Sickle Erythrocytes Inhibit Human Endothelial Cell DNA Synthesis

By Robert Weinstein, Meng-ai Zhou, A. Bartlett-Pandite, and Karen Wenc

Patients with sickle cell anemia experience severe vascular occlusive phenomena including acute pain crisis and cerebral infarction. Obstruction occurs at both the microvascular and the arterial level, and the clinical presentation of vascular events is heterogeneous, suggesting a complex etiology. Interaction between sickle erythrocytes and the endothelium may contribute to vascular occlusion due to alteration of endothelial function. To investigate this hypothesis, human vascular endothelial cells were overlaid with sickle or normal erythrocytes and stimulated to synthesize DNA. The erythrocytes were sedimented onto replicate monolayers by centrifugation for 10 minutes at 17 g to insure contact with the endothelial cells. Incorporation of \(^{3}H\)-thymidine into endothelial cell DNA was markedly inhibited during contact with sickle erythrocytes. This inhibitory effect was enhanced more than twofold when autologous sickle plasma was present during endothelial cell labeling. Normal erythrocytes, with or without autologous plasma, had a modest effect on endothelial cell DNA synthesis.

Vascular occlusive phenomena in patients with sickle cell anemia are manifested by a heterogeneous group of clinical events including pain crisis and stroke. Recent data indicate that interaction between sickle erythrocytes and the vascular endothelium may play a role in the pathogenesis of these vascular occlusive phenomena. Sickle erythrocytes adhere tenaciously to human, bovine, and rat vascular endothelial cells, and the adherence of sickle erythrocytes to human endothelial cells in vitro has been directly correlated with the clinical severity of vascular occlusive morbidity in individual patients. It has been suggested that adherence of sickle erythrocytes to microvascular endothelium in vivo may alter flow dynamics so as to facilitate trapping of dense, nondeformable sickle cells, leading to vessel obstruction, local hypoxia, and propagation of vessel occlusion. Primary trapping of rigid, nondeformable sickle erythrocytes at precapillary sphincters may also initiate a process of small vessel occlusion.

Alteration of endothelial function, or frank endothelial damage, may also be involved in the pathogenesis of vascular occlusive morbidity in sickle cell anemia. Morphologic changes consistent with endothelial injury have been described in the arterial and/or arteriolar circulation of the spleen and brain of sickle cell patients. Increased circulating endothelial cells have recently been detected in the blood of sickle cell patients studied during painful crisis. Whether these endothelial changes are a direct result of erythrocyte-endothelial interaction or are secondary to vascular occlusion and tissue hypoxia is currently not known. However, exposure of human vascular endothelial cells to sickle erythrocytes in vitro results in increased prostacyclin production by the endothelial monolayers, indicating that sickle erythrocytes can have a direct metabolic effect on the endothelial cell. This is potentially significant, because sickle erythrocytes are more adherent to damaged endothelial cells, and an area of damaged or dysfunctional endothelium may provide a nidus for development of occlusive lesions in large vessels.

The purpose of this study was to further test the hypothesis that interaction with sickle erythrocytes results in altered endothelial cell function. We characterize here the inhibition of human endothelial cell DNA synthesis by sickle erythrocytes, and the relationship of this inhibition to adherence of the erythrocytes to the endothelial monolayers. Endothelial cell DNA synthesis is an early event in repair of injured endothelium in vivo. Hence, inhibition of DNA synthesis may be a factor in development of vascular lesions in patients with sickle cell anemia.

MATERIALS AND METHODS

Vascular specimens, received from the National Disease Research Interchange, Philadelphia, PA, were used for harvesting of human endothelial cells from iliac artery and thoracic aorta as previously described. Primary cultures of human umbilical vein endothelial cells were generously supplied by Dr Michael Gimbrone, Boston, MA. All stock cultures were maintained as previously described. Human plasma fibronectin was prepared according to established procedures. Tissue culture grade endothelial cell growth factor (ECGF) was prepared from bovine brain as previously described. Culture medium 199 (M199), Dulbecco's Modified Eagle's Medium (DME), and Dulbecco's Phosphate Buffered Saline (DPBS) were from Gibco, Grand Island, NY. Fetal bovine serum (FBS) was from Sterile Systems, Logan, UT. Bovine serum albumin (BSA),
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In each experiment, ³H-thymidine labeling of endothelial cells was performed in the presence of BSA/DME with and without 50% (vol/vol) AA or SS plasma, in the presence and absence of AA or SS erythrocytes. To permit comparison among multiple experiments, DNA synthesis results are normalized, with 100% of maximal ³H-thymidine incorporation representing the counts incorporated in BSA/DME in the absence of erythrocytes. AA and SS plasma yielded similar results (P = .686, 49 determinations), and caused no inhibition of DNA synthesis by themselves (P = .619 v BSA/DME for AA plasma; P = .626 v BSA/DME for SS plasma) in the absence of erythrocytes. Therefore, experimental results are expressed in terms of percent of maximal counts incorporated by endothelial cells when erythrocytes were present, relative to counts incorporated when erythrocytes were not present, in a given incubation medium.

