Vascular Cell Adhesion Molecule-1 Is Involved in Mediating Hypoxia-Induced Sickle Red Blood Cell Adherence to Endothelium: Potential Role in Sickle Cell Disease

By B.N.Y. Setty and Marie J. Stuart

We investigated the effects of hypoxia on red blood cell (RBC)-endothelial cell (EC) adherence and the potential mechanism(s) involved in mediating this effect. We report that hypoxia significantly increased sickle RBC adherence to aortic EC when compared with the normoxia controls. However, hypoxia had no effect on the adherence of normal RBCs. In additional studies, we found that the least dense sickle RBCs containing CD36+ and VLA-4+ reticulocytes were involved in hypoxia-induced adherence. We next evaluated the effects of hypoxia on the expression of EC surface receptors involved in RBC adherence to macrovascular ECs, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and the vitronectin receptor (VnR). Hypoxia upregulated the expression of both VCAM-1 and ICAM-1, whereas no effect on VnR was noted. Potential involvement of VCAM-1 and ICAM-1 in mediating hypoxia-induced sickle RBC-EC adherence was next investigated using monoclonal antibodies against these receptors.

Whereas anti-VCAM-1 had no effect on basal adherence, it inhibited hypoxia-induced sickle RBC adherence in a concentration-dependent manner, with 50% to 75% inhibition noted at 10 to 60 μg/mL antibody (n = 6, P < .05 to P < .01). Anti-ICAM-1 (10 to 60 μg/mL, n = 8) had no effect on either basal or hypoxia-induced adherence. As noted in the bovine aortic ECs, hypoxia stimulated the adherence of sickle RBCs to human retinal capillary ECs, and this response appeared to be mediated via mechanisms similar to those observed with macroendothelium, i.e., via the adhesive receptor combination VCAM-1–VLA-4. Our studies show that hypoxia enhances sickle RBC adhesion to both macrovascular and human microvascular ECs via the adhesive receptor VCAM-1. Our findings are of interest because hypoxia is an integral part of the pathophysiology of the vaso-occlusive phenomenon in sickle cell anemia.

© 1996 by The American Society of Hematology.

The clinical hallmark of sickle cell disease is the periodic occurrence of the vaso-occlusive or painful crisis. Although the mechanism(s) of vaso-occlusion are not completely defined, they appear to be multifactorial, and include, among other etiologies, the adherence of sickle red blood cells (RBCs) to endothelium. Previous studies by Hebbel et al. have shown that increased sickle RBC-endothelial adherence correlates with the clinical severity of sickle cell disease. Although altered membrane properties of the sickle cell have been reported to play an important role in adhesiveness, adherence is further potentiated by plasmatic factors such as fibrinogen, fibronectin, thrombospondin, and von Willebrand factor. Because tissue hypoxemia is an integral part of the vaso-occlusive phenomenon, we have investigated the effects of hypoxia on sickle RBC-endothelial cell (EC) adherence and the potential mechanisms involved in mediating this effect. We show that hypoxia enhances sickle RBC adhesion to both macrovascular and human microvascular ECs and that the adhesive receptor, vascular cell adhesion molecule-1 (VCAM-1), is primarily involved in mediating this effect.

