Adhesion molecules and hydroxyurea in the pathophysiology of sickle cell disease

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Sickle cell disease (SCD) is a systemic disorder caused by a mutation in the gene encoding the β chain of hemoglobin. This mutation leads to the production of sickle hemoglobin (HbS) which is predisposed to polymerization while the hemoglobin is not oxygenated. HbS polymerization and Hb denaturation are thought to result in oxidant damage to the red blood cell membrane. Associated with such damage is abnormal erythrocyte cation homeostasis, which in turn can result in dehydrated dense cells and irreversibly sickled cells.[1] Red cell abnormalities result in both hemolysis and vaso-occlusion. Vaso-occlusion appears to be a complex event mediated by red cell and leukocyte adhesion, inflammation, injury to the endothelium, and activation of coagulation pathways. In addition, hemolysis probably contributes to dysregulation of nitric oxide. Vaso-occlusion is the major cause of morbidity and mortality in SCD, with occlusion of blood vessels followed by ischemia or infarction in various tissues, leading ultimately to progressive end organ damage.

The tendency of sickle red blood cells to adhere to the vascular endothelium is believed to be a major contributor to and possibly primary cause of the vaso-occlusive process.[2] Mediators of adhesion have, therefore, become a potential new target for pharmacological therapy to combat the complications of SCD. An article in this issue of Haematologica examines the effect of hydroxyurea on the expression of erythrocyte adhesion receptors in children with SCD. In a multicenter adult, trial hydroxyurea was shown to reduce the frequency of vaso-occlusive events.[3] Although initially thought to exert its beneficial effect primarily by raising HbF levels, and thereby decreasing red cell sickling, it has been suggested more recently that one of the mechanisms by which hydroxyurea produces clinical benefit is by decreasing erythrocyte adhesion, which presumably results in decreased vaso-occlusion.

The process of vaso-occlusion

Our understanding of vaso-occlusion has evolved over several decades. It was first thought that hemoglobin S polymerization resulted in the entrapment of sickled, poorly deformable erythrocytes that mechanically blocked small caliber vessels. It was later recognized that these damaged erythrocytes alone are not sufficient to produce the vaso-occlusion in larger vessels which can be observed in both humans and in animal models. End organ complications of SCD occur as a result not only of small vessel disease but also in post-capillary vessels and in arteries. This is particularly evident from the increased risk of cerebrovascular complications that tend to involve large cerebral arterial vessels in patients with SCD.[1] Damage similar to that seen in patients with atherosclerotic vascular disease has been seen in the large cerebral vessels of patients with SCD, including intimal hyperplasia, and fibroblast and smooth muscle proliferation. Endothelial damage and activation due to interactions between adherent sickle erythrocytes and the endothelium[4] are therefore thought to contribute to the vasculopathy involving small and large vessels in SCD.

Initial investigations into the pathogenesis of SCD concentrated on the role of abnormal erythrocytes and the consequences of hemoglobin S polymerization. Hoover *et al.* and Hebbel *et al.*[5,6] went on to demonstrate that HbS erythrocytes had a tendency to adhere to cultured vascular endothelial cells. Hebbel *et al.* also found that the increased adhesiveness of sickle erythrocytes *in vitro* seemed to correlate with the severity of clinical disease.[7] However, it has also been suggested that other factors contribute to the process of vaso-occlusion, including chronic vascular inflammation, leukocyte adhesion, platelet activation and aggregation, activation of coagulation, endothelial damage, and decreased nitric oxide bioavailability.[1]

Sickle erythrocytes have been shown to be abnormally adherent to at least three extracellular matrix proteins: thrombospondin (TSP), laminin, and fibronectin, of which laminin and thrombospondin seem to be the most important. Laminins are a family of proteins, each comprising a single α, β, and γ chain. Laminins with the α5 chain appear to interact with erythrocyte adhesion receptors. TSP is a multifunctional adhesive protein. Both TSP and laminin are extracellular matrix proteins that are also found in a soluble form in plasma. TSP is expressed by many cell types, including activated platelets and endothelial cells.[8,9]

