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**Research Article**

Increased adherence of sickle erythrocytes to vascular endothelium has been suggested by Hebbel and his colleagues to play a role in vasocclusive events of sickle cell disease. To define the role of cell membrane changes and plasma factors in cell adherence, a micropipette technique previously developed by us to obtain a direct, quantitative measure of cell adherence was used to evaluate the adhesivity of different morphologic classes of sickle cells to endothelial cells in various suspending media. Irregularly shaped, deformable sickle cells were four- to fivefold more adherent than discoid sickle cells, whereas rigid irreversibly sickled cells were least adherent. Sickle erythrocytes adhered to endothelial cells when suspended in autologous citrated or heparinized plasma but were totally nonadherent when suspended in autologous EDTA plasma. Removal of the divalent cation chelator and addition of calcium to EDTA plasma restored its ability to promote adhesion, implying an absolute requirement for divalent cations in sickle cell adherence. Sickle cells also did not adhere to endothelial cells in protein-free media containing divalent cations, suggesting an additional requirement for plasma proteins. Removal of collagen-binding proteins from citrated sickle plasma resulted in a three- to fivefold reduction in its ability to promote cell adhesion, suggesting an important role for these plasma proteins in sickle cell adherence. The results of this study imply that sickle cell adherence […]



# Sickle Erythrocyte Adherence to Vascular Endothelium

## Morphologic Correlates and the Requirement for Divalent Cations and Collagen-binding Plasma Proteins

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## Abstract

**Increased adherence of sickle erythrocytes to vascular endothelium has been suggested by Hebbel and his colleagues to play a role in vasocclusive events of sickle cell disease. To define the role of cell membrane changes and plasma factors in cell adherence, a micropipette technique previously developed by us to obtain a direct, quantitative measure of cell adherence was used to evaluate the adhesivity of different morphologic classes of sickle cells to endothelial cells in various suspending media. Irregularly shaped, deformable sickle cells were four- to fivefold more adherent than discoid sickle cells, whereas rigid irreversibly sickled cells were least adherent. Sickle erythrocytes adhered to endothelial cells when suspended in autologous citrated or heparinized plasma but were totally nonadherent when suspended in autologous EDTA plasma. Removal of the divalent cation chelator and addition of calcium to EDTA plasma restored its ability to promote adhesion, implying an absolute requirement for divalent cations in sickle cell adherence. Sickle cells also did not adhere to endothelial cells in protein-free media containing divalent cations, suggesting an additional requirement for plasma proteins. Removal of collagen-binding proteins from citrated sickle plasma resulted in a three- to fivefold reduction in its ability to promote cell adhesion, suggesting an important role for these plasma proteins in sickle cell adherence. The results of this study imply that sickle cell adherence to vascular endothelium is a complex process in which temporal changes in the numbers of cells identified to be most adhesive and the plasma concentration of protein(s) involved in the adhesive process determine the extent of in vivo sickle cell adherence.**

## Introduction

Chronic hemolytic anemia and recurring painful crisis as a result of vasocclusion are the two most common hematologic manifestations of sickle cell disease. Although substantial progress has been made in our understanding of the pathobiology of sickle erythrocytes, the cellular basis of the pathology of the disease remains to be fully defined (1–8). For example, although the number of circulating undeformable, irreversibly sickle cells seems to determine the hemolytic component of the disease, it cannot predict either the onset or the frequency of vasocclusive events (2, 9). In searching for other cellular alterations, which in conjunction with increased rigidity might lead to vasocclusion, Hebbel and his colleagues (7, 10, 11) found that sickle erythrocytes have an increased tendency to adhere to vascular endothelial cells, and that this increased adherence can be correlated with the clinical severity of vasocclusive events. In contrast, using a different assay system to quantitate cell adhesion, Wilkinson et al. (12) were unable to find significant differences between the adherence of normal and sickle erythrocytes to endothelial cells. Burns et al. (13) also reported that under laminar flow conditions, sickle cells did not exhibit an increased tendency to adhere to vascular endothelial cells, although these authors did find that, under disturbed flow conditions (e.g., at bifurcation points in capillary beds), an increased number of sickle cells did adhere to endothelial cells. Thus, the hypothesis that increased cell adherence contributes importantly to the pathophysiology of sickle cell disease has not found total acceptance.

The markedly different forces acting on the erythrocytes during measurement of cell adhesion in the various experimental systems may in part account for these disparate results. Different suspending media used in the experiments may also contribute to the conflicting data. To quantitate erythrocyte adhesion to endothelial cells under controlled conditions, we recently developed a micropipette technique to obtain a direct, quantitative measure of the adherence of individual erythrocytes to vascular endothelial cells. Using this technique, we were able to show in preliminary studies that sickle erythrocytes do exhibit enhanced adhesivity to endothelial cells (14). To further define the role of cell membrane changes and plasma factors in cell adherence, we have used this micropipette technique to explore the adhesivity of different morphologic classes of sickle cells to endothelial cells in various suspending media. We have found that irregularly shaped, deformable sickle cells are four- to fivefold more adherent than discoid sickle cells and that both plasma proteins and divalent cations are essential for cell adherence.

## Methods

Blood obtained from patients with homozygous sickle cell disease and from normal volunteer donors was collected into either acid citrate dextrose or EDTA-containing vacutainers. Some of the sickle cell patients were hospitalized for treatment of pain crisis, while others were in steady state when the blood was obtained. Cell-free plasma for use as a suspending medium was obtained by centrifuging a portion of each blood sample at 2,000 *g* for 10 min, and then at 10,000 *g* for 15 min to remove platelets.