Materials and Methods

Materials

Mouse monoclonal antibodies (MoAbs) against the thrombospondin receptor (CD36 [clones OKM5 and PA6.152]), the α-chain of the vitronectin receptor (VnR; CD51 [clone AMF7]), VCAM-1 (CD106 [clones 1G11 and BBIG-V1]), intercellular adhesion molecule-1 (ICAM-1; CD54 [clones 8A4H10 and BBIG-II]), and the isotypic control antibodies (clones B-Z1 and 117111.11) were obtained from Immunotec, Inc (Westbrook, ME), R&D Systems (Minneapolis, MN), Ortho Diagnostic Systems, Inc (Raritan, NJ), or Harlan Bio-products for Science, Inc (Indianapolis, IN). Besides their immuno-logic properties, these MoAbs have been shown to inhibit the functional activity of their respective receptors, i.e., these antibodies have been shown to block cell-cell adherence. Fluorescein isothiocyanate (FITC)-labeled anti-CD36, anti-CD49d (α-chain of the very late activation antigen-4 [VLA-4]) and isotypic control antibodies were purchased from Immunotec or Serotec (Washington, DC). Goat anti-mouse IgG conjugated with alkaline phosphatase and FITC-labeled goat antimouse IgG were obtained from Sigma Immunochemicals (St Louis, MO). Purified thrombospondin from human platelets and intereleukin-1 (IL-1) receptor antagonist (IL-1-RA) were obtained from Celsus Laboratories, Inc (Cincinnati, OH) and R&D Systems, respectively. 125I-Sodium chromate (400 to 1,200 mCi/mg) was purchased from New England Nuclear (Boston, MA). Tissue culture supplies were obtained from GIBCO Laboratories (Grand Island, NY).

Culture of ECs

Fetal bovine aortic ECs were isolated, identified, and cultured in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) as previously described. Human retinal capillary ECs were obtained from human donor retinas by collagenase digestion. The collagenase digest was filtered through Nytex screens, and the cells in the filtrate were pelleted by centrifugation. The cell pellet suspended in MEM supplemented with 20% FCS was plated on 60-mm^2 tissue culture dishes coated with collagen type I. Outgrowths with the morphologic characteristics of ECs were isolated, trypsinized, and plated. Cells were passaged at a ratio of 1:2 and maintained in MEM supplemented with 15% FCS. These cells expressed factor VIII-related antigen and synthesized plasminogen activator. Cells

From the Division of Pediatric Hematology-Oncology, St Christopher’s Hospital for Children, and Temple University School of Medicine, Philadelphia, PA.

Submitted January 4, 1996; accepted May 13, 1996.

Supported by National Institutes of Health, US Public Health Service Grants No. HL45969 and HL51497.

Address reprint requests to B.N.Y. Yamaja Setty, PhD, Division of Hematology-Oncology, St Christopher’s Hospital for Children, Erie Avenue at Front Street, Philadelphia, PA 19134-1095.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

0006-4971/96/8806-0.00/0

FIG 1. Effects of hypoxia on adhesion of RBCs to fetal bovine aortic EC monolayers. EC monolayers were incubated in the presence of 21% O₂ (normoxia) or 1% O₂ (hypoxia) for 24 hours and then tested in the adherence assay using ⁵¹Cr-labeled RBCs. The adherent RBCs were determined by ⁵¹Cr release as described. (Solid lines) RBCs from patients with homozygous sickle cell disease. (Dotted lines) RBCs from healthy donors.

Preparation of RBCs

After informed consent was obtained, venous blood anticoagulated with sodium heparin was obtained from healthy volunteers and patients with sickle cell disease (homozygous for hemoglobin S) and used within 24 hours. Blood was centrifuged at 120g for 20 minutes at room temperature, and platelet-rich plasma and buffy coats were removed. Packed RBCs were washed twice with 10% of Hank’s Balanced Salt Solution (HBSS) containing 1.3 mmol/L CaCl₂ and 0.5 mmol/L MgCl₂ buffered with 5 mmol/L HEPES (HBSS-HEPES buffer) and incubated with ⁵¹Cr-sodium chromate (100 μCi/mL packed cells) at room temperature for 60 minutes. Labeled RBCs were washed three times with 20 vol of saline and used at a final hematocrit of 10% in HBSS-HEPES buffer. For some experiments, ¹²⁵I-labeled RBCs were subfractionated into the reticulocyte-rich (the least dense fraction or top most layer), reticulocyte-poor (middle layer), and the most dense fraction (lower most layer) using a discontinuous arabinogalactan (Larex-LQ; Consulting Associates, Tacoma, WA) density gradient from 1.083 to 1.125 g/mL; isosmotic, 290 mOsml/L in an isotonic high potassium buffer as described by Sorette et al.¹¹ RBCs from the top (density, 1.083 g/mL), middle (density, 1.112 to 1.115 g/mL), and the lower (density, 1.120 g/mL) layers were sampled, diluted with Dulbecco’s phosphate-buffered saline (DPBS), washed free of gradient material, and used in appropriate experiments.

