Erythrocyte–Endothelial Cell Adherence in Sickle Cell Disorders

By Brian D. Smith and Paul L. La Celle

Detachment of individual sickle erythrocytes from cultured endothelial cell monolayers has been evaluated by a fluid-shearing technique in an effort to quantitate adherence at shear forces that would be anticipated in the in vivo circulation. Nonirreversibly sickled cells (non-ISC) were more adherent at normal oxygen tensions than control cells. More than 1% non-ISC remained attached to the monolayer at forces greater than physiologic shear stresses in capillary and venous circulations, and many of the most avidly attached cells, once separated, immediately reattached to adjacent endothelial cells. These data suggest that hemoglobin S–containing erythrocytes may have a higher frequency of adherene in vivo in regions of low shear stress where prolonged erythrocyte–endothelial cell contact could occur. Some of these cells detached by shear force would subsequently reattach in in vivo conditions. Plasma-enhanced attachment frequency and plasma from blood in a case of sickle crisis caused further increase. These observations further support the concept that sickle erythrocyte–endothelial cell interaction may be a significant factor in initiation of vascular occlusive events in sickle cell disease.

AN INCREASED FREQUENCY of erythrocyte adherence to the vascular endothelium has been postulated as a contributing factor in the vascular obstructive phenomenon of sickle cell anemia.[1,2] In this disorder the flow disturbances range from microvascular occlusive crises to arterial thromboses; these may result from both intrinsic erythrocyte abnormalities and plasma changes.[3] The well-known rheologic abnormalities of the sickle cell are manifested in vitro by the elevated viscosity of packed cell suspensions[3] and decreased filterability, and in vascular occlusive crises with attendant elevation of levels of reactive proteins the abnormalities are greater.[4,5] In small in vitro channels of the capillary size range, there is an observed decrease in the hematocrit value[6] with reduction of apparent viscosity, findings also noted in normal animal models.[7] The relatively low hematocrit value in sickle cell disease may result in a lower than normal capillary hematocrit value that would compensate for the increased viscosity and related increase in vessel wall shear stress that occur with the loss of cellular deformability[8] demonstrable in the irreversibly sickled cell (ISC) portion of sickle erythrocytes.[9] A decrease in wall shear stress particularly in vessels typified by lower flow velocity would tend toward increased blood cell–endothelial cell contact and favor adherence, and if the sickle cell surface characteristically were more adhesive, these flow dynamics and the sickle cell tendency for adherence could have important implications for the pathophysiology of the disorder. Adherence of erythrocytes to the endothelium would be expected to contribute to abnormal flow dynamics by changing the local vessel geometry and thereby favor further cell accumulation and resultant occlusion. The specific mechanism by which adherence of sickle cells to the endothelium may produce its effect in occlusion has not been demonstrated; however, in vitro observations indicate a correlation between increased adherence observed in vitro and clinical severity,[10] and during the crisis state, the rate of erythrocyte–endothelial cell adherence increases.[11,12]

The postulated increased frequency of an in vivo adherence to endothelium has been interpreted from in vitro observations of sickle erythrocytes binding to cultured endothelial cells. In these experiments, binding was assessed by sequential washing of isotope-labeled erythrocytes from endothelial monolayer cultures with the assumption that residual binding after washes reflects the likelihood of adherence in vivo. The studies of Hebbel et al showed that 0.1% to 1.0% of sickle cells remained adherent, thus demonstrating unique characteristics of sickle cell–endothelial cell interaction and strongly supporting the proposed mechanism.[10–12]

More recently, Mohandas and Evans[13] have developed a direct quantitive measure to examine sickle-endothelial cell adherence with results that demonstrated that 90% of sickle cells adhered in the presence of autologous plasma and one third of normal cells adhered in the presence of sickle plasma. The strength of sickle cell adhesion was nearly 50% greater than the control. Burns et al[14] did not detect significant differences between adherence properties of sickle cells and normal control cells in systems providing laminar flow conditions, but with vortex flow that promotes erythrocyte–endothelial cell contact, sickle cells were significantly more adherent than control cells.

