Demonstration of Endothelial Adhesion of Sickle Cells In Vivo: A Distinct Role for Deformable Sickle Cell Discocytes

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Different morphologic and density classes of sickle cells (SS) may play distinct roles in the generation of vasoocclusion, explaining the complexity of this phenomena. The densest SS red blood cells (RBCs) (SS4) can induce vasoocclusion in ex vivo microcirculatory preparations as well as in an intact animal model. Previous studies of the interaction of SS deformable discocytes with endothelial monolayers or the rat ex vivo mesecucum preparation have shown adhesion that is desmopressin (dDAVP)-stimulated, von Willebrand factor (vWF)-mediated, and limited to the small venules. However, in vivo adhesion of SS RBCs to the endothelium has neither been demonstrated nor characterized; and, in particular, the relation of adhesion to vasoocclusion is unknown. Using an intact animal model that involves injecting saline-washed, density-defined SS RBCs into the femoral artery of a rat, we find that: (1) Quantitative studies of RBCs retained in the rat thigh using ⁵¹ᵐ⁻TTc-labeled RBCs and γ camera imaging showed that dDAVP induces a threefold increase in retention of normal (AA) cells and deformable SS discocytes (SS2). (2) electron microscopy and Microfil injection show that the retention of SS2 cells is due to adhesion to the vascular endothelium with no evidence of obstruction. (3) H-1 magnetic resonance imaging showed that retention of SS4 cells induced a dose-dependent increase in tissue edema (presumably secondary to tissue hypoxia), while retention of AA or SS2 cells produced no change. We conclude that endothelial adhesion of deformable SS discocytes can be demonstrated in an in vivo animal model, that this adhesion is enhanced by dDAVP (presumably related to, but not necessarily limited to the release of vWF), and that this phenomenon per se does not lead to vasoocclusion. Nevertheless, adhesion of deformable SS discocytes may have consequences. We hypothesize that adhesion of SS discocytes could narrow the lumen of postcapillary venules and facilitate secondary trapping of SS4 cells and lead to subsequent vasoocclusion.

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ALTHOUGH VASOOCCLUSION at the microcirculatory level is a universal manifestation of sickle cell disease (SS), the frequency and severity of occlusive events is highly variable among SS patients. One factor that may modulate these events is the extent and type of SS red blood cell (RBC) heterogeneity. For example, we have defined four classes of SS RBCs on the basis of density and have shown that cells from these classes have characteristic morphologic and rheologic properties. RBC density is linearly correlated with the intracellular hemoglobin concentration (mean corpuscular hemoglobin concentration [MCHC]) and both the extent and rate of hemoglobin S (HbS) polymer formation under deoxygenated conditions are extremely sensitive to MCHC. We propose that cells from two density classes, dense cells and discocytes or deformable cells, may play distinct roles in microcirculatory vasoocclusion.

Irreversibly sickled cells (ISCs; which are a morphologically identifiable component of the SS dense cell category) have been observed obstructing precapillary sphincters and SS dense cells selectively disappear from the circulation during painful crisis. Because there is no direct evidence that RBCs are destroyed during crisis, trapping of cells with high MCHC or dense cells (SS4) at the site of vasoocclusion is the most likely explanation for the disappearance of these cells from the peripheral circulation. Using an animal model, we have shown that dense cells are much more effectively retained in the rat leg than normal (AA) or discocyte (SS2) cells, and that dense cells produce an immediate increase in the ratio of inorganic phosphate/phosphocreatine (Pi/PCr) that is indicative of anoxia. Subsequently, edema and changes in proton relaxation time detectable by magnetic resonance imaging occur. These data show that dense cells are indeed capable of inducing vasoocclusion. Nevertheless, failure to observe a correlation between the frequency of crisis and the percent dense or irreversibly sickled cells in the peripheral circulation suggests that the role of dense cells in vasoocclusion is not a simple one and that there are other participating factors.

