Modulation of fetal hemoglobin in sickle cell anemia

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Modulation of Fetal Hemoglobin in Sickle Cell Anemia

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Modulation of Fetal Hemoglobin in Sickle Cell Anemia

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
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Aan mijn ouders

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Chapter I

Sickle cell (SS) disease: a review

AW Saleh

Introduction

Sickle cell anemia is characterised by the presence of sickled red cells, first described by Herrick in 1910 as peculiar elongated and sickle - shaped red blood corpuscles¹. The main features of this inherited disease are chronic hemolytic anemia and recurrent pain episodes which are related to the presence of mutant hemoglobin S within the red blood cells.

Hemoglobins (Hb) F, Hb A and HbA₂ represent the normal hemoglobins in post-embryonal life. The hemoglobin molecule is constructed as a tetramer which consists of one pair of alpha chains and one pair of non alpha chains (β chain in HbA, γ chain in HbF and δ chain in HbA₂).

Hemoglobin S was discovered by Pauling and colleagues in 1949 who reported the different electrophoretic mobilities of HbS and normal adult hemoglobin HbA². The work of Ingram³⁻⁵ has shown that HbS is the result of replacement of glutamic acid by valine at position 6 in the β -globin chain.

HbS is inherited in a familial autosomal co-dominant manner which may result in homozygous SS disease, characterized by the presence of two β^S genes, and the asymptomatic heterozygous form (HbAS) which carries one normal β^A and one mutated β^S gene, known as 'sickle cell trait'.

Numerous other abnormal hemoglobins have been identified. Among these, HbC and E are found in high frequencies. The HbSC compound heterozygote may result in significant clinical disease resembling that of SS disease.

The other group of hemoglobin disorders are the thalassemias. These disorders may co-exist with HbS. Thalassemias are characterized by an inadequate rate of - qualitatively normal- α or β chain synthesis caused by an alteration in the genes encoding for these chains. The clinical severity is proportional to the degree of imbalance in β -globin (β -thal) or α -globin (α -thal) chain production.

The β -thalassemia genes are categorised into two groups namely those expressing no HbA at all (β^0 thalassemia) and those expressing reduced amounts of HbA (β^+ thalassemia). α -Globin genes normally occupy a double locus. In α -thalassemia different levels of disease severity result from deletions in one (α -thal-2) or both of the α gene loci (α thal-1).

The inherited hemoglobin disorders are among the most common single gene disorders in the world. They already place a significant burden on health services. Therefore, it is of importance that management of these diseases focuses on prevention of severe forms (genetic counseling and prenatal diagnosis) and on research of the pathophysiological and molecular mechanisms, thereby creating new treatment strategies.

Molecular basis of the α - and β -globin genes

Genes that determine the synthesis of globin chains are organized in two clusters on different chromosomes. In each cluster the genes are organized 5' $\rightarrow$ 3' in the same order that they are switched on during development. As the major site of erythropoiesis shifts from the yolk sac to the liver and then to the bone marrow, the globin synthesis switches from ζ and ϵ -globin to α and γ -globin, and at birth to α , β and δ -globin, respectively^{6,7}.

The β -like globin gene cluster covers a region of approximately 60 kb on the short arm of chromosome #11 (Figure 1.1). It contains 5 functional genes (the ϵ , γ , δ and β -genes) and one pseudo-gene $\psi\beta$ (pseudo-genes resemble functional genes but lack a critical component of the gene structure necessary for expression). The polypeptide chains produced by both γ genes differ in only one amino acid at position 136. The γ chain has glycine, whereas the δ chain has alanine. Sometimes a variant γ chain is detected which is characterized by an isoleucine $\rightarrow$ threonine replacement at position 75. Transcription of the β -like globin genes is regulated by distant sequences (so-called hypersensitive sites) on the same chromosome, known as the Locus Control Region (LCR), located 6 to 20 kb 5' to the ϵ -globin⁷. By interacting with proteins in its environment, the LCR appears to affect the chromatin organization of the β -globin gene cluster which determines the chronological transcription of the individual genes during ontogenesis⁷.

The α -like globin gene cluster is found on the short arm of chromosome #16 and has three functional genes: the embryonic ζ -globin gene, the $\alpha 2$ and $\alpha 1$ -globin genes (Figure 1.1). The synthesis of α -chains by both $\alpha 1$ and $\alpha 2$ genes differs only with respect to the rate of transcription and translation. Although the $\alpha 2$ gene has a more efficient transcription, the $\alpha 1$ gene has a more efficient translation, finally resulting in an almost equal production of α -globin chains. Regulation of expression of the α -globin genes is dependent on a region located 40 kb upstream to the ζ gene, named HS-40⁸.

The promoter region, about 100 nucleotides 5' to the transcription start site, is the element that initiates transcription by binding several transcription factors (TF-IIA,B,C,D,E and TF-III) and the enzyme RNA polymerase II. It contains three nucleotide sequences that are found in all globin genes (TATA box, CAT box and CAC box)^{7,9}.

The β -globin genes also have enhancers that upregulate gene expression irrespective of their location relative to the target gene. They bind specific regulatory factors (Sp1, GATA, NFE-2)^{7,9}. In addition, silencer elements have been identified that contribute to a negative regulation of gene expression^{10,11}. Processes such as DNA methylation and histone acetylation, that modify DNA after its synthesis appear to influence the expression of globin genes as well^{12,13}.

Figure I.1 Organisation of the α - and β -globin gene clusters. Dark boxes represent functional genes.

The rate of synthesis, the tissue specificity, and the modulation of developmental globin gene expression are considered to be the result of interaction of promoter, enhancer and /or silencer elements with hypersensitive sites within the 5' regulatory region. Various nuclear proteins (or trans-acting factors) as mentioned above, interact with these motifs to regulate globin gene expression.

Pathophysiology of sickle cell anemia

The basic abnormality of hemoglobin S, which is the substitution of valine for glutamic acid at the sixth position in the β - chain, is the product of a single base mutation (GAG $\rightarrow$ GTG) at codon 6 of the β -globin gene (β^S)⁶.

The amino acid mutation of hemoglobin S lies on the surface and normally causes little disturbance in the tetramer. However, especially in situations of low oxygen tension, it drastically changes the solubility of deoxygenated HbS. The $\beta 6$ valine molecules rapidly form hydrophobic bonds with complementary sites on β subunits

of other hemoglobin tetramers resulting in polymers or fibers made of large strands of tetramers. It has been shown that the rate of deoxygenation determines the shape and morphology of the red blood cells (RBC). Rapid deoxygenation results in multiple independent polymerization events producing a granular texture that does not alter the cell's shape. In contrast, when SS RBC are partially or slowly deoxygenated a single core of hemoglobin strands is established which continues to aggregate and align more molecules until fibers are formed that characteristically distort the red blood cell to a sickle shape¹⁴⁻¹⁶. The polymerization depends upon the concentration of HbS. In homozygous SS patients high levels of intracellular deoxygenated HbS lead to polymer formation whereas in heterozygotes (AS) the HbS level is usually insufficient to result in polymerization^{14,15}. The polymerization process is time-dependent showing a delay time prior to actual gelation. Polymer formation and breakdown (sickling and unsickling) will occur repeatedly as a consequence of switching between the oxy and deoxy configuration of the Hb molecule. This eventually results in irreversible RBC membrane damage and deformation as well as perturbation of several other erythrocyte properties which shorten red blood cell survival and promote vaso-occlusion^{14,15}.

The vaso-occlusive episodes are thought to result from increased adhesive tendency of sickle reticulocytes and young deformable sickle cells to endothelium causing capillary blood flow to slow down, increasing transit time, metabolic acidosis, deoxygenation and consequently intracellular polymerization leading to more sickling and further loss of deformability of red cells. Entrapment of dense, rigid and undeformable, RBC follows and gives rise to complete capillary vaso-occlusion¹⁷. The latter group of cells comprises the fraction of most dense and irreversibly deformed red cells which result from multiple sickling and unsickling episodes as well as a subset of cells with high occlusive affinity described as dense (high MCHC) discocytes^{18,19}. These discocytes are rigid and change shape very little during oxygenation. Although the irreversible sickled cells have been demonstrated to correlate with the rate of hemolysis they do not show a relationship with the clinical symptoms of SS patients.

Several other factors influence the rate of sickling and/or the adhesion of sickle cells to venular endothelium. These include the intracellular HbF and 2,3-DPG levels, leakage of potassium across the red blood cell membrane with influx of sodium and calcium, the Von Willebrand factor (vWF), low oxygen tension, decreased pH, increased temperature, advanced cell age, and increased intracellular HbS concentration²⁰⁻²².

Prevalence and geographical distribution of HbS

There is evidence that the sickle cell trait provides some protection against *Plasmodium falciparum* malaria and this explains the preferential selection of the β^s

gene^{23,24}. In accordance, the geographical distribution of sickle cell disease coincides with areas where malaria is or has been endemic. The β^S mutation probably took place in several locations in Africa and India millions of years ago. Today, sickle cell anemia is mainly found in Africa and among Afro-Americans in America and the Caribbean area. However, it is not limited to blacks. It is also encountered at lower frequencies in Southern countries of Europe, Eastern Europe, the middle east and India. In American blacks the frequency of HbS is approximately 8% and in Central Africa the frequency, depending on the area, may vary from 20 to 40%²³⁻²⁵.

The β^S haplotype

Distribution of β^S -haplotypes

While the β^S mutation is common to all SS patients it apparently occurred several times independently on chromosomes of different genetic backgrounds²⁵. Four specific β haplotypes have been recognised which relate to different areas of Africa. They are the Benin haplotype (type 19) present in Central West Africa and North Africa (Nigeria, Benin, Ivory Coast, Ghana, Algeria, Sudan), the Senegal haplotype (type 3) originating from Atlantic West Africa (Senegal, Gambia, Liberia, Guinea), the Bantu haplotype (type 20) in South-Central and Eastern Africa (Angola, Zaire, Kenya, Namibia, Tanzania) and the Cameroon haplotype (type 17) which is mainly restricted to an ethnic group in Cameroon. A fifth haplotype, named the Asian (type 31), is encountered in Saudi Arabia and India²⁵⁻³¹. The different β^S haplotypes of patients in America and the Caribbean likely disclose the origins of their African predecessors²³.

Association of β^S -haplotypes with hematological and clinical data

Although certain phenotypical differences have been noted between the haplotypes, a clear distinction has proven very difficult. Initial studies in Africa have been performed with small numbers of patients against a widely different environmental and genetic background. In developed countries, for example the United States, a predominant Benin haplotype is found together with a large group of heterozygous and atypical β^S - haplotypes rendering only small numbers of other homozygous haplotypes available for study. Therefore clinical and hematologic effects of haplotype in sickle cell anemia should be interpreted with caution. Some characteristics of the four most common haplotypes are shown in table I.1.

Table I.1. Hematological and clinical manifestations in relation to the most common β^S -haplotypes.

	BENIN (19/19)	BANTU (20/20)	SENEGAL (3/3)	ARAB- INDIAN (31/31)
Hemoglobin	↓	↓↓	↓	↓
HbF	= or ↑	=	↑↑	↑↑↑
Painfull crises	↑↑	↑↑	↑	↑
End organ damage	↑	↑↑*	↑	=**

=: not increased. * In particular high incidence of renal failure; ** Rarely priapism and leg ulceration; acute chest syndrome less frequent.

Diagnosis

Sickle cell anemia may be suspected on clinical grounds and the patient's hematologic profile (complete bloodcount, reticulocyte count and examination of peripheral blood smear). The presence of HbS may be confirmed by a sickle cell solubility test. This test relies on the fact that HbS precipitates in high osmolarity phosphate buffer at neutral pH when reduced with sodium hydrosulphite while normal Hb's do not precipitate³². The test is not useful in the newborn period because of the high amount of HbF within the red blood cells. A more accurate method of HbS detection is the immunologic procedure which is based on binding of antibody to the $\beta 6$ Glu → Val replacement³³.

The quantification of HbS, confirmation of sickle cell anemia and differentiation of HbS from other Hb types can be done by Hb electrophoresis, Isoelectric Focusing or by High Performance Liquid Chromatography (HPLC).

The Hb electrophoresis, usually in an alkaline medium (cellulose acetate), is the conventional method of confirming sickle cell anemia. The method relies on the migration of the negatively charged hemoglobin molecule towards the anode in an electrical field. The abnormal Hb has a different charge than Hb A and the two can be differentiated on the basis of their different mobilities in the electrical field. It remains popular because the method is simple, however, it fails in differentiating HbS from other Hb's (such as HbD) which show similar mobilities³².

Isoelectric Focusing (IEF) produces superior resolution of hemoglobins by fractionating proteins according to their isoelectric points along a pH gradient in an agar or polyacrylamide gel³⁴. In addition to detection of conditions like AS, SS, AC, AD and AG, more complicated combinations such as a β chain variant and a α chain variant are readily identified. In most centres IEF is the preferred procedure for newborn screening since the method clearly separates HbF from other minor quantities of adult Hbs.