Human albumin and human fibrinogen were obtained from Calbiochem-Behring Corp., La Jolla, CA. IgG was prepared from sickle plasma by affinity chromatography using Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ). Normal and sickle plasma was depleted of collagen-binding proteins by mixing plasma samples with Affigel-Gelatin beads (Bio-Rad Laboratories, Richmond, CA) in a ratio of 1 to 2 by weight. After incubating the mixture for 30 min at 37°C, the plasma was separated from the beads by centrifugation.

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*Vascular endothelial cell cultures.* For these experiments, a confluent monolayer of bovine aortic endothelial cells was grown on microcarrier beads, cytodex-3 (Pharmacia Fine Chemicals). In brief, dry microcarriers were swollen and hydrated in calcium-free and magnesium-free phosphate-buffered saline (PBS) for 3 h at room temperature. After being washed twice with PBS, the beads were washed once with 70% ethanol and left in 70% ethanol overnight. The beads were then washed with PBS. To grow vascular endothelial cells, the cytodex-3 beads at a final density of 100 beads/cm$^2$ were placed in tissue culture dishes containing confluent and resting cultures of vascular endothelial cells derived from adult bovine aortic arch (15). Once the beads had settled on the apical cell surface of the endothelial cell layer, the cells adhered to the beads within 3 h. During the next 3 d, the beads were fully covered by a layer of endothelial cells. They were then detached from the cell monolayer by gentle washing and were transferred to 10 cm bacteriologic petri dishes containing 15 ml of Dulbecco's modified Eagle's medium (H16 obtained from Gibco Laboratories, Grand Island, NY) supplemented with 10% calf serum (Sterile Systems Co., Logan, UT), 50 μg/ml gentamicin, 0.25 μg/ml Fungizone, and 100 ng/ml of fibroblast growth factor (16).

*Measurement of erythrocyte-endothelial cell adhesion.* Measurement of erythrocyte adhesion to endothelial cells was made by a multimicromanipulator microscope system. The system is centered around an inverted microscope with several micromanipulators mounted directly to the microscope stage (E. Leitz, Inc., Rockleigh, NJ). For each test, erythrocytes and cultured endothelial cells on microcarrier beads were injected in very small concentrations into a microchamber (1 cm × 2 cm × 0.1 cm) on the microscope stage. Erythrocytes were aspirated and maneuvered by small glass suction micropipettes attached to micromanipulators. The pipettes were prepared from 1-mm glass capillaries pulled to a needle point and then broken by quick fracture to obtain flat tips that had diameters in the desired range of 1 to 2 μm. The pipettes were coupled by continuous water systems to micrometer-positioned water manometers for zero pressure gradient. All measurements were carried out at 37°C. The microscope was equipped with a long working distance (×40 [0.6 NA]) objective. Each micromechanical experiment was simultaneously recorded on videotape; the pertinent data (e.g., suction pressure and time) were superimposed on the recording by means of video multiplexing.

For the erythrocyte-endothelial cell adhesion assay, single erythrocytes were aspirated with a small suction micropipette and were maneuvered close to the endothelial surface (Fig. 1 *A*). The erythrocyte was then gently pushed against the endothelial cell (Fig. 1 *B*), released from the pipette, and the pipette was pulled back (Fig. 1 *C*). If the contact between the erythrocyte and the endothelial cell was stronger than the natural gravitational sedimentation force ($10^{-9}$–$10^{-8}$ dyne), the erythrocyte remained in contact with the endothelial cell and the erythrocyte was considered to be adherent. Nonadherent erythrocytes slowly fell away from the endothelial cell surface. The nature of the adhesive contact between the cells was determined by reaspiration of the adherent erythrocyte in contact with the endothelial cell, followed by stepwise separation of the erythrocyte from the endothelial cell surface.

## Results

*Types of adhesion.* Three types of adhesive contact between erythrocytes and endothelial cells were noted during detachment of adherent erythrocytes from the endothelial surfaces and these are illustrated in Fig. 2. In type 1, the erythrocytes remained in contact with the endothelial cell surface without forming obvious areas of strong adhesive contact. These erythrocytes could be detached from the endothelial cell surface without disjoining any apparent adhesive site (Fig. 2 *A*). In type 2, the erythrocytes adhered to the endothelial surface by one small focus of strong adhesive contact. To detach these erythrocytes, it was necessary to break a single submicroscopic area of adhesive contact between the two cell surfaces (Fig. 2 *B*). During retraction of the pipette, a membrane filament was generated from the erythrocyte to the endothelial cell surface and complete detachment of the erythrocyte occurred when this filament broke. In type 3, erythrocytes adhered to the endothelial surface over large observable areas. Detachment of the erythrocyte involved disjoining a large region of contact between the two cell surfaces (Fig. 2 *C*). During retraction of the pipette, the erythrocyte membrane peeled away from the endothelial surface. When these erythrocytes were brought into contact with the endothelial cell, they readily spread over the endothelial surface and adhered over large areas. Since this type of strong attachment could potentially be the most deleterious in terms of causing vasocclusion, we quantitated the percentage of various erythrocyte populations that could form this type of adhesive contact in all subsequent experiments.