The purpose of the present study was quantitation of the retention of adherent sickle erythrocytes by endothelial cells as a function of applied fluid shear stress to determine whether erythrocytes remain attached at stresses typical of in vivo rheologic conditions. An abnormal frequency of adherence was observed at stresses typical of the calculated physiologic shear stresses of the microcirculation and arteries, thus providing support for the concept of a specific role for erythrocyte–endothelial cell interaction in the pathophysiology of sickle cell disease.

MATERIALS AND METHODS

Patients. Blood samples from five patients with sickle cell anemia confirmed by electrophoresis were studied. Two patients

From the Departments of Medicine and Biophysics, University of Rochester School of Medicine, NY.

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Address reprint requests to Dr P.L. La Celle, Box Biophysics, University of Rochester School of Medicine, 601 Elmwood Ave, Rochester, NY 14642.

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(I.D. and A.H.) had no history of vascular crisis, one (T.J.) had been admitted for treatment of vaso-occlusive crisis, and two (A.T. and T.W.) had previous experience of vascular crisis but were asymptomatic at the time of study. Two patients (D.H. and R.M.) had sickle-thalassemia, were asymptomatic, and had no history of vascular crisis. Tables 1 and 2 indicate the study group. Blood samples from 12 healthy volunteers served as controls. Informed consent was obtained in each instance.

Erythrocytes. Venous blood was collected with citrate or heparin as anticoagulant. There was no differnce in results obtained with one anticoagulant compared with the other, and no coagulation was apparent during the incubation experiments. Samples were centrifuged at 2,300 g for 15 minutes to separate cells and plasma, and the plasma was centrifuged subsequently at 6,500 g for 30 minutes to remove platelets. Some erythrocytes were suspended in autologous plasma and others washed three times in isotonic phosphate buffer at physiologic pH and resuspended in this buffer. Suspensions for layering on the endothelial monolayer, glass, or plastic surfaces contained washed or unwashed cells mixed with either phosphate buffer plus albumin (5 g/L) or autologous plasma. To aid visualization final hematocrit values were less than 1%; however, there was no change in adherence, with hematocrit values ranging from less than 1% to 25%.

Endothelial cell cultures. Human umbilical veins were cannulated and infused with phosphate buffer to remove erythrocytes, then filled with Pronase (Calbiochem-Behring Corp, San Diego) for 20 minutes at 37 °C to loosen endothelial cells. The endothelial cells were collected, washed twice in hypotonic buffer to lyse contaminating erythrocytes, mixed with McCoy's M-199 or 5A/1X powder culture media (GIBCO, Grand Island, NY), and layered on multiple 1-cm squares of plastic or glass. Thus, endothelial cells from the same cord were used for both control and sickle cell studies, which gave consistent, reproducible results. The culture medium was changed under sterile conditions at 24- to 48-hour intervals until the endothelium formed a confluent monolayer.

Quantitation of erythrocyte adherence. Erythrocytes in phosphate-albumin buffer were allowed to settle for 30 minutes at 37 °C, forming a layer two to five cells deep on an endothelial monolayer. Under direct microscopic visualization and closed circuit video recording for later analysis, adhesion was studied on ten fields, each with an endothelial surface area sufficient to provide contact for 100 erythrocytes. An initial local wall shear stress of 1 dyne/cm^2, an order of magnitude less than that normally found in the microcirculation, was induced by flow from a 12-μm (internal diameter) glass pipette filled with phosphate buffer, causing nonadherent cells to move out of the field. Remaining adherent cells were subjected to progressively higher shear stress until separation occurred (Fig 1).

In the studies the pipette was placed 10 μm from the adherent erythrocyte, and positive pressure was applied to the pipette to produce flow that impinged on the cell. The 10-μm distance was measured on the video screen (see Fig 1). Calculation of shear stress on the erythrocytes resting on the endothelial surface was based on the assumption that the parabolic distribution of the velocity profile in the pipette is maintained approximately over the short interval between the pipette tip and the individual erythrocyte under study. The flow velocity, a function of applied pressure, was determined by observation of the velocity of small axially flowing particles in the buffer in the pipette. Since the pipette axis is nearly parallel to the endothelial surface, the actual force on the cell is approximately equal to the product of the wall shear stress and the projected area of the erythrocyte. However, since the projected area or cross section of the cell perpendicular to the direction of flow is fairly uniform because of the similarity of erythrocyte diameter and volume, the wall shear stress proportional to the force is a practical expression of the force on the cell and permits estimation of in vivo dynamics from the in vitro experiment.

