

Pathophysiology and Treatment of Sickle Cell Disease

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Abstract: Current treatment and understanding of sickle cell disease require an appreciation for the complexity of its basic pathophysiology. The clinical manifestations of vaso-occlusion result from a dynamic combination of abnormalities in hemoglobin structure and function, red blood cell membrane integrity, erythrocyte density, endothelial activation, microvascular tone, inflammatory mediators, and coagulation factors. Existing and emerging therapies address each of these biologic alterations, individually and collectively. Examples include induction of fetal hemoglobin, modulation of erythrocyte hydration, augmentation of nitric oxide, chronic transfusion, stem cell transplantation, and gene therapy. Understanding the pleiotropic and epigenetic factors influencing disease phenotype may lead to more targeted application of these therapies.

Sickle cell anemia was among the first diseases to be understood at a molecular level, yet more than 50 years after the genetic basis of the disorder was first described many details of its primary pathophysiology are still not understood. While the proximate cause of the sickling disorders, the polymerization of hemoglobin S (HbS), has long since been elucidated mechanistically, exactly how this process results in the painful crises, acute chest syndrome, strokes, and other end-organ damage characteristic of the disease remains unclear. Research in recent years has refined the earlier model of microvascular obstruction and endovascular damage caused by trapped sickle erythrocytes. The new paradigm implicates dysregulated rheology, inflammation, coagulation, erythrocyte hydration, endothelial function, and vascular tone in a complex interplay that culminates in the familiar clinical manifestations of sickle cell disease (SCD). As the details of sickle cell pathophysiology have become more complicated, new potential targets have also begun to emerge for therapeutic interventions. This review will focus on the current understanding of the basic pathophysiology of SCD and the evolving treatment modalities that apply to this knowledge. While homozygous HbS disease (sickle cell anemia) is the most common type of SCD, the other sickling disorders, combining HbS with different β -globin gene mutations such as hemoglobin C (HbC) or β -thalassemia, share a similar basic pathophysiology. Although their clinical features may be distinct from sickle cell anemia, for the purposes of this review they will all be considered together collectively as SCD.

Keywords

Sickle cell disease, pathophysiology, treatment

Hemoglobin Polymerization

SCD results from a single base-pair mutation in the gene for the β -globin subunit of adult hemoglobin (HbA). An adenine-to-thymine substitution in the sixth codon replaces glutamic acid with valine in the resulting β -globin chain. The abnormal β^s globins paired with 2 normal α -globins yield the electrophoretically distinct sickle hemoglobin (HbS). In the deoxygenated conformation of HbS, the $\beta 6$ valine becomes buried in a hydrophobic pocket on an adjacent β -globin chain, joining the molecules together.¹ A collection of HbS molecules may aggregate in sufficient number to form a critical nucleus, with additional molecules stabilizing the polymer to grow into long, thin, helical fibers, which in turn become crosslinked by more molecules in an autocatalytic process.² Polymers exist in dynamic equilibrium with soluble hemoglobin tetramers, and in sufficient concentration form an insoluble gel, resulting in red blood cell (RBC) membrane damage, decreased deformability, and the sickle morphology for which the disease was named.³

A measurable time delay exists from nucleation to polymerization, influenced by several local factors including oxygen tension, pH, temperature, 2,3-diphosphoglycerate, and carbon monoxide levels. Erythrocyte HbS concentration exerts the strongest effect on the time to polymerization, which varies inversely as the 30th power of mean corpuscular hemoglobin concentration (MCHC).⁴ Thus, in its transit through the microcirculation, a discoid RBC will sickle if these factors produce a time delay too brief to traverse the terminal arterioles, capillary bed, and postcapillary venules. In these locations where oxygen saturation is reduced and where the small luminal diameter necessitates RBC deformability for passage, sickled erythrocytes may then cause microvascular obstruction through interactions with endothelial and other cells. Polymerization of HbS is therefore the central event in the pathophysiology of SCD. In the absence of any practical means to directly prevent polymer formation, indirect inhibition of this process through manipulation of hemoglobin concentration has been a major focus of recent therapeutic efforts. One approach has been through augmenting production of fetal hemoglobin (HbF).

HbF consists of 2 paired γ -globin and β -globin chains and is the major hemoglobin of the fetus, declining through the first year of life to adult levels of 0.5–1% as β -globin production increases to yield HbA and γ -globin production declines. HbF levels are variably increased and decline later in children and adults with SCD, with only part of the considerable heterogeneity explained by known factors such as β -globin gene haplotype and sex.⁵ It has long been recognized that higher HbF levels correlate with less severe clinical manifestations of SCD:

in certain Arab and Indian populations with elevated HbF, and in infants with physiologically elevated levels, disease manifestations tend to be relatively mild.⁶ Initial studies suggested that HbF levels of at least 25% are necessary to detect a therapeutic benefit.^{7,8} Later research has revealed that rates of pain crisis and death vary in a continuous inverse relationship with HbF level.⁹ It is now recognized that HbF interferes with HbS polymerization by 2 distinct mechanisms. First, as intracellular HbF percentage increases, the percentage of HbS must decrease if the total RBC hemoglobin concentration is constant. Reduced HbS concentration dramatically inhibits sickling by simply reducing the proportion of molecules capable of polymerization. Second, HbF tetramers can dissociate into dimers and mix with HbS dimers to form hybrids ($\alpha^2\beta^s\gamma$) that cannot participate in polymerization, whereas HbS/HbA hybrids can. Thus, HbF further reduces the amount of HbS available to aggregate, to a greater extent than HbA does alone by diluting HbS concentration.¹⁰