HPLC, a technically more difficult as well as expensive type of chromatography, allows separation of hemoglobins through application of cation exchange resin column. Three different methods are used³⁵⁻³⁷: 1. Cation exchange HPLC for separation of HbS. 2. Reversed phase HPLC for separation of globin chains and 3. Another type of reversed phase HPLC for the separation of fragments of globin chains obtained by proteolytic digestion. The technique provides an excellent resolution of the different hemoglobins and is useful in distinguishing the various forms of sickle cell disease (SS, SC, S- β -thal, and other rarer types). The HbF level as well as each of the different γ -chain levels can be accurately determined.

DNA analysis has enabled us to specify the molecular pathology of the different haemoglobinopathies. After isolating DNA from white blood cells, the DNA segment to be studied is amplified and sequenced, using synthetic oligonucleotide probes that hybridize specifically with the normal and mutated sequences^{38,39}. This technique enables identification of haplotypes of the β^S -globin gene and is now also used for prenatal diagnosis.

Clinical features of sickle cell disease

Infants before the age of 6 months do not show any sign of disease due to the predominance of HbF. However, as soon as the HbF level starts to decrease and the intracellular HbS concentration rises, symptoms of anemia and hemolysis follow.

One of the earliest signs of intravascular sickling in children is the hand-foot syndrome (dactylitis), a painful swelling of the hands and feet due to ischaemic inflammation⁴⁰. The clinical picture of the older SS patients is dominated by hemolytic anemia and recurrent vaso-occlusive episodes or crises. However, there exists an enormous variability in the clinical symptoms between individual patients. Also, the clinical features tend to change in the same SS individual during time.

The most common clinical manifestations are the **painful crises**. They are episodes of severe pain involving vaso-occlusion of marrow and or bone microcirculation⁴¹.

There are no specific laboratory findings in vaso-occlusive- or pain crisis. Diagnosis is based on clinical symptomatology. Pain episodes occur mostly in the extremities and the backbone, are often symmetrical and usually start in one place and extend distally. Pain events are rather stereotypical for a particular patient i.e. they occur frequently in the same site(s) and many times with the same sequence. Some patients suffer repeated acute and severe pain episodes on a background of chronic pains. Patients with a higher leucocyte count suffer more frequent painful episodes. The frequency and severity of these pain crises greatly influence a patient's quality of life. Although painful crises can occur at all ages, they are particularly common during adolescence, and between the ages of 20 and 40 years. After the third decade the rate of painful episodes decreases sharply which may be the result of bone marrow fibrosis due to recurrent infarctions⁴¹.

The acute chest syndrome (ACS), comprising chest pain, cough, fever and dyspnea with abnormal clinical and radiological chest signs, may be caused by a spectrum of infection, infarction, pulmonary sequestration of sickle red cells and fat embolism. The ACS may progress to a respiratory distress syndrome which places it among the commonest causes of death in adolescent and adult SS patients. Recurrent attacks of ACS may result in pulmonary fibrosis and chronic sickle cell lung disease^{42,43}.

Renal disease in young sickle cell patients is dominated by occlusion and infarction of vasa recta in the renal medulla. Earlier studies have shown that the tubular function becomes compromised and the potential to build up a high medullary interstitial solute concentration, necessary for urinary concentration, is gradually lost resulting in hyposthenuria^{44,45}. By the age of 15 all SS patients express hyposthenuria. The medullary ischemia also stimulates synthesis of renal prostaglandins which explains the increased glomerular filtration rate and effective renal blood flow found in patients under 40 years^{46,47}. Hematuria is occasionally seen due to micro-infarction of renal pyramids²⁷. As patients grow older renal blood flow and glomerular filtration rate tend to decline due to progressive glomerular sclerosis, tubular damage, infections and renal infarction (either by local vaso-occlusion or emboli from necrotic bone marrow) in some cases leading to renal insufficiency and dialysis⁴⁸.

Heart failure may occur as a consequence of chronic hypertrophy of the myocardium in very anemic older sickle cell patients ("high output" cardiomyopathy), or due to hemochromatosis of the heart secondary to multiple transfusions. Myocardial infarction due to sickling is highly unusual which is explained by the short transit time of red blood cells across the coronary system. Sickle cell vaso-occlusion of the myocardium resulting in cardiomyopathy has

never been demonstrated²⁷.

Sequestration crises are the result of massive trapping of erythrocytes in either spleen, liver or lungs. Sequestration in spleen or liver is clinically recognised by painful swelling of the organ with a significant decrease in hemoglobin level^{27,49}. The pain involved with liver sequestration crisis may give rise to confusion since it sometimes resembles other causes of the acute (right) abdomen. The liver sequestration presents a serious condition which may progress to fulminant hepatic failure and death. The spleen is particularly sensitive to recurrent vaso-occlusion, infarction and the sequestration phenomenon, which usually take place during the first 10 years of life. The organ gradually becomes fibrotic, regresses in size and its function is lost.

Infections

Sickle cell patients have a compromised immune system due to functional asplenia or splenic involution. Consequently, SS patients have a greater propensity for bacterial infections (and septicemia) in particular by *Pneumococcus*, *Haemophilus influenza* type b, *Escheria coli* and *Salmonella* spp. Interestingly, young SS patients with co-existent α -thalassemia have been shown to maintain a normal splenic function⁵⁰.

Other factors that add to the immunocompromised state of the SS patient are defects in complement system, granulocyte and B cell function. The cellular immunity may be diminished due to iron overloading by frequent transfusions^{27,49}.

Aplastic crises, caused by the destruction of erythrocyte precursors by human parvovirus infection may also complicate sickle cell disease. These attacks are characterized by a sudden decrease in hemoglobin (which falls by about 1 g/dl per day) with few or no reticulocytes^{27,49}. As long as adequate oxygen delivery to tissues is maintained by transfusions the outcome is good. The failure of erythropoiesis is self-limiting and bone marrow recovery usually starts after 7-10 days of aplasia.

Hyper-hemolytic crises are also recognised but many, in particular in younger individuals, may be due to splenic sequestration crises. Other hyper-hemolytic episodes usually accompany infections. Due to chronic hemolysis SS patients often reveal jaundice and many of them develop gallstones at a relatively young age⁴⁹.

Priapism resulting from blockage of the corpus cavernosum by sickle cells preventing the necessary outflow of blood from the penis, may be a particularly difficult problem. Many male patients when specifically questioned admit experiencing brief episodes of painful erections (stuttering attacks) which resolve

spontaneously. Major attacks of sustained erection for more than 24 hours almost invariably result in loss of penile erectile function²⁷.

Bone complications. Aseptic bone necrosis, especially of the femur but also of the humerus head, occurs in up to 30% of SS patients and occasionally leads to invalidity. Osteomyelitis is known to result from secondary infection of bone infarcts or avascular necrosis. Until recently, *Salmonella* has been considered to be the main causative organism, but, new evidence now shows a preponderance of *Klebsiella* and *Staphylococcus Aureus*^{27,49,51}.

Leg ulcers are common to SS individuals between 15 and 50 years and are almost always located at the medial side of the ankle. The leg ulcers may or may not occur in relation to trauma and are the consequence of skin infarction, poor woundhealing and poor granulation. They are seen more frequent in males. Individuals who experienced a leg ulcer are known to have a much greater risk for a new leg ulcer⁵².

Eye manifestations. Ischemia due to vaso-occlusion may damage several tissues of the eye. In general the most common serious ocular complications arise from retinal small vessel and capillary occlusion and neovascularization⁵³.

The central nervous system (CNS). Vaso-occlusive complications in the CNS system may vary from transient features such as speech, visual or hearing disturbances to permanent hemiplegia and death from stroke. Stroke occurs mostly in children and, similar to the leg ulcer, tends to re-occur in the same individual²⁷. Recurrence may be prevented by regular bloodtransfusions⁵⁴.

Multiorgan failure, which may complicate a period of recurrent painful episodes, presents a desperate clinical situation. It is probably caused by widespread fat embolization of extensive infarction and necrosis of bone marrow⁵⁵.

Pregnancy. During pregnancy, progesteron induced smooth muscle relaxation with slowing down of blood flow and increased levels of coagulation factors (in particular fibrinogen), provoke sickling and result in an increase in vaso-occlusive complications. Delivery presents extra risks due to stress, haemoconcentration, acidemia, hypotension (blood-loss) and hypoxemia⁵⁶.

Mortality. Average life expectancy of male sickle cell patients is 42 years, while females reach the average age of 48 years. Mortality correlates strongly to the number of painful episodes per year. This fact is also corroborated by the observation that adult SS patients in developed countries frequently die under

circumstances of hospitalization for severe and recurrent painful crises⁵⁷.

Univariate analysis of laboratory parameters has revealed that early mortality correlates directly with leucocyte and alkaline phosphatase levels and inversely with total hemoglobin and fetal hemoglobin (HbF) levels. Especially high HbF levels are associated with superior life expectancy^{57,58}.

Determinants of clinical severity of sickle cell disease

Among patients, disease expression varies enormously and varies in the same individual from time to time. As clearly shown in a large cohort study in the United States, the clinical course is highly influenced by specific organ damage sustained by SS patients during the years⁵⁹. The differences in clinical disease result from a great variety of genetic, vascular, plasma, red blood cell and environmental properties (Table I.2).

Table I.2. Factors that may contribute to sickle cell vaso-occlusion

HbS polymer
Sickle cell deformability
Sickle blood viscosity
Fraction of dense, irreversibly sickled cells
Sickle cell endothelial cell adhesion
Hemostatic activation
Platelets
White blood cells and cytokines
Vascular condition (anatomy, blood flow, endothelial condition; vaso active peptides)
The nervous system responses and environmental influence

Genetic factors

In addition to the crucial β^S mutation itself, other genetic variables or epistatic effects) such as regulators of the HbF level, total hemoglobin level and concomitant α -thalassemia influence the level of disease severity.

- *HbF modulating factors*. Elevated HbF is associated with a milder disease manifestation due to the formation of a soluble hybrid with HbS ($\alpha_2\gamma\beta^S$) which inhibits polymerization. At the molecular level this effect is explained by the presence of a glutamine residue at $\gamma 87$ position which prevents a critical lateral contact in the double strand of the sickle fiber⁶⁰.

Among others, specific mutations at the level of the promotor regions of the γ genes have been found to explain the differences in HbF levels and disease severity

between different haplotypes⁶¹⁻⁶³. Patients with the Asian and Senegal haplotype carry high HbF (15-30%) which is known to contribute to a less severe clinical outcome. Those with the Benin and Bantu type have lower HbF levels and reflect a more severe disease course⁶⁴⁻⁶⁹. Mutations described within the Locus Control Region of the β^S chromosome also influence HbF levels and thereby may alter the phenotypical expression of disease^{63,70}. Recently, the X-linked F-Cell Production (FCP) locus has been discovered⁷¹, which appears to be an even more important determinant of the level of HbF.

- *Hemoglobin level and whole blood cells.* The average hemoglobin level and packed cell volume of an individual patient being the result of several factors, is also known to influence the clinical disease state. High total hemoglobin raises viscosity of blood and is a risk factor for painful crises⁷², chest syndrome, hip necrosis and proliferative retinopathy⁷³. An elevated leucocyte count appears to be a risk factor for stroke⁷⁴, chest syndrome⁷⁵ and death in SS patients^{58,59}.

- *Concomitant α -thalassemia.* The combination of α -thalassemia (caused by deletion of either one or two genes) and the HbS homozygote produces microcytosis and a lower intra-erythrocytic concentration of HbS (lower MCHC values). Alpha thalassemia is also accompanied by increase in HbA₂ which is probably caused by greater affinity of the limited α chains for δ chains rather than for β^S chains. The lower MCHC and higher HbA₂ reduce the chance of polymer formation and intravascular sickling. The hemolytic rate is reduced and deformability of red cells is improved. The acute chest syndrome and leg ulceration are less common in SS - α -thal. There is no reduction in frequency of painful crises^{76,77}.

Vascular and plasma factors

Young deformable sickle red cells adhere to vascular endothelial cells much more than irreversibly sickled cells (ISC). Ballas et al.⁷⁸ have shown that there exists a significant positive correlation between red blood cell deformability and the frequency and severity of painful crisis. Deformable sickle cells may cause direct damage to endothelial cells and cause narrowing of the blood vessel lumen with consequent sludging or trapping of ISC and rigid dense cells resulting in vaso-occlusion. The interaction with endothelium also causes alterations in the levels of vaso-active peptides and other mediators^{79,80}. Exposure of human endothelial cells in culture to sickled erythrocytes resulted in a significant transcriptional induction of the gene encoding the vasoconstrictor endothelin-1⁸¹. Simultaneously, a suppression of transcripts, encoding the protein responsible for nitric oxide synthesis, was documented in the endothelial cells. Normal or unsickled sickle cells exposed to the same conditions had no effect on endothelin gene induction.

Imbalances in substances like nitric oxide, prostacyclins, platelet derived growth factor, endothelin and free oxygen radicals, all interacting with endothelium or with its direct environment, can cause local vasospasm or failure of vasodilatory mechanisms and contribute to sickle cell crises and organ damage⁸². On the other hand, the local microvascular complications may cause adaptive mechanisms to take effect. One such possibly adaptive mechanism, the periodic capillary flow, has been revealed by the use of Laser -Doppler velocimetry⁸³. In studies using this technique it has been observed that the microcirculatory flow in SS patients increases periodically. This results in increased perfusion pressure, which may help to restore the flow in plugged capillaries.