The second category of cells that may contribute to vasoocclusion by a different mechanism are the SS cells with a density similar to that of AA cells. These cells are also called deformable discocytes or SS2 cells in our notation and have been shown by Kaul et al to adhere to the venular endothelium of the rat mesocucum. Adhesion of SS cells to the endothelium, a phenomena first suggested by Hebbel et al, may be another contributor to vasoocclusion. Evidence that young cells and deformable discocytes are the most adhesive cells comes from three sources. (1) Mohandas and Evans, using a micropipette method applicable to individual sickle cells, found that irregular but deformable discocytes (not ISCs) were the most adhesive. (2) Barabino et al, using cultured endothelial cells, showed that low-density cells were more adherent than SS dense cells or AA cells. (3) Kaul et al (using video microscopy of a
rat mesocecum preparation perfused with human SS RBCs) observed that deformable discocytes (density fraction SS2) are more likely to adhere to the endothelium than dense cells (SS4). In addition, the finding of Kauf et al that adhesivity of RBCs to the vascular endothelium was limited to postcapillary venules, greatly strengthens the possibility of a specific role for SS discocytes in sickle cell vasocclusion.

Recent data implicate von Willebrand's factor (vWF) in sickle cell adhesion to endothelial cells. Barabino et al used endothelial monolayers grown on glass slides in a perfusion chamber to show that endothelial cell-conditioned media greatly enhanced the adhesion of sickle cells. Wick et al have shown that high multimers or ultra-large vWF (ULvWF) can dramatically increase adhesivity of SS cells and may play a role under physiologic conditions. A recent report by McIntire et al indicates that vWF-mediated adhesivity is a property of young cells, while Tsai et al have provided evidence that AA cells can also adhere to endothelial cells under shear flow conditions in the rat mesocecum after stimulation with dDAVP. Furthermore, Kauf et al showed that adhesion of SS cells to postcapillary venules in the rat mesocecum is dDAVP stimulated and inhibited by anti-vWF.

Hence, it is possible that both the obstructive properties of dense cells and the adhesive properties of SS discocytes participate in SS vasocclusion. In the artificially perfused, ex vivo microcirculatory preparation, Kauf et al have implicated adhesion of deformable SS cells followed by occlusion by denser SS cells in the initiation of microvascular occlusion. An important next step is to validate these findings in an animal preparation that includes neural, vascular, and humoral factors that might affect this phenomenon. In this report we will use the intact animal model to attempt to answer the following questions: (1) Is adhesion of SS RBCs demonstrable in an in vivo microcirculation? (2) Can factors that enhance adhesion in cultured cells and the mesocecum preparation enhance adhesion in vivo? (3) What cell types (discocytes versus dense cells) participate in adhesion in vivo? (4) What are the consequences of SS adhesion in vivo?

MATERIALS AND METHODS

Patient Material

Blood was drawn from 12 SS patients from the Heredity Clinic of the Bronx Comprehensive Sickle Cell Center after informed consent and characterized by two electrophoresis methods and a solubility test for HbS. All patients had been free of crisis for at least 1 month and were homozygous for HbS by Ms II digestion. Approximately half of the SS patients had concomitant a-thalassemia.

Preparation of Density Defined Cells

Percoll (colloidal silica coated with polyvinylpyrrolidone; Pharmacia Fine Chemicals, Inc, Piscataway, NJ) and Larex (arabinogalactan polysaccharide; Consulting Associates, Tacoma, WA) (prepared as described by Corash et al) gradients were formed from a mixture of Percoll and Larex (density, 1.207; determined from refractive index), water, and 10-fold balanced salts in a ratio of 3.5:3:0.2:8:0.7. The pH and osmolarity were adjusted to 7.35 and 290 mOsM, respectively. The procedure used was similar to that described in a previous publication. For preparation of 0.5 to 1.0 mL of packed cells of defined density (MCHC) classes of sickle cells, 0.5 mL of blood adjusted to hematocrit (Hct) 50 was added to 5.5 mL of the gradient mix. The mixture was centrifuged and the cells were aspirated from the formed gradients at predetermined depths in the tube with a pipette. They were washed three times with isotonic saline and either used immediately or stored in autologous plasma. The four density classes isolated consist of SS1, which contains reticulocytes and young cells with MCHC less than 33 g/dL (density, <1.076); SS2, which contains reticulocytes, young RBCs, and discocytes with a MCHC between 33 and 37 g/dL (density, 1.076 to 1.091); SS3, which consists of cells with a MCHC between 37 and 42 g/dL; and SS4, which consists of very dense discocytes and ISCs with MCHCs greater than 42 g/dL. That the density classes were sharply defined was shown by recentrifuging 50 µL of cells on an analytical gradient as described above. In general, density classes prepared by this technique have less than 5% of the cells exceeding the upper or lower density limits.