Erythrocyte surface adhesion receptors

There are several cell surface receptors that appear to be involved in mediating the interaction between HbS erythrocytes and the endothelium. These include: Lutheran blood group antigen, also known as basal cell adhesion molecule/Lu (BCAM/Lu, CD239); integrin associated protein (CD47); CD147; intercellular adhesion molecule-4 (ICAM-4, the protein bearing the LW blood group antigens); CD36 on reticulocytes; very late activation antigen 4 (VLA-4) on reticulocytes; and sulfated glycolipids.[10] Membrane damage to the HbS erythrocyte also leads to the exposure of phosphatidylserine, which is normally restricted to the inner surface of the mem-

brane lipid bilayer. This phosphatidylserine exposure is also thought to contribute to the adhesiveness of HbS erythrocytes to the endothelium.[1,8]

Over recent years, many studies have aimed to clarify the role of adhesion molecules in the interaction between the endothelium and sickle erythrocyte. BCAM/Lu is a protein expressed by a variety of cell types including the erythrocyte. It is a high-affinity receptor for laminins that contain the α5 chain. Both normal and HbS erythrocytes bear this laminin receptor, but HbS erythrocytes have increased expression of BCAM/Lu.[11,12] Zen *et al*. confirmed that BCAM/Lu is the most important receptor mediating HbS erythrocyte adhesion to laminin under flow and static conditions.[13] The BCAM/Lu interaction with immobilized laminin under flow conditions appeared to be upregulated by protein kinase A (PKA), downstream of β2 adrenergic receptors.[13,14]

While BCAM/Lu is found on both reticulocytes and mature erythrocytes, CD36 and VLA-4 expression, however, is limited to reticulocytes. CD36 and VLA-4 are among the most extensively studied adhesion receptors. VLA-4 is a member of the integrin family of cell surface adhesion receptors and is also known as α4β1 integrin. It is expressed by a variety of cell types, including early erythroid progenitors, but expression is lost during erythrocyte maturation, so that circulating mature erythrocytes do not normally carry this receptor. VLA-4 has been shown to be highly expressed on circulating HbS reticulocytes.[15] Ligands for VLA-4 include VCAM-1[16] and the extracellular matrix protein fibronectin.[17] VLA-4 also binds to a lesser extent to TSP.

VCAM-1 is expressed on the endothelium and is also found in increased amounts in the plasma of SCD patients. Endothelial expression of VCAM-1 is increased by cytokines such as tumor necrosis factor (TNF)-α, platelet activating factor (PAF) and interleukin-1 (IL-1). Reported data confirm that HbS reticulocytes show increased adhesion to endothelial cells stimulated by TNF-α and that this adhesion can be inhibited by antibodies to VCAM-1 and integrin. This suggests a role for the interaction of VLA-4 and VCAM-1 in vaso-occlusion,[16] and perhaps, in particular, in the setting of inflammation or infection.

CD36, or glycoprotein IV, is also an adhesion receptor limited to immature erythroid cells. CD36 can bind to endothelial receptors via von Willebrand factor and extracellular matrix proteins such as TSP. It can mediate adhesion to αVβ3 receptors on the endothelium via TSP. It has also been suggested that the interaction of CD36 with thrombospondin and αVβ3 is a potential mechanism by which HbS erythrocyte adhesion may contribute to vaso-occlusion.[18,19]

Another erythrocyte adhesion receptor for TSP is CD47, also known as integrin-associated protein (IAP). CD47 is expressed by both normal and HbS erythrocytes. However, normal erythrocytes do not adhere to TSP in the same way as HbS erythrocytes. The reason for this difference is unclear. However some investigators have suggested that one reason may be the abundance of reticulocytes in SCD patients, since reticulocytes express $\alpha_4\beta_1$ integrins, and CD47 is thought to function only in association with integrins. Brittain *et al*. showed that CD47 stimulates HbS erythrocyte binding to immobilized TSP, VCAM-1, and soluble fibronectin through activation of the α4β1 integrin on these cells.[20]

LW glycoprotein, or ICAM-4, is another erythrocyte adhesion molecule implicated in HbS red cell adherence to endothelium. It is a member of the immunoglobulin superfamily, is found mostly on erythrocytes, and binds to αV-integrin ligands. Kaul *et al*. first provided evidence that αVβ3 contributes to HbS erythrocyte-endothelial interactions and therefore to vaso-occlusion. They showed that antibodies to αV-integrin inhibited HbS erythrocyte adherence to rat endothelium treated with PAF and also improved microcirculation.[21] Zennadi *et al*. demonstrated that ICAM-4 was the red cell ligand for endothelial cell αVβ3 and can be activated by epinephrine through a PKA-dependent pathway, therefore also suggesting a role for physiological stress in triggering vaso-occlusive events.[22,23] In a later study, Kaul *et al*. also demonstrated that inhibition of ICAM-4-$\alpha_v\beta_3$ interactions by ICAM-4-derived peptides improved circulation of red cells,[24] thereby suggesting that this could be a potentially useful therapeutic target.