Note that during these experiments, the nature and strength of adhesive contact between the erythrocytes and the endothelial surface was quantitated by applying forces in a direction normal to the endothelial surface (Fig. 3 *A*). As such, adherent erythrocytes were removed essentially by "peeling" the erythrocytes off the endothelial cell surface in a normal direction. The detachment process did not entail generation of large extensional deformations of the erythrocyte membrane to remove the cell from the endothelial surface. Because adherent erythrocytes are primarily removed by application of fluid shear forces in a di-

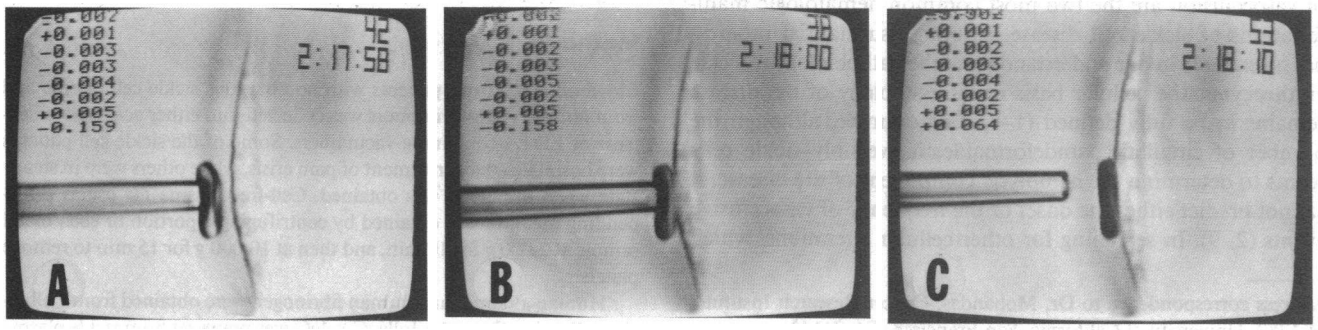


*Figure 1.* A sequence of videomicrographs showing various stages in the micromanipulation procedure used to determine erythrocyte adhesiveness to a vascular endothelial cell. Here, a normal erythrocyte with a small portion of its membrane aspirated into the micropipette is maneuvered into the proximity of a cultured endothelial cell on a microcarrier bead (*A*). The erythrocyte is then gently pushed against the endothelial cell and held in that position for a few seconds (*B*), after which it is released from the pipette by zeroing the suction pressure. The pipette is then gently pulled back (*C*). Adherent erythrocytes remain in contact with the endothelial cell surface indefinitely while nonadherent cells slowly settle away from the plane of focus.

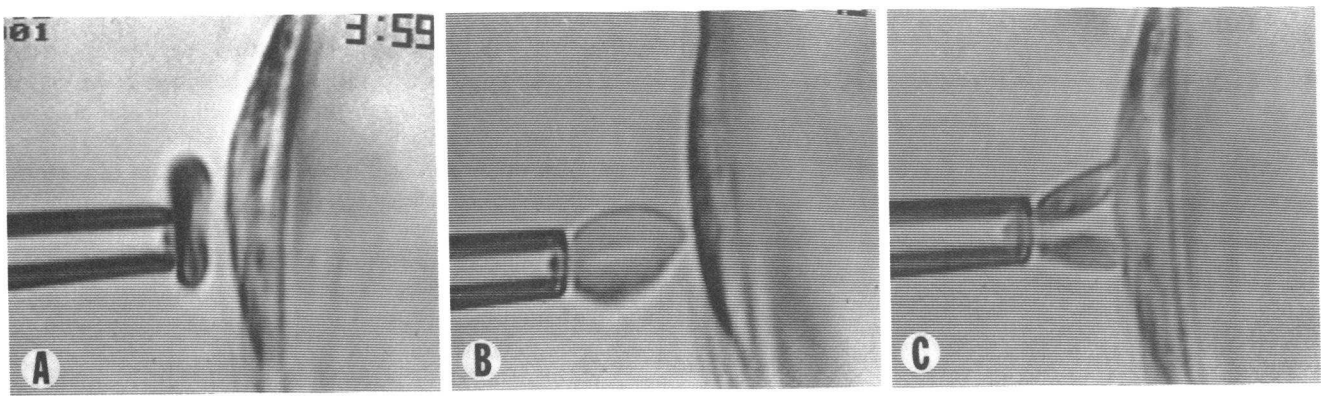


*Figure 2.* Videomicrographs showing the three types of adhesive contact between erythrocytes and endothelial cells observed during detachment of adherent erythrocytes from the endothelial surface. In type 1, the erythrocyte remained in contact with the endothelial cell surface without forming areas of adhesive contact and could be detached from the endothelial cell without having to break any apparent areas of strong adhesive contact (*A*). In type 2, the erythrocytes adhered to the endothelial surface by one small focus of strong adhesive contact and these erythrocytes had to be detached by breaking a single submicroscopic area of adhesive contact between the two cell surfaces (*B*). In type 3, erythrocytes adhered to the endothelial surface over large areas and erythrocyte detachment involved disjoining a large region of contact between two cell surfaces (*C*).

rection tangential to the endothelial surface in the circulation, we attempted to evaluate the magnitude of applied forces necessary for cell detachment in this mode (Fig. 3 *B*). Here, the forces applied in a direction tangential to the surface were as much as 100 times greater than the forces required to detach erythrocytes from the endothelial surface in the normal direction. Thus, if adherent cells were stiff and undeformable so that they could not be "peeled up" by the fluid shear forces in the circulation, very large forces would be necessary to "slide" the cells off the endothelium.

*Cell morphology and cell adhesion.* Data on adhesion of normal and sickle erythrocytes to endothelial cells are shown in Table I. When suspended in autologous citrated plasma, 8–10% of normal erythrocytes adhered strongly to endothelial cells. By comparison, in autologous citrated plasma, 20–40% of sickle erythrocytes from four individuals with homozygous sickle cell disease were strongly adherent, confirming earlier findings that sickle erythrocytes do indeed have an increased tendency to adhere to vascular endothelium. During this study we also found that adhesion-promoting activity of cell-free plasma prepared from either normal or sickle blood did not change during 4 d of storage at 4°C.