Wall shear stress calculations for in vivo model pressure and vessel dimension measurements[15] provided approximate ranges for arterioles, capillaries, and venules. Calculations from other animal models demonstrated similar or lower values for in vivo wall shear stress.[16]

RESULTS

The fluid shear stress required for separation of adherent sickle erythrocytes from the endothelial cell monolayer is

Table 1. Erythrocytes Suspended in Albumin Plus Isotonic Buffer. Erythrocyte Adherence to Cultured Endothelium for Individual Patients

Subjects	Percent Adherence at Various Shear Stresses (SE)				
	1 dyne/cm^2	10 dyne/cm^2	20 dyne/cm^2	30 dyne/cm^2	55 dyne/cm^2
Sickle cell patients					
I.D.	3.30 (0.73)	2.80 (0.63)	2.80 (0.63)	2.70 (0.65)	2.30 (0.59)
A.T.	1.38 (0.13)	0.44 (0.05)	0.25 (0.03)	0.22 (0.03)	0.09 (0.01)
E.W.	2.00 (0.99)	2.00 (0.99)	2.00 (0.99)	2.00 (0.99)	2.00 (0.99)
T.J.	0.10 (0.10)	0.10 (0.10)	0.10 (0.10)	0.10 (0.10)	0.10 (0.10)
A.H.	0.82 (0.24)	0.41 (0.12)	0.30 (0.09)	0.23 (0.07)	0.06 (0.02)
Sickle-thalassemia patients					
P.H.	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)	0.00
R.M.	0.43 (0.06)	0.29 (0.04)	0.29 (0.04)	0.14 (0.02)	0.07 (0.03)
Controls					
B.S.	0.60 (0.10)	0.21 (0.02)	0.15 (0.02)	0.10 (0.02)	0.08 (0.01)
C.S.	0.12 (0.04)	0.00	0.00	0.00	0.00
B.F.	0.00	0.00	0.00	0.00	0.00
D.K.	0.00	0.00	0.00	0.00	0.00
A.M.	0.30 (0.03)	0.05 (0.01)	0.03 (0.01)	0.02 (0.01)	0.02 (0.01)
C.C.	0.00	0.00	0.00	0.00	0.00
D.J.	0.00	0.00	0.00	0.00	0.00
E.K.	0.50 (0.11)	0.10 (0.02)	0.01 (0.02)	0.10 (0.02)	0.10 (0.02)
M.A.	(0.10) (0.05)	0.10 (0.05)	0.10 (0.05)	0.10 (0.05)	0.10 (0.05)
J.M.	(0.40) (0.07)	0.02 (0.01)	0.00	0.00	0.00
F.S.	(0.30) (0.04)	0.00	0.00	0.00	0.00
S.C.	0.00	0.00	0.00	0.00	0.00

SE, standard error.

Table 2. Erythrocytes Suspended in Autologous Plasma. Erythrocyte Adherence to Cultured Endothelium of Individual Patients

Subjects	Percent Adherence at Various Shear Stresses (SE)				
	1 dyne/cm²	10 dyne/cm²	20 dyne/cm²	30 dyne/cm²	55 dyne/cm²
Sickle cell patients					
I.D.	2.05 (1.12)	0.94 (0.71)	0.89 (0.66)	0.61 (0.61)	0.56 (0.55)
A.T.	1.37 (0.29)	0.90 (0.19)	0.32 (0.03)	0.23 (0.02)	0.09 (0.01)
T.J.	18.00 (4.00)	10.00 (5.00)	7.50 (5.50)	5.50 (4.50)	1.50 (1.50)
A.H.	2.09 (1.13)	0.83 (0.49)	0.83 (0.49)	0.58 (0.33)	0.33 (0.16)
Sickle-thalassemia patients					
P.H.	0.20 (0.05)	0.00	0.00	0.00	0.00
R.M.	2.25 (0.18)	0.92 (0.12)	0.84 (0.10)	0.67 (0.10)	0.50 (0.10)
Controls					
B.S.	1.58 (0.21)	0.87 (0.13)	0.61 (0.09)	0.43 (0.07)	0.12 (0.02)
C.S.	1.70 (0.33)	0.39 (0.12)	0.34 (0.10)	0.22 (0.06)	0.09 (0.02)
A.M.	1.11 (0.14)	0.50 (0.08)	0.36 (0.05)	0.27 (0.03)	0.19 (0.03)

