CHAPTER 13

Sickle cell disease

13.1 Pathophysiology and target pathways for novel therapies

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1. Introduction

A homozygous mutation in the gene for β globin, a subunit of adult haemoglobin A (HbA), is the proximate cause of sickle cell disease (SCD). Sickle haemoglobin (HbS) shows peculiar biochemical properties, which lead to it polymerising when deoxygenated. Studies of the kinetics of HbS polymerisation following deoxygenation have shown it to be a high order exponential function of haemoglobin concentration, thus highlighting a crucial role for cellular HbS concentration in sickling (1, 2). HbS polymerisation is associated with a reduction in cell ion and water content (cell dehydration), leading to increased red cell density which further accelerates HbS polymerisation (Figure 1) (1-3). Dense, dehydrated erythrocytes are likely to undergo instant polymerisation in conditions of mild hypoxia due to their high HbS concentration, and HbS polymers may be formed under normal oxygen pressure.

**Figure 1: Schematic diagram of pathogenesis of sickle cell disease**

*The deoxygenation-induced polymerisation of haemoglobin S (HbS) leads to erythrocytes morphological changes, generating sickled, dense, dehydrated red cells, which may be trapped into wider diameter vessels. Owing to decreased flexibility and increased tendency to adhere to abnormally activated vascular endothelium, sickled red cells can lead to vaso-occlusion with the concomitant development of pathological conditions such as acute ischaemic pain crisis, organ/endothelium damage and acute chest syndrome (modified from De Franceschi L et al. Haematologica 89: 348, 2004).*
Pathophysiological studies have shown that the dense, dehydrated red cells may play a central role in acute and chronic clinical manifestations of SCD, in which intravascular sickling in capillaries and small vessels leads to vaso-occlusion and impaired blood flow in a variety of organs and tissues (Figure 1) (2, 4). The persistent membrane damage associated with HbS polymerisation also favours the generation of distorted rigid cells (5) and further contributes to vaso-occlusive crisis (VOCs) and cell destruction in the peripheral circulation. These damaged, dense sickle red cells also show a loss of phospholipid asymmetry with externalisation of phosphatidylserine (PS), which is believed to play a significant role in promoting macrophage recognition and removal of erythrocytes (erythrophagocytosis), cell apoptosis and activation of coagulation. Although the percentage of dense erythrocytes does not predict the severity of the disease, it has been shown to increase prior to or during the first phase of the painful crisis and to decrease thereafter (4, 6, 7). Vaso-occlusive events in the microcirculation result from a complex scenario involving the interactions between different cell types, including dense, dehydrated sickle cells, reticulocytes, abnormally activated endothelial cells, leukocytes, platelets and plasma factors such as cytokines (8, 9) and oxidised pro-inflammatory lipids (6, 10, 11).

Hydroxyurea (hydroxyurea) is currently the only drug approved for chronic administration in adult patients with SCD to prevent acute painful crises and reduce the incidence of transfusion and acute chest crises (12). Long-term use of hydroxyurea has been demonstrated to produce dramatic reductions in mortality and morbidity in patients with SCD (13). Clinical use of hydroxyurea in paediatric and adult patients with SCD is discussed in the next chapter on clinical management (13.2). Decitabine has also been shown to be a promising agent for the ip-modulation on HbF in SCD (14). We will focus here on therapeutic strategies currently being considered for the treatment of SCD, which are not based on HbF modulation. They include:

- Use of agents which reduce or prevent sickle cell dehydration
- Use of agents which reduce sickle cell-endothelial adhesive events
- Use of nitric oxide (NO) or NO-related compounds
- Use of antioxidant agents.

2. Prevention of sickle red cell dehydration

One of the distinguishing characteristics of SCD is the presence of dense erythrocytes, formed as a result of cell dehydration and loss of potassium (K+). These dense red cells generally have a lower HbF content than the other red cells and include both reticulocytes and mature red cells (15). Usually, the dense fraction of erythrocytes
has a high percentage of irreversible sickle cells (ISCs), cells that maintain their sickle shape even when fully oxygenated. An inverse correlation has been demonstrated between the percentage of ISCs and erythrocyte survival. In vitro and in vivo studies in animal models for SCD have suggested a crucial role of dehydrated red cells in the pathogenesis of vaso-occlusive events; the dense, dehydrated red cells can be easily trapped in post capillary venules, promoting micro-vascular obstruction (16).