Erythrocyte-endothelial cell adherence assay. Human vascular endothelial cells were seeded at 3.5 to 4.0 x 10⁴/cm² in 24-well cluster plates in M199 containing 10% (vol/vol) FBS, heparin (100 µg/mL), and ECGF (150 µg/mL). Cultures were confluent within 72 to 96 hours, at which time they were used. For assay, the growth medium was removed by aspiration. The monolayers were washed with DME, and covered with BSA/DME, or BSA/DME containing 50% (vol/vol) AA plasma or SS plasma. Aliquots of AA or SS erythrocytes in BSA/DME were pipetted into triplicate wells to hemocrit of 5%, and sedimented onto the endothelial cell monolayers by centrifugation of the 24-well plates at 17g for 10 minutes at room temperature. Whether or not erythrocytes were added to the wells, the final volume was 0.5 mL. The cultures were incubated for 1 minute, 10 minutes, 1 hour, or 6 hours at 37°C in 5% CO₂ in humid air.

After incubation, the wells were filled to the top with BSA/DME and the plates were sealed with paraffilm, carefully inverted, and centrifuged at 4g for 5 minutes at room temperature. By this technique, a removal force of 10⁻⁴ dynes of relative centrifugal force was applied at the surface of the inverted endothelial monolayers. Mohandas and Evans have demonstrated that a force of this magnitude distinguishes strongly adherent SS erythrocytes, with a median density of approximately 1.1 g/mL, from nonadherent erythrocytes.16,27

Following centrifugation, the plates were uncovered while still inverted, and the media and nonadherent erythrocytes were drained out of the wells. Residual liquid media were removed by careful blotting and aspiration. The adherent erythrocytes were lysed by extracting the cultures with 0.05 mL of 0.5 N NaOH containing 0.1% Triton X-100, and 0.45 mL of 1% sodium dodecyl sulfate (SDS). For this extraction, the plates were turned upright, resealed with paraffilm, wrapped with aluminum foil, and shaken overnight at room temperature. After the extraction, the entire contents of each well were transferred to 8 mL of 50 mmol/L TRIS-HCl pH 7.5. The heme released by lysis of adherent erythrocytes was determined by measuring the absorbance of each sample at 385 nm using a spectrophotometer. With every experiment, AA and SS erythrocytes, from the specimens to be used that day, were suspended to a hematocrit of 5%, and sedimented onto the endothelial cell monolayers under controlled, predictable, and reproducible conditions. Although erythrocyte contact with endothelial monolayers, resulting in adherence and metabolic responses, occurs without initial centrifugation, Mohandas and Evans have pointed out the value of using a mild impinging force. In other experiments erythrocytes were centrifuged onto the monolayers, incubated with the monolayers at 37°C for 1 minute, 10 minutes, or for 1 hour, and then removed (as described below) before labeling with ³H-thymidine. Radionuclide was added after removal of the erythrocytes. After labeling, monolayers were washed twice with DME, followed by three 10-minute washes with 10% trichloroacetic acid at room temperature. Precipitated material was then solubilized with 0.5 N NaOH in 0.1% Triton X-100 for 10 minutes, with shaking, at room temperature. Double distilled water was added to each well, and shaking at room temperature was continued for 18 hours. The contents of triplicate or quadruplicate wells were then solubilized in Aquasol II for liquid scintillation counting.

bovine hemin (type I), and sodium heparin were from Sigma, St Louis, MO. Methyl-³H-thymidine (74 GBq/mmol/L) and Aquasol II were from New England Nuclear, Boston, MA. Ficoll-Paque was from Pharmacia, Piscataway, NJ. Streptan (arabinogalactan) was from Aldrich Chemical Co, Milwaukee, WI, or Sigma. Cell culture plasticware was from Falcon, Oxnard, CA, or GIBCO.