given for individual patients in Table 1 and depicted as the mean percent adherent cells *v* shear stress in Fig 2. The experiment in each case started 30 minutes after the erythrocytes in buffer were allowed to settle on the cultured surface, and since study of each cell required approximately three minutes, the total exposure time of each subsequent cell increased by approximately three minutes over the predecessor, ie, the exposure time of cells was not uniform. However, there was no trend in the data to suggest that increasing pressures were required to detach cells studied late in the experiment, and thus, no consideration for the point in time of study of specific cells was given in the assembly and interpretation of the data. The variation of the frequency of control cell adherence to differing cultures of endothelial cells was less than 1% at the lower shear rates in the case of buffer suspensions and less than 2.0% in plasma; thus data have been expressed as a percentage of the control or experimental cells adherent at each shear stress rather than in the ratio of experimental to control cell or in normalized form.

With gradually increasing shear stress, some sickle cells remained attached to the endothelial cells when subjected to calculated shear forces above the physiologic range as indicated by data from animal models. These persistently attached cells were located on the endothelial cell surface only, and thus the behavior appeared to reflect erythrocyte–endothelial cell surface interaction. Care was taken to avoid cells in regions of detectable surface artifacts since surface abnormality, eg, exposed glass surfaces, could explain resistance to detachment. Adherent cells that required large shear forces for separation frequently reattached instantly upon recontact with the endothelial surface, and equally large shear forces were necessary to separate cells from subsequent readhesions. More than 95% of the adherent cells from sickle cell patients were non-ISC. The ISC that were adherent required lower shear stress for separation from the endothelium than the non-ISC.

When erythrocytes were suspended in autologous plasma instead of buffer and albumin, adherence increased as shown in Table 2. The increased retention by the endothelial surface secondary to plasma was most pronounced in the low and intermediate shear stress ranges. For the three sickle cell patients not in vaso-occlusive crisis, the mean adherence in plasma was the same as the adherence in buffer with albumin

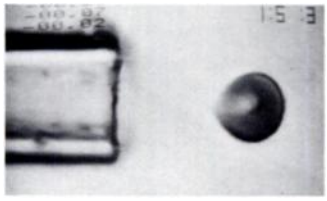

Fig 1. Photograph from a video monitor of an erythrocyte separating from a confluent endothelial monolayer in response to shear stress applied via a fluid-filled micropipette.

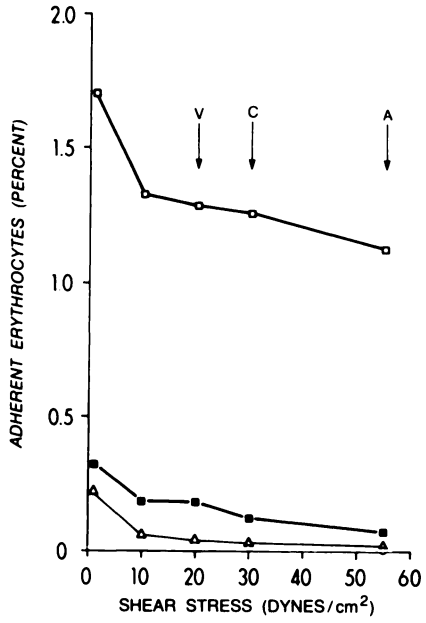

Fig 2. Mean percentage of erythrocytes suspended in isotonic buffer plus albumin that are adherent to cultured endothelial monolayers at various shear stresses. Key: V, maximum venule shear stress; C, maximum capillary shear stress; A, maximum arteriole shear stress; heavy solid line with open squares, hemoglobin S erythrocytes; heavy solid line with closed squares, sickle-thalassemia erythrocytes; light solid line, control erythrocytes.