In our first study (14), we reported that ~20% of normal and 80–90% of sickle erythrocytes adhered to endothelial cells when suspended in autologous citrated plasma. The reason for the lower values reported in the present study is related to our

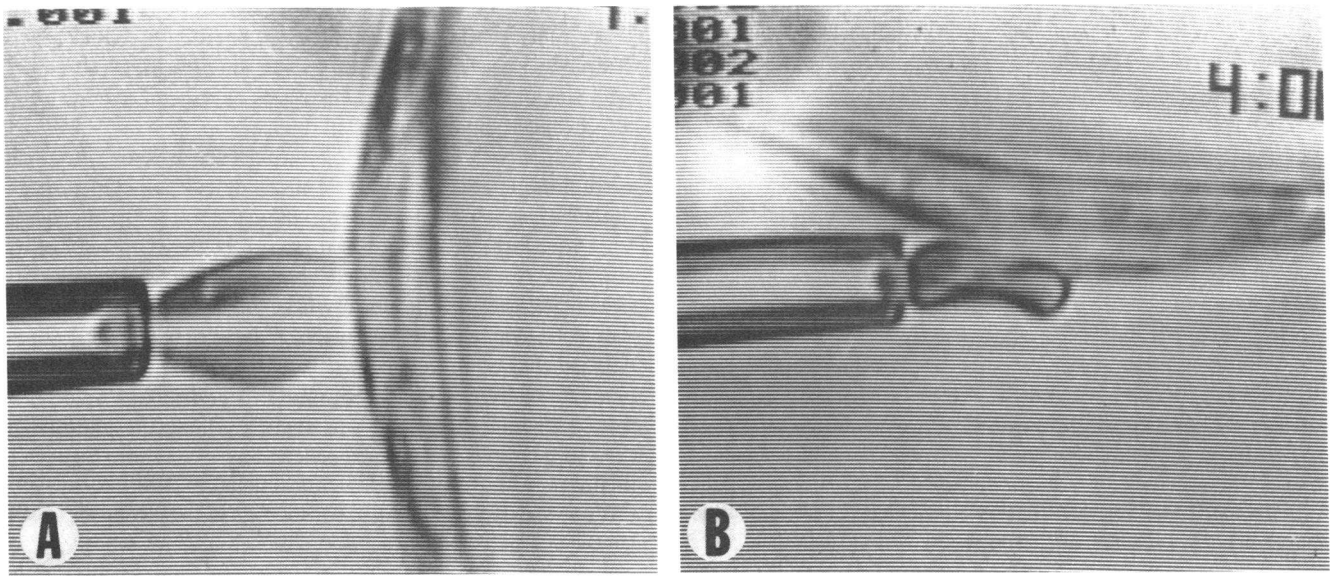


*Figure 3.* Videomicrographs showing detachment of adherent erythrocytes from the endothelial surface with forces applied in a direction normal to the endothelial surface (*A*) and in a direction tangential to the endothelial surface (*B*). Application of the detachment force in a normal direction results in "peeling" the erythrocyte off the endothelial surface, while application of force in a tangential direction results in "sliding" the erythrocyte off the endothelial surface. Forces necessary to detach erythrocytes from the endothelial surface, when applied in a direction tangential to the surface, are as much as 100 times greater than those necessary when applied in a normal direction.

*Table I. Adhesion of Normal and Sickle Erythrocytes to Vascular Endothelial Cells*

| | Total No. of cells studied | No. of adherent cells | Percent of total cells adhering |
|---|---|---|---|
| Normal cells | | | |
| Donor 1 | 250 | 19 | 8 |
| Donor 2 | 140 | 14 | 10 |
| Sickle cells | | | |
| Patient 1 | 166 | 38 | 23 |
| Patient 2 | 40 | 11 | 28 |
| Patient 3 | 60 | 23 | 38 |
| Patient 4 | 60 | 25 | 42 |

current ability to distinguish between the different kinds of adhesive contact between erythrocytes and the endothelial cell surface. While in this study only erythrocytes that were strongly adherent to the endothelial surface (type 3 adhesion) were quantitated, in our previous study weakly adherent (type 1 and 2 adhesion) as well as strongly adherent (type 3) erythrocytes were quantitated. We confined our attention to strongly adherent erythrocytes in the current study for two reasons. First, weakly adherent erythrocytes (type 1 and 2) can be pulled away from the endothelial surface with minimal detachment force, since little contact exists between the two surfaces. Hence this type of adhesion would not be expected to cause vasocclusion under prevailing flow conditions in the microcirculation. On the other hand, strongly adherent erythrocytes can only be detached by peeling away large areas of adhesive contact, which entails generation of large tangential forces at the sites of attachment in the circulation. Thus, strongly adherent erythrocytes have a much greater potential for causing vasocclusion. Second, erythrocytes attach to a number of different surfaces with a single point attachment and hence such contact cannot be viewed as a specific interaction between erythrocytes and the endothelial surface. The spreading of erythrocytes on the endothelial surface that occurs during type 3 adhesion, on the other hand, is not seen on other surfaces and hence is viewed as a specific interaction between these two surfaces.