Processing of blood. Venous blood from adult volunteers with homozygous sickle cell anemia, heterozygous sickle trait (hemoglobin SA), or from normal volunteers (hemoglobin A) was drawn into heparin. To accomplish this, aliquots of erythrocytes in BSA/DME were from New England Nuclear, Boston, MA. Ficoll-Paque was from Pharmacia, Piscataway, NJ. Streptan (arabinogalactan) was from Aldrich Chemical Co, Milwaukee, WI, or Sigma. Cell culture plasticware was from Falcon, Oxnard, CA, or GIBCO.

Endothelial cell DNA synthesis assay. All incubations were performed at 37°C in 5% CO₂ and 95% humid air. Human vascular endothelial cells were seeded at 1.4 x 10⁴/cm² in fibronectin-coated 24-well cluster plates. In M199 supplemented with 10% (vol/vol) FBS, heparin (100 µg/mL) and ECGF (150 µg/mL). Cultures were subconfluent after 72 hours. At this time, synchronization was begun by changing the culture medium to DME containing 2.5% FBS and heparin. After 22 hours, the medium was changed to DME containing 1% FBS. After 3½ to 4 hours, stimulation of the endothelial cell monolayers was begun with DME containing 5% FBS, heparin (100 µg/mL), and ECGF (150 µg/mL). After 18 to 21 hours, the monolayers were washed with DPBS and labeled with ³H-thymidine (0.074 MBq/mL) for 6 hours.
were incubated on human vascular endothelial cell monolayers for 2148 WEINSTEIN ET and adhered SSRBC (N) was AARBC in each culture well. When Materials and Methods). The relationship between erythrocyte adherence and the hemoglobin content (AsE) of the culture wells is shown monolayers was quantitated by measuring hemoglobin released by adherent erythrocytes after hemolysis with alkalai and SDS (see Materials and Methods). The relationship between erythrocyte adherence and the hemoglobin content (AsE) of the culture wells is shown in the inset to the figure. Adherence of cultures derived from aorta, iliac artery, and umbilical vein yielded

endothelial cells.

Sickle erythrocytes inhibit DNA synthesis by human endothelial cells. We investigated whether human vascular endothelial cell DNA synthesis is compromised by contact with erythrocytes from sickle cell patients by sedimenting sickle (SS) or normal (AA) erythrocytes onto stimulated endothelial cell monolayers at 17g for 10 minutes at room temperature and labeling the monolayers for 6 hours with 3H-thymidine (see Materials and Methods). As outlined in Table 1, 3H-thymidine labeling of human endothelial cells in BSA/DME was decreased to 59.32 ± 7.99% (% at 6 hours of incubation. In the presence of autologous sickle plasma (SS PPP), adherence of SSRBC was 1.2% at 1 minute, 11.1% at 10 minutes, 15.5% at 1 hour, and 49% at 6 hours of incubation. In the presence of autologous normal control plasma (AA PPP), normal control erythrocytes adhered less than 1% at 1 minute, 10 minutes, and 1 hour of incubation, and adhered 5.2% at 6 hours of incubation. Bars show means and standard errors (SEM).

RESULTS
Sickle erythrocytes inhibit DNA synthesis by human endothelial cells. We investigated whether human vascular endothelial cell DNA synthesis is compromised by contact with erythrocytes from sickle cell patients by sedimenting sickle (SS) or normal (AA) erythrocytes onto stimulated endothelial cell monolayers at 17g for 10 minutes at room temperature and labeling the monolayers for 6 hours with 3H-thymidine (see Materials and Methods). As outlined in Table 1, 3H-thymidine labeling of human endothelial cells in BSA/DME was decreased to 59.32 ± 7.99% (% at 6 hours of incubation. In the presence of autologous sickle plasma (SS PPP), adherence of SSRBC was 1.2% at 1 minute, 11.1% at 10 minutes, 15.5% at 1 hour, and 49% at 6 hours of incubation. In the presence of autologous normal control plasma (AA PPP), normal control erythrocytes adhered less than 1% at 1 minute, 10 minutes, and 1 hour of incubation, and adhered 5.2% at 6 hours of incubation. Bars show means and standard errors (SEM).