Thus, prevention of red cell dehydration represents an exciting possible new therapeutic strategy. Studies on membrane permeability in SCD have shown abnormalities in different specialised membrane-embedded transporters that carry cations, anions and water across the erythrocyte membrane. In the last two decades, studies on the nature and properties of the pathways mediating K+ loss in sickle cell erythrocytes have led to the development of new therapeutic tools to block K+ loss and dehydration.

The major pathways for K+ loss during sickle cell dehydration events are the Ca2+-activated K+ channel, known as Gardos channel, operating in parallel with the conductive Cl- pathway and the electroneutral K-Cl cotransport (Figure 2) (17-22).

2.1 The Ca2+-activated K+ channel (Gardos channel, KCNN4)
Sickle red cells are characterised by increased amounts of calcium, which is functionally and physically sequestered into intracellular vesicles, but maintained in normal concentration in the steady state. The cyclic deoxygenation and HbS polymerisation that occurs in sickle red cells has been shown to produce transient increase in free intracellular calcium, which is responsible for a large K+ loss, with associated Cl- and water loss. This effect is due to activation of a specific Ca-gated K+ channel that was first described by Gardos (23). The imidazole antimycotic clotrimazole (CLT) has shown to be a specific inhibitor of the Gardos channel and to prevent sickle cell dehydration in vitro (18). In a transgenic mouse model of SCD, oral administration of CLT was reported to specifically block the Gardos channel, increase the red cell K+ content and reduce red cell dehydration (24). The compound was further tested in normal humans (AA) and in sickle cell volunteers (SS), and was shown to be a powerful and effective inhibitor of the erythroid Gardos channel and of sickle red cell dehydration (25, 26). Further studies led to the development of a novel class of compounds based on the back-bone structure of CLT, which have conserved Gardos channel inhibitory power, but are devoid of the imidazole moiety of CLT, and thus of cytochrome P450 inhibitory effects (27). One of these compounds (ICA-17043) has been shown to have 10-fold greater potency than CLT in blocking the Gardos channel in vitro and in vivo to specifically inhibit Gardos channel and prevent K+ loss and red cell dehydration (28). Phase I studies in normal human
subjects and in sickle cell patients, showed significant blockade of the Gardos channel, in absence of any significant side-effects (29). A phase II study showed that ICA-17043 reduced haemolysis and the percentage of dense cells, with a significant amelioration of anaemia in patients with SCD (30). However, a phase III clinical trial showed no effect of ICA-17043 on the rate of painful events in SCD patients, which may reflect changes in blood viscosity due to increased survival of red cells. No other studies have been planned with this molecule.

Another therapeutic agent which has been recently shown to modulate the Gardos channel activity is L-arginine. Patients with SCD show a state of relative depletion of arginine, which is part of the nitric oxide pathway. L-arginine supplementation...
of transgenic sickle cell mice resulted in inhibition of erythrocyte Gardos channel activity and amelioration of red cell dehydration (16). A phase II study to test the effect of arginine supplementation has shown no major effects on Gardos channel function and erythrocyte hydration in patients with sickle cell disease (31, 32).

2.2 The K-Cl cotransport (KCC1/3/4)
Several forms of K-Cl cotransport (KCC) have been described in various human and mouse tissues. KCC2 expression seems to be limited to brain cells, while human and mouse erythrocytes seem to possess KCC1, KCC3 and KCC4 isoforms in different and still undetermined ratios. The K-Cl cotransport mediates red cell dehydration in SCD. Studies on K-Cl cotransport function have identified different triggers of activation, such as cell swelling, cell acidification, reduced cell magnesium (Mg) content, membrane oxidative damage and high concentrations of urea. Franco et al. (22) have also shown that K-Cl cotransport mainly contributes to dehydration of sickle reticulocytes and that deoxygenation of sickle red cells also stimulates K-Cl cotransport in isotonic solutions at pH 7.4 (Figure 2). The relative contribution of the Gardos channel and of the K-Cl cotransport pathway in generating dehydrated, dense sickle red cells is a complex and still unresolved issue.

K-Cl cotransport activity is modulated by red cell Mg content and low Mg levels are associated with abnormal activation of K-Cl cotransport. Some small studies have reported a reduction in red cell Mg content in SCD patients. Thus, oral Mg supplementation with the aim of increasing red cell Mg levels and inhibiting K-Cl cotransport activity may represent a possible therapeutic strategy for ameliorating SCD red cell dehydration (16, 17). Dietary magnesium supplementation in transgenic sickle cell mice has demonstrated that increasing erythrocyte Mg content can ameliorate red cell dehydration. Two uncontrolled trials of oral supplementation with Mg pidolate have been carried out in sickle cell patients, showing a reduction in K-Cl cotransport activity, an increase in red cell K and Mg content, an improvement in red cell dehydration and a reduction in the number of painful events (17, 33). A double-blind, placebo controlled crossover study with Mg pidolate supplementation in children with SCD did not demonstrate any significant changes in the haematological parameters studied; however, the Mg pidolate dosage was markedly lower than that used in the previous studies. In a phase I study, the combination of Mg pidolate with hydroxyurea has been evaluated in patients with HbSC disease, showing a significant reduction in the activity of the K-Cl cotransport after 3 months of supplementation (34).