Interestingly, we found that the adhesion of normal erythrocytes to endothelial cells was not significantly different when suspended in citrated normal plasma or in citrated sickle plasma (i.e., adherence of normal cells was 10% in normal plasma and 8% in sickle plasma); whereas adhesion of sickle erythrocytes was marked in citrated sickle plasma (42%). This finding suggests that membrane changes in sickle erythrocytes may be an important factor in their increased adherence. To define possible membrane changes in sickle erythrocytes responsible for their increased adherence, we classified these cells into three groups on the basis of their morphologic appearance and measured the adhesion to vascular endothelium for each group of sickle cells. The three morphologic groups identified were (*a*) discoid cells, (*b*) irregular discs, and (*c*) irreversibly sickled cells. Fig. 4 illustrates the morphology of these three groups of cells. Discoid sickle cells were indistinguishable from discoid normal cells and had smooth surface contours and normal deformability. In contrast, both irregular discs and irreversibly sickled cells had irregular surface contours and could be easily distinguished from discoid sickle cells. The cellular characteristic that distinguished irregular discs from irreversibly sickled cells was their shape: the irregular discs maintained their discoid shape, whereas irreversibly sickled cells acquired an elongated elliptocytic shape. Irregular discs appeared to have near normal deformability as judged by the small negative suction pressures needed to aspirate them into micropipettes and by their ability to rapidly recover their unstressed shape upon cessation of applied forces. In contrast, irreversibly sickled cells had markedly decreased deformability. Substantially higher suction pressures were needed to aspirate these cells into micropipettes. In addition, during the micromanipulation procedure it was observed that these cells were unable to undergo the deformations necessary to make intimate contact with endothelial cells.

We determined the percentages of these three morphologic groups of sickle cells in whole blood obtained from six patients. Discoid sickle cells constituted between 4 and 25%; irregular discs, between 48 and 60%; and irreversibly sickled cells, between 16 and 45% of the total circulating erythrocytes. When blood from two of these patients was separated on discontinuous Stractan density gradients, we found the discoid sickle cells present predominantly in the low density regions of the gradient (density range, 1.0785–1.0919 g/ml), whereas irreversibly sickled cells were found in the high density region of the gradient (density, >1.1149 g/ml). On the other hand, irregular discs were found to be distributed over a large range of densities (density range, 1.0785–1.1149 g/ml). The high density cell subpopulation enriched in irreversibly sickled cells contained only a small fraction of irregular discs.

The data on adhesion of the three morphologic groups of sickle cells to endothelial cells are shown in Table II. Four- to fivefold more irregular discoid cells adhered to endothelial cells compared with discoid cells. Moreover, in comparison to irregular discoid cells, a significantly smaller proportion of irreversibly sickled cells were found to be adherent. Again, it was evident that the increased rigidity of irreversibly sickled cells prevented them from forming uniform contact with the relatively flat endothelial surface. The data indicate that a subpopulation of sickle cells with normal deformability but altered membrane characteristics (as evidenced by changes in cell shape), are substantially more adhesive than the rest of the cell population. Although the data shown here are for erythrocytes from only three individuals with sickle cell disease, similar results have been obtained with all sickle cell blood samples studied.

*Plasma factors and cell adhesion.* Having shown that cell membrane changes play a role in sickle cell adherence to endothelial cells, we then began to explore the role of plasma factors in promoting cell adhesion. To maximize the sensitivity of the adhesion assay, we deliberately chose the group 2, irregular discoid sickle cells, as the test erythrocytes in all experiments designed to evaluate the role of plasma factors. Table III shows data on the requirement for divalent cations in sickle cell adherence to vascular endothelium. 88% of irregular discoid cells from a patient with sickle cell anemia adhered to endothelial cells when autologous citrated plasma was used as the suspending medium. Addition of the calcium chelator EDTA (5 mM) to this citrated plasma totally abolished its ability to promote adherence of sickle cells to endothelium. Similarly, when autologous EDTA plasma was used as the suspending medium, none of the irregular discs adhered to endothelial cells. When this EDTA plasma was dialyzed overnight against Hank's buffered saline solution containing 2.5 mM $Ca^{++}$, 2.5 mM $Mg^{++}$, and heparin, and then used as the suspending medium, sickle cell adherence was restored to values very similar to those observed