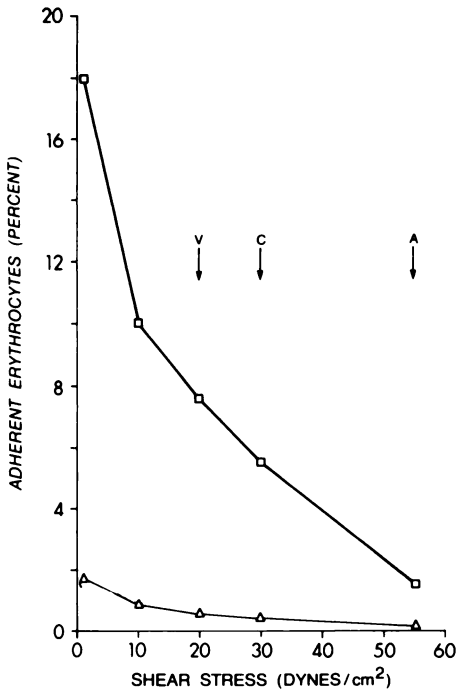

Fig 3. Percentage of erythrocytes suspended in autologous plasma that are adherent to cultured endothelial monolayers at various shear stresses. Key: heavy solid line, sickle crisis patient T.J.; light solid line, control patient B.S.

and resembled adherence of control cells in plasma. This implies that an intrinsic cellular abnormality promotes sickle cell endothelial adherence in buffer and albumin, and this abnormality is not normally influenced by plasma unless a vaso-occlusive crisis occurs. For patient T.J., who was in vaso-occlusive crisis, the presence of autologous plasma caused adherence to increase by more than two orders of magnitude. This is depicted in Fig 3, which is a representative experiment comparing cell adherence in a sickle patient with vaso-occlusive crisis with a control, each cell sample in autologous plasma. Addition of sickle crisis plasma to erythrocytes from a sickle patient not in crisis caused adherence of 12% at low shear stress with a progressive decrease to 1% adherence at maximum shear stress. When autologous plasma is incubated at 60 °C for one hour, it no longer augments adhesion. In studies of sickle and control cells, the presence of 600 mg/100 mL fibrinogen did not alter adherence. Since plasma pH might be expected to alter cell surfaces and plasma protein conformation, the effect of pH on erythrocyte adherence was studied. Variation of plasma or buffer pH over the range 6.5 to 8.0 did not influence retention of erythrocytes by the cultered cell surface (Table 3).

Adherence to surfaces beneath the endothelium exposed by potential breaches in the endothelial monlayer was studied. For control or sickle cells that were not washed, adherence to plastic or glass was nearly identical to adherence to endothelium. However, for washed cells there was a marked difference in adherence. Virtually 100% of washed cells resuspended in buffer and albumin became permanently adherent to glass or plastic surfaces, whereas adherence to endothelium was not affected by washing cells. This adherence was of such magnitude that the membrane remained attached even though the cell was destroyed by high shear force. When washed cells were reexposed to autologous plasma and suspended in buffer and albumin, only 3% demonstrated this irreversible adherence. These studies reemphasize the fact that the wash procedure may remove plasma protein from the erythrocyte surface and thus may contribute to behavior not expected of cells bathed in plasma proteins in the in vivo situation. Of interest, permanent adherence of washed cells to glass is eliminated by preexposing the glass or plastic to the supernatant from endothelial cultures. This suggests that potential breaches in the monolayer covering do not affect adherence characteristics and that endothelial cell products may play a role in modulating erythrocyte–endothelial cell interactions.

