§‖	9.81±7.66¶
Patient 7	6.16±5.08	12.76±5.98§	5.38±5.30¶
Patient 8	5.12±4.65‡	132.12±45.74§	8.77±9.85¶

Adhesion (RBC/mm^2) to human endothelial cell monolayers of washed red cells from normal donors and patients with sickle cell anemia resuspended in serum-free medium (SFM), human endothelial cell supernatant (EC SUP) containing unusually large vWF multimers, or EC supernatant depleted of vWF (vWF-DEP EC SUP). The values are mean±SD reflecting the variation of the 24 fields viewed and counted per slide.) (n = 48 fields analyzed, except where noted.

* n = 24.

‡ Same donor studied on different days. This patient has sickle cell β-thalassemia.

§ Increase in sickle RBC adhesion in EC SUP column compared with SFM column ($P < 0.001$).

‖ n = 72.

¶ Decrease in sickle RBC adhesion in vWF DEP-EC SUP column compared with EC SUP column ($P < 0.001$).

Discussion

EC supernatant contains only small quantities (< 12 U/dl) of vWF antigen. However, it includes unusually large vWF multimeric forms. EC supernatant, but not the largest vWF forms purified from cryoprecipitate, greatly increased the adherence of sickle erythrocytes to cultured human endothelial cells (Tables I and II). In contrast, only a slight augmentation of EC adhesion by the unusually large vWF forms of EC supernatant was observed for normal RBC (Table I). In the absence of the unusually large vWF multimeric forms, even a high concentration of vWF antigen (200 U/dl), containing the largest multimeric forms purified from the cryoprecipitate fraction of normal plasma, caused only a modest increase in the adhesion to endothelial cells of HbSS and HbSC RBC compared with increases observed in the presence of unusually large vWF multimers. The addition of fibronectin to sickle red cells suspended in SFM at 300 μg/ml also resulted in only small increases in adhesion (Table II) compared with the unusually large vWF. These data indicate that the unusually large vWF multimers derived from endothelial cells are optimally effective in promoting the binding of sickle red cells to endothelial cells.

The levels of fibronectin and vWF factor in normal pooled platelet-poor plasma are 300 μg/ml and 100 U/dl, respectively. The plasma fibronectin level remains relatively constant with time in both normal and sickle patients (34). This is not the

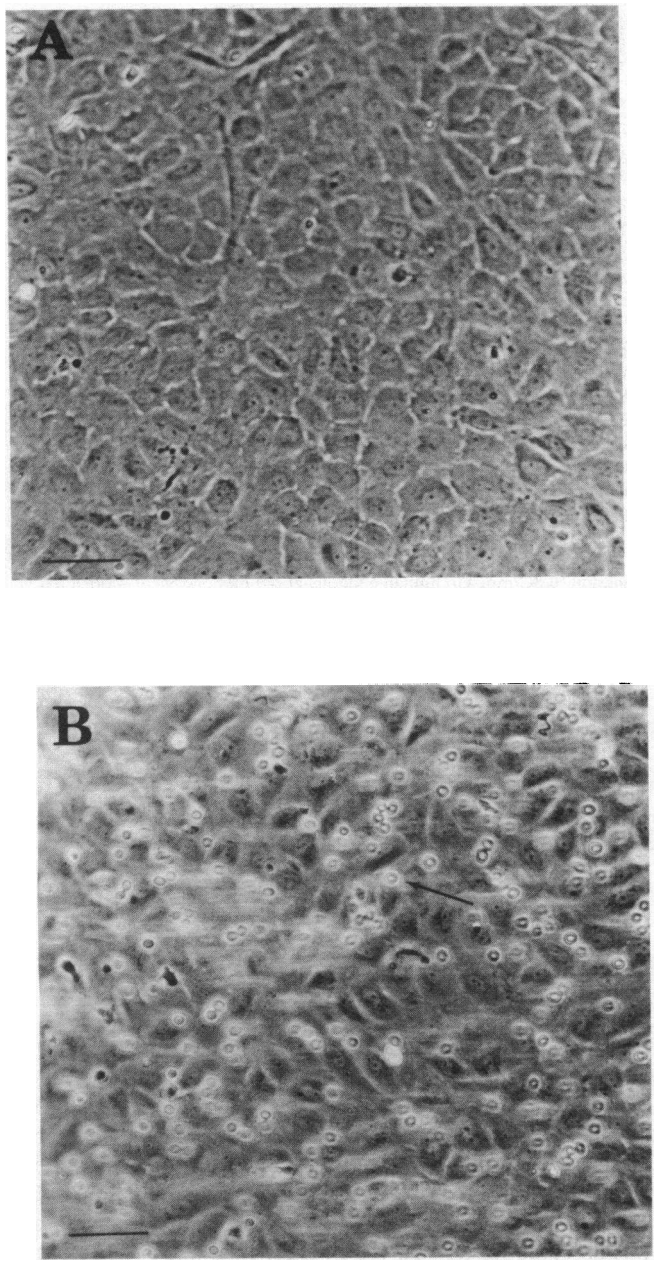

Figure 3. Adhesion of sickle red blood cells to endothelial cells. (*A*) EC monolayer being rinsed for 5 min by SFM before perfusion of sickle red cells. Flow is from left to right. Bar, 50 μ. (*B*) EC monolayer after 10-min perfusion of sickle red cells suspended in EC supernatant and a 20-min rinse to remove nonadherent cells with SFM. Arrow points to one of the many adherent red cells in this field. Flow is from left to right. Bar, 50 μ. Streaks are nonadherent cells flowing with the perfusion medium.