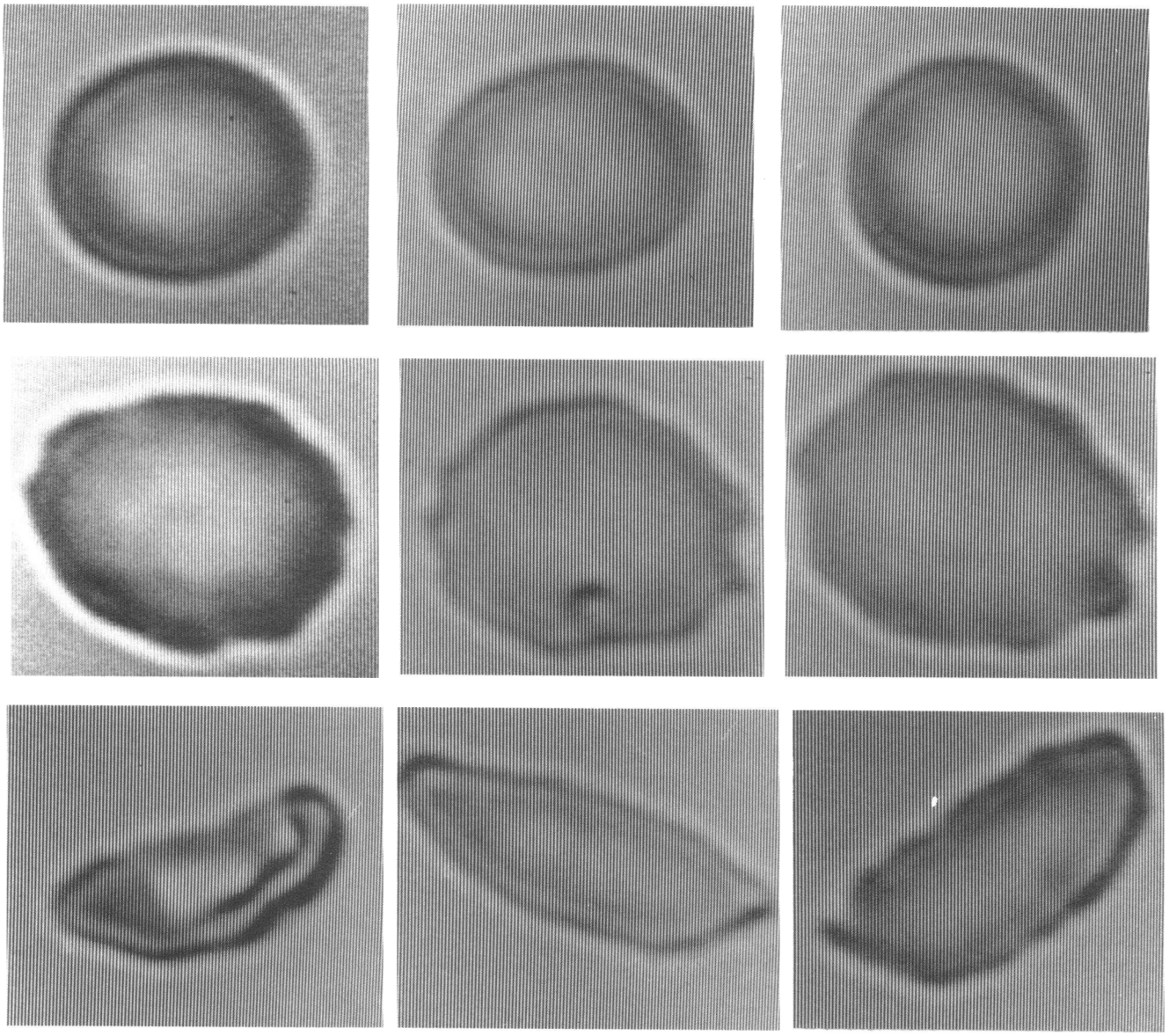

*Figure 4.* Videomicrographs illustrating the morphology of discoid sickle cells (*top*), irregular discoid sickle cells (*middle*), and irreversibly sickled cells (*bottom*). Discoid sickle cells have smooth surface contours, while irregular discoid sickle cells and irreversibly sickled cells have irregular surface contours. In addition, the irreversibly sickled cells are elongated and have elliptocytic morphology.

in autologous citrated plasma. Identical results were obtained in experiments with plasma samples from three other individuals with sickle cell anemia. These data imply a requirement for divalent cations in the suspending medium to promote sickle cell adherence.

To determine if other plasma factors besides divalent cations are required for sickle cell adherence, we measured cell adhesion in protein-free suspending media and suspending media reconstituted with purified plasma proteins. The data from these experiments are shown in Table IV. A population of group 2 cells (which showed marked adherence to endothelial cells when suspended in autologous citrated plasma ~88%) was found to be totally nonadherent (0%) when Hank's buffered saline solution was used as the suspending medium. Addition of 2.5 mM $Ca^{++}$ and 2.5 mM $Mg^{++}$ to Hank's buffered saline solution did not restore its ability to promote cell adhesion, implying that other plasma factors besides divalent cations are also necessary for sickle cell adherence. Direct confirmation for the requirement of additional plasma factors was obtained with our single cell transfer experiments. In these experiments, the discriminatory test of adherence of a selected sickle cell to endothelial cells in two different suspending media was performed by transferring the erythrocyte from a chamber filled with one suspending medium to another chamber filled with a different suspending medium. The micromanipulation procedure also allowed us to retransfer the cell back to the chamber with the first suspending medium to reaffirm the original adhesive characteristics. Using this procedure, we found that sickle cells that adhered strongly to endothelial cells in citrated plasma were totally nonadherent when transferred into Hank's buffered saline solution with calcium and magnesium. When retransferred back into citrated plasma, the sickle cells once again adhered strongly to endothelial cells. These single-cell transfer experiments provided strong direct evidence for the requirement for plasma proteins in sickle cell adherence.

Efforts to identify the plasma protein or proteins responsible

*Table II. Adhesion of Different Morphological Groups of Sickle Cells to Vascular Endothelial Cells*

| | Total No. of cells studied | No. of adherent cells | Percent of total cells adhering |
|---|---|---|---|
| Patient 1 | | | |
| Whole blood | 166 | 38 | 23 |
| Discoid cells | 53 | 8 | 15 |
| Irregular deformable discs | 52 | 26 | 50 |
| ISC | 61 | 4 | 7 |
| Patient 2 | | | |
| Whole blood | 60 | 25 | 42 |
| Discoid cells | 30 | 5 | 17 |
| Irregular deformable discs | 20 | 16 | 80 |
| ISC | 10 | 4 | 40 |
| Patient 3 | | | |
| Whole blood | 40 | 11 | 28 |
| Discoid cells | 8 | 1 | 12 |
| Irregular deformable discs | 17 | 10 | 59 |
| ISC | 15 | 0 | 0 |

for cell adhesion involved measurement of sickle cell adherence in buffered saline media reconstituted with purified plasma proteins. As shown in Table IV, irregular discoid sickle cells did not adhere to endothelial cells in buffered saline media containing albumin, fibrinogen, or immunoglobulin at concentrations normally present in plasma. Also, in three different experiments the adhesion of irregular discoid sickle cells to endothelial cells decreased from 88% to 0%, 50% to 4%, and 59% to 5% when autologous citrated sickle plasma was replaced by citrated normal plasma as the suspending medium. These data indicate that neither albumin, fibrinogen, nor immunoglobulin play a major role in sickle cell adherence to endothelial cells and that the adhesion-promoting protein or proteins are present at much higher levels in sickle plasma than in normal plasma.