The molecular mechanisms responsible for the normal shift in production from γ -globin to β -globin are not well understood. Expression of the β -globin gene cluster on chromosome 11 is under the remote control of upstream regulatory sequences known as the locus control region (LCR). DNA methylation in the promoter region is one important factor in silencing the γ -globin gene, and hypomethylation is associated with increased gene expression. A combination of *cis* and *trans* factors coordinated at the LCR appears to govern both an autonomous silencing of the γ -globin gene and a competition between genes such that β -globin becomes expressed preferentially, with continued small amounts of γ -globin production.¹¹ The HbF produced is not distributed evenly among RBCs, but limited to a small population of F-cells containing variable concentrations of HbF. Production of F-cells is higher in females and has been shown to be transiently augmented under conditions of hematopoietic stress, such as hemorrhage, hemolysis, and recovery from transient erythroblastopenia of childhood or myeloablative chemotherapy.¹²

Pharmacologic manipulation of HbF has been a major therapeutic goal in SCD research for the last 30 years. Initial studies investigated azacitidine as an agent that might affect γ -globin gene expression by hypomethylation, but its efficacy in augmenting HbF production was later thought to be related more to its cytotoxicity.^{13,14} Marrow stress presumably triggers secondary erythroid generation with a shift toward earlier progenitor cells, programmed to favor γ -globin expression. When this agent appeared toxic and difficult to use clinically, several other cytotoxic agents were investigated. The safest and most effective proved to be hydroxyurea (HU), an orally

Table 1. Pathophysiologic Targets of Sickle Cell Disease Therapy

Pathophysiology	Therapy	Mechanism
HbS polymerization	Hydroxyurea	HbF induction: stress erythropoiesis
	Decitabine	HbF induction: hypomethylation
	Butyrate, valproate	HbF induction: histone deacetylation
RBC membrane damage	Glutamate, vitamin C	Antioxidant
	Deferiprone	Membrane iron chelation
Hemolysis*	Transfusion therapy	Sickle RBC replacement
Iron overload	Desferoxamine	IV chelation
	Deferiprone, others	Oral chelation
	Apheresis, exchange transfusions	Reduced iron load
RBC dehydration	Clotrimazole, ICA-17403	Gardos channel inhibition
	Mg pidolate	K:Cl channel inhibition
	Dipyridamole	Cation flux inhibition
RBC endothelial adhesion	Polaxamer 188 (Flocor)	Surfactant
	Sulfasalazine	NFκB inhibition
	αVβ3 integrin monoclonal antibody	Thrombospondin blockade
	Dextran sulfate, chondroitin sulfate	Inhibits binding to glycolipids
Vascular tone	Inhaled NO	Vasodilation
	Arginine	NO substrate
	Hydroxyurea	NO donor
Inflammation	Hydroxyurea	Neutrophil reduction
	Steroids?	Anti-inflammatory
Hypercoagulability	Aspirin, ticlopidine	Platelet inhibition
	Heparin, enoxaparin	Anticoagulation
	n-3 fatty acids	Reduced tissue factor activity
	Lovastatin	Reduced tissue factor expression
	Tissue plasminogen activator	Thrombolytic
Genetic defect	Gene therapy	Gene replacement
	Stem cell transplantation	Bone marrow replacement

* Many other indications for transfusion exist.

HbF = fetal hemoglobin; HbS = sickle hemoglobin; IV = intravenous; NFκB = nuclear factor-kappa B; NO = nitric oxide; RBC = red blood cell.

available deoxyribonucleotide reductase inhibitor used in chronic myelogenous leukemia, polycythemia vera, and other malignancies. Its lack of direct effect on DNA or chromatin supports the idea that its effect on HbF production is through myelotoxicity, altering erythropoietic cell differentiation to favor more "fetal cells."⁶ A more sophisticated understanding of its mechanism is still lacking, but new clues are emerging that properties unrelated to HbF modulation may contribute to its clinical effect in SCD as well.

In a landmark clinical trial, the Multicenter Study of Hydroxyurea (MSH) followed 299 adults with SCD and found that HU decreased the frequency of hospitalization, vaso-occlusive crises (VOC), acute chest syndrome (ACS), and blood transfusion by nearly half. Hemoglobin, HbF, and F-cells increased on HU, while leukocytes, platelets, reticulocytes, and dense cells decreased.¹⁵ There was no change in the rate of stroke or death at the end of the study, but long-term follow-up revealed a 40% reduction in mortality.¹⁶ Subgroup analysis after 2 years of treatment showed a highly variable HbF and F-cell response, with significant increases in 50% of subjects, but little or no increment in the lower half of responders, and an inverse relationship between pain crisis rate and HbF response. As responsiveness was related to higher baseline reticulocyte and neutrophil counts, it was suggested that HU response is dependent on bone marrow "reserve" sufficient to tolerate the myelotoxicity of the drug.¹⁷ Studies in children and infants have been much more limited, but overall have revealed similar results, except that in contrast to adults, children with higher baseline HbF levels achieved higher levels with treatment.¹⁸ A recent study of 122 pediatric SCD patients given HU to maximum tolerated dose found that all patients responded to therapy, with a mean HbF of 20%, and that response was sustained over 7 years of treatment with minimal toxicity and no growth impairment.¹⁹ Lingering questions remain about the long-term safety of HU and its potential for carcinogenicity, but the available data so far are reassuring for overall safety in adults and children, and no increased rate of malignancy or mutagenesis has been detected. Current indications include SCD in adults and children (although it is not approved for pediatrics by the US Food and Drug Administration) with frequent VOC, ACS, other severe vaso-occlusive events, or severe symptomatic anemia.²⁰ One recent study reported that patients who died during HU treatment were significantly older at initiation of therapy and had lower hemoglobin and higher creatinine at baseline, despite similar HU dose, HbF response, and duration of treatment when compared with survivors, suggesting a need for earlier intervention.²¹ Ensuring compliance has been a challenge, but the most vexing problem with HU therapy remains the variability of response to

the drug, with up to 40% of patients demonstrating no increase in HbF.¹⁷ Furthermore, clinical improvement may not consistently correlate with HbF levels, suggesting that its therapeutic effect may derive from other mechanisms in addition to HbF augmentation.

