Review Article

Mechanisms of Disease

FRANKLIN H. EPSTEIN, M.D., *Editor*

PATHOGENESIS AND TREATMENT OF SICKLE CELL DISEASE

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IN 1949, the discovery that sickle hemoglobin ($\alpha_2\beta^S_2$) has an abnormal electrophoretic mobility prompted Linus Pauling and his colleagues to christen sickle cell anemia "a molecular disease."[1] The ensuing five decades have produced a wealth of information on the mechanisms by which a single base substitution in the gene encoding the human β-globin subunit, with the resulting replacement of β6 glutamic acid by valine, leads to the protean and devastating clinical manifestations of sickle cell disease. Until recently there was a disappointing lag in the application of this knowledge to the design of safe and effective forms of therapy. In the past few years, however, impressive progress has been made in supplementing time-honored supportive therapy with treatments directed to the disease's unique pathophysiology.

MOLECULAR PATHOGENESIS

The packaging of a very high concentration of hemoglobin (32 to 34 g per deciliter) into red cells requires that the protein be extraordinarily soluble. When sickle hemoglobin (hemoglobin S) is deoxygenated, the replacement of β6 glutamic acid with valine results in a hydrophobic interaction with another hemoglobin molecule, triggering an aggregation into large polymers. The polymerization of deoxygenated hemoglobin S is the primary event in the molecular pathogenesis of sickle cell disease, resulting in a distortion of the shape of the red cell and a marked decrease in its deformability. These rigid cells are responsible for the vaso-occlusive phenomena that are the hallmark of the disease.

When deoxygenated, cells containing hemoglobin S assume a variety of interesting shapes. In cells with a banana or sickle shape, transmission electron microscopy reveals the presence of bundles of fibers oriented along the long axis of the cell. In cells that assume a holly-leaf shape, bundles of hemoglobin S fibers point in the direction of each projection. The fiber's three-dimensional structure has been elucidated by high-resolution electron microscopy and novel methods of image reconstruction.[2] The twisted, rope-like structure is a polymer composed of 14 strands (Fig. 1). X-ray diffraction of crystals of deoxygenated hemoglobin S has shown that the primary structural unit is a double strand in which hemoglobin molecules are staggered. The hemoglobin tetramer is oriented in such a way that in one of the two β subunits, β6 valine forms a hydrophobic con-

Figure 1. Induction of Red-Cell Sickling by Polymerization of Deoxyhemoglobin S.

As red cells traverse the microcirculation, oxygen is released from oxyhemoglobin (red circles), generating deoxyhemoglobin (purple circles). The diagram at the left of the figure shows molecules of hemoglobin S, with the globular $\alpha_2\beta^S_2$ tetramer shown as a flat circle. Deoxygenation of hemoglobin S induces a change in conformation in which the β subunits move away from each other. The hydrophobic patch at the site of the β6 valine replacement, shown as a projection, can bind to a complementary hydrophobic site on a β subunit of another hemoglobin tetramer, shown as an indentation. This interaction is necessary for the formation of polymer, depicted as the interaction of three deoxyhemoglobin S molecules on one strand (dark purple) with three deoxyhemoglobin S molecules on another strand (light purple). At the bottom, a high-resolution model, prepared by Drs. Robert Josephs, S.J. Watowich, and L.J. Gross, shows the interaction of three deoxyhemoglobin S molecules on one strand with three deoxyhemoglobin S molecules on another strand. The α subunits are pale yellow-green, and the β subunits are gray (lighter in the foreground and darker in the background). The heme groups are shown as red spheres. Also shown are contacts between foreground β subunits involving β6 valine (blue) on one strand and the hydrophobic acceptor site (bright green) on the other strand. Only one of the two β6 valine sites in each hemoglobin S tetramer makes this contact.

The diagram in the middle shows the assembly of deoxyhemoglobin S into a helical 14-strand fiber, shown as a twisted rope-like structure. The equation shows the time that elapses, or delay time (t_d), between the deoxygenation of hemoglobin S and the concerted formation of polymer. The delay time is inversely proportional to the intracellular hemoglobin concentration (C), raised to about the 15th power; k denotes an experimental constant. The photograph at the bottom is a high-resolution electron micrograph of a fiber, provided by Dr. Stuart Edelstein.