RBC Adherence Assay

ECs were plated at a density of 100,000 cells per well into wells of 12-well plates and grown to confluence. Confluent cell monolayers were incubated in 37°C either in the presence of 21% O₂ and 5% CO₂ (normoxia) or in the presence of 1% O₂ and 5% CO₂ for 6 hours (hypoxia-6) or 24 hours (hypoxia-24) in air-tight humidified incubation chambers. pO₂ levels in media from cells exposed to normoxia, hypoxia-6, and hypoxia-24 were 65 ± 5 (mean ± SE, n = 8), 31 ± 3, and 30 ± 3 mm Hg, respectively. pH values of the media differed by less than 0.1 after these treatments. After normoxia/hypoxia treatment, cells were tested in a static adherence assay as described by Hebbel et al. in the absence of plasma. In brief, EC monolayers were washed twice with 2 mL of HBSS-HEPES buffer, and 0.5 mL of the labeled RBC suspension was layered on the cell monolayers. Incubations for 45 minutes at 37°C were conducted and the nonadherent RBCs were removed. The monolayers were then washed five times with 0.5 mL HBSS-HEPES buffer containing 0.5% bovine serum albumin. Adherent RBCs were determined by ⁵¹Cr release after cell lysis. To evaluate the involvement of adhesive receptors on hypoxia-induced RBC adherence, EC monolayers were pretreated for 60 minutes with varying concentrations of MoAbs against the EC adhesive receptors VCAM-1, ICAM-1, CD36, or the isotypic controls and then used in adherence assays. Individual data points represent the mean of triplicate values.

Analysis of Adhesive Receptors on ECs

Adhesive receptors evaluated in the macrovascular EC system included the VnR, VCAM-1, and ICAM-1, whereas in the microvascular system, the VnR, CD36, VCAM-1, and ICAM-1 were assessed. Effects of hypoxia on adhesive receptor expression was evaluated by both fluorescence enzyme-linked immunosorbent assays (ELISAs) and flow cytometry.

Analysis by fluorescence ELISA. After exposure to either normoxia or hypoxia, ECs (grown in 12-well plates) were incubated with anti-CD51, anti-CD36, anti-VCAM-1, anti-ICAM-1 (20 μg/mL), or an equivalent amount of an isotypic control antibody for 60 minutes. Cells were washed and incubated for an additional 60 minutes with a second antibody, goat antimouse IgG conjugated with alkaline phosphatase (1:25 in DPBS). Washed cell monolayers were then incubated for 15 minutes with the fluorogenic alkaline phosphatase substrate (AttoPhos; JBL Scientific, San Luis Obispo, CA), the reaction was terminated by the addition of EDTA, and the fluorescence was measured in a Millipore Cytofluor 2350 System (Millipore Corp, Bedford, MA) using 450-nm excitation and 580-nm emission filters. Isotypic control antibody-treated ECs processed in a manner similar to the anti-CD51-, anti-CD36-, anti-VCAM-1—, or anti-ICAM-1—treated cells served as a measure of nonspecific fluorescence.