Recent studies have found that HU contributes *in vitro* and *in vivo* to production of nitric oxide (NO), a potent vasodilator of great potential importance in SCD.²² Additionally, HU and other NO donors have been shown to increase γ -globin gene expression in erythroid precursors, suggesting a possible genetic mechanism of globin gene regulation involving NO.²³ While neutropenia is an expected toxicity of HU, it has been suggested that reducing the neutrophil count may actually be part of its therapeutic benefit.²⁴ Elevated leukocyte count is known to be associated with more severe disease, although decreased neutrophil count may not necessarily correlate with reduced mortality, and markers of dysregulated neutrophil activation were reduced to near normal in SCD patients on HU.^{16,25,26} Reduction of erythrocyte-endothelial adhesion may be an additional mechanism of action, supported by *in vitro* studies and by reduced levels of vascular cell adhesion molecule-1 (VCAM-1) in SCD patients given HU.²⁷⁻²⁹ Other suggested mechanisms include reduction of dense cells and "stress reticulocytes," methemoglobin production, and erythropoietin induction.³⁰

In addition to HU, several other agents to augment HbF production have been investigated for use in SCD. Decitabine is a less toxic analog of azacitidine, and encouraging results in recent trials have led to renewed interest in this agent. It appears to work by hypomethylation of the γ -globin gene promoter, with associated changes in histone acetylation and chromatin structure, but not through primary cytotoxicity.^{31,32} In a pilot study, 8 patients who previously responded poorly to HU received low-dose intravenous decitabine for 2 weeks and showed significant increases in HbF, F-cells, and total hemoglobin.³³ Longer treatment over 36 weeks produced similar results, with mild transient neutropenia the only side effect.³⁴ A more recent trial used weekly subcutaneous dosing in 8 patients and found increases in HbF and F-cells from 6.5% and 38.1% to 20.4% and 71.4%, respectively. Bilirubin and reticulocytes decreased, hemoglobin increased, and mild neutropenia with thrombocytosis were noted. Additionally, D-dimer, VCAM-1, and von Willebrand factor (vWF) levels all decreased, and erythrocytes demonstrated reduced adhesion.³² Correlation with clinical severity and larger controlled trials are still lacking, and concern remains about carcinogenicity. Some studies have shown mild mutagenicity, little reactivation of other suppressed genes, and a surprising tumor-suppressive effect in animal models.^{35,36}

Butyrate is a short-chain fatty acid known to induce gene expression by inhibiting histone deacetylase and changing chromatin structure. It has been shown to prevent and partially reverse the fetal to adult hemoglobin gene switch in animals, and is postulated to be the cause of delayed γ -globin gene switch with elevated HbF levels in infants of diabetic mothers.³⁷⁻³⁹ Early trials of continuous arginine butyrate infusions in patients with SCD and β -thalassemia were inconsistent, but later trials using intermittent infusions were more encouraging, with sustained HbF response and no cross-resistance to HU, although a minimum baseline HbF level appears to be necessary for response.⁴⁰⁻⁴² Other short-chain fatty acids and their analogs are being investigated, including the anticonvulsant valproic acid, which is known to increase HbF levels.⁴³ Erythropoietin, while not itself indicated for treatment of anemia in SCD, has been shown to cause an additive increase in HbF when combined with HU, above that expected with monotherapy.⁴⁴ Combination therapy with drugs of different mechanisms such as butyrate, HU, decitabine, and erythropoietin offers the appealing possibilities of synergistic effect and benefit to patients who fail to respond to a single therapy alone. Such combinations are currently being investigated, but none of the above HbF modifiers, excluding HU, can be recommended at this time outside of research protocols.

RBC Membrane Damage

While the polymerization of HbS is the origin of the molecular pathophysiology in SCD, it is actually through erythrocyte membrane damage that HbS produces most of the clinical features of disease, from hemolytic anemia to microvascular obstruction.⁴⁵ Apart from its capacity to polymerize, HbS is more unstable than HbA, with a tendency to autooxidize and interact with the inner surface of the RBC membrane. Through electrostatic interaction with the lipid bilayer inner leaflet, HbS precipitates to form methemoglobin, which then denatures releasing hemichromes and free heme, which in turn promote lipid peroxidation and release of free iron, inducing further oxidative damage. This process leads to Heinz body formation, affecting the membrane itself and causing aggregation of transmembrane proteins such as ankyrin and band 3.⁴⁶ Autologous immunoglobulin G (IgG) and complement have been shown to bind to the clustered band 3 protein, coating sickle RBCs and facilitating their destruction.⁴⁷ HbS precipitation and oxidation also damage the erythrocyte cytoskeleton, leading to portions of membrane shedding by vesiculation, and possibly contributing to hypercoagulability.⁴⁸ Cytoskeletal damage causes decreased deformability and increased membrane rigidity even under fully oxygenated conditions. These

properties contribute to altered rheology and tendency to occlude the microvasculature, exemplified by irreversibly sickled cells (ISCs), a subpopulation of dense, dehydrated cells with membranes so rigid they retain their sickled shape even when fully oxygenated without HbS polymer.⁴⁹ ISCs have a low percentage HbF and very short survival in circulation. Their relative numbers vary among and within SCD patients but do not appear to correlate with disease severity, although they do correlate with hemolysis. These cells are thought to play a critical role in the process of vaso-occlusion, perhaps becoming trapped among lighter, more deformable RBCs that have entered the microcirculation but adhered to the vascular endothelium.