Sickle cells as well as endothelial cells both express surface molecules that mediate intercellular adhesion (Figure I.2). Studies indicate that the CD 36 and the group of very late activation(VLA-4) receptors, also referred to as the $\alpha 4 \beta 1$ integrin receptors of the erythrocyte membrane, are specifically involved in endothelial adhesion^{84,85}. On the endothelial side the integrin $\alpha v \beta 3$ and vascular adhesion molecule-1 appear to play a specific role in the interaction with red blood cells. Immature reticulocytes show the greatest expression of adhesion receptors and this decreases as the cells become more mature^{86,87}. Several cytokines (i.e. TNF- α , IL-1 and IL-6), when induced by external factors such as microorganisms or toxins may enhance expression of endothelial adhesion receptors, activate leucocytes (i.e. IL-8), the complement system and coagulation, and stimulate production of acute phase proteins⁸⁸⁻⁹⁰. Certain plasma proteins (so-called ligand proteins) may augment sickle cell-endothelial adhesion by acting as bridging molecules between the abovementioned receptors. Among these proteins are fibrinogen, thrombospondin, von Willebrand factor multimers and fibronectin⁹¹⁻⁹⁴. In addition, thrombospondin also binds to sulphated glycans on SS red cells⁹⁵. Fibronectin (FN) is a large glycoprotein dimer which may be found in plasma as well as in the interstitium. It's main source of production are the hepatocytes (for the plasma FN), fibroblasts, macrophages and endothelial cells (for the cellular FN). Fibronectin contains multiple binding sites for cell membrane integrins, fibrin, collagen and heparin⁹⁶.

Red blood cell properties

In addition to the abovementioned factors, the increased red blood cell turnover and hematopoietic stress cause changes in the circulating erythroid progenitors (BFU-E). Their number increases, they react more sensitive to cytokines and their capacity to proliferate increases⁹⁷.

An effect which does not appear to be specifically related to young erythrocytes, is the enhanced oxidative stress within red blood cells, which probably results from an increase in methemoglobin formation and reduction. The oxidative stress damages cytoskeletal proteins which causes cell rigidity and alters transport proteins²⁷.

Figure 1.2 Vascular factors and cellular mechanisms involved in adhesion of blood cells to endothelium and vaso-occlusion. * Receptor up-regulation by cytokines.

It has also been shown that abnormalities in composition and function of phospholipids (sulfated glycans) in the membrane of the sickle cell serve as a target for macrophage recognition, may cause complement activation or express procoagulant activity (binding of thrombospondin) and could therefore influence the vaso-occlusive processes^{95,98,99}.

Patients with associated red blood cell G-6-PD deficiency may be more at risk for repeated hemolytic crisis¹⁰⁰.

Red blood cell volume alterations, as in SS - α -thal or acquired because of a concomitant vitamin deficiency or iron loss, as well as by changes in hydration status¹⁰¹, also modulate disease severity.

Leucocytes

Since leucocyte numbers correlate well with early mortality in sickle cell disease and the severity of sickle cell crisis⁵⁸, it is conceivable that abnormal leucocyte-endothelial cell adhesion plays a role in the pathogenesis of the vaso-occlusive crisis. Indeed, during sickle cell crisis increased adhesive properties of leucocytes have been observed in addition to the commonly noted leucocytosis¹⁰²⁻¹⁰⁴. Also, Hofstra et al.¹⁰⁵ have shown that polymorphonuclear leucocytes may be activated as a result of specific sickle RBC binding via an IgG-mediated process. Leucocytes could initiate a vaso-occlusive crisis in two ways (Figure 1.2): Either mechanically block the capillary system, especially when the vascular bed is already constricted or partially obstructed by adherent red cells, or adhere to venous or capillary endothelium through specific leucocyte-endothelial ligand -receptor interactions (i.e. fibronectin)^{96,103,104,106}. With respect to the latter mechanism, it has also been shown that hypoxia promotes white blood cell adhesion to endothelium, prior to any ischemic tissue damage¹⁰⁷. The adhered and/or obstructing leucocytes inhibit blood flow and oxygen delivery to the tissues and may release cytokines which activate many other cells giving way to massive intravascular sickling and ischemic tissue damage. Furthermore, activated leucocytes could traverse the endothelial border, releasing inflammatory mediators and oxygen radicals in the interstitium, causing significant deterioration of tissue inflammation and ischemic damage¹⁰⁸.

Platelets

Thrombocytosis is common to sickle cell crisis, but is also seen in steady state. The thrombocytosis has been attributed to the progressive splenic infarction¹⁰⁹. In addition to thrombocytosis a marked increase in circulating platelet aggregates and platelet factors (such as thrombospondin, vWF and β -thromboglobulin) have been observed in the steady state^{92,94,110}. Platelets, being thus readily activated, may enhance sickle red cell binding to endothelium (Figure 1.2). Although hypothetically individual differences in platelet activity could result in differences in severity of disease, this remains to be proven clinically.

Environmental factors

There are various environmental factors that have their influence on the severity of disease. They are not only responsible for differences in disease expression

between individuals, but also may modulate disease in the same individual. One of the most important environmental factors that may induce painful crises is cold temperature as shown by a study performed in Jamaica⁴¹. Among the other factors are malaria (in particular in many tropical areas), proper health care, nutrition, intake of folic acid and the iron levels. Nutrition promotes growth and strengthens the immune system. Folic acid protects against megaloblastic changes¹¹¹. A low iron level inhibits HbS polymerization by lowering the mean cell hemoglobin concentration (MCHC) and may actually ameliorate some aspects of sickle cell disease¹¹².

Sickle cell trait

In comparison to the homozygous SS patients, the red blood cells of the heterozygous AS individuals carry approximately 45% HbS. The AS individual usually does not exhibit sickling, is asymptomatic and has a normal life expectancy. However, under extreme conditions i.e. a combination of hypoxemia, hypotension acidosis and slowing down of blood flow, sickling may occur. This is particularly true for the renal medulla where the sluggish blood flow and hypertonic environment promote sickling in the vasa recta. Therefore AS individuals may develop renal abnormalities during the years. Also, occasionally splenic infarction has been observed at high altitudes^{27,113}. Sudden death occurs more frequently in persons with sickle cell trait and has particularly been described in military recruits exposed to heavy exertion and heat stress¹¹⁴. Sickle cell trait confers protection for malaria. Individuals with the sickle trait show reduced mortality from *Plasmodium Falciparum* infection probably due to early elimination of infected sickle cells from the bloodstream⁸².

Treatment

Therapy for acute crises

Painful crises are usually treated empirically with hydration and analgesics. Due to the extreme character of the pain morphin is often administered. Oxygen is frequently administered, but not routinely indicated except in the setting of hypoxemia or cardiopulmonary disease. Simple transfusion is commonly used to treat life-threatening situations although good evidence for its use is difficult to obtain. Retrospective analysis and uncontrolled data indicate that in multiorgan failure as well as stroke and chest syndrome in children, transfusions to low levels of HbS (approximately 30%) are associated with increased chance of

survival^{55,115-119}. Some studies have shown a beneficial effect of transfusion in lessening the severity of vaso-occlusive crises^{115,116}. A study comparing conservative versus aggressive preoperative transfusion showed no differences in outcome except for an increase in transfusion related adverse events in the group of aggressively transfused patients¹²⁰. The Hb levels aimed at with transfusion may be too high since a Hb level in excess of 8 g/dl may result in hemodynamic and rheological deterioration¹²¹.

Red blood cell exchange transfusion offers advantages over simple transfusion such as eliminating the risk of volume overload and increase in viscosity and reducing the chance of iron overload. It's major role is in the management and /or prevention of life or organ-threatening events. Based on information from numerous anecdotal reports in the past decade it is now recommended for deteriorating chest syndrome, acute or impending vascular episodes, hepatic or multisystem failure and retinal arteriolar vaso-occlusion¹¹⁸.

The most significant reduction of the mortality rate in sickle cell anaemia over the past decades has been achieved due to the advent of antibiotic treatment. Treatment of vaso-occlusive episodes that may involve infection should include early application of antibiotics.

Therapies directed at prevention

Several drugs have been tried over the years in an attempt to reduce the rate of vaso-occlusive episodes in SS patients. Based on the beneficial effects of HbF on HbS polymerization agents such as hydroxyurea, butyrate analogues and erythropoietin have been administered. The effects of these agents as well as the results of hydroxyurea therapy and isobutyramide are discussed in detail in the next chapters of this thesis.

- **Drugs with antisickling effect.** Antimycotic agents, such as clotrimazole, have demonstrated the ability to inhibit sickling in mice, by lowering corpuscular Hb through inhibition of calcium activated loss of K^+ through the Gardos channel in the erythrocyte membrane¹²². Oxygen affinity increasing products are also under investigation¹²³. Combinations of the latter agents are expected to be studied clinically in the near future¹²⁴. During the past two decades many other agents that were claimed to be of benefit in the treatment of sickle cell anemia, either due to an antisickling effect or by promoting capillary flow, were studied (pentoxifylline, cetiedil, piracetam, dicoumarol, aspirin, calcium antagonists and defibrinating agents). The beneficial effects of most of these agents were not confirmed in controlled trials¹²⁵⁻¹³⁴. For some, the data from ongoing controlled trials are not yet available.

- **Allogenic bone marrow transplantation** in sickle cell disease is feasible and may be curative in young patients with severely symptomatic sickle cell disease¹³⁵⁻¹³⁹. However, some patients reject the marrow allograft and the sickle cell disease reoccurs. Mortality of the procedure still carries a risk of 10% as well as a risk of infertility. Furthermore, data on the induction of malignancies by high dose chemotherapy, involved in ablation of bone marrow before transplantation, are not yet available¹⁴⁰.
- **Gene therapy.** Addition of a normal β gene into the genome of the haemopoietic stem cell of the SS patient is considered by many as the golden standard of treatment. Researchers have been able to transfer the human β -globin gene with the LCR region to erythroid cells, using recombinant adeno-associated virus as a vector¹⁴¹. The newly transduced cells showed an accurate regulation of human globin expression. Although these techniques have not reached beyond the level of the laboratory, they may become feasible in clinical practice in the near future.
- **Intervention in blood cell-endothelium interactions.** The growing insight into pathophysiologic mechanisms of adhesion of different blood cells to endothelium may offer new possibilities to directly reduce or prevent vaso-occlusive events^{142,143}.

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Chapter II

Sickle cell disease in Curaçao

AW Saleh

Introduction

Sickle cell anemia in Curaçao was first described in 1943 by van der Sar¹. He observed the case of a pregnant woman with this disease. Over the past decades, many studies have provided data on morbidity and mortality of local sickle cell anemia as well as data on pathophysiological aspects of the disease. In the fifties and sixties, research produced valuable epidemiological data and investigations on the properties of the abnormal hemoglobin¹⁻⁶. Studies in the seventies and eighties offered new insights into the pathophysiology of sickle cell nephropathy⁷⁻¹⁹ as well as information on calcium, lipids, fatty acids and trace elements in sickle cell anemia²⁰⁻²³. In the past ten years, several studies have been published on clinical aspects of the disease in Curaçao, such as ocular findings²⁴ and the acute chest syndrome²⁵; new data on the incidence of hemoglobinopathies have been reported^{26,27} and research has focused on the pathophysiology of adhesive mechanisms, which seem to play an important role in sickle cell vaso-occlusion²⁸⁻³². Moreover, several therapies have been tried in an attempt to improve the symptoms of the disease³³⁻³⁷.

Epidemiological aspects

SS and SC disease

The population of Curaçao, like many other islands in the Caribbean, is predominantly black, originating mainly from the west coast of Africa. The number of patients with sickle cell disease (SS + SC) in Curaçao has been estimated through studies by van der Sar², Jonxis³, van Zanen⁴, and more recently by Muskiet and van der Dijs^{26,27}. In the latter study, during three months, 67.2% of all (748) newborns were screened for their hemoglobin pattern. The authors report a β^S gene frequency of 0.0258 and a β^C gene frequency of 0.0347. The Hb AS incidence was found to be 4.6%. Considering an island population of 145.000 and applying the appropriate equations ($[\beta^S \text{ gene frequency}]^2 \times \text{population no.}$) for SS patients and ($\beta^S \text{ gene frequency} \times \beta^C \text{ gene frequency} \times 2 \times \text{population no.}$) for SC patients, an estimate prevalence of 100 SS patients and 260 SC patients may be reached. The actual number of SS patients will be lower due to the shorter life span of these patients. Compared to the most common Hb S regions in Africa where the proportion of Hb S carriers is over 15%, the percentage of Hb S carriers in Curaçao is relatively low. The discrepancy may be explained by 2 reasons:

1. Racial admixture. This was first brought about by mingling of European plantation owners with black female slaves. Thereafter, at the beginning of the 20 th century, immigrants of other nations were attracted to the island by the oil refinery and also mixed to some degree with the black population. One could speculate on a further decrease of the local Hb S carriers in view of the recent

increase in immigration of black individuals to the Netherlands.