Analysis by flow cytometry. ECs were inoculated at a density of 2.5 × 10⁶ cells per 75-cm² flask containing 25 mL of MEM supplemented with 10% FCS and grown to confluence. After exposure to either normoxia or hypoxia, cell monolayers were dissociated either by incubating washed monolayers with 5 mL of nonenzymatic cell dissociating reagent (Sigma Immunochemicals) for 15 minutes or with 2 mL of trypsin (0.05%)-EDTA (0.53 mmol/L) for 60 seconds at 37°C, as previously described.¹² Ten milliliters of MEM supplemented with 10% FCS was added, and the suspension was triturated to break up cell clumps and centrifuged at 1,500 rpm for 10 minutes to pellet cells. After two washes in MEM, cells were suspended to a final density of 20 × 10⁶ cells/mL in HBSS-HEPES buffer. One
HYPOXIA INCREASES SICKLE RBC-EC ADHESION

Fig 2. Involvement of reticulocytes in hypoxia-induced adherence of sickle RBCs to fetal bovine aortic EC monolayers. ⁶⁷Cr-labeled sickle RBCs were separated into the reticulocyte-rich, reticulocyte-poor, and irreversibly sickled RBCs using a discontinuous arabinogalactan density gradient. Density-separated RBCs were then analyzed for reticulocyte levels and surface antigens including CD36 and VLA-4 (A) or tested in the adherence assay using EC monolayers that had been subjected to either normoxic or hypoxic treatment for 24 hours (B), as described. The results presented in both panels are the mean ± SE from five different experiments.

Flow Cytometric Analysis of Surface Receptors on Sickle Erythrocytes and Reticulocyte Enumeration

One million unfractionated RBCs or density separated cells (suspended in a total volume of 50 μL DPBS containing 2% FCS and 0.1% sodium azide) were incubated with 20 μL of FITC-labeled anti-CD36, anti-CD49d, or negative isotypic control antibody for 30 minutes at room temperature. The incubation mixture was diluted with 1.0 mL of DPBS, and cells were pelleted at 5,000 rpm for 1 minute in an Eppendorf centrifuge. After an additional wash in DPBS, cells (suspended in 100 μL of DPBS) were incubated with 10 μL FITC-labeled goat antimouse IgG for 30 minutes in the dark at room temperature. Washed antimouse IgG FITC-labeled cells were suspended in DPBS and analyzed immediately in a Becton Dickinson Flow Cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a 15-mW, 488-nm, air-cooled argon-ion laser and formatted for one-color analysis at a flow rate of 1,000 to 1,500 cells per second. Data from 20,000 events were collected and analyzed on a Hewlett-Packard 9000 series, 340 computer with Pascal 3.22 operating system and Consort 32 program (Hewlett-Packard Co, Wilmington, DE).

Data Analysis

Results were expressed as the percentage of control response to compare concentration-dependent effects of test compounds among replicate experiments. Statistical evaluation was performed by the paired Student's t-test when only one treatment group was involved. The significance of difference in a treatment series was determined by analysis of variance (F-ratio). Individual treatments in a treatment series were then compared with the control using the Dunnett's...
RESULTS

Studies of Hypoxia With Macrovascular Endothelium

Effects of hypoxia on RBC adherence to aortic ECs. In six different experiments we initially compared the time-dependent effects of hypoxia on sickle RBC adherence to aortic endothelium. Hypoxia significantly increased RBC adherence, with an increase over baseline of 27% ± 4% (mean ± SE, P = .05) and 68% ± 14% (P < .01) in the 6- and 24-hour hypoxia-treated ECs, respectively (data not shown). For all our subsequent studies, we have used ECs exposed to 24-hour hypoxia. Although hypoxia had no significant effect on the adherence of normal RBCs (Fig 1, dotted lines, 101% ± 11% of normoxic controls, n = 8), sickle RBC adherence to ECs exposed to hypoxia was enhanced by 66% ± 13% (Fig 1, solid lines, n = 9, P < .006). We next tested whether the increased adherence of sickle RBCs to hypoxic endothelium was due to the presence of a unique subpopulation of erythrocytes in this patient group. Sickle RBCs were separated into the least dense cells, middle dense cells, and the most dense cells using a discontinuous arabinogalactan density gradient. These cell fractions corresponded to the reticulocyte-rich, reticulocyte-poor, and irreversibly sickled RBCs, respectively. Density-separated RBCs were then analyzed for reticulocyte levels and surface antigens including CD36 and VLA-4 reported to be present on a subpopulation of reticulocytes. These cells were also concomitantly evaluated for their adhesive potential. As seen in Fig 2A, the least dense cell fraction was enriched with reticulocytes by 240% ± 49% (n = 5) when compared with the unfractonated whole RBC preparation. CD36* and VLA-4* cells were also enriched in this fraction by 181% ± 67% and 205% ± 64%, respectively. When these density-separated erythrocytes were analyzed for their adhesive property, RBCs present in the reticulocyte-rich fraction were the most adherent to both normoxic (and hypoxic) endothelium when compared with the cells present either in the reticulocyte-poor or the irreversibly sickled cell fraction (Fig 2B). Hypoxia stimulated the adherence of the cells present only in the reticulocyte-rich fraction (49% ± 8% stimulation compared with normoxia, n = 5, P < .0025). No effect on the adherence of cells contained either in the reticulocyte-poor or the irreversibly sickled RBC fraction was noted.