Another example of erythrocyte membrane damage with probable clinical significance is the loss of phospholipid asymmetry in the lipid bilayer. Normal cells display primarily phosphatidylcholine and sphingomyelin in their outer monolayer, with phosphatidylserine (PS) exclusively in the inner layer. Maintenance of this composition is an active process, catalyzed by adenosine triphosphate (ATP)-dependent translocases.⁵⁰ Sickle RBCs display significant alteration of this asymmetry, with exposure of PS on the outer membrane, induced by repeated cycles of polymerization.⁵¹ Exposure of this anionic phospholipid contributes to hypercoagulability, increased adherence to monocytes, macrophages, and endothelial cells, and activation of the complement pathway.⁵² The extent to which this phenomenon contributes to the pathophysiology of vaso-occlusion is not known, but this is an area of active research and potential for future pharmacologic intervention.

Among the consequences of RBC membrane damage is a chronic hemolytic anemia. This relates primarily to the decreased survival of dense cells and ISCs and is mediated by the spleen until splenic infarction has occurred, then by macrophage and monocyte erythrophagocytosis, which is facilitated by binding of IgG and complement and by PS exposure.⁵³ Clinically, most patients are asymptomatic from chronic anemia itself, although risk of stroke and eventually of heart failure are increased by severe anemia, and hemolysis may result in bilirubin gallstones. Interestingly, anemia may actually be protective against vaso-occlusive complications, as a low hematocrit moderates the increase in viscosity associated with deoxygenation and sickling at high shear rates in the microcirculation.⁴⁹

No specific treatment yet exists to modify erythrocyte membrane damage or its consequent hemolysis and vaso-occlusion. Antioxidants such as glutamine, vitamin C, vitamin E, and vanillin may be of potential benefit, but research to date has been scant. Deferiprone, an experimental oral iron chelator, was shown in one study to effectively chelate RBC membrane iron deposits

in vitro from SCD and thalassemic patients, and in vivo in 6 patients with thalassemia.⁵⁴ Although transfusion therapy is not always intended to correct hemolysis or anemia per se, this treatment modality does affect the various consequences of damaged sickle erythrocytes by diluting them with a population of normally functioning cells. Physiologically, the goal is to increase the oxygen-carrying capacity of the blood, enhance tissue perfusion, and decrease the percentage of cells capable of sickling. Indications for episodic transfusions include acute severe anemia (splenic sequestration, aplastic crisis, hyperhemolysis), pain crisis accompanied by symptomatic anemia, ACS, acute stroke, and preparation for surgery.²⁰ Chronic transfusion therapy is indicated for secondary prevention of stroke after a first cerebral infarction or transient ischemic attack, and may be indicated for recurrent severe VOC, recurrent ACS, pulmonary hypertension, and other conditions.²⁰ The importance of primary stroke prevention in children was demonstrated by the Stroke Prevention Trial in Sickle Cell Anemia (STOP): chronic transfusion therapy effectively prevented a first stroke in children at risk by abnormal screening with transcranial Doppler ultrasonography.⁵⁵ The appropriate length of time to continue chronic transfusions is not known. A small recent study reported a successful transition from chronic transfusions to HU therapy in secondary stroke prevention. A transfusion program of gradually reduced intensity was overlapped with escalating-dose HU therapy and phlebotomy in place of chelation; this regimen was effective in lowering the risk of recurrent stroke in the children studied.⁵⁶

The complications of transfusion therapy include iron overload, alloimmunization, and autoimmunization. Among the new therapies emerging to manage these complications are the use of RBC apheresis with exchange transfusion and the development of effective oral iron chelators, although the former is complicated by issues of vascular access, time, and resources, and the latter are still investigational and not yet clinically available.⁵⁷ A new approach to prevent alloimmunization and possibly autoimmunization is the use of polyethylene glycol polymers to coat erythrocytes and mask antigenic sites.⁵⁸ Generally, transfusions should always be with phenotypically matched, leuko-reduced, sickle-negative blood, and should attain a posttransfusion hematocrit of $\leq 36\%$ to prevent hyperviscosity.²⁰

Erythrocyte Dehydration

Patients with SCD have a higher proportion of dense, dehydrated erythrocytes than do normal individuals.⁵³ This dense cell fraction includes ISCs and cells with more extensive membrane damage, and thus plays an important

role in vaso-occlusion, as described above. The percentage of dense cells has been shown to decrease during VOC, but does not correlate with the incidence or onset of pain crises.^{59,60} Because HbS polymerization is so closely related to intracellular hemoglobin concentration, the mechanisms of dehydration and dense cell formation have been the subject of extensive research, and new therapeutic targets have begun to emerge from this knowledge.