A clue to the nature of the plasma protein (or proteins) responsible for cell adhesion was obtained when sickle plasma from which collagen-binding proteins were removed by treatment with Affigel-Gelatin was used as the suspending medium. As shown in Table V, when citrated sickle plasma pretreated with Affigel-Gelatin for 30 min at 37°C was used as the suspending medium, the percentage of sickle cells adhering to endothelial cells was greatly decreased. A three- to fivefold decrease in sickle cell adhesion was noted upon removal of collagen-binding proteins from sickle plasma, suggesting a role for these proteins in cell adhesion. The identity of the specific collagen-binding protein responsible for sickle adherence remains to be defined.

*Table III. Requirement for Divalent Cations for Sickle Cell Adherence to Vascular Endothelial Cells*

| | Total No. of cells studied | No. of adherent cells | Percent of total cells adhering |
|---|---|---|---|
| Autologous citrated plasma | 40 | 35 | 88 |
| Autologous citrated plasma + EGTA | 40 | 0 | 0 |
| Autologous EDTA plasma | 40 | 0 | 0 |
| EDTA plasma dialyzed against $Ca^{++}$, $Mg^{++}$, and heparin | 40 | 30 | 75 |

## Discussion

Data obtained during this study has enabled us to establish clearly that both erythrocyte membrane changes and plasma factors play a crucial role in determining the nature and extent of adhesive contact between sickle erythrocytes and vascular endothelial cells. We have found that membrane changes, as manifested by acquisition of an irregular discoid shape, produced an erythrocyte surface that was able to form strong adhesive contact with endothelial surface. In addition, we have shown that divalent cations and collagen-binding plasma proteins are essential to generate strong erythrocyte-endothelial cell interactions, and in the absence of either of these plasma components the interactions are weak.

As the adherent characteristic of erythrocytes was evaluated on a cell-by-cell basis in the micromechanical technique used, it was possible to determine directly the shape and deformability characteristics of the most adherent population of sickle cells. In all the sickle blood samples studied, a population of cells characterized by irregular discoid shape and near-normal deformability was found to be the most adherent. Once these cellular characteristics were defined, it was easy to identify this population of cells in whole blood. Although the manifestation of irregular discoid shape by this population suggests altered membrane characteristics, the actual membrane changes responsible for changes in cell shape or increased tendency to adhere have not as yet been defined. Interestingly, irreversibly sickled cells, which have previously been shown to exhibit deranged membrane organization, were not very adherent (17). The increased rigidity of these cells prevented them from undergoing the marked membrane deformations necessary for forming a uniform contact with the relatively flat endothelial surface. Thus, it seems that although cell surface changes responsible for increased adherence may be exhibited by irreversibly sickled cells, additional changes such as cellular dehydration and increased membrane rigidity prevent them from forming strong adhesive contacts. The implication of these findings is that even though irreversibly sickle cells, by virtue of their decreased deformability and increased polymer content at reduced oxygen tensions, may account for increased erythrocyte destruction, their role in causing vasocclusion through adherence to endothelium may be very limited. They further imply that a subpopulation of sickle cells, which by virtue of their normal deformability properties would not be expected to cause vasocclusion, may indeed do so through their increased ability to adhere to vascular endothelium.

The data defining the requirement for the presence of plasma factors for sickle cells to adhere to vascular endothelial cells point out that in understanding the rheological basis for sickle cell disease, in addition to hemoglobin polymerization-induced erythrocyte changes, we need to consider changes in plasma fac-

*Table IV. Requirement for Plasma Factors for Sickle Cell Adherence to Vascular Endothelial Cells*

| | Total No. of cells studied | No. of adherent cells | Percent of total cells adhering |
|---|---|---|---|
| Autologous citrated plasma | 40 | 35 | 88 |
| Hanks buffered saline | 60 | 0 | 0 |
| Hanks buffered saline + 2.5 mM $Ca^{++}$ and 2.5 mM $Mg^{++}$ | 60 | 0 | 0 |
| Hank's buffered saline with $Ca^{++}$ and $Mg^{++}$ + 40 mg/ml human albumin | 60 | 0 | 0 |
| Hank's buffered saline with $Ca^{++}$ and $Mg^{++}$ + 5.0 mg/ml human fibrinogen | 60 | 0 | 0 |
| Hank's buffered saline with $Ca^{++}$ and $Mg^{++}$ + 10 mg/ml human IgG | 60 | 0 | 0 |

tors as well. None of the hundreds of sickle erythrocytes studied adhered to endothelial cells when suspended in EDTA plasma, implying an absolute requirement for divalent cations. The finding that the addition of calcium-specific chelator EGTA can totally abolish the adhesion-promoting activity of plasma that previously promoted adherence suggests that calcium may be the important divalent cation. The finding that both heparinized and citrated plasma promote strong sickle cell adherence while EDTA plasma promotes none further indicates that only small concentrations of calcium are required for cell-cell interactions, since free ionized calcium concentrations of heparinized plasma, citrated plasma, and EGTA plasma are ~2.5 mM, ~25 $\mu$M, and ~15 nM, respectively.