The exposure of phosphatidylserine on erythrocytes has also been shown to contribute to erythrocyte adhesion to endothelium.[8,25] The distribution of phospholipids in the normal erythrocyte membrane is asymmetrical, with phosphatidylserine located exclusively in the inner leaflet. In SCD, erythrocyte damage leads to loss of the normal phospholipid arrangement, and phosphatidylserine is exposed at the surface.[26] Manodori *et al*. showed that phosphatidylserine exposure on HbS erythrocytes leads to phosphatidylserine-mediated erythrocyte adhesion to the extracellular TSP of a cultured endothelial monolayer. This adhesion was reduced when phosphatidylserine was blocked by annexin V.[8]

The adhesion molecule CD147, a highly glycosylated cell surface protein, is also expressed by mature circulating erythrocytes, is expressed throughout erythroid development and by leukocytes, platelets and endothelial cells. It is the carrier molecule for the blood group antigen Ok^a. Soluble recombinant forms of this protein bind to various cells, including endothelial cells, and the lack of CD147 has been implicated in RBC trapping in the spleen.[27] However, it is as yet unclear how this adhesion molecule might contribute to vaso-occlusion.

Role of leukocytes in vaso-occlusion

Clinical observations that leukocytosis correlates with poor outcome and increased severity of disease in some patients suggest that leukocytes play a role in the pathogenesis of SCD. A high steady state leukocyte count is a risk factor for acute chest syndrome, stroke, and mortal-

ity. In a multicenter study of hydroxyurea in adults, the clinical benefits correlated with a reduction in the neutrophil count, even without the expected increase in hemoglobin-F levels.[3,28] *In vitro* studies have also demonstrated that HbS erythrocytes bind to neutrophils in a static adhesion assay.[29] It has been suggested that activation of the vascular endothelium promotes leukocyte recruitment, activation and adhesion, culminating in adhesive interactions between circulating erythrocytes and adherent leukocytes. The HbS erythrocytes may then become trapped, and this promotes deoxygenation and sickling of the erythrocytes. Vaso-occlusion may then occur as the flow of the HbS erythrocytes is impaired. Neutrophils in SCD patients have also been shown to be less deformable than those in patients without SCD. This makes it more likely that adherence of these cells could contribute to vascular obstruction.[1]

Leukocyte adhesion to vascular endothelium is mediated by the interaction of leukocyte adhesion molecules L-selectin (CD62L), αMβ2 integrin (CD11b/18) and αLβ2 integrin (CD11a/18) with endothelial adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin.[28] These endothelial adhesion molecules are also implicated in vascular inflammation. A role for CD64 has also been demonstrated specifically in the adhesion of neutrophils to the endothelium.[30] Turhan *et al*. demonstrated that recruitment of leukocytes was reduced in sickle mice lacking P- and E-selectins, resulting in protection from vaso-occlusion and increased survival.[31]This and other studies clearly suggest a role for leukocyte adhesion in vaso-occlusion.

Conflicting data have been published regarding the expression of integrins on the surface of neutrophils in SCD. Fadlon *et al*. and Benkerrou *et al*. reported no difference in leukocyte integrin expression between SCD patients and healthy controls.[30,32] However, in a later study, Okpala *et al*. found that steady state expression of αMβ2 integrin by neutrophils and of αLβ2 by lymphocytes was higher in SCD patients compared with healthy controls.[28] All these studies agree that expression of L-selectin, which seems to be primarily responsible for the contact between leukocytes and the blood vessel wall, is increased in SCD patients experiencing vaso-occlusive crises.[28,30,32] Animal data suggest that the leukocyte subclass predominantly recruited to the endothelium and participating in the vast majority of interactions with circulating HbS erythrocytes is composed of neutrophils.