2. The absence of malaria in Curaçao.

It has been shown that, in areas endemic for malaria, the frequency of sickle cell carriers (trait) is higher³⁸. Their survival rate is increased compared to healthy individuals because they develop malaria less often and less severe. Consequently, in the absence of malaria, the sickle cell trait will not be positively selected and its frequency will be lower.

The relative high prevalence of SC disease is explained by the predominant descent of the population from West Central Africa (Ghana/Gold Coast) where a relative high frequency of Hb C is found². In general, data from the black population of other islands in the Caribbean are rather similar to that from Curaçao, although some areas in Jamaica and Surinam show proportions for Hb S carriers similar to Africa due to the presence of malaria. Moreover, the SC frequency in the region is somewhat lower which may be explained by the fact that the ancestry came from different parts of Africa^{2,3,39}.

Sickle cell -thalassemia disease

With respect to the presence of thalassemia van Zanen⁴ was the first to document so-called non-classical thalassemia among Hb S carriers in Curaçao. This form of expression of thalassemia was later ascribed to α -thal-2 which is typical for the African population. In their study Muskiet and van der Dijs^{26,27} estimate that 4,1 % had either Hb S or Hb C with 8,1

α -thalassemia-2. These findings are in general agreement with data from the Afro-American population in the region and the United States with the exception of some population groups in Jamaica and Surinam that carry the α -thal-1 type deletion which is typical of their Asiatic background.

Data from African areas with a high β^S gene frequency reveal a low prevalence of β -thalassemia⁴⁰. Similarly, only small numbers of patients with sickle cell - β -thal have been reported among Jamaicans of West African origin⁴¹. So far no attempt has been made to detect compound heterozygotes for HbS and β -thalassemia in Curaçao.

A few other hemoglobin variants and compound Hb S heterozygotes have been incidentally documented but a discussion of these is not within the scope of this thesis.

Age and sex distribution

Before the sixties the average life-span of SS patients was reported to be 20 years. The average age of the SC patient was longer and approached that of normal individuals which lead to the conclusion that SC disease was much more benign than SS disease². During the recent years no new local data have been acquired. However, the Cooperative Study of Sickle Cell Disease in the USA has estimated

that 85% of SS patients now live beyond the age of 20 years compared with 50% two decades ago⁴². This most likely holds true for Curaçao since compared to the USA, equivalent advances in health care (antibiotics, education, improved supportive measures) have been established during the past two decades. Data on sex distribution in sickle cell disease in Curaçao are very limited. The frequency of the male sickle cell trait has been shown to be consistently higher than that of the female when looking at their local distribution as well as in the region or Surinam^{2,4}. Recent data from Africa indicate that these differences occur mainly during teenage years⁴³. The explanation for this discrepancy remains unclear.

β^S -Haplotype distribution in Curaçao

Prior to the studies performed in this thesis no information was available on the local β^S haplotype distribution. Of the 18 local SS patients described herein, thirteen had their β^S haplotype and α -gene number determined (Table II.1). All patients were SS individuals registered at the outpatient clinic for internal medicine. The β^S haplotypes were determined by Dot-Blot DNA hybridization analysis⁴⁴ and the α -globin genotypes were analysed by screening for the - α (3.7 kb) deletion using a modified polymerase chain reaction method⁴⁵. Fourteen patients revealed the Benin haplotype or type 19/19. Two carried hybrid haplotypes (Benin/Bantu or 19/20 and Benin/Senegal or 19/3). One patient had a combination of sickle cell anemia with α -thalassemia. She revealed a relatively low MCV and MCHC which explain the less severe hemolysis typical of co-existent α -thal. Another patient was a carrier of the α -thalassemia trait ($-\alpha/\alpha\alpha$). Because of her relatively high Hb F level this patient was also assumed to have some type of deletional hereditary persistence of fetal hemoglobin (HPFH). The finding that the majority are of the Benin haplotype is consistent with historical facts. In the 17th and 18th century Curaçao was a center through which slaves from Africa were distributed over neighbouring countries. Most slaves in Curaçao originated from Ghana, the Gold Coast and neighbouring countries which regions are typical for the Benin haplotype (Figure II.1). Accordingly, it has also been shown that the most common haplotype in Surinam, another former Dutch colony in the region, is the Benin haplotype⁴⁶.

Clinical SS disease in Curaçao

Tables II.1 to II.3 summarize the clinical picture as well as the laboratory data of 18 SS patients during a period of ten years prior to the studies performed in this thesis. Clinical data were collected by studying the patient's records at the polyclinic for internal medicine and at the medical archive of the St. Elisabeth Hospital. Patients were also requested in an interview to give a summary of their complaints and complications during the past 10 years. The specific data that were

looked at included the number of painful crises, pulmonary and renal complications, priapism, bone complications, leg ulceration, eye problems, hemochromatosis, and complications related to pregnancy. All patients showed signs of hemolytic anemia with hemoglobin levels varying from 4.5 g% to 9.6 g% with increased LDH. Overall, individuals with high fetal hemoglobin experienced a less complicated clinical course.

Figure II.1 Distribution of β^S haplotypes in Africa and the Mediterranean area, and the origin of the 19/19 haplotype (Benin type) in Curaçao (adapted from ref. 20).

Table II.1 Hematological data and Haplotypes from the sickle cell (SS) population screened prior to trial accrual.

Patient	Sex-Age	Hb g/dl	PCV l/l	RBC 10 ¹² /l	MCV fl	MCH pg	MCHC g/dl	HbF %	No. α Genes	β^s Haplotypes	Serum Creatinine mg/dl	LDH u/l	Serum Fe mg/dl	Fe Saturation %
1 JZ	M-42	6.1	0.197	2.00	99	30.5	31.0	*	$\alpha\alpha/\alpha\alpha$	19/19	1.8	1105	123	79
2 RF	M-33	6.2	0.215	2.06	104	30.1	28.8	2.7	$\alpha\alpha/\alpha\alpha$	19/20	0.7	748	56	18
3 RM	M-35	9.6	0.307	3.36	91	28.6	31.3	10.2	$\alpha\alpha/\alpha\alpha$	19/19	0.8	590	55	18
4 SH	F-24	8.0	0.253	2.50	101	32.0	31.6	6.9	$\alpha\alpha/\alpha\alpha$	19/19	0.5	620	129	49
5 AH	M-18	8.0	0.252	2.67	94	30.0	31.7	7.3	$\alpha\alpha/\alpha\alpha$	19/19	0.7	579	**	**
6 IR	F-30	6.8	0.205	2.19	94	31.1	33.2	3.2	$\alpha\alpha/\alpha\alpha$	19/19	0.6	1030	101	38
7 LF	F-46	7.4	0.240	2.87	84	25.8	30.8	13.5	α/α	19/19	0.6	478	65	21
8 EP	M-58	5.4	0.169	1.54	110	35.1	32.0	7.3	$\alpha\alpha/\alpha\alpha$	19/19	1.0	1112	68	22
9 EM	F-62	9.0	0.309	3.56	87	27.8	32.0	20.8	$\alpha/\alpha\alpha$	19/19	0.6	461	179	86
10 MM	F-37	9.2	0.284	2.81	101	32.7	32.4	12.0	$\alpha\alpha/\alpha\alpha$	19/19	0.7	632	113	38
11 MA	F-48	7.8	0.227	2.18	104	35.8	34.4	14.4	$\alpha\alpha/\alpha\alpha$	19/3	0.7	603	162	78
12 GH	F-41	6.3	0.167	1.67	100	37.7	37.6	2.7	$\alpha\alpha/\alpha\alpha$	19/19	0.7	1198	125	40
13 GL	F-30	5.2	0.134	1.45	93	36.0	39.0	4.8	$\alpha\alpha/\alpha\alpha$	19/19	0.8	1930	86	35
14 SW	M-30	8.7	0.248	2.98	83	28.0	34.0	4.6	ND	ND	0.8	1288	ND	ND
15 EV	M-31	6.6	0.193	2.11	92	31.3	34.1	7.3	$\alpha\alpha/\alpha\alpha$	19/19	1.0	2324	ND	ND
16 MV	F-41	4.5	0.120	1.22	100	37.0	37.0	NA	ND	19/19	1.5	1616	128	74
17 RC	F-36	6.8	0.198	2.34	85	29.0	34.0	4.0	ND	ND	0.5	ND	64	33
18 JC	M-17	9.3	0.266	2.90	92	32.0	35.0	7.5	ND	19/19	0.6	788	70	25

Ref. values: serum Creatinine 0.5-1.1 g/dl, serum Fe 35-140 μ g/dl, LDH 200-550 u/l, Fe saturation 20-40%.* Patient was transfused one month prior to screening; ** no Fe level available, Ferritin 78 ng/ml ($n = 15-280$ ng/ml).
ND not determined

Table II.2 Incidence* of the most common complications per SS patient, based on a retrospective study of 10 years.

Patient	Sex-Age	Painful Crisis	Leg Ulcers	Acute Chest Syndrome	Aplastic Crisis	Osseous Complications	Priapism	Liver Sequestration Crisis	Hematuria	Stroke
1	M-42	4	1	1	1			1		
2	M-33	10	4	1						
3	M-35	12	2							
4	F-24	8								
5	M-18	14					1			
6	F-30	18		1						
7	F-46	2								
8	M-58						1			
9	F-62		6			1				
10	F-37	2								
11	F-48	0								
12	F-41	2			1					
13	F-30	1	6		2					
14	M-30	10		1		2				
15	M-31	8		2			1		1	1
16	F-41	4	3	1	2					
17	F-36	10		1	1					
18	M-18	4								
Total No.		109	22	8	7	4	2	1	1	1

* Number of events per 10 years. Blank spaces represent zero events.

Table II.3 Summary of clinical complications resulting in permanent organ damage based on a 10 year retrospective study of 18 SS patients varying in age from 19 to 64 years.

Clinical complications		Mechanism	No. of patients
Renal damage	Hyposthenuria	Obliteration of vasa recta	18
	Reduced Glomerular filtration rate (patients over the age of 40 yrs)	Glomerular sclerosis, tubular damage, infection or renal infarction	3
Splenic involution	Propensity for bacterial infections	Chronic intrasinusoidal sickling, infarction and fibrosis	17*
Jaundice	Elevated LDH and bilirubin	Chronic hemolysis	18
Gallstones	Asymptomatic or right abdominal pain, small bilirubin stones	Enhanced excretion of bilirubin	11"
Hemochromatosis	Liver or endocrine dysfunction, cardiac failure	Excessive tissue iron accumulation due to transfusions or increased iron intake	4
Death	Multiple organ failure	Extensive renal/myocardial damage	1

* All patients, except for one assumed to have normal splenic function due to the presence of α -thalassaemia;

" 8 cholecystectomies

Painful crises

Pain episodes, at times difficult to quantify taking into consideration the different factors that play a role in pain expression of patients, were recorded when patients had to be admitted to the hospital or when failure of non-opiate analgesics was apparent. The painful crisis was the most common clinical manifestation (Table II.2) occurring in particular during the second and third decade of life which is in agreement with international data.

Acute chest syndrome (ACS)

Eight episodes of ACS were counted in 7 individuals during the 10 years of retrospective survey (Table II.2). None of these patients died as a consequence of ACS. The survey done by van Agtmael et al in Curaçao²⁵ revealed 81 episodes of acute chest syndrome in 53 patients over a 12 year period with a case fatality rate of 6%, making the chest syndrome the most common cause of death in sickle cell patients in Curaçao.

Hemochromatosis

Based on clinical findings, serum iron and ferritin levels, 4 of the 18 SS patients screened were suspected to have some degree of hemochromatosis (Table II.1). Three patients (Patient J.Z., E.M. and M.V.) received many transfusions for low hemoglobin and related complaints during the past years; one only received 14 transfusions but erroneously used oral iron supplements for a long time.

Priapism

Only two of the patients studied in this thesis had a serious episode of priapism (Table II.2). The patients were not questioned on the incidence of minor attacks.

Bone complications

One patient suffered some deformation of the medial femur condyl due to bone necrosis which eventually needed a correction by an orthopedic surgical procedure. Osteomyelitis is known to result from secondary infection of bone infarcts or avascular necrosis, as was the case for 2 of our SS patients. One of these patients even experienced two episodes of osteomyelitis.

Leg ulceration

During the 10 years of retrospective survey of the SS individuals leg ulceration was common. The fact that leg ulcers are known to reoccur in the same individuals was confirmed by the finding of 22 events of leg ulceration in 6 SS patients (Table II.2).

Ocular abnormalities

None of the patients were specifically screened for eye complications, but there were no complains related to their eyesight except for a limited and temporary visual perturbation in one patient experiencing a light stroke. Ocular abnormalities in sickle cell disease on Curaçao were described and reviewed by Van Meurs in 1990²⁴. He confirmed the general finding that proliferative retinopathy is less common in SS than in SC individuals and reported an incidence of severe visual loss of 2% in SS patients.

Renal complications

All patients showed hyposthenuria, defined as a specific density equal to or less than 1,010 g/ml in an early morning urine portion. This was likely since the patients were all older than 15 years (the time at which concentrating capacity in SS patients is significantly impeded due to destruction of vasa recta in the kidneys).