Recently, it has been reported that infusion of Mg sulphate reduces the length of stay of sickle cell patients hospitalised during vaso-occlusive crises (16).
2.3 The Cl\textsuperscript{-} permeability pathway
Studies on the conductive Cl\textsuperscript{-} pathway indicate that for red cell dehydration the movement of K\textsuperscript{+} must be accompanied by that of chloride (or other monovalent anions) to maintain electroneutrality (Figure 2). Elegant sets of studies demonstrate that movement of K\textsuperscript{+} and dehydration via the Gardos channel can be blocked if the Cl\textsuperscript{-} conductive pathway is inhibited. A specific inhibitor of Cl\textsuperscript{-} conductance has been recently developed (NS3623). NS3623 has been tested in transgenic sickle cell mice and was found to reduce \textit{in vivo} sickle cell dehydration, with a mild echinocytosis at the highest doses. Unfortunately, NS3623 was not further developed for clinical use because of undesirable side effects observed in human subjects (16, 19).

3. Anti-adherence therapy in sickle cell disease
Vaso-occlusive episodes are central events in the pathophysiology of SCD, causing the clinical manifestations and leading to acute and chronic organ damage. The abnormal adhesive interactions between erythrocyte, reticulocytes, endothelial cells, platelets and/or soluble mediators may represent a possible new therapeutic target. In addition, SCD patients showed abnormally activated circulating endothelial cells which increase during acute vaso-occlusive crisis suggesting the presence of chronic vascular endothelial damage which further worsens during acute events (4, 35-37). The end-point of anti-adherence therapy is to interfere with the initialisation and/or amplification of adhesive events. Although anti-adherence therapy has been mainly studied during acute painful events, its mechanisms of action are only partially known (4, 6).

In SCD, the available anti-adherence therapeutic strategies can be divided into:
- Molecules interfering with chemical-physical processes during erythrocyte-endothelial adhesion events
- Molecules interfering with sickle cell-endothelial adhesive mechanisms
- Molecules modulating inflammatory pathways involved in sickle cell-endothelial adhesion
- Nitric oxide (NO)-based therapies.

3.1 Molecules interfering with chemical-physical processes during erythrocyte-endothelial adhesion events
A non-ionic surfactant copolymer such as RheothRx (Poloxamer 188) can lower viscosity and frictional forces, thus improving microvascular blood flow. RheothRx has been shown to block hydrophobic adhesive interactions (cell-cell, cell-protein or protein-protein interaction) in blood, resulting in a reduction of erythrocyte
aggregation and red cell adherence to vascular endothelium, with a hypothetical improvement in microvascular flow (38). Phase II studies have shown a limited favorable effect in treatment of acute pain crises, when combined with hydroxyurea (HU) in children with SCD. However, no further clinical development studies are planned for this compound. The possibility of delivering oxygen directly to sickled red cells entrapped in partially obstructed vessels has also been explored. Perflubron-based fluorocarbon emulsion (PFE) decreases the peripheral vascular resistance ex vivo in the mesoecaecal vasculature of rats, due more to its ability to dissolve oxygen than to its ability to modify the vascular tone (39).

3.2 Molecules interfering with sickle cell-endothelial adhesive mechanisms
Recent studies on the sickle cell-endothelial adhesive mechanism have identified different interactions which may have particular therapeutic relevance (Figure 3):

- The integrin α4β1 receptor of fibronectin and the vascular adhesion molecule-1 (VCAM-1), E-selectin and P-selectin
- The thrombospondin and/or collagen and receptor CD36, present on the surface of endothelial cells, platelets and reticulocyte-rich subpopulations of normal and sickle erythrocytes
- The sulphate glycolipids, which bind thrombospondin, von Willebrand factor multimer and laminin (2, 40, 41)
- The Lutheran blood group proteins (BCAM/LU), whose expression is increased in red cells from SCD patients that bind to α5 subunit of laminin, a component of extracellular subendothelial matrix (42, 43)
- The ICAM-4 (Landstein-Weiner blood group glycoprotein- LW), which binds αVβ3 integrin receptors on endothelial cells (44-47)
- The exposure of phosphatidyl-serine (PS), detectable in a subpopulation of sickle red cells, which participates in sickle cell adhesion to activated endothelium (48-52).