Data also suggest that neutrophil activation is increased in SCD. Fadlon *et al*. have reported that neutrophils expressing CD64, a marker of neutrophil activation, were increased in SCD patients both during steady state and during crisis. $CD64^+$ neutrophils were shown to be highly adherent to untreated vascular endothelium, and adhesion was enhanced by pre-treatment of the cultured endothelial monolayers with TNF-α. However, attempts to inhibit leukocyte adhesion using CD64 antibodies gave inconsistent results.[30]

Role of platelets in vaso-occlusion

The role of platelets in vaso-occlusion has been less clear, although there is certainly evidence to suggest platelet involvement in SCD pathogenesis. Circulating platelets in SCD patients are abnormally activated during both steady state and vaso-occlusive crisis.[33] This can be seen from increased levels of platelet contents such as thrombospondin and IL-1 in the plasma, as well as increased platelet P-selectin and von Willebrand factor (vWF). Increased expression of P-selectin is thought to be an initial step in the development of vascular disease, since P-selectins are involved in promoting platelet adhesion to damaged vascular endothelium and initiating thrombosis.[1,4,8]

Damaged HbS erythrocytes demonstrate abnormal phospholipid asymmetry, with phosphatidylserine exposed on their outer surface, and activated platelets also circulate in the same state. Exposed phosphatidylserine provides a platform for coagulation factors and promotes procoagulant activity. It has not yet been clearly demonstrated how these processes contribute to vaso-occlusion-but they may serve as a bridging mechanism for the adhesive interactions between circulating sickle erythrocytes, leukocytes, and the endothelium.[1]

Endothelial factors in vaso-occlusion

The endothelial proteins involved in abnormal adhesion in patients with SCD include VCAM-1, ICAM-1, E-selectin, P-selectin, laminin, TSP, fibronectin, and αVβ3 integrin. These proteins interact with adhesion receptors on sickle erythrocytes and leukocytes. Specific interactions are summarized in Table 1. Asymptomatic SCD patients have been found to have increased circulating levels of soluble VCAM-1, ICAM-1, P-selectin, laminin, and TSP. During vaso-occlusive episodes, patients have increased numbers of circulating activated endothelial cells that express ICAM-1, VCAM-1, P-selectin, and E-selectin.[4,34] There is also some evidence that HbS erythrocytes activate or damage endothelial cells. Brown *et al*. demonstrated increased expression of VCAM-1, E-selectin, and ICAM-1 when cultured endothelial cells were exposed to HbS erythrocytes. E-selectin and ICAM-1 are thought to be important for promoting the inflammatory response observed after ischemia and reperfusion injury, which would be a consequence of vaso-occlusion.[4]

The cell-to-cell and cell-to-endothelial ECM interactions that have been identified and which are thought to contribute to vaso-occlusion present potential targets for pharmacological approaches to ameliorate the severity of disease in patients with SCD. Finnegan *et al*. investigated the use of a small peptide molecule to block adhesion mediated by αVβ3 integrin. This endothelial integrin appears to have multiple interactions besides that with ICAM-4/LW. It has been thought to bind to CD36/TSP,

Table 1. Red blood cell adhesion receptors and their adhesive interactions.

Adhesion molecule and alternative names	*Ligand/adhesive function*
Expressed by reticulocytes only and proven active on SS reticulocytes	
CD36 (platelet glycoprotein IV, Naka [platelets])	Fibronectin, thrombospondin, von Willebrand factor
VLA-4 ($\alpha 4\beta 1$ integrin, CD49d/CD29)	VCAM-1, fibronectin, thrombospondin
Expressed by immature and mature erythrocytes and proven active on sickle red blood cells	
BCAM/Lu (Lutheran, CD239)	Laminin ($\alpha 5$ containing variants only), $\alpha 4\beta_1$ integrin
ICAM-4 (LW, CD242)	Multiple integrins, including endothelial $\alpha_V\beta_3$, leukocyte integrins (LFA-1 [$\alpha_L\beta_2$], Mac-1 [$\alpha_M\beta_2$], $\alpha_V\beta_1$, $\alpha_V\beta_5$), platelet integrin $\alpha IIb\beta_3$
CD47 (integrin-associated protein, Rh-related protein)	VCAM-1, thrombospondin, fibronectin
Expressed by immature and mature erythrocytes, but not yet proven active on sickle red blood cells	
CD44 (Indian, In(Lu)-related p80)	Hyaluronan, fibronectin, CD44
Lymphocyte-associated antigen-3 (LFA-3, CD58)	CD2
CD99 (MIC2 gene product)	Lymphocyte CD99 necessary for formation of T-cell rosettes
JMH (semaphorin K1, SEMA7A, CD108)	May have role in adhesion of activated lymphocytes
CD147 (Ok^a, neurothelin)	Type IV collagen, fibronectin, laminin in other tissues
Scianna (ERMAP)	Putative adhesive function
MER2 (CD151)	Forms laminin-binding complexes with integrins