Table 1. Effect of Erythrocytes on Human Endothelial Cell DNA Synthesis

<table>
<thead>
<tr>
<th>Incubation Medium*</th>
<th>Erythrocytes†</th>
<th>n</th>
<th>% DNA Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>55</td>
<td>59.32 (7.99)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>59</td>
<td>92.01 (3.03)</td>
</tr>
<tr>
<td></td>
<td>SS</td>
<td>55</td>
<td>22.80 (2.42)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>59</td>
<td>80.65 (2.61)</td>
</tr>
</tbody>
</table>

*BSA/DME = 0.5% BSA in DME. Plasma = 50% (by volume) autologous sickle or normal control (respectively) platelet-poor plasma in BSA/DME. Plasma and erythrocytes used in each individual experiment were obtained from the same whole blood specimen.
†SS, erythrocytes from 10 patients with homozygous sickle cell anemia, AA, erythrocytes from 10 hematologically normal volunteers.
‡Incorporation of 3H-thymidine into endothelial cell DNA in the presence of erythrocytes, expressed as the percent of 3H-thymidine incorporation achieved in the absence of erythrocytes, in the respective incubation media. Actual counts per minute in the media controls were normalized to 100% to facilitate comparison of data among n measurements as indicated.

The influence of plasma factors on the inhibition of endothelial cell DNA synthesis by sickle erythrocytes. In vivo, interaction between erythrocytes and the endothe-

Data analysis. Treatment groups were compared by t-test or analysis of variance using PC Statistician (Human Systems Dynamics, Northridge, CA). Graphical analysis was performed using Sigma-Plot (Jandel Scientific, Sausalito, CA). Endothelial cell cultures derived from aorta, iliac artery, and umbilical vein yielded similar results. Hence, data obtained using cultures derived from these diverse sources were pooled for analysis and presentation. Cultures were not used beyond the sixth passage, therefore human blood samples, which were obtained sequentially, were not all assayed using the same endothelial cell culture. Similar results were obtained for each treatment using SS or AA blood, respectively; hence, the results for donor groups were pooled for presentation.

The influence of plasma factors on the inhibition of endothelial cell DNA synthesis by sickle erythrocytes. In vivo, interaction between erythrocytes and the endothe-

Fig 1. Erythrocyte adherence to human endothelial cells. Sickle erythrocytes (SSRBC) and erythrocytes from normal controls (AARBC) were incubated on human vascular endothelial cell monolayers for 1 minute, 10 minutes, 1 hour, or 6 hours as indicated on the abscissa. Nonadherent erythrocytes were then removed by inverted centrifugation (see Materials and Methods). Adherence of erythrocytes to the monolayers was quantitated by measuring hemoglobin released by adherent erythrocytes after hemolysis with alkalai and SDS (see Materials and Methods). The relationship between erythrocyte adherence and the hemoglobin content (AsE) of the culture wells is shown in the inset to the figure. Adherence of 100% is set at an absorbance reading (A100) of 2.106, corresponding to the starting hematocrit of 5% in each culture well. When 0.5% BSA was the only protein supplement in the incubation media (BSA/DME), adherence of SSRBC and AARBC was approximately 1%, regardless of the incubation time. In the presence of autologous sickle plasma (SS PPP), adherence of SSRBC was 1.2% at 1 minute, 11.1% at 10 minutes, 15.5% at 1 hour, and 49% at 6 hours of incubation. In the presence of autologous normal control plasma (AA PPP), normal control erythrocytes adhered less than 1% at 1 minute, 10 minutes, and 1 hour of incubation, and adhered 5.2% at 6 hours of incubation. Bars show means and standard errors (SEM).
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Table 1. The combination of autologous plasma and erythrocytes resulted in further inhibition of endothelial cell DNA synthesis compared with the effect of erythrocytes in albumin alone.

Endothelial DNA synthesis during contact with SS erythrocytes in the presence of autologous SS platelet-poor plasma was only 22.8 ± 2.42% (P < .001) of maximum. During contact with AA erythrocytes in the presence of autologous AA plasma, the monolayers exhibited 80.65 ± 2.61% (P < .001) maximal incorporation of ³H-thymidine into DNA. Thus, plasma factors enhance the ability of SS and AA erythrocytes to inhibit human endothelial cell DNA synthesis. However, whereas the inhibitory effect of AA erythrocytes and AA plasma is relatively modest, the inhibitory effect of SS erythrocytes and autologous SS plasma is striking. In heterologous AA plasma, contact with SS erythrocytes resulted in 40.53 ± 5.47% (P < .001) maximal DNA synthesis (not shown in Table 1).