case with vWF antigen levels, which often increase in times of crises (35), suggesting that endothelial cell stimulation or damage occurs during these clinical events.

In two preliminary experiments, addition of 6.81×10^{-8} M of the tetrapeptide Arg-Gly-Asp-Ser to sickle red cells suspended in EC supernatant (a molar concentration 100 times that of plasma fibronectin) decreased by 99% the binding of

Table II. Effect of Plasma Proteins on Adhesion of Sickle RBC to Endothelial Cells

	Adhesion in SFM	Adhesion in SFM + FN	Adhesion in SFM + Plasma vWF
Patient 1	7.29±5.33	12.76±6.62	15.71±6.56
Patient 2	3.47±3.37	5.64±3.70	6.33±3.84
Patient 3	1.39±2.16	3.47±2.76	2.43±2.96

Adhesion of washed red cells (RBC/mm^2) from patients with sickle cell syndromes resuspended in SFM to human endothelial cell monolayers. The largest plasma forms of vWF (200 U/dl) and/or human FN (300 μg/ml) were added as indicated. The values are mean±SD reflecting the variation of 24 fields viewed and counted per slide (48 total fields analyzed for each trial). Both columns were significantly different ($P < 0.01$) from the SFM adhesion column. Patients 1 and 2 had sickle cell anemia (HbSS) and patient 3 had HbSC disease.

sickle red blood cells to endothelial cells that was mediated by the unusually large vWF multimers. Red cells from sickle patients 5 and 8 (Table I) were incubated in the EC supernates containing the peptide for 30 min and analyzed for adhesion in the flow chamber as previously described. Adhesion of the red cells suspended in EC supernatant decreased from 35.94±24.43 to 6.16±6.55 RBC/mm^2 and from 132.12±45.74 to 6.68±6.75 RBC/mm^2 for patients 5 and 8, respectively, in the presence of the peptide. The observed adhesion values in the presence of the peptide were not significantly different from the adhesion values in the SFM control experiments for each of the donors. Thus, either the endothelial cells or the sickle red blood cells have binding sites similar to the glycoprotein IIb/IIIa complex in platelets that were able to recognize the Arg-Gly-Asp-Ser sequences in unusually large vWF multimeric forms. That type of binding site has recently been demonstrated in erythroleukemia cells (36) as well as in human endothelial cells (37). It has also been reported recently that human endothelial cells contain receptors similar to glycoprotein Ib (38), the other binding site for large vWF forms on human platelets.

Sickle red cells, in contrast to normal red cells, may have incipient receptors for vWF on their surface because they are relatively young cells, or because of alterations in their membrane structure induced by cycles of HbS polymerization-depolymerization in conjunction with shear forces in the blood. Shear forces have been shown to effect the exposure or topography of platelet surface receptors for large and unusually large vWF multimers (12).

Sickle cell disease is characterized clinically as a pathologic red cell alteration leading to periodic, localized, vaso-occlusive crises. These crises often occur in conjunction with increased stress, vigorous exercise, infection, or pregnancy, which may lead to mechanical or chemical stimulation of endothelial cells. Endothelial cell stimulation could occur in the region of the microcirculation where the small capillary diameter allows for intimate contact of closely apposed red cells and endothelial cells. Here, mechanical stimulation or lysis of endothelial cells could even be provoked by direct contact with rigid, sickled erythrocytes. Our data suggest that unusually large vWF multimers mediate the adhesion of sickle RBC to endothelial

cells and contribute to vaso-occlusive episodes. These episodes may occur when endothelial cells are intensely stimulated to release their content of unusually large vWF from Weibel-Palade bodies. Locally high concentrations of unusually large vWF multimers (in excess of the capacity of plasma to process or remove these huge forms [39]) may result in the concurrent binding of unusually large vWF multimers to sickle RBC and endothelial cells. The bonds formed may be strong enough to withstand the fluid shear forces in the microcirculation, or at least those forces in the postcapillary venules simulated in our experiments. Vaso-occlusion may then occur, with adherent sickle red cells combining locally with the most dense, irreversibly sickled RBC to occlude portions of the microvascular circulation in a cycle of lengthened transit times, deoxygenation, HbS polymerization, unusually large vWF-mediated sickle RBC attachment to the endothelium, and vascular obstruction.

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