Pregnancy

None of the pregnancies (total of 3) were complicated, only the newborns showed a

low birth weight. No abortions were recorded.

Mortality

No data are readily available on life expectancy and mortality rate. As mentioned above the acute chest syndrome is the major cause of death among adult sickle cell patients in Curaçao. None of the 18 patients screened died prior to start of the studies in this thesis. One patient (JZ), who did not meet the selection criteria for the studies, died a year later at age 43. Frequent painful crises combined with deterioration of renal function and cardiac failure resulting from high output cardiomyopathy as well as possible hemochromatosis of myocardium (due to many transfusions) attributed to the cause of his death.

Local environmental influence

In Curaçao painful crises are not likely to occur in relation to temperature changes since the climate is stable, dry and warm. However, it is the general impression that crises do happen more frequent during rainy seasons when flu episodes are common. Malaria, known to have a significant impact on the clinical course of sickle cell disease in the African continent, is not present in Curaçao and therefore does not constitute a threat to the local sickle cell population.

Due to the dry climate local agriculture is not pursued on a large scale. Therefore, adequate nutrition on the island depends partly on imported food products which can be relatively expensive. Even so adult sickle cell patients in Curaçao do not normally present with major nutritional deficiencies. In addition, because of chronic hemolysis, most patients are routinely prescribed a supplement of folic acid.

Psychosocial aspects

Although social class is known for its influence on growth and physical development in a normal population this is not clearly the case for SS patients⁴⁷. It is known that specific factors linked to social class may play a role in psychological development. However, in Curaçao most pediatric patients irrespective of their social class perform rather well in school (FAJ Muskiet, personal communication). With respect to the job, most adult SS patients without a permanent handicap, perform reasonably well as long as the work does not involve an especially high physical activity.

Management

Sickle cell patients in Curaçao receive medical care on an outpatient basis in several polyclinics and most patients with acute illness are admitted to the Sint Elisabeth Hospital, a 700 bed facility.

Although recently a multicenter study in the United States has shown hydroxyurea to be of some value in the prevention of painful crises⁴⁸, management of adult sickle cell patients is still mainly supportive. Medical care of the SS patient in Curaçao abides by international guidelines consisting of measures such as proper hygiene and counseling, early antibiotics in case of suspected infection and adequate analgesia for painful episodes. Local pediatricians do practice preventive medicine by routinely administering vaccines (Pneumovax and HIB) to their pediatric patients as well as by giving antibiotic prophylaxis to the patients with frequent infections.

If deemed necessary patients are admitted to the hospital for rehydration, administration of oxygen and other more specific treatments depending on the type of complication. In the event of extreme anemia or aplastic crisis, transfusions are offered. When life threatening complications occur, exchange transfusions are commonly performed.

Conclusions

The majority of SS patients screened for their β^S haplotype in this thesis appear to carry the Benin (19/19) type. This type is known in general for a rather severe clinical course. Today, despite the fact that local medical care is up to international standards, several important data on the disease are still not being accurately registered; possibly, due to a lack of a sickle cell clinic where care is centralized. Most importantly, the financial costs involved in the current management of sickle cell disease are not known. They need to be disclosed in order to determine the burden of sickle cell disease on local health care and to be able to select ways to improve care of the sickle cell patient and to reduce expenses. Several means, such as routine screening of newborns, antenatal diagnosis and new drug therapies, could be pursued to reduce the incidence as well as the financial costs and human suffering that accompany sickle cell disease. Early diagnosis has been shown to produce better chances of survival for individuals inflicted with sickle cell disease⁴⁹ and antenatal diagnosis by chorionic villus sampling, utilizing DNA amplification techniques and probes that identify the specific nucleotide change of sickle cell anemia during the early months of pregnancy, offers parents the possibility to avoid sickle cell disease within their offspring. However, these procedures should not be established without first determining their local feasibility and effectiveness through a careful cost/benefit analysis against a background of the general local health care.

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Chapter III

Pharmacological induction of fetal hemoglobin synthesis in sickle cell disease

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Summary

In the present review the role and regulation of fetal hemoglobin (HbF) in sickle cell disease as well as the pharmacological modulation of HbF are discussed. Enhanced levels of HbF have an inhibitory effect on sickling and ameliorate the clinical course of sickle cell disease. This knowledge has incited a search for pharmacological agents capable of stimulating HbF production. Cytostatic drugs, in particular hydroxyurea, but also other agents such as erythropoietin, butyrate and its analogues have been shown to induce HbF production. The mechanisms of action and the clinical effects of these agents are summarized. Only hydroxyurea has shown significant clinical effects in terms of reduction of paincrises, chest syndrome and transfusions in sickle cell patients. However, not all patients experience clinical amelioration and it appears difficult to predict which patient will gain from hydroxyurea therapy. Further studies need to expand the clinical experience with hydroxyurea, other HbF inducing agents and combinations of these drugs. Moreover, they will hopefully provide criteria to enable appropriate selection of those sickle cell patients that will benefit clinically from HbF stimulation, also taking into account the risks and costs of long-term pharmacological therapy.

Introduction

Sickle cell anemia is a hereditary disorder characterized by an abnormality in the hemoglobin molecule. The molecular abnormality is caused by substitution of valine for glutamic acid, at the sixth residue of the β -chain. The sickle hemoglobin (HbS) when in deoxygenated state results in formation of molecular aggregates (polymerization). The process of polymerization distorts the red blood cell to a rigid sickle shape. The homozygous form, sickle cell (SS) disease, is characterized by chronic hemolysis that is stable and moderately debilitating, and episodic vaso-occlusive crises that cause organ failure and account for most of the morbidity and mortality associated with the disease. The heterozygous form (HbAS) or sickle cell trait is mostly asymptomatic due to the presence of more than 50 percent of normal HbA.

Deoxy HbS polymerization is also reduced in the presence of other Hb-types within the red cell. Fetal hemoglobin (HbF), normally lower than 2 percent but occurring at higher levels in sickle cell disease, is particularly beneficial in this respect because it forms a soluble complex with HbS, which results in a considerable prolongation of the time prior to polymerization and thereby may prevent red blood cells from sickling and improve their survival. This advantage has incited a search for products that modulate the HbF production in sickle cell disease.

The goal of this paper is to provide an overview on the regulation and role of fetal hemoglobin in sickle cell disease and to discuss mechanisms of action as well as clinical results of several pharmacological agents that stimulate HbF production.

Regulation and role of fetal hemoglobin level

Fetal hemoglobin is composed of two α and two γ chains. The duplicated γ genes are localized on the short arm of chromosome #11. They differ slightly which results in two types of γ chains namely the $^G\gamma$ and $^A\gamma$ chain. Hemoglobin synthesis follows a developmental stage and tissue specificity. In the embryonic period ϵ chains are synthesized, but as soon as hematopoiesis shifts to the fetal liver in the 6th week, production of HbF is initiated. When the bone marrow becomes active in the second trimester and eventually becomes the most important hematopoietic site, production gradually switches from γ chains to the adult β (HbA) and δ (HbA₂) chains. At birth babies with SS as well as matched AA controls still carry HbF levels between 60 and 80% with the ratio between the two γ chains being 70% $^G\gamma$ and 30% $^A\gamma$. In the first six months a second switch takes place involving reversal of the $^G\gamma$ and $^A\gamma$ ratio from 70 : 30 to 40 : 60 (Figure III.1). Simultaneously, the HbF level declines to an average of 1-2% at the age of 6 months. However, infants with sickle cell anemia show a much slower decrease of HbF^{1,2}.

Figure III.1 Developmental switching of β -like globin genes [adapted from ref. 56].

The proces of developmental hemoglobin switching appears to be controlled at the level of gene transcription. Regulation is exerted by promoter, enhancer and silencer elements (consisting of DNA sequences in close proximity to the structural globin genes) and by distant DNA sequences within the same chromosome interacting with certain regulatory nuclear proteins. The promoter elements play an important role in the initiation of the transcription proces and modulate the efficiency of transcription. Enhancer and silencer elements influence the level of transcription. The distant DNA sequences are named the Locus Control Region (LCR). These sequences preferentially interact with γ - and β -globin genes (Figure III.2).

Figure III.2 Schematic presentation of the β -globin gene cluster of chromosome no. 11 showing organisation and sequential activation of the globin genes by interaction with the locus control region (LCR). The letter P denotes the promoter regions.

The LCR organizes the globin locus into an active chromatin domain and enhances globin gene transcription. The interaction of either the γ promoter or the β promoter with the LCR, and hence the developmental expression of γ - or β -globin genes, is a competitive process which is thought to depend on the presence of developmentally regulated (but still mostly unidentified) nuclear proteins^{2,3}.

Distribution of HbF among red blood cells

The HbF in SS patients and normal adults is heterocellular and is mainly confined to erythrocytes named F cells. The levels of F- cells and HbF are considerably increased in sickle cell anemia. Due to preferential survival of F-cells the HbF level enhances even further. The relative proportion of F cells in a given SS individual is remarkably constant and genetically determined^{1,2} but the HbF levels in peripheral blood show a considerable inter-individual variation³, which may be attributed to differences in the production of F-cells, variation in the amount of HbF per F-cell and to the preferential survival of F-cells⁴.

Genetic factors influencing the HbF synthesis

The broad distribution of the HbF levels in SS patients suggests that various factors control HbF production. Among these are the β^S haplotype with mutations in promotor regions of the γ and β -globin genes, co-existent α -thalassemia (α -thal), mutations which have been identified in the LCR region on chromosome 11, age and the X-linked F-cell production locus.

Five distinct sickle cell (SS) sub-types or β^S haplotypes linked to the β^S -globin gene are recognised and they probably originate from the β^S mutation occurring several times independently in individuals of different backgrounds. These haplotypes are named the Senegal, Cameroon, Benin, Bantu, and Saudi Arabia-India type or number 3, 17, 19, 20 and 31 (Figure III.3). Studies by Gilman and Huisman as well as others⁵⁻⁸ have shown that a single nucleotide base mutation (C->T) within the promotor region at position - 158, 5' to the γ -globin gene, is associated with high γ and higher HbF levels. This mutation is characteristic of the Senegal (#3) and Saudi Arabian(#31) haplotype. It has been established that the presence of at least one chromosome with haplotype No 3. is sufficient to increase γ and HbF levels and to improve the hematological profile.

In SS disease with co-existent α -thalassemia the HbF levels are decreased. Dover et al.⁹ have demonstrated an inverse correlation between the number of α -globin genes and non-F cells. They concluded that the lower HbF levels in α -thal reflect improved survival of non-F cells and are not due to intrinsic differences in F cell- or HbF-production. In α -thal, the decrease in HbF levels is presumed to be the result of preferential formation of $\alpha\beta^S$ dimers instead of $\alpha\gamma$ dimers. The improved survival of non-F cells in SS patients with co-existent α -thalassemia can be

attributed to the lower mean corpuscular hemoglobin concentration. Certain mutations in LCR region, characteristic for haplotype No. 3, are also associated with increased C_γ and HbF levels¹⁰. When studying the effects of age and sex on HbF levels it is noted that newborns and children with sickle cell anemia up to the age of 5 years express high HbF levels (20 - 40%) and thereafter HbF decreases gradually. After the age of 10 years, female sickle cell patients show consistently higher HbF in all age groups. Over the age of 40 years HbF levels remain stable in male SS patients while in female patients an increase of HbF is observed¹. Most recently, an X-linked locus which regulates F- cell production (FCP locus) has been identified¹¹. The FCP locus is probably responsible for most if not all sex difference in HbF levels. Multiple regression analysis, including all abovementioned variables, has shown that the FCP locus is the most important predictor of the HbF level, accounting for 40% of the HbF variation. The β -globin haplotype, α -globin gene number and age account for less than 10% of the variation¹¹. The factors responsible for the other 50% of the variation in HbF levels still remain unknown.

Figure III.3 DNA enzyme restriction sites identifying the five major SS haplotypes [from ref 1,57].

Clinical significance of HbF and the rationale of HbF induction in sickle cell disease

The antisickling effect of HbF was first recognized by Watson in 1948. He observed that red blood cells from infants with sickle cell anemia showed much less evidence of sickling as opposed to almost all cells of their sickle cell mothers.

This observation was supported thereafter by several reports of compound β^S heterozygotes with higher HbF and hereditary persistence of HbF (S-HPFH), who showed much less signs of sickle cell disease. Perrine et al.¹² reported that sickle cell patients from Saudi Arabia had relatively mild sickle cell disease due to HbF levels of 10-26%. Later, a cohort study in Los Angeles revealed that patients with HbF levels above 10% were protected from major vaso-occlusive crises such as stroke or aseptic necrosis and patients with HbF levels in excess of 20% experienced much less painful crises or pulmonary disorders¹³. Furthermore, a study by Platt et al.¹⁴ has demonstrated that life expectancy of sickle cell patients that carry HbF levels in excess of 8.6 percent approaches that of black male individuals without hemoglobinopathy and is practically equal to the life expectancy of males with HbSC disease.