Femoral Artery Injection of Cell Suspensions

Wistar female rats (200 to 225 g) were anesthetized with pentobarbital (35 mg/kg). A 1.5-cm incision was made in the femoral triangle, the artery was exposed by blunt dissection, and was dilated by topical application of 5% Cyclaine Hydrochloride (Merck, Sharp, and Dohme, West Point, PA). Blood flow was temporarily stopped with a hemostatic clip and 0.5 mL of a Hct 30 suspension of RBCs in saline was injected into the femoral artery using a 30-gauge needle. After hemostasis was achieved (usually between 5 and 10 minutes), the site of injection was gently cleaned with saline and the wound was sutured. The animal was then observed by the techniques described.

Cannulation of the Femoral Artery

After the femoral artery was exposed, it was cannulated with PE10 tubing and the cells infused via cannula. This procedure is particularly suitable for studying the earliest events in occlusion because the injection can be made in the gamma camera. In the rat the effects of ligation of the femoral artery are less severe than they would be in other species because of the extensive collateral circulation to the leg.

Injection of Desmopressin (dDAVP)

We administered dDAVP to rats intraperitoneally at a rate of 1 µg/kg 15 minutes before injection of Tc-labeled human cells.

Technetium

Cells for ⁹⁹Tc imaging were prepared using the method of Bardy et al and a Mallinckrodt pyrophosphate kit (Mallinckrodt Diagnostic Products Division, St Louis, MO). RBCs suspended at Hct 50 in isotonic saline were reacted with 0.5 µg/mL of stannous pyrophosphate in isotonic saline for 10 minutes. The pertechnetate (⁹⁹Tc O₄⁻) stock solution was then added and this solution was reacted for another 10 minutes. Unreacted technetium was removed by washing the cells three times in sterile, nonpyrogenic isotonic saline (NEN Medical Products, Billerica, MA). After the last wash, the cell suspension was spun in a microhematocrit tube, the tube was broken at the cell-supernatant interface, stored for 24 hours, the tops and bottoms were counted separately, and the percent label was determined using % T = (100 × RBC)/(RBC + plasma). The percent free technetium was less than 5% for AA cells and between 5% and 15% for SS4 cells.

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Gamma Camera Imaging

One hour after injection of $^{99m}$Tc-labeled RBCs the animals are imaged with a large field of view Anger camera, peaked for the 140 keV photon of $^{99m}$Tc with a 20% window and a high resolution parallel hole collimator. Static images are collected with a PDP 11/34 dedicated minicomputer in 64 x 64 word mode and stored, which allows the activity of $^{99m}$Tc in selected areas to be determined. The equivalent volume of packed cells retained was calculated from $^{99m}$Tc computer matrix images, selected from areas of interest (such as leg, bladder, and liver-spleen-kidney) and compared with the activity of a suspension of known Hct and volume of the same cells used for injection. Appropriate (small) background corrections were made based on the size of the area of interest. Under these imaging conditions, attenuation due to passage through the tissue of the animal is expected to average between 10% and 15% per centimeter. Due to the uniform weight (200 to 225 g) and small size of the animals, this correction has not been applied to the present data.

During kinetic experiments, images were obtained every 15 seconds for the first 5 minutes and then at 10-minute intervals for the next hour. In some cases, images were taken at hourly intervals for 6 hours. A total of 13 animals were examined by injection in the $\gamma$ camera: three SS2 without dDAVP, six SS2 with dDAVP, three SS4 without dDAVP, and one SS4 with dDAVP.

H-1 NMR

Proton images were obtained at 0.18 Tesla using a superconducting prototype imaging system developed in the Department of Diagnostic Radiology at Yale University. Images were obtained 10 to 20 minutes after the injection of sickle cells. A 2D Fourier single slice imaging technique was used to obtain coronal images from 8-mm slices at 128 x 128 resolution. All 180$^\circ$ pulses were nonselective and TRs (repetition rates) of 120, 170, 270, 470, 670, 1,070, and 1,570 msec were used with a TE (echo time) = 15 msec to obtain $T_1$, data by saturation recovery. A saddle-shaped radio frequency coil (6 cm in diameter) was used as the receiver and the animal was attached to a Plexiglas bed with space for two reference vials between the legs. The bed was centered in the coil by means of a smaller Plexiglas cylinder. $T_1$, was calculated from the multiple saturation recovery data by computer fit to the data from selected regions of interest. For $T_2$ determinations, a multi-echo Carr-Purcell sequence with eight echoes at 20-msec intervals and TR = 2,000 msec was used in combination with the imaging sequence to generate 8-mm axial slices. This sequence uses balanced dephasing gradient pulses before and after each 180$^\circ$ pulse to remove artifactual ("ghost") components of transverse magnetization.