As deoxyhemoglobin S polymerizes and fibers align, the red cell is distorted into an elongated banana or "sickle" shape, as shown in the diagram at the right. The photograph at the bottom is a scanning electron micrograph of a reversibly sickled cell, provided by Dr. James White.

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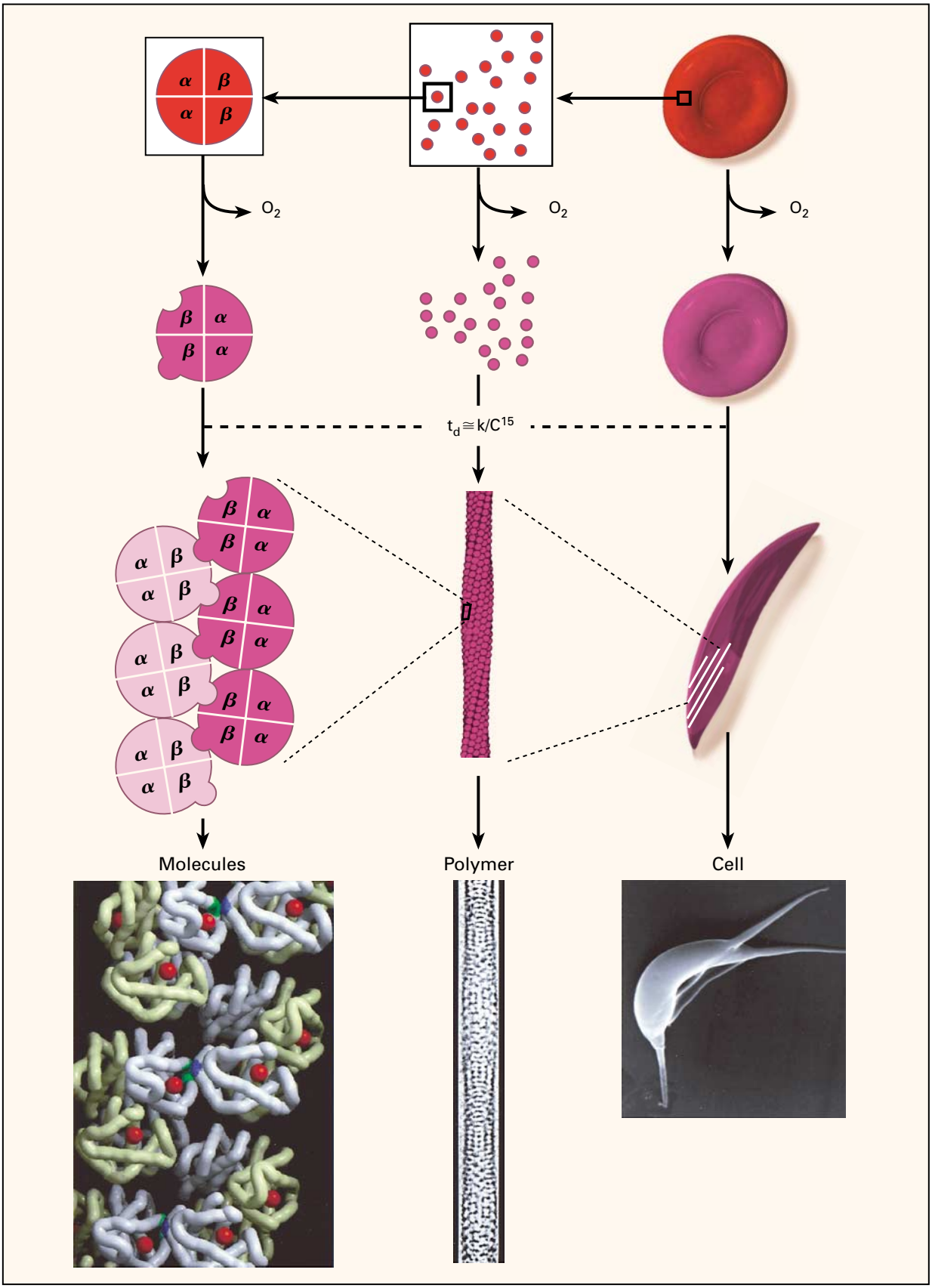
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tact with a complementary or acceptor site on a β subunit of the partner strand (Fig. 1). The molecular contacts in the hemoglobin S fiber, as determined by x-ray analysis, are in excellent agreement with the findings from earlier studies of the gelation and solubility of mixtures of hemoglobin S with hemoglobin A and A_2, fetal hemoglobin (hemoglobin F), and a large number of hemoglobin mutants.[3-5]