The sparing effect of HbF on the sickling process has prompted a search for agents that can stimulate HbF production. Some cytostatic agents, in particular 5-azacytidine and hydroxyurea, were found to have these properties. Because of its leukemogenic action, further studies of 5-Azacytidine were abandoned. Erythropoietin and butyrate analogues have also been shown to induce HbF production and recently it has been demonstrated that granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-3 (IL-3) and in particular stem cell factor (SCF) induce F-reticulocytosis in primates and human erythroid cell cultures.

Mechanism of action and clinical effects of pharmacologic agents that stimulate HbF synthesis

Hydroxyurea

This agent has mainly been used as a cytotoxic drug to control various myeloproliferative disorders. In fast dividing cells, hydroxyurea has effects on DNA by inhibiting the enzyme ribonucleotide reductase, blocking the conversion of ribonucleotides into deoxyribonucleotides. The mechanism of action of HbF induction, although not fully elucidated, is ascribed to selective proliferation of early erythroid progenitors which preserve the program for γ -chain synthesis but remain relatively inactive in the normal adult marrow, where erythropoiesis is dominated by mature progenitors carrying the β -chain synthesis pattern (Figure III.4). In situations of acute bone marrow stress, such as recovery from the cytotoxic effects of hydroxyurea, the early erythroid progenitors are selectively recruited and proliferate maximally, resulting in an enhanced proportion of HbF in the new red cell population¹⁵. Furthermore, it has been shown that treatment with 5-azacytidine or hydroxyurea is associated with non-random hypomethylation of DNA around the γ and β -globin gene complex and increase in γ mRNA^{16,17}. Hypomethylation of DNA can usually be demonstrated near active genes and hypermethylated DNA is found in the vicinity of inactive genes.

In addition to the action on HbF production, hydroxyurea also induces

macrocytosis and improves red cell membrane deformability aswell as hydration status of erythroid cells¹⁸. Where the improvement of sickle cell hydration probably is an indirect result of the increase in HbF, the macrocytosis and red cell deformability are most likely a unique property of the early erythroid progenitors. Furthermore, in vitro studies show that hydroxyurea affects morphology and modulates expression of vascular adhesion molecules in endothelial cells¹⁹ suggesting that a separate effect of hydroxyurea on endothelium-red blood cell interaction should be considered.

Figure III.4 Diagram showing proliferation of erythroid progenitors. In situations of hematopoietic stress, proliferation of early erythroid progenitors, which carry the program for γ globin synthesis is preferred, resulting in an enhanced proportion of F-cells and HbF. The action of cytokines, Erythropoietin (Epo), Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF) and Stemcell Factor (SCF), maximizes production of different stages of the erythrocyte lineage.

In several clinical studies²⁰⁻²⁵ most sickle cell patients treated with hydroxyurea characteristically showed decrease of hemolytic rate, increase of total hemoglobin, macrocytosis and varying increments in HbF (Figure III.5).

Figure III.5 Levels of HbF, total Hb and MCV of six SS patients treated with Hydroxyurea [adapted from ref. 25].

The cause of this variability is unknown. No genetically determined differences in metabolism of hydroxyurea have been reported. In most patients HbF levels increase at low dosage of hydroxyurea whereas in some individuals a (minimal) response is only seen after long periods of therapy or at myelotoxic doses. The average dose of hydroxyurea used to stimulate HbF was 20 mg/kg/day. Intermittent or continuous dosing regimens have not resulted in significant differences in HbF response. In primates the HbF response also varied and a nucleotide difference in the γ -gene promoter was found, which accounted for the varying HbF response. No nucleotide sequence differences have been detected in γ -gene promoter region of humans²⁶. However, it is likely that other still undefined genetic factors together with the severity of hematopoietic stress may explain the variability in HbF response. Although clinical symptoms of some patients treated with hydroxyurea seemed to improve, no clear answers were available on the frequency of painful crises or other vaso-occlusive events during treatment. However, a recent multicenter trial of hydroxyurea in nearly 300 patients with sickle cell anemia in the United States²⁷ showed that this drug can ameliorate the clinical course of some

adult sickle cell patients. Painfull crises, chest syndrome and blood tranfusions were significantly decreased, while no differences were observed in stroke events, hepatic sequestration crises or mortality. Maximum tolerated doses of hydroxyurea were not required to reduce the incidence of painfull episodes. Some degree of myelo-suppression occurred in most patients but this did not result in serious complications. No deaths could be attributed to hydroxyurea. Still, it is noted that some patients that show significant increase of HbF do not appear to benefit from this therapy and may even fare worse^{25,28}. One reason for this finding could be that the clinical improvement of a sickle cell individual is not solely dependent on HbF induction and reduction of HbS polymerization, but is also determined by the increase in total hemoglobin and packed cell volume. The latter changes could give rise to deleterious effects in the microcirculatory blood flow of especially prior damaged organs. Other explanations for the variable clinical response include the abovementioned effects of hydroxyurea on red cell volume, hydration status, the red cell membrane and a direct effect on endothelium.

So far, hydroxyurea treatment has not demonstrated any evidence of carcinogenesis in sickle cell patients. However, there are reports of longterm administration of hydroxyurea in myeloproliferative disorders that suggest that the drug may be involved in leukemogenesis^{29,30}. Also, the effects of hydroxyurea on offspring, growth and development in children as well as longterm effects of hydroxyurea on progression of organ damage and mortality remain unresolved.

Erythropoietin (Epo) and other hematopoietic growth factors

A number of cytokines, in particular IL-3, GM-CSF and SCF have been demonstrated to stimulate HbF production in erythroid cell cultures and animals³¹⁻³⁵. SCF related superior increments in HbF compared to other cytokines but required the presence of Epo. Probably, SCF causes expansion of the pool of stem cells which in the presence of Epo differentiate and mature into F-cells. The mechanism of intracellular γ -gene activation still has to be unraveled, but erythroid transcription factors such as GATA-1 are thought to be involved as well as the so-called Homeobox genes and their proteins, that may recognize specific regulatory regions of γ -globin gene³⁶.

Thus far none of these cytokines has been administered to SS patients with the purpose of HbF induction, except for Epo. High dose pulse therapy with Epo stimulates F-cell formation and HbF production in animals as well as in humans³⁷⁻³⁹. Erythropoietin is a growth factor that induces red cell formation by stimulating proliferation, differentiation, and maturation of erythroid progenitors and precursors. The exact mechanism by which erythropoietin induces HbF production at high doses is unclear but probably the resultant rapid regeneration of erythroid progenitors stimulates the formation of F-cells. While cooperative treatment of sickle cell patients with erythropoietin and hydroxyurea only produced moderate

increase in HbF levels at best³⁹, the combination of Epo with hydroxyurea and iron supplementation produced a much more significant response⁴⁰. It is doubted that this regimen, in view of the extremely high costs involved, would ever be of benefit to SS patients in general. Nevertheless, studies are currently underway to further expand the experience on cooperative Epo and hydroxyurea induction of HbF in SS patients.

Butyrate and butyrate derivatives

Butyric acid, a 4-carbon fatty acid, occurs naturally in the body and is formed by hydrolysis of ethylbutyrate. Studies show that this fatty acid affects morphology, growth rate and gene expression in mammalian cell cultures⁴¹. Infants, born to mothers with poorly controlled diabetes mellitus, have been noticed to have a delayed $\gamma \rightarrow \beta$ gene switch, which appears to be associated with high serum levels of α -amino-n-butyric acid^{42,43}. Subsequently, it has been shown that infusion of butyrate in the umbilical vein impedes the normal $\gamma \rightarrow \beta$ gene switch in sheep fetuses⁴⁴ and that butyrate stimulates γ -globin genes in cultured mammalian cells⁴⁵. In addition to this action, butyrate displays other effects such as induction of cell differentiation, activation of cyclic AMP, effects on DNA methylation and growth inhibition^{41,46}. These effects should be distinguished from the action on γ -globin synthesis. Gamma-globin synthesis is thought to be controlled at the level of transcription. Increases of γ -globin messenger RNA and globin protein production have been demonstrated in patients on butyrate, while at the same time a decrease in β^S -globin messenger RNA was detected⁴⁷. Gene assays in erythroid cell lines have already identified the region on the γ -globin gene which represents the butyrate-response promoter elements⁴⁸. These findings imply that butyrate may act to increase HbF production in a complete different way than hydroxyurea. A pilot study by Perrine et al.⁴⁷ reported remarkably rapid stimulation of γ -globin gene expression following intravenous arginine butyrate. F-reticulocyte levels increased within days and fetal hemoglobin synthesis increased significantly within three weeks. In another study with intravenous butyrate⁴⁹ less impressive results were seen, but this difference could have resulted from the higher dosing regimen which is known to cause suppression of erythropoiesis⁵⁰. However, because butyrate is cleared by the liver and rapidly metabolized, its therapeutic use requires frequent or prolonged intravenous infusions. This practical disadvantage and the observation that butyrate metabolites could also induce HbF production⁵¹, resulted in identification of oral bioavailable butyrate derivatives capable of HbF induction. During the recent years there have been only a few reports on trials with butyrate analogues in sickle cell patients. Trials with the analogues sodium phenylbutyrate and isobutyramide show induction of γ chains or HbF synthesis in patients⁵²⁻⁵⁴. However, the increments are moderate and rapid tolerance is observed. Also, there are concerns about neurological as well as gastrointestinal side effects. Another

structurally related compound, valproic acid, known for its use in epilepsy, has recently been shown to induce HbF but the data are still very limited⁵⁵.

Conclusions

It is known that clinical disease expression varies greatly between SS patients. This, among other factors, is dependent on the levels of HbF. The presence of the X-linked FCP locus has been shown up until now, to be the most significant predictor of the HbF level. In general high HbF levels appear to ameliorate the severity of SS disease and because of this knowledge several pharmacological agents that induce HbF production have been studied and are still being studied. Hydroxyurea stimulates HbF production but the response is highly variable. In clinical studies, hydroxyurea imparts a reduction in painful crises, chest syndrome and transfusions to some patients, but others do not experience improvement and may even fare worse. The addition of iron supplements and high dose pulse therapy with Epo to hydroxyurea increases HbF levels even further but there are very few data on the clinical effects. Moreover, the administration of hydroxyurea still carries an unknown risk of carcinogenicity and teratogenicity while the longterm effects of hydroxyurea on mortality remain unresolved. These findings indicate that strict laboratory and clinical terms have to be established to enable careful selection of those SS patients that will truly benefit from treatment with hydroxyurea. Butyrate analogues stimulate HbF production by a different mechanism compared to hydroxyurea. They have the theoretical advantage of not being potentially carcinogenic drugs. However, they will probably not replace hydroxyurea but instead may be of additive or synergistic value to treatment with hydroxyurea or other drugs.

Several ongoing studies as well as future trials will continue to expand on the clinical experience with HbF inducing agents in SS patients. Although progress is also made in other treatment modalities such as bone marrow transplantation and gene therapy, pharmacological ways of stimulating γ genes will continue to improve and remain a valuable option to the treatment of sickle cell disease in the next decennium.

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Chapter IV

Aims of the study and thesis outline

AW Saleh

Aims of the study and thesis outline

Many drugs have been tried and advocated for ameliorating the clinical severity of sickle cell disease during the past three decades. None of these has been so extensively investigated as the group of agents that confer a reduction in the polymerization rate of sickle cells by induction of HbF production. This latter group, consisting of hydroxyurea, the short chain fatty acids (butyrates) and erythropoietin, cause induction of HbF in erythroid progenitors by different mechanisms which are discussed in chapter II. The net effect of these agents on HbF production is very variable. The cause of this variability is not clear and requires further analysis to enable appropriate patient selection and avoid unnecessary treatment. Certain sickle cell patients may reveal a relatively benign course of disease due to several factors, such as genetic, vascular or environmental determinants, and may not benefit from treatment that involves HbF induction. Moreover, treatment would probably have to be administered on a long-term basis, which theoretically poses a risk of carcinogenicity and teratogenicity for agents such as hydroxyurea. In this respect further studies on the effects on HbF production by oral butyrate derivatives could establish worthwhile alternatives to hydroxyurea.

Not only does the inter-individual HbF production differ significantly, but the clinical effects of hydroxyurea treatment in sickle cell patients cannot always be correlated to the changes in HbF. This suggests that, in addition to the HbF induction and changes in hematologic profile, other effects of hydroxyurea may play a role in the final therapeutic outcome. As stated in chapter I of this thesis, red blood cells, leucocytes, platelets and endothelium are intricately involved in the mechanism of sickle cell vaso-occlusion. The adhesion process is also heavily influenced by several plasma factors such as certain cytokines and procoagulant factors. Actions of hydroxyurea on some of the determinants of cellular adhesion, vascular endothelium and cytokine production may play an important role. In fact, apart from reducing the number of leucocytes, hydroxyurea has been shown to cause specific changes in endothelial cells *in vitro* and to alter red blood cell properties.

Also, cytokines are vital to hematopoiesis, and even more so to stress hematopoiesis. In sickle cell anemia the interactions between erythropoietin (Epo), stem cell factor (SCF), interleukin-3 (IL-3), granulocyte-macrophage stimulating factor (GM-CSF) and the erythroid pool are of critical importance. However, their behaviour during hydroxyurea treatment is not known.