HbS polymerization and cell sickling have been shown to increase cytosolic calcium levels in sickle RBCs by transiently and nonspecifically increasing membrane permeability. Recent work shows this calcium influx to be a stochastic process, randomly affecting a population of cells to greater or lesser degrees.⁶¹ Exactly how HbS polymers produce this effect is still unknown, but is presumed to involve membrane damage. Embedded in the membrane of all human erythrocytes are calcium-activated potassium channels, first described by Gardos. When exposed to calcium transients in sickled cells, the Gardos channel facilitates potassium loss, with passive loss of chloride and water. Acidification accompanies this process, as increased cellular H⁺ concentration accompanies the decrease in Cl⁻ concentration. This mechanism of dehydration is supported by the observation of increased calcium levels retained in cytoplasmic vacuoles in the densest sickle cells, a record of multiple episodes of sickling-induced calcium fluxes and subsequent dehydration.^{62,63}

Another mechanism of potassium and water loss is through membrane K:Cl cotransport channels present in erythrocytes. This channel is most active in normal reticulocytes, but remains active in sickle cells of all ages. The channel is activated by cell acidification, by positively charged hemoglobins including HbS and HbC, and by deoxygenation-induced HbS polymerization. It is known to be inhibited by increased cytoplasmic magnesium, suggesting a possible therapeutic intervention. The relative importance of the Gardos and K:Cl channels in mediating cell dehydration is not known, but they each appear to play their own role in the process and may work synergistically to enhance water loss in the formation of dense cells. Sodium and potassium fluxes induced by deoxygenation may also play a role in dehydration, mediated by membrane Na-K ATPase.^{62,63}

Prevention of erythrocyte dehydration is an appealing alternative to HbF induction as a means of reducing cellular HbS concentration, thereby inhibiting sickling and dense cell formation.^{30,64} Over 20 years ago, attempts were made to induce osmotic swelling of sickle cells through hyponatremia by administering DDAVP (1-deamino-8-D-arginine vasopressin, also known as desmopressin) and hydration to patients with SCD.⁶⁵ In vitro reduction in sickling was demonstrated, but such a clinical regimen could not be maintained. Imidazole antimycotic drugs

were discovered to be specific Gardos channel inhibitors, with clotrimazole proving the most potent. After encouraging results in transgenic mice, oral clotrimazole was given to 5 patients with SCD and again demonstrated Gardos channel blockade, increased erythrocyte potassium content, and reduced MCHC and RBC density.⁶⁶ Side effects were mild but included nausea, dysuria, and mild hepatotoxicity. A new compound based on clotrimazole metabolite structure, ICA-17403, with greater potency and fewer side effects, was successful in phase I studies and is currently in phase II/III trials.⁶² Another potential means of inhibiting Gardos channel function is blockade of chloride ion membrane conductance; preventing chloride movement prohibits potassium passage through the Gardos channel and thus dehydration. Of the compounds in development, one reduced sickle cell dehydration in mice, but showed undesirable side effects in humans.⁶² Attempts to inhibit the K:Cl channel with magnesium supplements have been promising. Again after success in transgenic mice, a small uncontrolled clinical trial of oral magnesium pidolate in SCD patients showed increased erythrocyte magnesium and potassium, reduction in K:Cl transporter activity, and fewer dense RBCs.⁶⁷ Another longer trial found similar biochemical effects, correlated with reduced frequency of pain crisis.⁶⁸ Larger, placebo-controlled trials of Mg pidolate are now underway. The antiplatelet drug dipyridamole affects the Na-K ATPase and has been shown to inhibit cation fluxes in deoxygenated sickle cells, offering another possible means of preventing dehydration.⁶⁹

Endothelial and Vascular Function

Occlusion of the microvasculature by sickled erythrocytes is thought to underlie many of the clinical manifestations of SCD, including painful crises, ACS, stroke, and ultimately damage to nearly every organ system. The mechanisms involved in vaso-occlusion are still being elaborated, but a wealth of experimental evidence implicates abnormal erythrocyte adhesion to endothelium early in the process.^{70,71} Sickle RBCs are shown to be abnormally adherent to vascular endothelial cells in a variety of experimental models, and it has been demonstrated that increased adherence correlates with clinical severity of VOC.⁷²⁻⁷⁴ The light density fraction of RBCs, including reticulocytes and young cells, is increased in SCD patients, and appears to be the most adherent subpopulation, despite their increased deformability compared with the dense fraction.⁷⁵⁻⁷⁷ This observation has led to the “log jam” or “Trojan horse” concept, wherein the light cells adhere to microvascular endothelium and remain long enough to sickle when deoxygenated, thereby trapping the less adherent but more rigid dense cells and

propagating the obstruction.^{49,78} Ex vivo experiments in animal models have supported this hypothesis.^{77,79} The molecular mechanisms contributing to altered sickle cell adhesiveness are complex and still poorly understood, but a growing list of factors appears to mediate abnormal erythrocyte-endothelial interaction. Thrombospondin facilitates adhesion by bridging CD36 expressed on reticulocytes and several putative receptors on endothelial cells; thrombospondin levels are also elevated in some SCD patients during painful episodes.⁸⁰⁻⁸³ Sickle reticulocytes overexpress $\alpha_4\beta_1$ integrin, which mediates adhesion binding to VCAM-1 expressed on stimulated endothelial cells, and is elevated in SCD.⁸⁴⁻⁸⁷ vWF is elevated in SCD patients even in steady state, and in vitro promotes sickle cell adhesion through glycoprotein Ib/V/IX receptors on endothelial cells, although no vWF receptor on erythrocytes has yet been identified.^{88,89} Laminin is a constituent of subendothelial matrix and binds to the Lutheran antigen (BCAM/Lu), which is expressed on sickle RBCs in greater quantity than on normal RBCs.⁹⁰ Other factors include fibrinogen, fibronectin, Ig-Fc receptor interaction, and direct erythrocyte-endothelial cell adhesion via clumped surface charges or exposed PS.⁴⁹

There is also evidence to support a role for increased endothelial injury and activation in SCD. A transgenic mouse model now supports the idea that intermittent vaso-occlusion induces “ischemia-reperfusion” physiology, with vascular damage from oxidative stress and inflammation during the reoxygenation that follows obstruction.⁹¹ Increased numbers of circulating endothelial cells in SCD patients during crisis suggest endovascular injury, and these cells additionally exhibit abnormal stimulation, with altered expression of VCAM-1, ICAM-1, P-selectin, E-selectin, and tissue factor.^{92,93} Patients have also demonstrated variably elevated levels of endothelial stimulators including tumor necrosis factor (TNF), platelet activating factor, lipopolysaccharide, phospholipase A2, and a variety of cytokines and other inflammatory mediators.^{49,94} The relative importance of all the above molecules and interactions is not yet understood, but this is an area of continued research and future therapeutic potential.