When a concentrated solution of deoxygenated hemoglobin S is examined with the use of various physical and chemical probes, large polymers (fibers) and free tetramers are readily demonstrated, whereas species of intermediate size cannot be detected. This phenomenon indicates that the polymerization of hemoglobin S is extremely cooperative and can be regarded as a simple crystal–solution equilibrium.[6] The solubility of a mixture of equal amounts of hemoglobin S and hemoglobin F is about twice that of hemoglobin S alone. Hemoglobin F inhibits polymerization, primarily owing to the glutamine residue at γ87,[3] which prevents a critical lateral contact in the double strand of the sickle fiber.

Kinetics of Hemoglobin S Polymerization

The polymerization of sickle hemoglobin is a remarkably dynamic event. The kinetic features of polymer formation are critical determinants of the shape and morphology of cells.[7,8] When deoxygenation is rapid, multiple, independent polymerization events result in a granular or cobblestone texture that does not alter the cell's disk-like shape. In contrast, when SS red cells are slowly or partially deoxygenated, a single nucleus of aggregated molecules of deoxygenated hemoglobin S is formed. This nucleation is followed by the growth and alignment of fibers, transforming the cell into a classic sickle shape. The distortion of the shape of the cell by projections of aligned hemoglobin S fibers (Fig. 1) has a critical role in perturbing the structure and function of the membrane in SS red cells, mediated in part by oxidant stress.[9]

The rate and extent of polymer formation in a circulating SS red cell depend primarily on three independent variables: the cell's degree of deoxygenation, the intracellular hemoglobin concentration, and the presence or absence of hemoglobin F. The lag period required for the concerted formation of polymer is designated as the delay time (T_d). Because the range of transit times in the microcirculation is short in relation to the range of delay times for SS red cells, polymers do not form in most of the cells (over 80 percent) during their flow through arterioles and capillaries.[7]

Rigorous measurements of the kinetics of polymer formation, in hemoglobin solutions as well as in sickle erythrocytes,[7] have provided critical insights into the pathogenesis of the vaso-occlusive events that play such an important part in sickle cell disease.[10] In contrast, equilibrium measurements greatly overestimate intracellular polymerization in vivo.[11,12] If polymer formation were at equilibrium at the oxygen tensions in the microcirculation, virtually all cells would contain polymer. The resultant marked decrease in deformability would lead to generalized vaso-occlusion and death.

Dysregulation of Red-Cell Volume

Although the mean intracellular hemoglobin concentration and mean density of the overall population of SS red cells are close to those of normal red cells, the density distribution of SS red cells is unusually broad. The increase in the least dense SS cells is due primarily to a high number of reticulocytes with a relatively low intracellular hemoglobin concentration. The presence of a substantial population of very dense cells is the result of polymerization-induced membrane damage leading to enhanced dehydration. The final stage of this process is the irreversibly sickled cell, one that has the characteristically elongated shape even though it has been fully oxygenated and lacks polymer. Since the rate of polymerization of deoxygenated hemoglobin S is dependent on the hemoglobin concentration[13] (Fig. 1), dense SS cells are much more likely to become distorted and rigid and thus contribute disproportionately to the vaso-occlusive and hemolytic aspects of the disease. This accelerated in vivo dehydration is the most relevant pathophysiologic consequence of the membrane lesion in SS red cells.

The most important contributors to the dehydration of SS red cells are potassium–chloride cotransport and Ca^{++}-activated K^+ efflux[14] (Fig. 2). In normal AA red cells, the former transport mechanism is active only in reticulocytes. The rates of potassium–chloride cotransport are much higher in CC red cells[15] and SS red cells,[16,17] a finding that cannot be attributed solely to hemolysis with an increase in young red cells.[18] Potassium–chloride cotransport is induced by cell swelling and also by acidification. The latter stimulus probably occurs in vivo, particularly at sites of stagnant circulation. Enhanced potassium–chloride cotransport appears to have a major role in the marked dehydration not only of SS erythrocytes but also of those in CC[15,17] and SC[17] diseases, resulting in a common and prominent morphologic feature, target cells.