The blood from four patients with sickle trait (hemoglobin SA) behaved similarly to AA blood in these experiments. The blood from one patient with sickle C disease (hemoglobin SC) behaved similarly to SS blood in these experiments (data not shown). Analogous behavior of these heterozygotes has been described vis a vis adherence to endothelial monolayers, perhaps suggesting that the erythrocyte alterations resulting in these effects are a function of the polymerization tendency of the hemoglobin.

The effect of transient contact with sickle erythrocytes on subsequent endothelial cell DNA synthesis. The results described above were obtained under conditions which maintained contact between the erythrocytes and the endothelial monolayers continuously during 6 hours of ³H-thymidine labeling. These conditions probably do not mimic the situation in vivo where multiple brief encounters between RBCs and an area of endothelium may be more likely than long periods of contact. The effect of SS erythrocytes on endothelial cell DNA synthesis should persist beyond the transient period of contact between them if these interactions are clinically significant. To test this hypothesis, SS erythrocytes were sedimented onto stimulated endothelial monolayers at 17g for 10 minutes at room temperature. These mixed cultures were then incubated at 37°C for 1 minute, 10 minutes, or 1 hour. The erythrocytes were then removed from the cultures by inverted centrifugation, and the endothelial monolayers were then labeled for 6 hours with ³H-thymidine (see Materials and Methods).

The results of these experiments, summarized in Table 2, indicate that endothelial DNA synthesis was reduced to 60% to 70% of maximum following transient contact in the presence of autologous SS plasma. Hence, the inhibitory effect of SS erythrocytes on human endothelial cell DNA synthesis persists after removal of the erythrocytes from the culture wells. This effect on endothelial DNA synthesis is rapid in onset as well as persistent, given that the inhibitory effect after 1 minute of contact is essentially equivalent to the inhibitory effect following 1 hour of contact (P = .256, 1 minute v 1 hour). Without plasma in the culture media (BSA/DME), endothelial cell DNA synthesis was nearly 100% of maximal, despite contact with SS erythrocytes (see

Table 2. Effect of Transient Exposure to Sickle Erythrocytes on Subsequent DNA Synthesis by Human Endothelial Cells

<table>
<thead>
<tr>
<th>Incubation Medium</th>
<th>Incubation Time</th>
<th>% DNA Synthesis Mean (SEM)</th>
<th>n</th>
<th>Media Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1 min</td>
<td>69.06 (4.45)</td>
<td>12</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>BSA/DME</td>
<td>1 min</td>
<td>96.96 (5.11)</td>
<td>12</td>
<td>P = .316</td>
</tr>
<tr>
<td>Plasma</td>
<td>10 min</td>
<td>65.89 (3.15)</td>
<td>54</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>BSA/DME</td>
<td>10 min</td>
<td>96.32 (2.68)</td>
<td>53</td>
<td>P = .159</td>
</tr>
<tr>
<td>Plasma</td>
<td>1 h</td>
<td>59.47 (3.81)</td>
<td>54</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>BSA/DME</td>
<td>1 h</td>
<td>96.95 (2.07)</td>
<td>53</td>
<td>P = .149</td>
</tr>
</tbody>
</table>

*BSA/DME = 0.5% BSA in DME. Plasma = 50% (by volume) autologous sickle platelet-poor plasma in BSA/DME. Sickle plasma and erythrocytes were obtained from the same whole blood specimen used for each individual measurement.

†Sickle erythrocytes were sedimented onto endothelial monolayers at 17g for 10 minutes, incubated with endothelial monolayers for the indicated time periods, then removed (see Materials and Methods) before labeling of the endothelial monolayers with ³H-thymidine.

‡Incorporation of ³H-thymidine into endothelial cell DNA following exposure to sickle erythrocytes, expressed as the percent of ³H-thymidine incorporation achieved without exposure to erythrocytes for each incubation medium. Actual counts per minute in the media controls were normalized to 100% to facilitate comparison of data among n measurements as indicated.

Table 2). DNA synthesis by endothelial monolayers preincubated with AA erythrocytes was between 80% and 90% of maximal in autologous AA plasma, and there was also no inhibition of DNA synthesis by monolayers preincubated with AA erythrocytes in the presence of BSA alone (AA erythrocyte data not shown).