DISCUSSION

The key observation of the study is the demonstration that some sickle erythrocytes remain adherent to the cultured endothelial surface at shear stresses greater than those expected in the in vivo circulation at regions where obstructive phenomena occur. Since these experiments were performed in the absence of platelets, the observation supports the earlier interpretation[2] that, in addition to intrinsic rheologic abnormalities of non-ISC, their tendency to adhere to endothelium may contribute as well to initiation of obstruction. It is consistent with the conclusions of Kakukcelebi et al[17] in studies of umbilical cord vessels perfused with sickle cells. Based on these data, increased adhesion in the low-flow rate venous portion of the circulation would be likely, a suggestion that fits the concept of venous obstruction as either an actual one, the result of a rheologic plug of erythrocytes and platelets, or rheologically functional obstruction resulting from cell adherence to the venous wall and increased cell-to-cell interactions that increase resistance to flow. This also fits the prediction of Hochmuth et al[18] that erythrocyte–endothelial cell adherence would be most feasible in regions of intermittent low shear stress.

Table 3. Control Erythrocyte Adherence to Cultured Endothelium as a Function of pH at Various Shear Stresses

	Percent Adherence at Various Shear Stresses*				
pH	1 dyne/cm²	10 dyne/cm²	20 dyne/cm²	30 dyne/cm²	55 dyne/cm²
7.37	0.16	0.04	0.03	0.03	0.02
7.20	0.15	0.03	0.03	0.03	0.02
6.98	0.16	0.04	0.03	0.03	0.03
6.50	0.04	0.01	0.01	0.01	0.00

*Minimum of 1,000 cells observed for each shear stress.

Approximately 5% of sickle cell patients experience occlusive episodes involving the CNS[19]; these correlate with large-vessel arterial disease.[20] In the high-velocity portions of the circulation, increased frequency of sickle erythrocyte movement from the axial stream to slower movement in the plasma layer adjacent to the endothelium might offer opportunity for adhesion; however, vascular abnormalities augmenting such cell movement to the vessel surface and also the presence of platelets probably are necessary for initiation of thrombous formation.

An important observation is the enhancement of erythrocyte–endothelial cell adhesion by the presence of plasma; this is similar to the studies of Mohandas and Evans.[13] The greater influence of plasma from blood obtained during sickle crisis clearly supports the earlier conclusion of Hebbel et al[11] from in vitro studies of sickle cell adherence to cultured endothelial cells.

The specific basis for the abnormal retention of sickle cells by the cultured endothelial cells is not clear. Earlier suggestions that sickle cells were deficient in sialic acid, the principle determinant of surface charge, have not been confirmed,[21] and the suggestion that the triggering feature is an abnormal distribution of sialic acid on the membrane surface has not been sustained by experiments.[22] Thus, although the phenomenon must involve the erythrocyte's outer membrane surface, definition of a molecular mechanism to explain the avidity is not yet available. It is of interest that pH has no important effect, since pH change probably influences binding of plasma proteins to the cell surface. Attachment in these experiments occurs during the 30-minute incubation period when erythrocytes are resting on the endothelial surface, and during this interval in which the two membrane surfaces are very closely approximated, many biochemical processes may occur, the net result of which is to effect attachment. Although the initiating factors of the surface-to-surface interaction are not obvious, it is of interest that many adherent cells, after being separated from the endothelium, immediately reattach, suggesting that the significant phase of this interaction occurs very early in the contact of the cell surfaces or that the prior changes render the cells more liable to reattachment.

These studies may be interpreted to suggest that both the features of the sickle cell surface and plasma contribute to significant erythrocyte–endothelial cell adherence in sickle cell anemia. As reasoned by Hebbel et al[12] the plasma factors may be the modulator of adherence in the pathophysiologic process, since erythrocyte membrane surface characteristics would be presumed to be relatively constant after the reticulocyte stage. Reduced sickle cell deformability leading to relative obstruction or slowing of flow, membrane characteristics predisposing to adherence, and plasma factors may combine to produce the vascular phenomena typical of the disease. It seems probable that flow must be slowed sufficiently or local flow dynamics modified to produce opportunity for the sickle cell–endothelial cell contact leading to adhesion. This latter suggestion is supported by the observation of Burns et al[14] that vortex flow promotes significant erythrocyte–endothelial cell adherence for sickle cells.

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