Effects of hypoxia on adhesive receptor expression on aortic ECs. Because recent studies have shown that the EC adhesive molecules, including VCAM-1, ICAM-1, and the VnR, are involved in mediating RBC adherence to macroendothelium, we investigated whether any of these adhesive receptors were involved in mediating the effects of hypoxia. Methodologies used included fluorescence ELISA (analyzed using intact monolayers) and flow cytometry (analyzed using cell suspensions). The effects of hypoxia on the expression of adhesive receptors as assessed by the fluorescence ELISA assay are shown in Fig 3. In these studies, hypoxia upregulated the expression of VCAM-1 (150% stimulation, n = 11, P < .001) and ICAM-1 (60% stimulation, n = 7, P < .01) when compared with normoxia-treated ECs. No effect on VnR expression was noted (95% of normoxia control, n = 5). Results similar to those observed with fluorescence ELISA were also noted when these experiments were repeated using flow cytometric analysis (Fig 4). When the data from the representative experiment depicted in Fig 4 were analyzed for adhesive receptor-positive cells (measured as antigen-positive events) and cell surface receptor density (measured as mean fluorescence), hypoxia markedly increased not only the number of VCAM-1–positive cells, but also VCAM-1 antigenicity on the EC surface.

Effects of MoAbs against VCAM-1 and ICAM-1 on sickle RBC adherence to aortic ECs. Because hypoxia upregulated the expression of VCAM-1 and ICAM-1 on the aortic EC surface, we next investigated whether these adhesive receptors were involved in mediating hypoxia-induced sickle RBC adhesion using adhesion blockade-specific MoAbs against these target receptors. The effects of anti–VCAM-1 on hypoxia-induced sickle RBC adherence is shown in Fig 5A. Whereas anti–VCAM-1 had no effect on basal adherence, hypoxia-induced adherence was inhibited in a concentration-dependent manner, with 50% ± 15%, 65% ± 12%, and 75% ± 10% inhibition noted at 10, 30, and 60 μg/mL antibody, respectively (n = 6, P < .05 to P < .001). As opposed to the effect of anti–VCAM-1, when an antibody to ICAM-1 (10 to 60 μg/mL, n = 8) was used, no effect was noted on either basal or hypoxia-induced adherence (Fig 5B). Responses similar to those observed with anti–ICAM-1 were also noted with the negative isotopic control antibody.