Sickle erythrocyte adherence and endothelial cell DNA synthesis. The adherence of sickle erythrocytes to vascular endothelial cells is a well-studied cellular interaction which may correlate with the clinical severity of sickle cell anemia. To examine how the adherence phenomenon may relate to sickle cell inhibition of endothelial cell DNA synthesis, we measured the adherence occurring under the conditions of our DNA synthesis assay (see Materials and Methods). As shown in Fig 1, nearly 50% of SS erythrocytes were adherent after 6 hours of contact in the presence of autologous SS plasma. Adherence of SS erythrocytes was 15.5% after 1 hour, 11.1% after 10 minutes, and 1.2% after 1 minute of contact in autologous plasma. In the absence of plasma (BSA/DME), SS erythrocyte adherence was approximately 1% or less at all incubation times. The relationship between SS erythrocyte adherence and endothelial DNA synthesis is shown by considering the 1-minute time point. After a 1-minute incubation, SS erythrocyte adherence was equally scanty in SS plasma or BSA/DME (P = .687). However, as shown in Table 2, endothelial cell DNA synthesis was significantly inhibited after 1 minute of contact with SS erythrocytes in SS plasma compared to BSA/DME (P = .003).

These data demonstrate that strong adherence of sickle erythrocytes to the endothelial monolayers occurs in our assay system when sickle plasma is present. However, the effect of sickle erythrocytes on endothelial DNA synthesis is not dependent on extensive adherence. Consistent with this is the observation that endothelial cell DNA synthesis was equivalently inhibited by contact with sickle erythrocytes in
BSA/DME for 6 hours (~1% adherence) and contact with sickle erythrocytes in autologous sickle plasma for 1 hour (~15% adherence) (P = .313, see Tables 1 and 2). Adherence of AA erythrocytes in BSA/DME or autologous plasma was meager compared with SS erythrocytes at all incubation times (see Fig 1).

**DISCUSSION**

Painful crisis in sickle cell anemia results from microvascular obstruction. Recent data support a pathogenesis which includes adherence of sickle erythrocytes to the endothelium of small postcapillary venules, resulting in trapping of dense erythrocytes and irreversibly sickled cells in venules and capillaries. Direct obstruction of capillaries and precapillary arterioles by dense sickle erythrocytes may also play a role. Injection of dense sickle cells into rat femoral artery results in physiologic alterations in the rat thigh muscles that are consistent with microvascular obstruction and development of pain. In contrast to pain crisis, the cerebrovascular complication of sickle cell anemia is not readily attributable to microvascular obstruction. The reported incidence of stroke in children (under age 20) with sickle cell anemia ranges from 6% to 17%. Approximately 70% of strokes in sickle cell patients are caused by cerebral infarction. Radiologic studies of the cerebral circulation of such patients have shown discreet stenoses and occlusions of the major intracranial arteries, including the internal carotid, anterior cerebral, and middle cerebral arteries. Histopathologic studies of such lesions at autopsy have shown intimal damage and luminal compromise due to intimal thickening (with fibromuscular hyperplasia) with or without mural or transluminal thrombosis. Similar lesions, characterized by endothelial damage and intimal thickening, have been described in the arterial circulation of the spleen in sickle cell patients.

The etiology of these large vessel lesions in the cerebral circulation of sickle cell patients is unknown. Stockman et al, without histologic data, hypothesized that the intimal damage in intracerebral arteries resulted from occlusion of the vasa vasorum by sickle cells. However, as pointed out by Rothman et al, the intracerebral arteries most commonly affected do not possess vasa vasorum. It has been suggested that the initial insult may be alteration of arterial flow due to adherence of sickle erythrocytes to the arterial endothelium, although adherence has not been experimentally demonstrated under arterial shear forces. However, sickle erythrocytes move slowly over endothelial surfaces and can form focal contacts with endothelial cells that do not result in tenacious adherence, but may provide an opportunity for biochemical interaction between erythrocytes and endothelial cells.