CD47/TSP, sulfated glycolipids/TSP, and sulfated glycolipids/vWF. A synthetic peptide based on the $\alpha V\beta 3$ binding domain of ICAM-4 decreased HbS erythrocyte adhesion and vaso-occlusion in PAF-stimulated *ex vivo* vasculature.[18] Though these results are promising, further study is needed before a drug with clinical benefit can be developed. So far, hydroxyurea is the only drug shown to decrease the frequency of vaso-occlusive episodes and provide clinical benefit for patients with SCD in a large trial.

Effects of hydroxyurea

Several studies have investigated the mechanisms by which hydroxyurea decreases the severity of disease in patients with SCD. The increase in hemoglobin-F observed in patients taking hydroxyurea almost certainly decreases sickling and hemolysis. However, other mechanisms are probably also responsible for producing benefit since some patients experience clinical improvement without an increase in hemoglobin-F.[3] Various studies have also shown that hydroxyurea decreases HbS erythrocyte adhesion. The first study to report a decrease in adhesion receptor expression with hydroxyurea therapy was by Styles *et al*. They found that HbS reticulocyte expression of both VLA-4 and CD36 measured by flow cytometry decreased within ten weeks of hydroxyurea therapy, with a more pronounced decrease in VLA-4 than CD36.[35] This finding was duplicated by Covas *et al*.[36] using flow cytometry and by Gambero *et al*.[19] who measured gene expression using real-time PCR. Covas *et al*. also found a decrease in phosphatidylserine expression on the surface of erythrocytes and platelets in patients on hydroxyurea therapy.

Hillery *et al*. investigated the effect of hydroxyurea therapy on HbS erythrocyte adhesion to extracellular matrix proteins TSP and laminin. They demonstrated a sustained decrease in adhesion under low shear flow conditions for patients receiving hydroxyurea. The effect on adhesion was seen prior to maximal increase in hemoglobin-F.[37] In addition to the effect of hydroxyurea on adhesion to the ECM proteins TSP and laminin, Brun *et al*. showed that hydroxyurea therapy downregulated the expression of VCAM-1 and endothelin-1 which are both increased in plasma in the setting of inflammation.[38] These studies therefore support a role for hydroxyurea in decreasing vaso-occlusive events by decreasing adhesive interactions. However, the mechanism by which hydroxyurea affects adhesion molecule expression is still not clear and further study is warranted.

In this issue of the journal, Odiévre and colleagues[39] examine the effect of hydroxyurea on adhesion receptors and on cultured erythroid progenitors. The adhesion receptors included in this study were: CD47, CD147, ICAM-4, BCAM/Lu, CD36 and VLA-4. Somewhat surprisingly, they found increased expression of BCAM/Lu, CD47, and CD147 following hydroxyurea therapy, with no significant change in ICAM-4 expression. However, expected decreases in CD36 and VLA-4 were seen. When erythroid progenitors were cultured in the presence or absence of hydroxyurea, VLA-4 and ICAM-4 expression were decreased and BCAM/Lu and CD47 expression increased in the presence of hydroxyurea. No significant change was seen in CD36 and CD147 expression, although they continued to be expressed at high levels. Odièvre *et al*. propose that the effects of hydroxyurea on erythroid progenitors observed in their study suggest that another mechanism other than modulation of membrane expression of adhesion molecules may mediate the effects of hydroxyurea on adhesion. This is the first published report of the effect of hydroxyurea on BCAM/Lu expression, and further study is therefore needed to confirm these findings.

The vaso-occlusive process in SCD is responsible for much of the morbidity and mortality observed in this disease. While there is growing evidence for the role of adhe-

sion molecules in vaso-occlusion, the mechanisms responsible for the adhesive interactions between blood cells and the endothelium are still not known. It has already been demonstrated that hydroxyurea, a drug with proven clinic benefit, can affect adhesion, which presumably contributes to the reduction of vaso-occlusive episodes. However, Odièvre *et al*. suggest that the mechanism by which hydroxyurea affects adhesion may not be straightforward. As the authors suggest, hydroxyurea may reduce HbS erythrocyte adhesion by affecting signaling pathways leading to receptor activation rather than by reducing adhesion receptor expression. Further studies are needed to clarify these issues.

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