Studies of Hypoxia With Microvascular Endothelium

Effects of hypoxia on sickle RBC adherence to retinal capillary ECs. Because microvascular capillary endothelium is the surface most likely to encounter RBCs in vivo,
HYPOXIA INCREASES SICKLE RBC-EC ADHESION

Fig 4. Flow cytometric analysis of the effects of hypoxia on surface expression of adhesive receptors on fetal bovine aortic ECs. After 24 hours of exposure to either normoxia or hypoxia treatment, EC monolayers were dissociated, suspended in HBSS-HEPES buffer, and then incubated with anti-VCAM-1 (top panels), anti-ICAM-1 (middle panels), anti-CD51 (bottom panels), or an equivalent amount of a negative isotypic control antibody (30 μg) for 30 minutes. Cells were washed and incubated for an additional 30 minutes with FITC-labeled goat antimouse IgG. Washed antimouse IgG FITC-labeled cells were then analyzed by flow cytometry. Results presented are from a representative experiment repeated four times with similar results. VnR was measured as CD51 antigen. The left and right panels depict cells from normoxia and hypoxia treatments, respectively. Cells stained with the negative isotypic control antibody had a fluorescence peak between 1 and 20 fluorescence units (data not shown). Results presented in this figure were analyzed for antigen-positive events (APE) and mean cell fluorescence (MCF) using the statistical package provided by the manufacturer.

We extended our initial studies to include microvascular ECs from the human retina. Effects similar to those observed with the macrovascular ECs were observed when the effects of hypoxia were evaluated using microvascular endothelium. Hypoxia stimulated the adherence process by 50% ± 8% (n = 8, P < .001) when compared with normoxic control cells (Fig 6).

Effects of hypoxia on adhesive receptor expression on retinal capillary ECs. The effects of hypoxia on the surface expression of the adhesive receptors known to be involved in mediating RBC microvascular EC adhesion was evaluated. Further studies were then performed to identify which of these receptors specifically modulated hypoxia-induced adhesion. As seen in Fig 7, hypoxia stimulated the expression of VCAM-1, ICAM-1, and CD36 by 145%, 48%, and 32%, respectively (n = 5 to 10, P < .05 to .001). As noted in the experiments using macrovascular ECs, no effect on VnR expression was seen (104% of normoxia control, n = 7).

Effects of MoAbs against VCAM-1, ICAM-1, and CD36 on sickle RBC adherence to retinal capillary ECs. In experiments similar to those conducted with macroendothelium, the effects of antibodies against VCAM-1, ICAM-1, and CD36 on hypoxia-induced adhesion of RBCs was studied. The effects of anti–VCAM-1 on adherence is shown in Fig 8A. Results very similar to those observed with the macrovascular cells were observed with inhibitions of 40% to 65% noted at antibody concentrations between 10 and 60 μg/mL (n = 6, P < .05 to P < .01). Anti–ICAM-1 (10 to 60 μg/mL, n = 6) had no effects on hypoxia-induced adherence (Fig 8B). Results presented in Fig 9 show that thrombospondin (n = 4) stimulated sickle RBC adherence to both normoxic and hypoxic capillary endothelium. Anti-CD36 completely inhibited thrombospondin-induced adherence to both normoxic and hypoxic capillary ECs. No effects on control or hypoxia-induced adherence was observed with anti-CD36.

Effects of IL-1-RA on hypoxia-induced sickle RBC adherence and VCAM-1 expression on retinal capillary ECs. Because previous studies have documented that hypoxia stimulates the production of IL-117 and that IL-1 stimulates the expression of VCAM-1 on the EC surface,18 we investigated whether IL-1 was involved in mediating hypoxia-induced
adherence. Potential involvement was assessed by an evaluation of the effect of the IL-1-RA on both hypoxia-induced sickle RBC adherence and VCAM-1 expression on ECs. The effects of IL-1-RA on the surface expression of VCAM-1 on retinal capillary endothelium and adherence of sickle RBCs are shown in Fig 10. IL-1-RA inhibited in parallel both hypoxia-induced sickle RBC adherence and VCAM-1 expression in a concentration-dependent manner. Whereas the expression of VCAM-1 was inhibited by 50% to 90%, the adherence process was blocked by 30% to 75% at IL-1-RA concentrations of 50 to 200 ng/mL.