Ex vivo and in vitro experimental studies have shown that thrombospondin- and von Willebrand factor-mediated interaction between sickle red cells and endothelium via αVβ3 integrin might be blocked by monoclonal antibodies against αVβ3 integrin receptors (40, 46, 53). Short synthetic peptides interfering with ICAM-4 and αVβ3 integrin binding have recently been evaluated in an ex vivo system, showing a reduction in sickle erythrocyte adhesion to activated endothelial cells, which suggests that the blocking of this adhesion pathway could be a possible therapeutic new strategy in the treatment of acute sickle cell events (46, 53). The binding between thrombospondin (TSP), von Willebrand factor and laminin, which mediates sickle cell-endothelial adherence, could be blocked by anionic
polysaccharides such as high molecular weight dextran sulfate or chondroitin sulphate (2, 40, 41).

An additional therapeutic approach for blocking sickle cell adhesion to endothelial cells is heparin, which might interfere with sickle cell adhesion to endothelial cells through P-selectin (54-57) or binding to TSP, which can mediate the interactions between sickle erythrocytes and the vascular endothelial surface. A double blind randomised trial with tinzaparin in SCD patients during acute VOCs has documented a reduction of severity and duration of the VOCs (49, 58).

3.3 Molecules modulating inflammatory pathways involved in sickle cell-endothelial adhesion

A chronic inflammatory state has been described in SCD patients, characterised by increased plasma levels of acute phase proteins and soluble cytokines such as IL-1β, IL-6, TNF-α and endothelin-1 (ET-1), which are further elevated during acute VOCs. These factors are involved in leukocyte chemotaxis, modulate vascular tone and contribute to sickle cell related tissue damage. Thus, it has been suggested that...
anti-inflammatory therapy could interfere with the inflammatory storm and so reduce abnormal vascular activation (59). Sulfasalazine is an anti-inflammatory molecule and can inhibit the transcription of nuclear factor (NF)-κB and so interfere with endothelial cell activation (60-63). Transgenic sickle cell mice treated with sulfasalazine show a reduction in activated circulating endothelial cells, and in the expression of VCAM-1, ICAM and E-selectin by vascular wall endothelial cells. In a pilot study, the administration of sulfasalazine to sickle cell patients resulted in a reduction in the abnormal endothelial activation (4). Another possible strategy aimed at reducing the adhesion of sickle red cells to vascular endothelium is the inhibition of interactions between leukocytes already adherent to endothelium and sickle red cells during vaso-occlusive events (64). Based on the *in vitro* evidence that immunoglobulin (Ig) significantly reduces the binding of sickle red cells to neutrophils in transgenic sickle cell mice, the infusion of Ig *in vivo* was shown to inhibit the interaction between sickle red cells and leukocytes in the cremasteric venules, suggesting that Ig may act either by inhibiting the interactions between sickle red cells and leukocytes and/or by reducing the number of adherent leukocytes (64). In humans, three out of four sickle cell patients treated with infusion of Ig showed some beneficial effect, whereas in the fourth case the treatment accelerated a vaso-occlusive crisis (64, 65). Since Ig infusion may be related to severe side effects such as renal toxicity and thrombosis, it should be used with caution in sickle cell patients.

Recent studies in a mouse model for SCD have shown the important role of ET-1 in acute sickle cell related VOCs (66, 67). The block of ET-1 actions was obtained directly by the ET-1 receptor antagonist, Bosentan, evaluating its effects on SCD mouse kidney as target organ, and indirectly by the inhibition of phosphodiesterase-4 with Rolipram in a model of early pulmonary hypertension (66, 67). Bosentan is currently under evaluation in a phase III clinical trial in SCD patients with pulmonary hypertension (68).

### 3.4 Nitric oxide based therapies

Nitric oxide (NO) is a potent vasodilator and inhibitor of vascular remodeling and also affects the multi-step cascade of events involved in leukocyte, platelet and endothelial activation. NO is generated from L-Arginine by endothelial cells via constitutive (eNOS) and inflammatory inducible nitric oxide synthases (iNOS). SCD is characterised by a relative reduction in NO bioavailability which contributes to abnormal activation of endothelial cells and SCD organ damage. In addition chronic haemolysis leads to an increase in plasma levels of haemoglobin, which acts as an efficient NO buffer, and so contributes to reducing NO levels in SCD.