Another area of intense research in the pathophysiology of vaso-occlusion is altered vascular tone. NO is a potent vasodilator expressed by constitutive and inducible enzymes, and is the major endothelial relaxing factor in normal physiology. Growing evidence supports a model of dysregulated NO metabolism in SCD, with important therapeutic implications.⁹⁵ In transgenic mice, baseline NO synthase levels are elevated, but unable to further increase production when stimulated, due to accelerated NO catabolism.⁹⁶⁻⁹⁸ Clinical studies suggest a similar scenario in patients, although many details are still being elucidated. NO metabolites have been found to

correlate inversely with baseline pain scores in VOC, but not with changes in pain over time.^{99,100} Adults with SCD have been reported to have increased NO metabolites and decreased levels of arginine, the precursor to NO synthesis.^{101,102} Arginine deficiency may be age-dependent and variable; children with steady-state SCD had normal levels and low levels during VOC. NO metabolites were normal at presentation but decreased during hospitalization for VOC and ACS.¹⁰³ There is now evidence for increased NO catabolism by superoxide and by hemoglobin, which is elevated by hemolysis in SCD.¹⁰⁴ Arginine deficiency also results in NO synthase production of superoxide instead of NO, further reducing its availability.¹⁰⁵ Arginase activity may be altered in SCD as well, limiting arginine availability as substrate for NO.¹⁰⁶ VCAM-1 and endothelin-1, mediators of endothelial adhesion and vasoconstriction, respectively, are elevated in SCD and are both reduced by NO through its inhibition of the transcription factor nuclear factor-kappa B (NFκB).^{107,108} Nitric oxide may have additional direct effects by inhibiting platelet and leukocyte activity, by inhibiting the Gardos channel, and even by augmenting HbF production.⁹⁵ Current evidence suggests a model of constitutively elevated NO production due to ongoing vaso-occlusion and hemolysis, leading to relative arginine deficiency which becomes acute in the face of illness, with impaired synthesis and bioavailability plus increased destruction leading to deficiency of NO activity at the local level, potentiating vasoconstriction and vaso-occlusion. In addition to mediating pain crises and ACS, NO likely plays a significant role in the specific pathophysiology of pulmonary hypertension, another recognized complication of SCD.

A few therapeutic agents intended to reduce sickle cell adhesion are under investigation. Poloxamer 188 is a non-ionic surfactant copolymer that blocks hydrophobic adhesive interactions between erythrocytes and endothelium, improving microvascular circulation. In phase II trials the duration of painful episodes was reduced, particularly in younger patients and in those receiving HU.^{109,110} Sulfasalazine is known to inhibit NFκB, which normally promotes transcription of VCAM-1 and other endothelial factors. In transgenic mice, sulfasalazine reduced expression of VCAM-1, ICAM, and E-selectin; in SCD patients circulating endothelial cells showed decreased activation.¹¹¹ In an *ex vivo* model monoclonal antibodies to the αVβ3 integrin receptor for thrombospondin blocked sickle RBC adhesion.¹¹² Dextran and chondroitin sulfate also blocked sickle cell adhesion in a rat mesoconium preparation, by inhibiting binding of vWF, thrombospondin, and laminin to sulfated glycolipids.¹¹³

NO currently offers another exciting area for potential pharmacotherapy of SCD. Case reports describe the use of inhaled NO in 3 patients with ACS, resulting in

reduced pulmonary hypertension and improved oxygenation.^{114,115} It has also been shown to inactivate circulating plasma hemoglobin, thereby improving availability of endogenous NO by reducing its scavenger.¹⁰⁴ Because inhaled NO is potentially dangerous and difficult to use, other mediators of NO production are under active investigation. Oral arginine has recently been shown to reduce pulmonary artery pressure in SCD patients with pulmonary hypertension.¹⁰⁶ In sickle cell mice, arginine and inhaled NO both reduced erythrocyte dehydration by inhibition of Gardos channel.^{116,117} In a small clinical investigation, oral L-arginine administration in steady-state SCD patients caused a paradoxical decrease in NO metabolites, but a dose-dependent increase during VOC.¹¹⁸ Recently the same investigators demonstrated an increase in NO metabolites among steady-state SCD patients coadministered arginine and HU, further suggesting a role for HU in NO metabolism and the potential for dual-modality therapy.¹¹⁹ HU itself is an NO donor and may also enhance NO synthase activity, as well as stimulating HbF production by generating NO.^{22,23} Inhaled NO and oral arginine for SCD are currently in clinical trials, and other NO mediators, including sildenafil are being investigated as well.