In addition to divalent cations, an absolute requirement also exists for plasma protein(s) since in protein-free suspending medium with calcium and magnesium, sickle cells did not adhere to endothelial cells. Addition of albumin, IgG, and fibrinogen to suspending medium at the highest concentrations found in plasma did not restore adhesion, implying that these plasma proteins are not involved in the process. Moreover, the adhesion-promoting protein(s) seems to be present in significantly higher concentrations in sickle cell plasma compared with normal plasma, since sickle cells were much more adherent in sickle plasma.

Although the identity of adhesion-promoting plasma protein(s) has not been resolved in this study, the finding that removal of collagen-binding protein(s) from sickle plasma effectively reduces its adhesion-promoting activity suggests that this protein(s) is characterized by its ability to bind to collagen. The major collagen-binding plasma protein is fibronectin, and, because of its established involvement in cell-cell interactions in other systems, its role in promoting sickle cell adherence deserves serious consideration. However, our preliminary data, which show that the addition of purified fibronectin isolated from sickle plasma does not induce sickle cell adherence, suggest that fibronectin may not be the key plasma component. Further studies are needed to define the nature of collagen-binding protein(s) responsible for sickle cell adherence to vascular endothelium.

*Table V. Effect of Collagen-binding Proteins on Sickle Cell Adherence to Vascular Endothelial Cells*

| | Total No. of cells studied | No. of adherent cells | Percent of total cells adhering |
|---|---|---|---|
| Patient 1 | | | |
| Citrated plasma | 150 | 76 | 50 |
| Affi-gelatin treated citrated plasma | 60 | 11 | 18 |
| Patient 2 | | | |
| Citrated plasma | 40 | 30 | 75 |
| Affi-gelatin treated citrated plasma | 40 | 9 | 23 |
| Patient 3 | | | |
| Citrated plasma | 40 | 33 | 83 |
| Affi-gelatin treated citrated plasma | 40 | 5 | 13 |

The micromechanical technique used during this study to assay adhesion of single erythrocytes to endothelial cells offered several insights into the adhesion process, which previously used techniques were unable to document (10, 12, 13). For example, only sickle cells that spread over the endothelial cell surface and form multiple areas of strong adhesive contact have the potential to create serious problems in the microcirculation. Previously used methods could only determine if a cell adhered or not, but could not distinguish between various types of adhesive contact. The micromechanical technique we used not only enabled us to document various types of adhesive contact between sickle cells and endothelial cells, but also allowed us to quantitate the fraction of sickle cells that formed strong adhesive contact. In addition, it permitted us to study the adhesion of the same sickle cell to endothelial cells in two different suspending media by single-cell transfer, thus enabling us to obtain strong, direct, and unequivocal evidence regarding influence of plasma factors on sickle cell adherence.

The micromechanical technique also allowed us to document two other important factors that might influence the adherence and detachment of sickle cells in the microcirculation: first, the impinging force necessary to induce initial intimate contact between the erythrocyte and the endothelial cell to produce adherence, and second, the detachment force necessary to remove an adherent cell from the endothelial surface. For the erythrocyte to adhere, it must come into close contact with the endothelial surface. It was observed during the present study that sufficiently large forces need to be applied to the erythrocyte to achieve this type of contact. In the micromechanical method, as the erythrocyte is physically pushed against the endothelial surface, sufficient forces are made to act on the cell so as to permit it to come into close contact with the endothelial surface. In contrast, in previous studies, the erythrocytes were allowed to settle onto

a monolayer of endothelial cells and the force bringing the two cell surfaces together was natural gravitational force, which is quite small by comparison ($\sim 10^{-8}$ dynes). This might explain in part why a much smaller fraction of sickle cells were found to be adherent in previous reports (10, 18). The requirement for a sufficiently large impinging force for strong cell-cell interaction implies that sickle cells can adhere to vascular endothelium only in areas of circulation where the two cells come into intimate contact (e.g., bifurcation points in the microcirculation) (4, 13).

As long as adherent erythrocytes remain deformable, they can be detached from the endothelial surface by the fluid forces in the microcirculation, and the consequences of cell adhesion to vasocclusion will be minimal. If, however, the deformable adherent sickle cells become deoxygenated before they can be detached, they will lose their ability to deform. As shown in the present study, very large forces would be required to "strip" these undeformable cells from the endothelial surface; adherent cells that cannot be detached by the fluid forces in circulation will have the potential to cause vasocclusion.

The results of this study imply that sickle cell adherence to vascular endothelium is a complex process. Efforts to relate adhesion to vasocclusive events need to take into account multiple factors that regulate this interaction. Both temporal changes in the number of cells identified to be most adhesive, and plasma concentration of protein(s) involved in the adhesive process will determine the extent of in vivo sickle cell adherence. Our finding of an important role for plasma proteins in sickle cell adherence supports Hebbel's (7) earlier suggestion that variations in plasma factors may play a major role in the onset of vasocclusion and painful crisis. In this context, the role of elevation of various coagulation factor activities (which have been reported to occur during sickle crisis) in promoting sickle cell adherence and vasocclusion deserves careful attention (19, 20). Moreover, the finding that a significant impingement force is necessary to induce strong sickle cell adherence to vascular endothelium implies that this interaction can occur only in certain areas of the microcirculation.

In conclusion, we have shown that sickle erythrocytes exhibit increased adherence to endothelial cells and that this increase is mediated by cellular and plasma factors. Although it is reasonable to suggest that increased erythrocyte adherence may play an important role in vasocclusion, to imply a direct role for it in the pathophysiology of sickle cell disease will require a complete understanding of the factors regulating cell-cell interactions. The results of the present study constitute a beginning in our understanding of the complex process of erythrocyte-endothelial cell interactions.

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