SS red cells have increased amounts of calcium that is compartmentalized within intracellular vesicles,[19] with normal steady-state concentrations of Ca^{++} in the cytosol. When the cell membrane is distorted by sickling, however, there is a transient increase in cytosolic Ca^{++}. This increase is sufficient to trigger the Ca^{++}-dependent (Gardos) K^+ channel, thereby providing a second pathway for sickling-induced loss of K^+ and water and leading to cell dehydration.

Interaction of SS Red Cells and Vascular Endothelium

The most mysterious and challenging aspect of sickle cell disease is the episodic and unpredictable nature of the vaso-occlusive events, both temporally and spatially. Since the potential for a sickled cell to initiate a vaso-occlusive event depends primarily on whether the rate of polymer formation is within the range of the capillary transit time,[10] anything that retards the transit of SS red cells in the microcirculation can have a critical effect on the pathogenesis of vaso-occlusion in sickle cell disease. Accordingly, there has been considerable interest in studies of the interaction between SS red cells and vascular endothelium. Measurements performed under both static[20-22] and dynamic[23] conditions have demonstrated that SS red cells have a sticky surface and attach more readily than normal cells to monolayers of cultured endothelial cells. The relevance of the initial in vitro experiments has been buttressed by ex vivo perfusion studies in rats[24] and transgenic mice.[25] The degree of adherence is strongly correlated with the severity of the disease in patients with SS disease or other sickle genotypes.[20]

Recent studies have begun to delineate the molecular interactions responsible for the adhesion of SS red cells to endothelium (Fig. 3). Reticulocytes, especially those from patients with SS disease, have on their surface the integrin complex $\alpha_4\beta_1$, which binds to both fibronectin[26] and vascular-cell adhesion molecule 1,[27,28] a molecule expressed on the surface of endothelial cells, particularly after activation by inflammatory cytokines such as tumor necrosis factor α. In addition, both microvascular endothelial cells and a subpopulation of sickle reticulocytes have CD36, which binds to thrombospondin secreted by activated platelets.[29,30] Thrombospondin also binds to sulfated glycans on SS red cells.[31,32] In addition to thrombospondin, several other plasma proteins, including very-high-molecular-weight forms of von Willebrand factor,[33] may make an important contribution to adhesion. During inflammatory stress, the adhesion of SS red cells to endothelial cells may be increased as a result of increases in the above-mentioned plasma proteins as well as increased expression of vascular-cell adhesion molecule 1 on endothelial cells. Increased binding of SS neutrophils to fibronectin may also contribute to vaso-occlusive episodes.[34]

APPROACHES TO THERAPY

On the basis of our current understanding of the molecular pathogenesis of sickle cell disease, as summarized above, a number of independent approaches to therapy have been proposed and developed. Three of these approaches have undergone thorough laboratory and clinical investigation: chemical inhibition of hemoglobin S polymerization, reduction of the intracellular hemoglobin concentration, and pharmacologic induction of hemoglobin F.

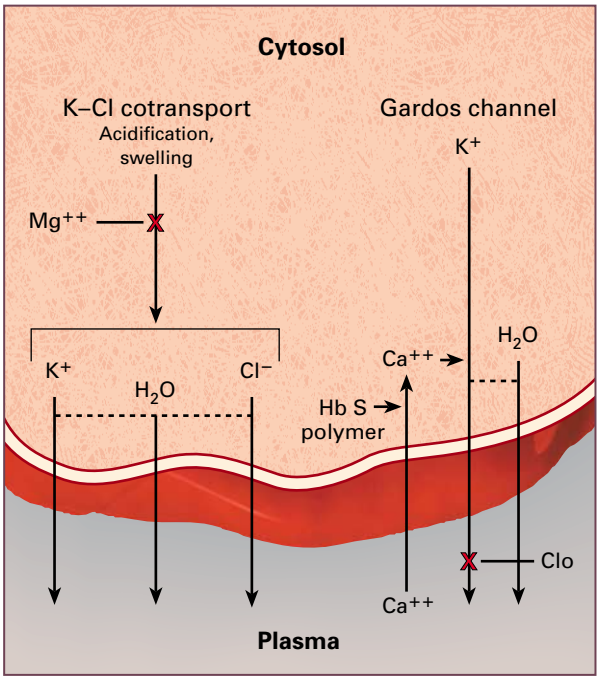

Figure 2. Principal Mechanisms Responsible for Potassium and Water Loss in Sickle Red Cells.