Preparation of Sample for Electron Microscopy

Either SS4 or SS2 cells were infused after pretreatment with dDAVP via a femoral artery catheter. Five minutes after the injection was complete, 120 mL of isotonic saline was gently infused via the vena cava using a firmly tied cannula in the direction of the heart and 2 mL of saline was very gently introduced via the femoral artery catheter. This was followed immediately by 60 mL of freshly prepared 2% glutaraldehyde in phosphate-buffered saline in the vena cava, which was cut below the site of cannulation before the saline flush. Finally, 2 mL 2% glutaraldehyde was gently infused via the femoral catheter. Care was taken at all times to avoid excess pressure. Immediately after this treatment, thin slices of the skeletal muscle from several locations in the thigh were sampled and submerged in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer for 1 hour. After a 15-minute wash in 0.1 mol/L cacodylate buffer, the tissue was postfixed with 1% osmium tetroxide/0.1 mol/L cacodylate buffer (pH 7.4) for 1 hour, followed by a buffer rinse for 15 minutes and a 15-minute rinse in distilled H$_2$O. The tissue was then transferred to 1% uranyl acetate for 1 hour, dehydrated in a graded series of ethanol solutions, and embedded in EPON LIX12 (Ladd Research, Burlington, VT). Sections (80 nm) were cut using a Reichert Ultracut E ultramicrotome and stained with uranyl acetate, followed by lead citrate and viewed in a JEOL JEM 1200 EX electron microscope (JEOL [USA] Inc, Peabody, MA). A total of six animals injected with SS cells were examined: three with SS2 with dDAVP and three with SS4 without dDAVP. In addition, two animals that had been injected with AA cells were examined.

Visualization of the Microcirculation With Microfil

Either AA, SS2, or SS4 cells were injected via femoral catheter and 5 minutes later Microfil was introduced via the same catheter. In the set of experiments illustrated in Fig 1, dDAVP was administered (as described above) 15 minutes before the introduction of cells via the catheter. Microfil is a low-viscosity silicon rubber that subsequently polymerizes. The tissue was then fixed in 3.7% formaldehyde for 2 weeks, bleached in hydrogen peroxide, washed, dehydrated in alcohol and benzol, and cleared in methyl salicylate. This process results in a semitranslucent tissue, without cellular detail, in which the Microfil-perfused vessels stand out sharply. Finally, it is examined by light microscopy using transillumination, which causes the Microfil-perfused vessels to appear black and stand out sharply. Some tissue structures and RBCs remain visible, but translucent. A total of four animals were examined: SS2 with and without dDAVP and SS4 with and without dDAVP.

RESULTS

The Effect of dDAVP on Retention of Cells

We administered dDAVP (which stimulates the release of UL-WF in rats) and may possibly stimulate the release of other substances) intraperitoneally at a rate of 1 pg/kg 15 minutes before the injection of Tc-labeled human cells. The cells, which were obtained from crisis-free patients, were injected directly into the femoral artery via a 30-gauge needle. Figure 2 illustrates the number of cells retained in the rat thigh at 1 hour postfemoral artery injection for AA, SS2, and SS4 (density-defined sickle dense cells) in the presence and absence of prior treatment of the animal with dDAVP. Quantification of the number of cells retained was performed by $\gamma$ camera imaging of technetium-labeled RBCs. When administered 15 minutes before the injection of RBCs, dDAVP increased the retention of the more deformable AA (normal) RBC from 0.54 ± 0.2 µL to 1.43 ± 0.2 µL (N = 4 and N = 5, P < .0005; mean ± standard deviation, number of animals) and SS2 (density-defined, sickle discocytes) RBC from 4.4 ± 1.4 to 12.2 ± 6.3 (N = 6 and N = 19, P < .026), while no statistically significant effect was observed with SS4 cells, which increased from 12.7 ± 7.5 to 15.9 ± 7.4 (N = 33 and N = 11, P < .22).