Inflammation

SCD is now recognized as a condition of chronic inflammation, related to the repetitive microvascular damage inflicted by sickled erythrocytes. Numerous inflammatory markers are elevated in SCD patients even at baseline, including C-reactive protein (CRP), TNF-α, and interleukin-1 and -8, in addition to the mediators of endothelial activation such as VCAM-1 and endothelin-1, discussed earlier.^{94,107} Apart from this observation, however, little is known about the specific role played by these inflammatory mediators in the pathophysiology of SCD and its complications. Leukocyte count has been shown to correlate with many manifestations of SCD, including stroke and ACS.¹²⁰ Baseline granulocyte counts are elevated in patients with sickle cell anemia, and leukocytosis is a risk factor for increased mortality.^{9,121} Evidence is mounting to suggest that leukocytes are more than a surrogate marker of disease activity, but rather are an integral part of the pathophysiology. Several reports have described severe vaso-occlusive episodes associated with administration of granulocyte colony-stimulating factor or granulocyte-macrophage colony-stimulating factor to patients with SCD.^{122,123} Neutrophils in SCD demonstrate increased adherence to endothelium, and they can bind sickle RBCs *in vitro*.^{26,124} Several markers of neutrophil activation are elevated in SCD patients at steady state, and signifi-

cantly increased during painful episodes.^{125,126} Sick cell neutrophils overexpress CD64, an endothelial adhesion factor, suggesting one mechanism for their adherence.²⁶ Intravital microscopy in sickle cell mice demonstrated in vivo interactions between RBCs and leukocytes adherent to venule endothelium, which increased after administration of TNF- α , and resulted in complete vascular obstruction.¹²⁷ Another report found increased leukocyte adherence and extravasation in sickle mice exposed to hypoxia followed by reoxygenation, reflecting ischemia-reperfusion injury, and abrogated by the addition of an anti-P selectin antibody. Thus, inflammation appears to play an important role in vaso-occlusion, with cytokine attraction and activation of leukocytes, adherence to activated endothelium, and interactions between sickle red cells and white cells. No specific agents have yet been employed to counteract inflammation or leukocytes in SCD, although the inflammatory cascade offers a variety of possible targets for therapeutic interventions. It has been suggested, but not established, that decreasing neutrophil count is partly responsible for the clinical efficacy of HU.²⁴ A small clinical trial reported steroids to be beneficial in reducing length of hospitalization, analgesics, and oxygen requirement during ACS, but this therapy requires further investigation and extreme caution.¹²⁸

Hypercoagulability

Thrombosis is part of the clinical spectrum of SCD, manifesting as silent and overt stroke, avascular necrosis of large joints, and frequent pulmonary thrombi found at autopsy. Almost every aspect of hemostasis is abnormal in SCD, including platelet function, procoagulant, anticoagulant, and fibrinolytic systems.¹²⁹ Sickle erythrocytes themselves are procoagulant, owing to their altered PS asymmetry with anionic PS exposure and to membrane shedding of microvesicles.^{130,131} Antiphospholipid antibodies are often elevated in patients with SCD, particularly against PS, and correlate with markers of activated coagulation.^{132,133} Tissue factor expression is markedly increased, and is abnormally expressed on monocytes and circulating endothelial cells in SCD patients.^{93,134} In transgenic sickle mice, tissue factor expression appears limited to the pulmonary veins.¹³⁵ High levels of other procoagulant proteins such as vWF and factor VIII are also seen in patients.⁸⁸ Protein C and S levels are often decreased, particularly in patients with a history of stroke.^{133,136} There is increased generation of thrombin, as evinced by elevated D-dimers, prothrombin fragment 1.2, thrombin-antithrombin complexes, and reduced factor V levels; fibrinolytic activity also appears to correlate with frequency of pain crises.¹³⁷⁻¹³⁹ Thrombocytosis is common in adolescents and adults, likely due to asplenia, and platelet aggregation appears to be increased

as well, although these findings are not usually present in children.^{88,140-142} There is also good evidence for increased platelet activation in adults and children with SCD.^{143,144} Most of the abnormalities above are known to be altered at steady state; it is unclear whether hypercoagulability is further increased during episodes of vaso-occlusion, but some evidence suggests enhanced platelet consumption during crises.¹²⁹ As with inflammation, it is still unknown if hypercoagulability is more a cause or a result of sickle cell vaso-occlusion, but in either case it appears likely to play some significant role in the pathophysiology of the disease.

Overall, trials of anticoagulants and antiplatelet agents in SCD have been limited, small, and poorly controlled, providing insufficient information about their efficacy. Among antiplatelet agents, a few studies of aspirin and ticlopidine have yielded mixed results.¹⁴⁵⁻¹⁴⁹ Warfarin reduced pain crises in an early study, but with significant bleeding complications.¹⁵⁰ A pilot study of low-dose acenocoumarol found reduced thrombin generation and fibrinolysis, but no change in frequency of pain episodes.¹⁵¹ A long trial of mini-dose heparin reduced the rate of pain crises without complications, but only 4 subjects were included.¹⁵² A prospective trial of low-molecular-weight heparin in SCD is ongoing. Dietary supplementation with n-3 fatty acids may impede thrombosis by reducing tissue factor activity, and a recent clinical trial demonstrated fewer pain episodes and decreased fibrinolysis and thrombin generation.¹⁵³ The use of fibrinolytic agents such as tissue plasminogen activator for stroke in SCD has not been studied; although SCD is not itself a contraindication for its use in adults who would otherwise be candidates, many clinicians remain reluctant to begin thrombolytics due to a perceived increased risk of hemorrhage. Lovastatin, a cholesterol-lowering agent with multiple anti-inflammatory effects including NO synthase induction, was recently reported to eliminate tissue factor expression in clinically severe and posthypoxic sickle mice, possibly through NO-related inhibition of NF κ B.¹³⁵ Statins have also been reported to inhibit tissue factor expression in monocytes and endothelial cells, and to protect rodents from ischemic stroke and renal injury.¹⁵⁴⁻¹⁵⁷