The observations reported in this study suggest that contacts between sickle erythrocytes and human endothelial cells do result in biochemical alteration of the endothelial cells. Contact between sickle erythrocytes and human endothelial monolayers resulted in marked inhibition of endothelial cell DNA synthesis. Although this inhibition was most pronounced when sickle erythrocytes were present during the entire 6 hours of endothelial cell thymidine labeling, shorter exposures of the monolayers to sickle erythrocytes, before the onset of labeling, still resulted in significant inhibition of endothelial DNA synthesis. As brief as 1 minute of contact with sickle erythrocytes resulted in over 30% inhibition of endothelial cell thymidine incorporation during the subsequent 6 hours, and the effect of 1 minute of contact was not different from the effect of 1 hour of contact. These results suggest that the inhibitory effect on endothelial DNA synthesis begins rapidly on cell-cell contact and is persistent. This is potentially relevant to the in vivo situation where contacts between individual erythrocytes and vascular endothelial cells are likely to be very brief. It should be noted that the assay used in these studies was designed to optimize the detection of alterations in endothelial DNA synthesis following contact with erythrocytes. The in vivo correlates to the measured effects may be more subtle. The persistence of the inhibitory effect beyond the period of contact implies that there may be a cumulative effect of sequential contacts with successive erythrocytes on a given area of endothelium, particularly at bifurcations where deviation from laminar flow may enhance the contact and adherence between erythrocytes and the endothelium. The static nature of our assay system precludes its adaptation to contacts briefer than 1 minute, but we will test this hypothesis using a controlled flow model.

The presence of plasma during contact with erythrocytes was required for sickle cell inhibition of endothelial DNA synthesis when the RBCs were removed before thymidine labeling. Even when the RBCs were allowed to remain throughout the entire labeling period, the presence of plasma enhanced the inhibitory effect of sickle erythrocytes approximately twofold. The specific nature of the plasma factor(s) involved is not currently known. When heterologous normal plasma was substituted for autologous sickle plasma, the inhibitory effect of sickle erythrocytes was still enhanced compared with their effect in albumin, but the enhancement in normal plasma was about half the enhancement obtained using autologous sickle plasma. These results may suggest that the enhancing factor(s) in sickle plasma is a normal plasma component that is quantitatively different in sickle plasma compared with normal plasma. Alternatively, the factor(s) may exert a different or greater effect on sickle than on normal erythrocytes.

We observed adherence of sickle erythrocytes to the endothelial monolayers under the experimental conditions used to measure endothelial DNA synthesis. This adherence was strongly dependent on the presence of autologous sickle plasma, a finding noted by Mohandas and Evans, who used similar forces to dislodge sickle erythrocytes from the endothelial surface. The inhibitory effect of sickle erythrocytes on endothelial DNA synthesis occurred in the absence of significant adherence. Evidence for this conclusion is provided by the results of 1-minute incubations in the presence of autologous sickle plasma versus BSA: in albumin, erythrocyte adherence was about 1% and DNA synthesis was not inhibited; with plasma present, erythrocyte adherence was still about 1%, but the inhibition of endothelial DNA synthesis was significant. In addition, by maintaining contact between erythrocytes and the endothelial monolayers during the full 6 hours of thymidine labeling, significant inhibition of DNA synthesis was achieved in albumin alone, but...
adherence was still about 1%. This lack of dependence on adherence for the inhibitory effect on endothelial DNA synthesis is potentially important because it provides for the possibility that sickle cell inhibition of endothelial DNA synthesis occurs in the arterial circulation where shear forces may prevent contacts between erythrocytes and the endothelium from resulting in significant adherence.5,18

The clinical significance of the effect of sickle erythrocytes on endothelial cell DNA synthesis is uncertain. However, the normal endothelial response to injury involves DNA synthesis as an early step.16,21,37 A delay in onset of endothelial cell DNA synthesis impairs healing of experimental endothelial wounds in vivo20,21 and in vitro.38 Conceivably, endothelial damage, perhaps due to inflammatory mediators or neutrophil-derived oxidants,39 or inefficient repair in the cerebral circulation of some patients with sickle cell anemia. The result may be intimal proliferation, luminal stenosis, and stroke. Contact with sickle erythrocytes may also provide a primary mechanism of endothelial damage, but this requires confirmation.

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The specific mechanism by which sickle erythrocytes inhibit endothelial DNA synthesis is not yet determined. Hemoglobin S has been shown to undergo auto-oxidation,41 which may relate to the abnormal amount of free heme detected on sickle cell membranes44 and in sickle erythrocyte cytosol,43 as well as the spontaneous generation of oxidant radicals by sickle erythrocytes.44 In preliminary fashion, we have found42 that micromolar concentrations of hemin, in the range measured in sickle erythrocyte cytosol,43 inhibit human endothelial cell DNA synthesis to an extent similar to that seen using intact sickle erythrocytes. The potential involvement of sickle erythrocyte heme in the observed effects on endothelial DNA synthesis is currently under investigation in our laboratory.

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