DISCUSSION

Recurring episodes of vaso-occlusion are one of the major clinical problems in patients with sickle cell disease. Sickle RBCs with altered deformability play a major role in the initiation of vaso-occlusion in small arterioles and precapillary vessels, with the process being further exacerbated by components of vasoconstriction in these vessels. Further factors contributing to the initiation and propagation of the vaso-occlusive process include biochemical properties of sickle cells, including membrane surface properties, microvascular hemodynamics, tissue hypoxia, and plasma cellular factors including platelet- and leukocyte-endothelial interactions. Because previous studies have documented that the degree of sickle RBC adherence to endothelium correlates with overall clinical vaso-occlusive severity, this sickle cell-endothelial interaction has been the focus of intensive investigation. Although the role of both sickle RBC membrane properties and plasma adhesinogenic proteins on the adherence process have been previously evaluated, the effect of hypoxia on this process has not been studied to date. In this study, we show that hypoxia stimulates sickle RBC adherence to endothelium. Our initial studies were conducted using fetal bovine aortic ECs, because previous investigators have obtained analogous adherence results using both bovine aortic and human umbilical vein endothelium. Our final studies were performed using human microvascular capillary endothelium, because this is the surface most likely to encounter erythrocytes in vivo. Moreover, recent studies by Brittain et al have uncovered dissimilarities in adherence properties between microvascular and macrovascular endothelium isolated from tissues from the same species. For example, von Willebrand factor potentiated sickle RBC adherence to human umbilical venous endothelium, but had no effect on adherence to dermal microcapillary endothelium.
We show that hypoxia stimulates the adherence of sickle RBCs to both macrovascular and microvascular ECs and that the receptors involved appear to be similar in both cell types.

Previous studies have documented that sickle RBCs are more adhesive to endothelium when compared with normal erythrocytes. The adhesinogenic potential of sickle cells is further increased by several plasmatic factors or adhesinogenic molecules, such as fibrinogen, von Willebrand factor, and thrombospondin. A recent study by Sugihara et al has shown that the sickle RBC population that is involved in the abnormal thrombospondin-induced adhesiveness to normal endothelium is the least dense erythrocyte fraction containing reticulocytes. This abnormal thrombospondin-induced reticulocyte adherence appears to be unique to sickle reticulocytes, because thrombospondin had no effect on the adherence of reticulocytes from patients with other hemolytic diseases with high reticulocyte counts. In additional experiments, these investigators have shown that the abnormal adhesiveness of sickle reticulocytes in this patient group was due to the presence of a large proportion of a subclass of reticulocytes termed stress reticulocytes, which were CD36+ by flow cytometric analysis. A second study by Swerlick et al has shown that approximately 25% of the sickle reticulocytes express VLA-4 antigen. This property is not selective to sickle reticulocytes, because VLA-4-positive reticulocytes were also found in erythrocyte preparations from other hemolytic diseases with high reticulocyte counts. However, when compared with reticulocytes from patients with sickle cell disease, only a smaller percentage of reticulocytes in this latter high reticulocyte control patient group were VLA-4 positive. Finally, a third study by Joneckis et al showed that both CD36 and VLA-4 receptors are present on sickle stress reticulocytes. These studies have shown that a subpopulation of sickle reticulocytes that are CD36+ and VLA-4-positive are the most adherent RBCs and are involved in both plasma-dependent and plasma-independent adhesion to endothelium. Our present study has reconfirmed these previously documented observations and further shown that sickle reticulocytes are involved in the increased adherence to hypoxic endothelium.