Genetics

Although SCD was the first monogenetic disease described, the single β 6 A \rightarrow T mutation is clearly insufficient to explain the considerable heterogeneity of disease phenotype. Thus SCD has come to be viewed as a multigenic disorder, subject to the effects of pleiotropic genes involved in secondary pathophysiologic processes, and epistatic genes whose contribution to primary pathophysiology is polymorphic.^{158,159} Epistatic genetic

effects include β -thalassemia, β -globin gene haplotypes, and factors relating to HbF expression. Pleiotropic effects are even more varied, and include the multitude of genes involved in the pathophysiologic events described above: RBC membrane damage and dehydration, modulators of endothelial activation and vascular tone, inflammatory mediators, and hypercoagulability factors. Another example is concomitant α -thalassemia, which is associated with lower MCHC, higher hematocrit, decreased hemolysis, and less retinal disease, but more frequent pain crises and osteonecrosis.¹⁶⁰ A few single-nucleotide polymorphisms have been identified in genes that may affect SCD phenotype, including VCAM-1 and TNF- α , but new advances in laboratory techniques now afford the opportunity to more efficiently investigate the variation and expression of the thousands of genes thought to play a role in the variability and pathophysiology of SCD.¹⁵⁹ One major goal of this research is the early identification of genetic and clinical risk factors for specific disease complications, in order to target patient therapy without overtreating or undertreating, in a model analogous to modern cancer staging and risk assessment for chemotherapy.

Gene therapy offers great promise for actual cure of genetic diseases such as SCD, but substantial work remains before any clinical application of this modality. Low efficiency of gene transfer to stem cells, the requirement for high globin gene expression with proper regulation, difficulty developing appropriate animal models, and perennial safety concerns have hampered progress in the last 20 years, but significant advances are now being made.^{161,162} Murine oncoretroviral vectors have been used to correct several hematologic disorders in transgenic mice, but results in other animal models have been less successful. More recent use of lentiviral vectors derived from the human immunodeficiency virus (HIV) genome has solved some problems associated with the older vectors, with more efficient transduction of human cells. Other retroviral vectors are also in development, including human foamy virus and adeno-associated virus. Advances are also being made in several techniques to selectively amplify the transfected stem cell population *in vivo*. Two transgenic mouse strains with human Hb SS disease have been transduced using lentiviral vectors with a globin gene linked to regulatory elements of the LCR, resulting in decreased ISCs, reduced hypoxia-induced sickling, and improved renal concentrating capacity, associated with human β -globin synthesis at 10–50% of the level of endogenous β -globin production.¹⁶³

A different approach to curing SCD is now both clinically available and highly effective, but associated with significant risks. Allogeneic stem cell transplantation was initially performed in a sickle cell patient with a coexisting hematologic malignancy, and proved that SCD could be

cured by this method.¹⁶⁴ With improving experience and outcomes, transplantation has been expanded to include patients with severe clinical features or poor prognostic factors, and current efforts now focus on reducing toxicity and expanding the pool of available donors.¹⁶¹ Results have been best among children with human lymphocyte antigen-identical sibling donors; adults and recipients of unrelated or mismatched donors have fared more poorly.¹⁶⁵ Overall disease-free survival has approximated 80–85%, with protection from hallmark SCD complications up to 8 years post-transplant, and recovery of splenic function in younger patients.¹⁶⁶⁻¹⁶⁸ Complications of transplant have resulted in 5–10% mortality, however, most often from graft-versus-host disease (GVHD). Graft rejection remains another significant problem, with autologous bone marrow recovery and SCD recurrence in 10% of patients. Current indications for transplantation among SCD patients under 16 years include stroke or associated impairment of neuropsychologic function, recurrent ACS, and recurrent vaso-occlusive episodes; other considerations include lung disease, nephropathy, multiple joint osteonecrosis, and RBC alloimmunization on chronic transfusion therapy.¹⁶¹ Earlier identification of candidates by known predictors such as early dactylitis and severe anemia may eventually improve outcomes by treating patients before exposure to multiple blood products, thereby reducing risk of graft rejection. Stable mixed chimerism is a common event after conventional transplantation, but even partial engraftment appears to be beneficial; among patients with lower proportions of donor cells, HbS levels were similar to donor levels and resulted in clinical cure of SCD.¹⁶⁷ This observation has led to recent investigation of nonmyeloablative “mini-transplant” regimens with a goal of decreasing toxicity and GVHD, but with an increased risk of at least partial graft rejection.^{169,170} Umbilical cord blood contains more hematopoietic progenitors than adult bone marrow and is immunologically naive, resulting in less GVHD with unmatched transplants. Experience has been limited in SCD, but results suggest similar outcomes to conventional transplant with the potential benefit of decreased GVHD and an expanded donor pool.¹⁷¹

Conclusions

The pathophysiology of SCD is considerably more complex than its monogenetic origin would suggest, involving abnormalities in the function of hemoglobin, RBC membrane, erythrocyte hydration, endothelium, vascular tone, inflammatory response, leukocytes, and coagulation. The dynamic combination of these factors culminates in altered cell interactions, producing hemolysis and microvascular obstruction, and leading ultimately to damage

and dysfunction of nearly every organ system. Current and evolving treatments attempt to target each of these pathophysiologic processes, through induction of HbF, reduction of RBC dehydration, augmentation of NO, and inhibition of inflammation and coagulation. Other modalities attempt to treat the primary pathophysiology, via transfusion, stem cell transplantation, and gene therapy. Improved stratification of genetic and clinical risk factors for disease severity may help identify which patients are candidates for each of these therapies, individually or in combination.

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