The potassium–chloride cotransport pathway, shown on the left, is activated by either acidification or cell swelling and is inhibited by magnesium ions. The Gardos channel, shown on the right, is activated by a transient influx of calcium ions resulting from membrane distortion by hemoglobin S (Hb S) polymer. The Gardos channel is inhibited by clotrimazole (Clo).

Inhibition of Hemoglobin S Polymerization

Detailed information on the three-dimensional structure of the hemoglobin S polymer has facilitated studies of compounds that inhibit polymerization.[35-37] The development of a safe and effective inhibitor of hemoglobin S polymerization poses a formidable challenge. The ideal agent would be readily absorbed through the gastrointestinal tract, circulate in the plasma without binding strongly to plasma proteins, readily penetrate the erythrocyte membrane, and bind strongly and specifically to hemoglobin S in a way that would inhibit polymerization. It should not affect physiologic oxygen transport or bind to other biologically important molecules. A large amount of drug would be needed to bind to the approximately 400 g of hemoglobin in patients with SS disease. Unfortunately, no antisickling drugs tested thus far have a ratio of efficacy to toxicity that is high enough to merit clinical use.

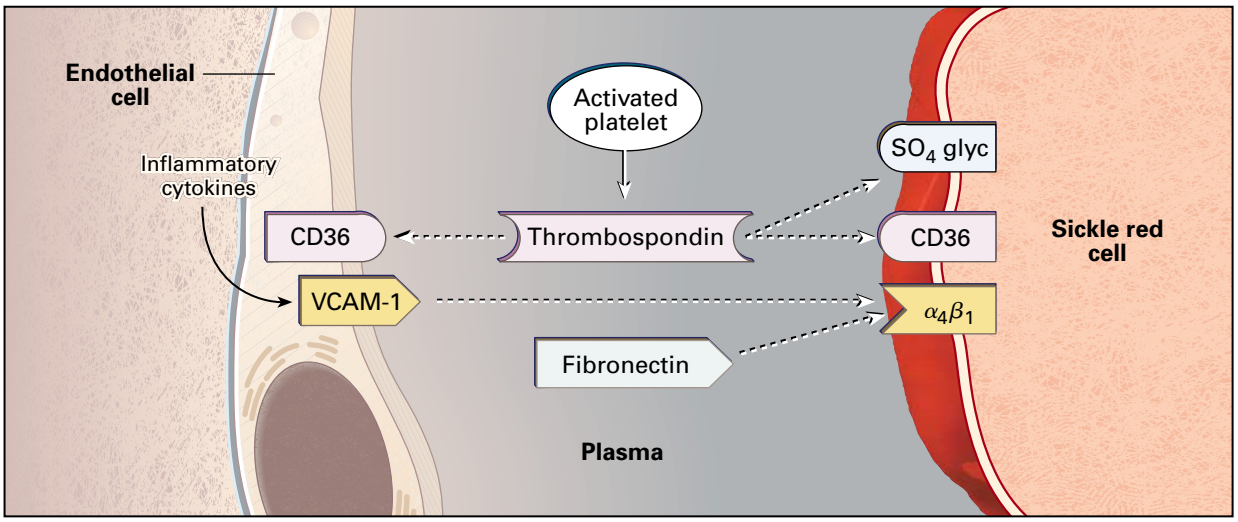

Figure 3. Principal Interactions Responsible for the Adhesion of a Sickle Red Cell to the Microvascular Endothelium.
Activation of platelets releases thrombospondin, which can act as a bridging molecule by binding to a surface molecule, CD36, on an endothelial cell and to CD36 or sulfated glycans (SO_4 glyc) on a sickle reticulocyte. Inflammatory cytokines induce the expression of vascular-cell adhesion molecule 1 (VCAM-1) on endothelial cells. This adhesive molecule can bind directly to the $\alpha_4\beta_1$ integrin on the sickle reticulocyte.