If dDAVP is mixed into the cell suspension when it is injected, retention of cells is not enhanced, which is consistent with the observation that the release of vWF occurs with a 10 to 15 minute delay after the exposure of endothelial cells.
Examination of longer series, such as the 33 cases in which SS4 only was injected, shows that both donor to donor and animal to animal variation occurs.

Rate of Release of SS2 and SS4 Cells in Presence and Absence of dDAVP Pretreatment

Short-term effects. If cells are injected via a femoral catheter while the animal is observed in the gamma camera, both the rate of release of cells from the microcirculation of the leg and the ultimate fate of the cells in the body can be observed. We find that after a relatively constant maximum number of cells in the leg is reached, cells begin to enter the vena cava, probably via arterial-venous shunts leading to the femoral vein, reach the lungs, and are briefly retained, and then are taken up by the liver, kidney, and spleen and held tightly.

The kinetics of uptake and release in the leg are illustrated in Fig 3. Dense cells (SS4) rapidly reach a maximum level during the bolus injection and are then slowly released. In contrast, a larger number of discocytes (SS2) are briefly retained and then rapidly released; however, if the animal is pretreated with dDAVP, the rate of release of SS2 cells is slowed.

If short time segments are examined, the kinetics of release are approximately exponential. Examination of release over long periods of time with inclusion of early time points shows multieponential behavior that we have not attempted to characterize mathematically.

Long-term effects. Trapped SS4 cells are slowly eluted from the leg with half of the cells lost in the first 8 hours. AA and SS2 cells appear to be eluted more rapidly with half of the cells lost in the first 4.5 hours. Loss of activity (as determined by gamma camera imaging of the Tc-labeled cells) from the leg is accompanied by increased activity in the liver. Comparison of the rate of release of SS2 cells in the presence and absence of dDAVP pretreatment shows...
that, over a 6-hour period, treatment with dDAVP significantly slows the rate of loss of cells from the thigh (Fig 4).

Edema Develops at the Site Where Dense Cells Are Retained

Femoral artery injection of 100 to 200 µL of dense SS red cells (SS4) results in retention of 10% to 20% of these cells in the microcirculation of the thigh. The remaining cells return, possibly via arterio-venous shunts, to the vena cava and enter the general circulation where they are first taken up by the lungs and then by the reticuloendothelial system (RES) in the liver, kidney, and spleen. There is strong correlation between the area in the thigh in which the

Comparison of Change in MRI (Edema) and Number of SS2 or SS4 Cells Retained in Leg

The number of cells retained in the thigh is variable from animal to animal for all cell types; however, statistically significant trends can be detected. As indicated by Fig 2, the largest number of retained cells were observed after injection of SS2 in the presence of dDAVP and SS4 irrespective of whether dDAVP was administered. The proton magnetic resonance spin-lattice relaxation time (H-1 T1) can be correlated linearly with increased tissue water content or edema (these experiments will be described in a separate publication). When H-1 T1 was measured 18 to 28 hours after the injection of sickle cells as a function of the number of cells retained in the thigh, the results for animals injected with SS2 and SS4 cells were quite different. The time interval was chosen based on the previous observation that the maximum change in T1 occurred at this time. To minimize animal to animal variation of T1, all results were normalized by dividing the T1 in the injected leg by the T1 in the control leg of the same animal:

\[ T_{\text{norm}} = \frac{T_{\text{inj}}}{T_{\text{control}}} \]

In animals injected with SS4 cells there is a linear relation between the number of cells trapped and the ratio of T1inj/T1control, which is in agreement with our previously reported results. In contrast, in animals injected with SS2 cells, no elevation in the ratio of T1inj/T1control was seen at any level of retained cells (Fig 6). No elevation in the ratio of T1inj/T1control was seen in animals injected with AA cells or SS2 cells without dDAVP (results not shown); however, the number of cells retained in both of these instances was much smaller than in either of the cases described above and therefore no attempt was made to interpret these results.
SS RED CELL ADHESION IN VIVO

SS2 cells are retained by adhesion and that the vessel lumen remains open, which allows the rat’s RBCs and plasma to be washed out during the saline perfusion phase. However, as soon as the saline wash was performed, active perfusion with oxygenated RBCs ended, which has been found to result in profound anoxia in microcirculatory beds within 2 minutes or less.33 Because the delay time (the time between deoxygenation and polymer formation) is very short, usually of the order of milliseconds and at most seconds, for SS RBCs,34 the presence of polymer does not indicate that the tissue was anoxic before saline perfusion was begun.