The receptor combinations that have been reported to be involved in the adherence of sickle RBCs to macrovascular endothelium include CD36-thrombospondin-αvβ3 and VLA-4-VCAM-1. Receptor pairs involved in microvascular adhesion include CD36-thrombospondin-αvβ3 and CD36-thrombospondin-CD36. Because VCAM-1 has also been shown to be present on microvascular capillary endothelium, the receptor combination VLA-4–VCAM-1 could potentially also have been involved in sickle RBC-micro EC adherence. Therefore, in the present study, we explored the possible involvement of these receptor combinations in hypoxia-induced adherence of sickle RBCs to both macrovascular and microvascular endothelium. Our results show that the receptor combination VCAM-1–VLA-4 is involved in mediating hypoxia-induced adherence of sickle RBCs to macroendothelium. This conclusion was supported by the findings that hypoxia upregulated the expression of VCAM-1 on the EC surface and that adherence blockade-selective MoAbs against this receptor inhibited hypoxia-induced adhesion.
herence of sickle RBCs in a concentration-dependent manner. Whereas hypoxia also upregulated the expression of ICAM-1 on the macrovascular EC surface, absence of an inhibitory effect with anti–ICAM-1 on RBC adherence excluded the involvement of this receptor in mediating the hypoxia-induced effects. The findings that hypoxia had no effect on the VnR expression and that hypoxia stimulated the adherence process in the absence of plasma or plasma adhesinogenic proteins indicated that the VnR is not involved in hypoxia-mediated adherence. In further studies, we have also documented that hypoxia stimulated sickle RBC adherence to human microvascular ECs via mechanisms similar to those involved with macroendothelium, ie, via VCAM-1–VLA-4. Finally, we have also shown that only the cells contained in the reticulocyte-rich fraction were involved in the enhanced adherence to hypoxic endothelium.

Many inflammatory mediators are produced and released
HYPOXIA INCREASES SICKLE RBC-EC ADHESION

by ECs during hypoxia, including IL-1,17 platelet-activating factor,38 and vascular EC growth factor.39 Studies have previously documented that IL-1 stimulates the expression on the EC surface of adhesive receptors that belong to the Ig supergene family.17,40 Recently, Shreenivas et al17 have shown that the hypoxia-induced upregulation of ICAM-1 on human umbilical vein ECs was mediated via endogenous IL-1. In the present study, we have shown by the use of an IL-1-RA that both hypoxia-induced VCAM-1 upregulation and its functional consequence, ie, increased sickle RBC-EC adherence, are mediated in major part by this inflammatory mediator.

Our results are of potential interest because not only is hypoxia an integral part of the pathophysiology of the common complication in sickle cell disease, ie, the vaso-occlusive crisis, but also could play a role in the rapidly progressive potentially lethal complication of acute chest syndrome. In this latter entity, pulmonary involvement, which may be initiated by pulmonary infiltrates on chest x-ray, can rapidly proceed to white out on chest x-ray with concomitant pulmonary insufficiency.41,42 Although an infectious cause has been carefully sought, definitive involvement of bacterial, viral, or mycoplasma etiologies has been unsatisfactorily documented. The rapidly progressive pulmonary involvement seen in acute chest syndrome could be justifiably hypothesized to be related in part to hypoxia-induced endothelial expression of VCAM-1 and massive intrapulmonary sickle RBC-EC adherence as a consequence of such hypoxemia.

ACKNOWLEDGMENT

The authors thank the staff members of the division of Hematology-Oncology, St Christopher’s Hospital for Children for their cooperation in obtaining blood samples from patients with sickle cell disease, all volunteers for their generous donation of blood samples, and S. Setty for typing this manuscript. Human retinal capillary ECs used in this study were kindly provided by Dr J.E. Graeber.

REFERENCES


5. Simmons D, Makgoba MW, Seed B: ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. Nature 331:624, 1988


32. Brittain HA, Eckman JR, Wick TM: Sickle erythrocyte adherence to large vessel and microvascular endothelium under physiologic flow is quantitatively different. J Lab Clin Med 120:538, 1992
34. Joneckis CC, Ackley RL, Orringer EP, Wayner EA, Parse LV: Integrin \(\alpha_\beta\) and glycoprotein IV (CD36) are expressed on circulating reticulocytes in sickle cell anemia. Blood 82:3548, 1993
Vascular cell adhesion molecule-1 is involved in mediating hypoxia-induced sickle red blood cell adherence to endothelium: potential role in sickle cell disease

BN Setty and MJ Stuart