Reduction of the Intracellular Hemoglobin Concentration

Because the rate of polymerization of sickle hemoglobin is so dependent on the hemoglobin S concentration (Fig. 1), any treatment that lowers the mean corpuscular hemoglobin concentration even slightly has a sound rationale. The simplest approach and the first to be investigated in clinical trials is the induction of hyponatremia, with concomitant osmotic swelling of red cells.[38] Although this treatment appears to be effective, it requires meticulous laboratory monitoring and is therefore too cumbersome and risky to be adapted to long-term outpatient use.

Considerable progress has recently been made in the development of drugs that inhibit K^+ and water loss from SS red cells and thus cause a reduction in the intracellular hemoglobin concentration. The Gardos channel is specifically inhibited by the widely used antifungal drug clotrimazole. The efficacy of this drug has been demonstrated by in vitro incubation experiments,[39] studies in transgenic mice with sickle cell disease,[40] and observations in patients with SS disease.[41] At much lower doses than those used for antifungal therapy, clotrimazole treatment in five patients with SS disease resulted in a prompt and striking reduction in dense and irreversibly sickled cells and an increase in intracellular K^+, accompanied by a small but reproducible increase in the hemoglobin concentration and a significant decrease in the serum unconjugated bilirubin concentration, indicating an amelioration of hemolysis.[41]

There is no known pharmacologic inhibitor of potassium–chloride cotransport, the other major pathway for K^+ efflux and dehydration. However, intracellular divalent cations, particularly Mg^{++}, effectively retard K^+ and water loss from SS red cells in vitro.[42] Recently, administration of magnesium supplements in transgenic mice with sickle cell disease and in 11 patients with SS disease resulted in approximately 50 percent inhibition of potassium–chloride cotransport accompanied by a significant decrease in dense erythrocytes and an increase in the hemoglobin concentration.[43]

Induction of Hemoglobin F

The biochemical evidence that hemoglobin F is a very potent inhibitor of the polymerization of deoxyhemoglobin S is amply supported by observations in patients. Among Bedouin Arabs from the Saudi Arabian peninsula and certain tribes from central India, patients homozygous for sickle cell disease have relatively high amounts of hemoglobin F and relatively mild clinical manifestations. The inhibitory effect of hemoglobin F extends to patients of African ancestry who have sickle cell disease. In a cooperative study of the natural history of SS disease in patients in the United States, the frequency of painful crises was inversely correlated with the hemoglobin F concentration.[44]

Because of the compelling biochemical and clinical evidence that hemoglobin F inhibits sickling, drugs that increase the production of hemoglobin F would be expected to benefit patients with sickle cell disease. The first drug to be tested, 5-azacytidine, an antineoplastic drug that inhibits mainte-

nance methylation of DNA, caused a marked increase in hemoglobin F in baboons.[45] These results prompted limited clinical trials that demonstrated significant, albeit less dramatic, induction of hemoglobin F production in patients with sickle cell disease.[46,47] Subsequently, other antitumor drugs, in particular hydroxyurea, were also shown to increase the production of hemoglobin F in nonhuman primates as well as in patients with SS disease.[48-50] The molecular mechanism through which these agents stimulate hemoglobin F production is not known.

Hydroxyurea is currently the only drug in widespread use to stimulate hemoglobin F production. It is relatively nontoxic, its myelosuppressive effects are readily reversible, and it is not known to induce tumors. Most patients with SS disease who are given doses sufficient to cause mild myelosuppression have marked increases in the number of hemoglobin F–containing red cells and the percentage of hemoglobin F, as well as in the level of hemoglobin F per F cell.[49-54] This effect is accompanied by a reduction in hemolysis and a slight increase in the hemoglobin concentration. A significant decrease in the number of irreversibly sickled cells and dense cells is accompanied by improvement in measurements of sickling in vitro.[53] A national, multicenter clinical trial of treatment with hydroxyurea for two years in 299 adults with sickle cell disease showed that the drug was relatively nontoxic and was effective in reducing the frequency and severity of painful crises, as well as in reducing the incidence of the acute chest syndrome and the need for transfusions.[55,56] Similarly, in a recent crossover study involving 22 children with sickle cell disease, treatment with hydroxyurea resulted in a substantial reduction in clinical events requiring hospitalization.[57]