In contrast, after the injection of SS4 cells (Fig 7C), only about half of the cells contain polymer. The rest of the cells must be rat RBCs that are trapped retrograde to the blocked portions of the microcirculation. All SS4 cells would form polymer under these experimental conditions because they have an MCHC in excess of 42 g Hb/dL and are depleted of F cells (cells with high HbF content), which are the only SS cells that are resistant to polymerization. In addition to rat RBCs, in many instances the lumen also contained plasma, which suggests that the saline wash was unable to reach these vessels. Expanded interstitial space around the blood vessels was seen for tissues infused with both SS2 and SS4 cells. This finding suggests that partial blockage was present and resulted in exudate around the vessels either before or during the saline wash phase of sample preparation. Damaged mitochondria were also observed in tissue perfused with SS4 cells.

Visualization of the Microcirculation With Microfil

RBCs are not usually detected in blood vessels after Microfil injection. We find that after the injection of SS2 cells following pretreatment with dDAVP, most of the microcirculation remains accessible to Microfil, but that in small vessels with a diameter at least twice that of the RBCs

Transmission Electron Microscopy

Injection of AA cells resulted in little retention of cells, the lumen of all capillaries was free of plasma, and the morphology of the fixed tissue was normal with intact mitochondria and no widening of the interstitial space around capillaries (Fig 7A).

In contrast, after the injection of SS2, examination of larger vessels showed cells adhered to the walls of venules or arterioles (distinction between vessel types was not possible in these samples). All of the RBCs found in large vessels contained polymer and were therefore human sickle cells (Fig 7B). Smaller vessels also contained polymerized human RBCs and were free of plasma. This indicates that

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**Fig 5.** Comparison of localization of cells by gamma camera (A) with the appearance of edema detected by proton MRI at 0.18 Tesla (B). (A) Bright areas result from the detection of γ-emission from 99mTc-labeled RBCs that are localized in the thigh, liver, and kidney. In the proton MRI (B), bright areas are due to prolonged proton relaxation time in this T₁-weighted image. Prolonged relaxation times have been correlated with elevated tissue water content in this model. Note that edema appears in the same area as that in which cells are retained and not at the site of the incision and femoral artery injection (arrow).

**Fig 6.** The effect of dDAVP-induced cell retention on tissue edema for SS discocytes (SS2) and SS dense cells (SS4). The abscissa represents the ratio of the longitudinal proton relaxation time $T_1$ in the injected leg (which is proportional to tissue water content) over the $T_1$ in the control leg. The number of cell retained in the injected leg as determined by γ-camera imaging are displayed on the ordinate. Dense cells in the absence of dDAVP (○), dense cells after dDAVP pretreatment (●), and discocytes after dDAVP pretreatment (▲). The results for discocytes in the absence of dDAVP are not displayed because they cluster around the origin. Note that retention of large numbers of discocytes does not result in edema.
Fig 7. Appearance of the microcirculation of skeletal muscle in the rat thigh 5 minutes after femoral artery injection of normal human RBCs (AA) (A), SS discocytes (B), or SS dense cells (C). Note that after injection of either AA or SS2 cells, the lumen (L) is clear, in contrast to (C), in which the lumen contains plasma. After the injection of AA cells, the mitochondria (M) are intact; whereas after injection of SS dense cells the mitochondria are swollen and disrupted. Similarly, the collagen layer (C) surrounding the vessels is compact after injection of AA cells, but is disrupted after injection of both SS discocytes and dense cells. HbS polymer (P) is detected in all cells found after injection of SS discocytes; however, after injection of SS dense cells, rat RBCs (R) are also trapped.

and larger (which may be postcapillary venules), cells line the walls and only a narrow passage remains open. In vessels with a diameter similar to that of an RBC, adhesion and obstruction due to poor deformability cannot be distinguished. Some extreme narrowing suggesting vaso-oedema was also observed.

After the injection of SS4 cells following pretreatment with dDAVP, one of two cases prevailed: (1) the microcirculation (including capillaries) remained open and uncompromised, or (2) entire regions were totally inaccessible to Microfil and hence unperfused. A picture of patchy obstruction emerges with perfused and unperfused areas existing side by side. These areas may remain anoxic and account for the subsequent edema observed by MRI. In contrast to our observation with SS2 cells, very few SS4 cells can be detected in larger vessels, although cells can clearly be detected in small vessels that have a diameter similar to that of an RBC.