Recent studies have focused on inhaled NO for the treatment of tissue damage in...
various ischaemic syndromes, including cardiovascular disease, pulmonary hypertension, and acute lung distress syndromes. The possible therapeutic role of inhaled NO has been studied in different animal models of lung injury induced by ischaemia/reperfusion. Inhaled NO prevents leukocyte migration and reduces the permeability of the peripheral microvasculature. In association with surfactant, inhaled NO alleviates alveolar oedema and reduces bronchoalveolar leukocyte and neutrophil infiltration in animal models of ischaemic lung injury. A placebo-controlled randomised clinical trial of inhaled NO in SCD has recently reported beneficial results in the treatment of acute vaso-occlusive crisis, although the mechanism of action remains unknown. Plasma NO metabolites are decreased in SCD patients during vaso-occlusive crisis associated with severe pain and also in acute chest syndrome (32). A decrease in exhaled NO has been reported in sickle cell patients, suggesting a role for NO in the pathogenesis of the pulmonary complications (69). In a transgenic mouse model of SCD, it has been shown that inhaled NO provides protection during ischaemia/reperfusion lung injury, in which endothelial NO production is reduced (70, 71).

In addition NO-donors such as polynitroxy-albumin and nitrox-albumin have been shown to be effective in reducing inflammatory state in a SCD mouse strain and to reduce the hypoxia-induced lung damage in another mouse model of acute VOCs (72, 73).

Another possible therapeutic strategy for increased NO production in SCD is supplementation of L-Arginine. Morris et al. showed that L-Arginine supplementation alone induces an unexpected decrease in NO metabolite production (11, 74). In a subsequent pilot study, an increase in NO metabolites was observed when L-Arginine was co-administrated with HU, suggesting that the combination treatment may have a synergistic effect on NO production (2, 31). A phase II trial of L-Arginine supplementation in SCD however showed no effects on NO levels or on erythrocyte features.

4. Antioxidant agents

SCD is characterised by a pro-oxidant environment due to high production of reactive oxygen species (ROS) related to increased levels of free iron and haem groups associated with a reduction in antioxidant systems such as GSH (75-78). Studies in vitro on SCD red cells have shown that iron chelation by deferiprone (L1) reduces the susceptibility of the sickle red cell membrane to iron mediated oxidative damage (75-79). An in vivo study on SCD patients supplemented with L-glutamate to increase GSH and glutamate levels has shown some improvement of chronic pain (76).
5. Conclusion
In conclusion, the emerging picture for the treatment of SCD is that abnormalities ranging from membrane cation transport pathways to the structure and function of red cell membrane proteins, or red cell-endothelial adhesive events, may constitute new pharmacological targets for treating SCD. Prospective therapies for SCD need to combine molecules with different pharmacological targets in order to increase their therapeutic efficacy and to reduce their side effects (e.g. inhibitors of dehydration and either hydroxyurea or anti-adhesive molecules).

References


31. Morris CR, Kato GJ, Poljakovic M et al. Dysregulated arginine metabolism, hemolysis-


Multiple Choice Questionnaire

To find the correct answer, go to http://www.esh.org/iron-handbook2009answers.htm

1. All the agents listed below have been tested in humans, based on a inhibitory effect on the Ca-activated K channel, except:
   a) Clotrimazole .................................................................
   b) Ica-17043 (senicapoc) ....................................................
   c) Arginine ........................................................................
   d) L-glutamate .................................................................

2. All the following adhesive interactions have particular therapeutic relevance for the vasculopathy of sickle cell disease, except:
   a) E-selectin ......................................................................
   b) P-selectin ......................................................................
   c) Lutheran ......................................................................
   d) ICAM-4 .......................................................................  
   e) Glycophorin C ..............................................................

3. Please identify the anti-inflammatory molecule which can inhibit the transcription of nuclear factor (NF)-κB and interfere with endothelial cell activation:
   a) Aspirin ........................................................................
   b) Infliximab ....................................................................
   c) Ibuprofen ....................................................................
   d) Sulfasalazine ................................................................
   e) Acetaminophen ............................................................

4. Please identify the agent that has shown promise for increasing HbF in sickle cell disease:
   a) L-glutamine .................................................................
   b) Decitabine .................................................................
   c) Heparin ........................................................................
   d) Mg sulfate ....................................................................
   e) Atorvastatin ...............................................................
5. Please identify the divalent cation which is deficient in sickle erythrocytes and has shown promise in preventing the sickle cell dehydration:
   a) Ca
   b) Cu
   c) Zn
   d) Mg
   e) Mn