It is usually assumed that the benefit of treatment with hydroxyurea in patients with sickle cell disease is due to the induction of hemoglobin F. However, a detailed multivariable analysis of data from the above-mentioned multicenter study showed that the percentage of F cells was inversely correlated with the rate of painful crises only during the first three months of therapy.[56] In contrast, there was a strong correlation between the neutrophil count and the rate of painful crises throughout the two-year study. Thus, the slight neutropenia that accompanies treatment with hydroxyurea may contribute to the drug's efficacy. This finding may be linked to the experimental observation (discussed above) that SS neutrophils have enhanced binding to fibronectin.[34] In addition, the benefit of hydroxyurea therapy may be due in part to a reduction in the number of reticulocytes and young, low-density SS red cells, since these cells are particularly likely to adhere to vascular endothelium. Indeed, in one study, the adhesion of red cells to cultured endothelial cells decreased markedly within two weeks after the initiation of treatment with hydroxyurea, long before there was a significant induction of hemoglobin F.[58] Since treatment with hydroxyurea also partially suppresses erythropoiesis, the reduction in the number of young adherent cells is accompanied by only a slight increase in the red-cell mass. A full compensatory response to the reduction in hemolysis might have adverse rheologic consequences.

As experience with the long-term administration of hydroxyurea in patients with sickle cell disease increases, there is appropriate concern about the possible induction of tumors. The risk appears to be small in patients with myeloproliferative disorders who have taken the drug for up to 10 years. Despite two years of treatment with hydroxyurea, no increase in chromosomal abnormalities in bone marrow cells was detected in 32 patients with sickle cell disease.[59]

Because of concern about the long-term administration of an antitumor drug in patients with a congenital, nonmalignant disorder, there is considerable interest in identifying safe alternatives for inducing hemoglobin F production. Although initial studies suggested that recombinant human erythropoietin stimulated hemoglobin F production in patients with sickle cell anemia,[60] subsequent clinical studies with recombinant human erythropoietin given alone or in combination with hydroxyurea have yielded conflicting results.[53,61,62]

There is considerable interest in the physiologic and pharmacologic roles of butyric acid and its analogues in the regulation of hemoglobin F production. In infants of diabetic mothers, the switch from hemoglobin F to hemoglobin A is delayed in association with increased serum concentrations of amino-*n*-butyric acid.[63] This metabolite as well as sodium butyrate enhanced the expression of the γ-globin gene in erythroid progenitors from patients with sickle cell disease or β-thalassemia.[64] Infusions of butyrate in sheep fetuses[65] and normal baboons[66] resulted in increased hemoglobin F production. However, there are conflicting data on the efficacy of butyrate therapy in patients with β-thalassemia or sickle cell disease.[67,68] Analogues of butyrate, as well as acetate[69] and other short-chain fatty-acid derivatives,[70,71] also appear to induce hemoglobin F. More studies are needed to assess the efficacy and safety of these potentially nonteratogenic and nonmutagenic means of inducing hemoglobin F production.

Finally, increasing attention is being paid to bone marrow transplantation as a cure for sickle cell disease.[72,73]

FUTURE PROSPECTS

New therapies for sickle cell disease can now be evaluated with more objectivity and confidence than were possible previously, owing in part to the development of highly relevant transgenic-mouse models and in part to improvements in the design and execu-

tion of clinical trials. The opportunity for effective therapeutic interventions at different points in the pathogenetic pathway strongly suggests that the combination of two or more drugs, each with a different mechanism of action, would be additive and perhaps synergistic. Admittedly, such an approach is empirical, like multidrug regimens for the treatment of hypertension and cancer. A definitive cure seems far out of reach. Gene therapy for sickle cell disease is especially formidable, owing to difficulty in transducing hematopoietic stem cells and the necessity for erythroid-specific, high-level, balanced expression of the globin genes. Even if this goal eludes us, the recent progress in treatment and the prospects for further improvements are still cause for optimism.

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