DISCUSSION

The results reported here reflect the effect of dDAVP on the disparate interaction of discocytes and dense cells with the microcirculation in an intact animal model and can be interpreted in terms of adhesion by discocytes and obstruction by dense cells. More specifically, these results allow us to draw the following conclusions.

Pretreatment With Desmopressin (dDAVP) Increases the Retention of Deformable SS Discocytes and AA RBCs to the Microcirculation of the Thigh and This Retention Is Adhesion-Mediated

We conclude that adhesion of SS discocytes and normal RBCs to the endothelium of the microcirculation is the mechanism for retention of these human RBCs in the rat thigh for the following reasons. (1) Cells are observed by electron microscopy attached to the endothelium in vessels containing no plasma, showing that the lumen was accessible to the saline wash and effectively patent to flow. (2) The RBCs retained were almost exclusively human RBCs based on the observation of polymerization in most cells when SS discocytes were perfused. (3) Microfil injection clearly shows individual RBCs adhering to blood vessels with diameters greater than twice that of an RBC after the injection of SS2 cells in both the presence and absence of dDAVP. Nevertheless, the proximal portions of these vessels remain accessible to Microfil, which implies that the vessel is patent. (4) The absence of MRI alterations after the injection of discocytes is compatible with the vessel lumen remaining open (as is expected if RBC retention is due to adhesion), perfusion possible, and a lack of subsequent hypoxic changes in the tissue where SS discocyte adhesion takes place. This result explains the failure of SS2 cells to cause edema and altered intensity in H-1 MRI (Fig 6).

We interpret these results to mean that dDAVP increases the adhesion of normal (AA) and deformable SS discocytes (SS2 RBCs) and that this adhesion (in the absence of other type of SS cells) does not involve obstruction of the vessel.

That dDAVP enhances adhesion is consistent with, but does not prove, that RBC adhesion in the in vivo microcirculation is mediated by vWF. In support of this interpretation are the findings of Tsai et al.27 who showed that treatment of rats with dDAVP results in the release of vWF. This interpretation is in agreement with the results obtained with cultured endothelial cells20,21,35,36 and in the rat mesooccum.25,24,27 Nevertheless, caution should be exercised because dDAVP is involved in the release of other proteins, such as GMP-140,37,38 which may be involved either in adhesive processes of RBCs or other cells such as platelets or neutrophils, and may induce other functional changes in endothelial cells. No statistically significant effect of dDAVP
on SS4 retention was observed under these experimental conditions. This result does not preclude adhesion of some cells in the SS4 density fraction; indeed, Kaul et al report that adhesion of SS4 is substantially weaker than that of SS2 cells, but is clearly present.

*dDAVP Pretreatment Decreases the Rate of Release of SS Deformable Discocytes From Adhered Sites*

Two aspects of these results merit emphasis. First, the retention (by adhesion or occlusion) of RBCs in the microcirculation of the rat leg is a reversible process. Second, the rate of release of deformable RBCs is decreased by dDAVP pretreatment. This effect is reminiscent of the role of vWF in platelet adhesion and spreading. Platelet adhesion occurs transiently in the absence of vWF, but only in the presence of vWF does spreading and strong adhesion occur.

**Retention of SS Dense Cells (SS4) Is dDAVP Insensitive and Is Characterized by Electron Microscopy and H-1 MRI Features Compatible With Vasoocclusion**

As shown in Fig 2, the retention of dense SS cells (SS4 density fraction) in the microcirculation of the thigh is not significantly enhanced by dDAVP under these experimental conditions. This feature in itself suggests that the retention of dense cells by the microcirculation is controlled by a different pathophysiologic mechanism than that of deformable cells.

Electron microscopy of the microcirculatory beds of the thigh presents a different picture from that seen after the injection of SS2 cells. After injection of SS4 cells (Fig 7c), only about half of the cells trapped contain polymer (the majority of cells without polymer are rat RBCs) and the lumen of the vessel frequently contains plasma. We conclude that, in the case of dense cells (SS4), vessels in the area of cell retention are obstructed because the lumen cannot be washed free of rat RBCs and plasma. Microfil presents a similar picture in that the walls of intermediate sized vessels are smooth and free of adhered cells even after pretreatment with dDAVP; however, numerous blunt ends were noted, which is compatible with obstruction. Failure to see evidence of individual RBCs in vessels with a diameter more than twice that of an RBC is the strongest evidence against a major role for dDAVP-mediated, endothelial adhesion in the retention of SS4 cells; however, cell to cell interactions may account for the relatively slow release of SS4 cells once they have become lodged in the microcirculation. In these experiments, it is impossible to distinguish between obstruction due to poor deformability and adhesion in smaller vessels where the cell can touch both sides.