The specific aims of the studies described in this thesis are:

- * To study the effects on HbF production in sickle cell (SS) patients of the oral butyrate analog, isobutyramide, which has the theoretical advantage of lacking the carcinogenic potential like cytostatic drugs such as hydroxyurea. (Chapter VI)
- * To examine whether hydroxyurea causes increases of HbF production in homozygous sickle cell patients standardized for the same β^S -haplotype. (Chapter VII)
- * To study if hydroxyurea treatment in SS patients results in clinical improvement, counted as reduction in painful crises or other specific vaso-occlusive crises. (Chapter VII)
- * To determine if hydroxyurea therapy in SS patients influences the levels of IL-3, GM-CSF, Epo, SCF, as well as various soluble adhesion molecules and to study whether HbF levels and HbF induction are in any form related to plasma cytokine activity. (Chapter VIII)
- * To study the levels of endothelial (sVCAM-1, fibronectin, IL-8), neutrophil (myeloperoxidase, sIL-6 receptor α , SL-selectin) and platelet (vWF) derived factors in SS patients during hydroxyurea therapy. (Chapter IX)

Chapter V

Materials and Methods

AW Saleh

This chapter provides general information on the patients who cooperated and the techniques that were used in these studies.

Patients

Of the 18 SS patients that were screened prior to the start of the studies in this thesis, 11 patients cooperated with one or more studies. The inclusion criteria for participation were a Benin haplotype with four intact α -globin genes, normal serum creatinine level, normal liver function and no pregnancy. Two patients were excluded because of an abnormal serum creatinine. Of the remaining 14 patients three did not wish to participate. Table V.1 shows specifically which patients participated in each of the studies. Before participation informed consent was obtained from each patient. Charts and hospital records were reviewed for their medical history and laboratory data. These are summarized in chapter II. The clinical features of each patient individually are mentioned in the studies described in this thesis. During the study patients visited an outpatient clinic, once every month, for physical exam. During these visits clinical symptoms (especially painful crises) and complications were registered. In order to obtain the most information, patients were encouraged to record their symptoms in a diary. In the event of a severe painful crisis or serious complication, patients were admitted to the St. Elisabeth Hospital for further treatment. Painful crises were defined as severe pains in the bones of the extremities, back, abdomen or chest which were not relieved by non-narcotic analgesics (acetaminophen).

Of the 18 patients that were screened, 16 had their haplotype determined at the laboratory of the Comprehensive Sickle Cell Center in Augusta, GA, USA (Director: Prof THJ Huisman).

Table V.1. Allocation of patients to each of the studies

Study	Patients (Initial)
Isobutylamide therapy in patients sickle cell anemia (chapter VI)	A (MA), B (RM), C (EP), D (IR)
Hydroxyurea therapy in sickle anemia patients in Curaçao, Netherlands Antilles (chapter VII)	A (EP), B (IR), C (RM), D (MM), E (MA), F (AH), G (GH), H (RF), I (GL)
Cytokines and soluble adhesion molecules in sickle cell anemia patients during hydroxyurea therapy (chapter VIII)	A (EP), B (IR), C (MM), D (MA), E (GH), F (RF), G GL)
Levels of endothelial-, neutrophil- and platelet- specific factors in sickle cell anemia patients during hydroxyurea therapy (chapter IX)	A (EP), B (IR), C (RM), D (MA), E (GH), F (RF), G (EV), H (JC)

Blood collection and shipment

About 10-20 ml of blood for haematological analysis was obtained by venepuncture into vacutainers with EDTA as anticoagulant. Also, 5-10 ml of blood was obtained for chemical analysis. Samples for haplotype and HbF determination at the laboratory in Augusta were shipped on ice by courier service the same day and always arrived at destination within 2 days.

Haematological data

CBC's and red cell indices were determined with a fully automated Onyx Coulter Counter.

Hb Analyses

RBC's were washed with isotonic saline and haemolysed with 1.5 volumes of water and 0.4 times the volume of CCl₄ for 15 minutes at room temperature. Stroma was removed by centrifugation at 20,000 at 4°C for 20 minutes. These haemolysates were used for electrophoretic and chromatographic studies.

Hb Electrophoresis

Diagnosis of SS disease was made by Hb electrophoresis. The principle of this technique is based on a charge change in the hemoglobin molecule that occurs with certain amino acid substitutions. The replacement by valine of glutamic acid in HbS results in a more net positively charged Hb relative to HbA. With hemoglobin movement- in the appropriate medium- towards the anode, HbS moves more slowly than HbA. Red cell haemolysates were applied to cellulose acetate as alkaline medium with tris-EDTA-borate as buffer system and Hb separation was obtained by electroforesis for 20 minutes at 350 V¹.

Determination of HbF, $\alpha\gamma$ and $\beta\gamma$ levels

The HbF levels were determined by high-performance liquid chromatography or HPLC². This technique allows complete separation of HbF from other Hb types in less than 50 minutes. The chromatographic procedure was performed on a weak cation exchange resin in a PolyCath A column (4.6x200 mm with particle size of 5 mm; PolyLC, Columbia MD, USA) in a Waters HPLC system (Waters Chromatography division, Milford, MA, USA). Two eluents, A and B, were used which contained respectively 35 mM Bis-Tris, 3 mM NH₄-acetate, 1.5 mM KCN, pH 6.47 (Developer A) and 35 mM Bis-Tris, 1.5 mM KCN, 150 mM Na-acetate, 16.85 mM NH₄-acetate, pH 7.0 (Developer B). 50-200 μ g Hb in red cell lysates was applied to the column that was subsequently eluted with a linear gradient of 25:75 (v/v) eluent A:B to 15:85 (v/v) eluent a:B in 85 min at a flow rate of 0.8

ml/min. The absorbance of the effluent was continuously recorded at 415 nm. The different hemoglobins appear in the following order: HbF, HbA, HbA₂ and HbS. The individual peaks are quantitated (as area%) by a data module (Figure V.1).

Figure V.1. Quantitation of HbF by HPLC and $\alpha\gamma$ and $\lambda\gamma$ by reversed HPLC procedure in an adult homozygous sickle cell patients. Minor discrepancies in HbF levels between these procedures may result from slight contaminations of $\alpha\gamma$ with α chains. The ratio of $\alpha\gamma$ to $\lambda\gamma$ (63.4 : 36.6) in this sample (patient MA) reflects the presence of a Senegal gene.

The $\alpha\gamma$ and $\lambda\gamma$ levels were determined in Augusta, GA, USA, by a reversed phase HPLC, using a large -pore Vydac C₄ column and two water-acetonitrile-TFA developer as originally described by Shelton et al³ and Kutlar et al⁴. A low pH of 2 is chemically established which causes hemoglobin to dissociate in heme and globin chains. The chromatogram was developed by applying 50-800 μ g Hb in red cell lysates to the column at a flow rate of 1 ml/min, after having established a gradient of 50:50 (v/v) eluent A:B to 30:70 (v/v) eluent a:B. The effluent was continuously recorded at 215 nm and the peaks were calculated as mentioned above.

Separation of the globin chains depends primarily on differences in their

hydrophobicities. The globin chains appear in the following sequence: β^C , β^S , β^A , δ , α , γ^T , γ and γ^I (Figure V.1).

The γ^I and γ^T chains are the results of a polymorphism at the aminoacid position 75 of the γ globin chain (Isoleucine-I- for the common γ type and Threonine -T- for the other γ type).

The levels of HbF ($= \gamma + \gamma^I + \gamma^T$) are calculated as percentage of total β -like chains ($\delta + \beta + \gamma^T + \gamma + \gamma^I$).

Samples that contained less than 20 % HbF in their red cell lysates required an initial enrichment of the HbF fraction. This was done by performing a anion exchange DEAE-cellulose chromatography. Depending upon the % HbF in the red cell lysate, 20-60 mg Hb in the haemolysate were applied to a column (30x1,5 cm); the chromatogram was developed for 15 minutes at a flow rate of 50 ml/hour. The top of the column containing the HbF fraction was removed and poured into a microcolumn (7x 0.5 cm) and eluted with a stripping buffer⁵.

Determination of β^S haplotype and α -globin gene number

(Comprehensive Sickle Center, Augusta, GA, USA)

After isolating DNA from white blood cells by the method of Poncz et al⁶, the modified procedure of Southern^{5,7} was used for the determination of the β -globin gene haplotype and for the evaluation of α -globin genes. The gene mapping procedure for the β -globin gene cluster was done by first digesting the genomic DNA with different restriction enzymes (HincII, Xmn I, Hind III, Ava II, HpaI, and Bam HI). The resulting DNA fragments were separated by electrophoresis on an agarose gel. Following electrophoresis the DNA in the gel was stained with ethidium bromide and photographed under long wavelength UV light. Then the DNA was depurinated by soaking the gel in 0.1 N HCL, denatured in a neutralization solution (3M NaCl, 1.5 M Tris-HCL, pH 7.0), transferred to a nylon membrane and baked in a vacuum oven at 70-80°C. Next, the membranes were prehybridized with Flavell modified solution and denatured salmon sperm DNA followed by hybridization using denatured α -³²P-labeled probes. These probes are different fragments of β -globin gene cluster, previously cloned in plasmid vectors, that hybridize specifically with their complementary DNA sequences. Finally, the membranes were washed until a minimum of background radioactivity was measured (by Geiger counter) and exposed to a Kodak XAR X-ray film. The films were developed by Kodak X-ray film processor and the positions and sizes of the particular sequences of DNA were evaluated.

The entire procedure was followed in the same way to determine the α -globin gene number utilising restriction enzymes Eco RI, Bgl II and Bam HI, and a α probe (containing the entire α -globin gene), ζ -globin gene probe and θ -globin gene probe.

Quantitation of adhesion molecules and cytokines

These were performed in serum by Enzyme Linked Immuno-Sorbent Assays (ELISA) according to the manufacturer's guidelines. Interleukin-6: CLB, Netherlands. Interleukin-3, granulocyte macrophage-colony stimulating factor (GM-CSF) and soluble Intercellular Adhesion Molecule-1 (sICAM-1): Medgenix, Belgium. Soluble Vascular Adhesion Molecule-1 (sVCAM-1), sE-Selectin, sP-Selectin, sL-Selectin, soluble IL-6 receptor- α Fibronectin, Erythropoietin and Stem Cell factor: R & D-systems, UK.

Liver and Spleen scintigraphy

The reticulo-endothelial function of the patients was determined by injecting intravenously 99m Technetium-labelled colloid. Radionuclide images of posterior, lateral and anterior views of the spleen and liver were obtained after 30 minutes. The spleen activity was interpreted as positive (normal or partial visualization) or negative (no visualization).

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Chapter VI

Isobutyramide therapy in patients with sickle cell anemia

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Abstract

We have administered Isobutyramide as a suspension over a period of 3 months, from a starting dose of 50 mg/kg/day up to 150 mg/kg/day, to four adult sickle cell (SS) anemia patients. The maximum dose was maintained for 3 weeks. The blood counts remained stable and the HbF levels decreased slightly. The G γ levels increased at the end of the trial, suggesting activation of the G γ gene at the highest dose of Isobutyramide. Three patients showed a stable rate of hemolysis, while in one patient, an increase of lactate dehydrogenase occurred. None of the patients experienced pain crisis or organ-specific crisis, but all four complained about mild epigastric burning and a bitter taste. After the first month of treatment one patient complained about intolerable epigastric discomfort which was relieved by Omeprazole. Another patient complained about increasing dyspepsia in the 12th week leading to the termination of the trial. Oral Isobutyramide administration does not qualify as an effective treatment of SS patients.

Introduction

It is well known that the clinical course of sickle cell (SS) disease is improved in patients with high levels of fetal hemoglobin (HbF)¹. Moreover, a study of the natural history of sickle cell disease reveals that any increment in the % HbF, even at low levels, will have an ameliorating effect². Consequently, clinical research has focused on the pharmacological manipulation of HbF and F cells. Chemotherapeutic agents like hydroxyurea can induce γ chain synthesis³ but pose the risk of cytotoxicity and mutagenicity. Intravenous butyrate, a naturally occurring compound which lacks cytotoxicity, has been reported to stimulate HbF production⁴, its effect being explained by an action on the promoter of both γ -globin genes. Butyrate is rapidly metabolized by the liver which limits its use to (prolonged) intravenous administration. Isobutyramide, a derivative of butyrate, has been produced as an oral alternative and has been shown to increase F cell production⁵. Here we report our preliminary findings about Isobutyramide in a small group of SS patients.

Methods

Four SS patients, two males and two females, were admitted to the study which was conducted with the approval of the local board of the Food and Drug administration and the ethical board of the St. Elisabeth Hospital, Curaçao, the Netherlands Antilles. Their mean age was 42 years. Three had haplotype 19/19, while one carried a hybrid 19 haplotype. All had four α -globin genes ($\alpha\alpha/\alpha\alpha$). All four had been treated with Hydroxyurea in a previous trial, until 3 months before the start of this study. None took any medication other than folic acid, except for one female patient who was on Acenocoumarol because of a liver sequestration crisis 6 months earlier. None experienced symptoms suggesting pain crisis or any organ-specific crisis at the start of the study nor had they received a transfusion within 6 months of the start of this study.