**Comparison of Change in H-1 MRI Longitudinal Relaxation Time (Which Is Correlated With Edema) and the Number of SS2 or SS4 Cells Retained in Leg Provide Further Proof of the Differences in the Mechanism Involved in the Retention of These Two Types of SS Cells**

Prolonged proton relaxation (T1 and T2), which can be correlated with tissue edema, is observed in the thigh after femoral artery injection of dense cells. This finding, which in conjunction with decreased PCR and acidification strongly suggests vasoocclusion, contrasts with the absence of these changes when deformable discocytes are injected.

We have previously shown that the T1 in the injected leg increases in proportion to the number of SS dense cells trapped in the leg. Moreover, the ratio of T1/PCR increases in proportion to the increase in the P/PCR ratio that can serve as an indicator of tissue anoxia.

When AA or deformable SS discocytes (SS2) cells are injected in the absence of dDAVP, very few cells are retained. Under these conditions, there is no edema, which may be due to the small number of retained cells.

We find here that even when the number of discocytes retained after pretreatment with dDAVP was comparable to the number of dense cells with or without dDAVP, there was still no elevation in T1 or T2 (Fig 6). This result implies that these cells did not cause anoxia and further strengthens the contention that when only SS discocytes are injected, they are retained as the result of adhesion and do not induce vascular obstruction per se.

**The Fate of the SS RBCs Injected in the Femoral Artery**

The animal model used here has been previously described in detail. An accounting of the fate of all the cells injected in the femoral artery of the rat shows that more than 90% of the cells not retained in the thigh are retained in the liver, kidney, or spleen. This result and the observation of intact cells by electron microscopy both indicate that the results described here are the effect of intact sickle cells on the microcirculation. That intact cells are eventually released from the microcirculation can be shown by monitoring the uptake of radio-label by the liver, which occurs only in the case of intact cells. This conclusion is based on the following observations: (1) When human RBCs are introduced into the circulation of the rat, those which are not retained by the microcirculation downstream from the point of injection are very effectively removed by the liver. (2) The cells that remove foreign RBCs are Kupfer cells, which retain intact RBCs or cell membranes, but not Hb. (3) Technetium labeling is primarily located on Hb, as shown by Bardy et al.

Failure to observe an increase in edema after prolonged retention of deformable discocytes shows that retention of a large number of human RBCs in the rat microcirculation is not in itself sufficient to induce edema or any other inflammatory response. This further strengthens the conclusion that edema after the injection of dense cells is due to obstruction followed by anoxia and is not due to the presence of large numbers of human RBCs in the microcirculation of the rat.

**Conclusions and Significance**

Pretreatment with dDAVP causes a statistically significant increase in the number of AA RBCs and SS discocytes retained in the rat leg in contrast to the absence of significant increase in the number of cells retained when
dense (SS4) cells are infused. Although retained SS dense cells result in ischemia and subsequent edema detectable by increased H-1 MRI relaxation time, dDAVP-induced retention of a similar quantity of SS2 cells does not. These findings, in addition to electromicroscopy and Microfil experiments and the retarded rate of SS discocyte release after dDAVP pretreatment, allow us to conclude that dDAVP enhances retention of AA and SS2 cells in the microcirculation of the rat by adhesion to the vascular endothelium. However, adhesion of SS discocytes is not sufficient to cause vasoocclusion or edema. In contrast, when SS dense cells are perfused alone, retention of these cells leads to vasoocclusion and trapping of rat RBCs.

These findings are consistent with the hypothesis that adhesion of deformable SS cells can lead to enhanced vasoocclusion if nondeformable cells such as SS dense cells are also present. In this scheme, adhesion of deformable SS cells will precede trapping of dense cells, which then results in blockage of the vascular lumen. Indeed, there is clear experimental evidence for this type of phenomena in the ex vivo microcirculatory preparation model. The challenge of the next phase is to test this hypothesis in the intact animal model.

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Demonstration of endothelial adhesion of sickle cells in vivo: a distinct role for deformable sickle cell discocytes

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