Isobutyramide was prepared as a suspension, sweetened, and artificially flavored. The patients were treated on an ambulatory basis. After written informed consent was obtained, Isobutyramide was administered starting at a dose of 50 mg/kg/day and was increased to 150 mg/kg/day over a period of 3 months. The maximum dose was maintained for 3 weeks. Blood counts, reticulocytes, lactate dehydrogenase (LDH), bilirubin, HbF, and $^G\gamma$ levels were assessed at weekly intervals. HbF was quantitated by high performance liquid chromatography (HPLC)⁶ and the $^G\gamma$ levels by reversed phase HPLC⁷. Patients were visited at home once a week, at which time compliance was evaluated and symptoms or complaints were noted.

Results and discussion

During the entire study the hematological data remained the same (some are listed in table VI.1). A slight decrease in HbF was noted which may well reflect the residual effect of Hydroxyurea which was given as a part of another trial and was stopped 3 months prior to the start of this study. The $\alpha\gamma$ levels remained essentially unchanged at first, suggesting no preferential activation of γ genes. However, at the highest dose (150 mg/kg/day) of Isobutyramide an increase in $\alpha\gamma$ was observed in all patients (Figure VI.1). Because of increasing complaints of epigastric discomfort the study was terminated after 3 months, making it impossible to evaluate if the level of $\alpha\gamma$ gene activation would result in a significant increase in HbF. Three patients showed stable LDH levels and reticulocyte counts (Table VI.1). Patient A showed a slight increase of LDH and in reticulocytes at the highest dose of Isobutyramide due to an increase in the rate of hemolysis, which was likely caused by a direct (toxic?) effect of the Isobutyramide, since signs of infection or vaso-occlusive episodes were not observed. Kidney function and liver parameters remained unaffected during the study. None of the patients experienced a pain crisis or other vaso-occlusive episodes. Two patients did not have any undesirable symptoms other than some epigastric burning and a bitter taste in the mouth at the time of ingestion of the drug. Patient C complained about intolerable epigastric discomfort after 1 month. He refused endoscopy and was given Omeprazole (20 mg twice /day) with good results. Patient A developed an increasing dyspepsia in the 12 th week of the study.

Figure VI.1 The relative levels of $\alpha\gamma$ chain (in % of total γ) in four SS patients treated with oral Isobutyramide.

We conclude that oral Isobutyramide may activate the $\alpha\gamma$ -globin genes in vivo at extremely high doses but the effect appears to be weak at best. The use of oral Isobutyramide as “monotherapy” in SS patients has no clinical value but it may be

that the use of Isobutyramide in combination with other drugs will be more effective in stimulating γ chain production.

Table VI.1 Laboratory Data for Four SS Patients Treated With Isobutyramide

Patient	Age (yrs)	Dose (mg/kg/day)	Hb (g/dl)	PCV (l/l)	RBC ($10^{12}/l$)	HbF (%)	Retics (1,000/mm ³)	LDH (μ/l)
A	46	0	8.8	0.260	2.38	17.0	274	386
		50 ^a	8.6	0.265	2.38	14.8	350	422
		100 ^b	8.6	0.252	2.32	15.6	260	416
		150 ^b	8.8	0.266	2.45	15.4	343	501
B	35	0	7.8	0.233	2.58	10.7	464	633
		50 ^a	9.6	0.290	3.08	8.8	400	709
		100 ^b	9.6	0.272	3.03	9.3	336	817
		150 ^b	9.5	0.275	3.09	8.3	569	572
C	56	0	5.9	0.171	1.77	6.3	246	1.099
		50 ^a	5.6	0.172	1.70	5.7	207	1.103
		100 ^b	5.9	0.174	1.86	4.1	285	1.197
		150 ^b	5.6	0.172	1.83	3.2	271	992
D	31	0	7.0	0.206	2.32	4.2	432	933
		50 ^a	7.3	0.222	2.44	3.9	310	909
		100 ^b	7.9	0.222	2.55	2.6	268	975
		150 ^b	7.5	0.225	2.59	2.6	518	1.016

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Chapter VII

Hydroxyurea therapy in sickle cell anemia patients in Curaçao, the Netherlands Antilles

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Abstract

We have treated nine patients with sickle cell anemia (SS) with hydroxyurea (HU). All nine patients carried four α -globin genes and the β^s -globin haplotypes 19/19 (Benin/Benin), except for one who had haplotype #19 together with type #3 (Benin/Senegal). Six patients received HU for 10 months and were again treated with the drug for five months after an intermission of one year. One patient was given HU for 22 consecutive months. A record was kept of hematological and biochemical data, HbF and $\alpha\gamma$ levels, as well as possible clinical complications. Our data show that HU generally improves the hematological and biochemical values and the level of HbF, and reduces painful crises in some patients. However, although the clinical symptoms ameliorate in some patients during HU therapy, the older patients did not observe changes in their general condition; the same is the case for the patient with haplotype 19/3. One patient also experienced a life-threatening liver sequestration during treatment. We conclude that the selection of patients who may benefit from HU therapy needs further evaluation.

Introduction

Clinical surveys conducted during the past decade show that SS patients apparently have a milder disease when their fetal hemoglobin (HbF) level is increased^{1,2}. Consequently, the question has been raised: Would a pharmacological induction of HbF production in SS patients ameliorate their disease? Hydroxyurea (HU), a cytostatic agent, has been shown to increase HbF levels^{3,6}. The mechanism of action of HbF induction, although not fully elucidated, is perhaps explained by a cytotoxic action on cycling erythroblasts with preferential survival of non-cycling erythroid progenitors which may undergo accelerated differentiation and maturation. These precursors have an enhanced capacity for γ -globin synthesis. A definitive insight into the mechanisms of the effect of HU on fetal erythropoiesis is still lacking. Apart from the action on HbF production HU induces macrocytosis and improves cell deformability as well as the hydration status of erythroid cells⁷.

A recent multicenter trial of HU in nearly 300 patients with SS in the United States⁸ showed that this drug can ameliorate the clinical course in some adult patients. Painful crises, chest syndrome, and the requirement for blood transfusion were significantly decreased, while no differences were found in stroke events, hepatic sequestration crises, or mortality. The most important side effect was myelosuppression which occurred in almost all patients but this did not give rise to any serious complications. No deaths could be attributed to the HU treatment. Still, questions remain concerning the teratogenic and carcinogenic potential of HU, its effects on growth and development in children, and the long-term effect of HU on progression of organ damage and mortality.

We have studied the hematological and clinical effects in response to HU treatment in adult SS patients during a 10-month period (from august 1992 till june 1993). After one year without HU the same patients, except two, were again treated with HU for a period of five months. One patient was treated for 22 consecutive months. Our data show that HU improves hematological and biochemical values in most patients during both treatment periods. However, although clinical symptoms ameliorated in some patients, others did not observe an improvement in their general condition, and one patient experienced a life-threatening liver sequestration.

Methods

Eight patients, four males and four females and ranging in age from 20 to 58 years (mean 38 yrs), participated in the study. Each patient had four α -globin genes ($\alpha\alpha/\alpha\alpha$), had their β^S haplotypes determined, and expressed different levels of disease severity. Detailed explanation of the study and possible effects of HU treatment were given on two successive visits in the presence of a relative. At the

second visit all patients gave their written consent. The study was conducted with the approval of the Ethical Board of the St. Elisabeth Hospital, Curaçao, The Netherlands Antilles. Before starting the HU therapy, base line CBC values, reticulocyte counts, lactate dehydrogenase (LDH), unconjugated bilirubin, kidney function, HbF and G_γ levels, were determined. HbF was quantitated by cation exchange high performance liquid chromatography (HPLC)⁹ and the G_γ levels by reversed phase HPLC¹⁰. None of the patients took any medication other than folic acid. Iron levels were measured and patients were given an iron supplement if considered necessary. Pregnancy was ruled out. Creatinin clearance of each patient was within normal limits and none of the patients was addicted to narcotics.

After the start of HU treatment, the various blood tests were done at least twice a month. Patients were ambulatory and visited the policlinic twice a month at which time compliance was evaluated and symptoms and complaints were registered. Painful crises were defined as severe pains in the bones of the extremities, back, abdomen or chest which were not relieved by non-narcotic analgesics (500 mg acetaminophen); some made a hospital admission necessary. During the second treatment period, radio isotope (technetium) scans of the liver and spleen were made before and after five months of therapy. The starting dose of HU in the first treatment period was 15 mg/kg/day, to be taken during four consecutive days a week. The dose was increased by 5 mg/kg/day at four-week intervals until bone marrow suppression or other significant side effects appeared, or to a maximum of 30 mg/kg/day. In the event of bone marrow suppression (absolute reticulocyte count $< 40,000$ per mm^3 , a leucocyte count < 4000 per mm^3 or a platelet count $< 150,000$ per mm^3) the dose was adjusted downward with 5 mg/kg/day. In the second treatment period most patients were started and maintained on the ideal dosage determined during the first treatment period.

Patients (Table VII.1)

All participants, except for patient E and H, carried haplotype #19 on each chromosome (19/19 or Benin/Benin) as well as four α -globin genes ($\alpha\alpha/\alpha\alpha$). Patient E was found to have a combination of haplotypes #19 and #3 (19/3 or Benin/Senegal) with four α -globin genes. Patient H carried a combination of Haplotypes #19 and #20 (Benin/Bantu) and had a normal number of α -globin genes. Patient A, a 58-year-old male, has a remarkably benign clinical disease. He had peptic ulcer disease 3 years before the study but has not experienced a painful crisis for over two decades. He is employed as a bus driver. Patient B, a 33-year-old female, has been admitted to the hospital about twice a year for pain crises. She frequently complains about pain in her lower abdomen possibly due to minor intestinal crises or irritable bowel syndrome. Ultrasound examination and colonoscopy did not detect any abnormalities. Patient C, a 37-year-old male, experienced approximately two painful crises a year during the previous five years,

but is otherwise well. Patient D, a 37-year-old female, has had two painful crises during the past five years, cholecystectomy in 1988 and eclampsia with birth of a premature but healthy infant in 1987. Patient E, a 48-year-old female, was free of pain crises for over 15 years and is in good general condition.

Patient F, a 20-year-old male, has an average of three painful crises per year but no other complications. Patient G, a 41-year-old female, experienced only two painful crises during the past 10 years. However, she did receive a total of 12 transfusions due to hemolytic crises, in conjunction with an otherwise uncomplicated delivery and cholecystectomy for symptomatic cholelithiasis. The latter occurred one month prior to the HU treatment. Patient H, a 35-year-old male, suffered about two painful crises per year over the past 10 years, localised in either his extremities and back or abdomen. During this period he was hospitalized once for acute chest syndrome and has been treated on an ambulatory basis for leg ulcer. He has not been able to maintain full-time employment. The history of patient I, a 31-year-old female, who participated in the second phase of the HU treatment only, reveals a relatively low hemoglobin level secondary to chronic hemolysis and possibly aplastic crisis. She received 23 units of blood during the past 15 years. She had chronic ulcers on both legs for over one year prior to the HU treatment but did not experience any painful crisis during the previous years.

Table VII.1 Laboratory data for 8 SS patients treated with HU during the first phase of the study.

Patient sex-age	Months of R _x	HU ¹ mg/kg/day	Hb g/dl	MCV fl	HbF %	G _γ %
A	0	0	6.1	101	6.0	40.9
M-53	10	15	7.3	118	20.7	47.0
B ²	0	0	7.2	85	2.3	34.5
F-33	10	20	8.4	104	11.0	40.2
C	0	0	10.1	89	8.8	39.2
M-37	10	30	10.9	101	18.4	44.0
D	0	0	9.1	99	10.3	43.8
F-37	10	30	9.9	113	19.7	42.4
E ³	0	0	7.8	103	13.4	63.6
F-48	10	17.5	10.1	120	21.7	67.0
F	0	0	8.6	89	5.0	33.2
M-20	10	30	9.8	102	12.3	34.3
G ²	0	0	6.1	102	2.7	34.3
F-41	22	25	7.5	123	10.4	31.1
H	0	0	7.6	84	1.3	42.2
M-35	10	30	8.9	93	5.2	40.2

All patients were homozygous for β^S haplotype 19, except patient E who was 19/3 and patient H who was 19/20. All had four α-globin genes.

¹ 4 days/week; ² patients B was transfused in the 6th month of HU treatment and patient G was transfused one month prior to HU treatment; ³ HU increased to 25 mg/kg, interrupted for 2 weeks in the 5th month and reintroduced at a dose of 17.5 mg/kg/day.

Results

Dosage

During the first treatment period the dosage for patients C, D, F and H, was monthly increased to 30 mg /kg/day (average 1,500 mg/day) for four days a week, without signs of bone marrow suppression. The dosages in patients A, B and G were not increased beyond 15, 20, and 25 mg/kg/day, respectively, since a doubling of the HbF level was observed at these dosages. Patient E developed recurrent epistaxis (without trombocytopenia) after four months. HU treatment was interrupted for 2 weeks and subsequently restarted and maintained at 17.5 mg/kg/day. Except for patient G, all patients were treated for 10 months during the first trial period. Patient G received uninterrupted HU treatment for up to 24 months. Patient C was lost to follow-up after the first trial.

In the second treatment phase patients B, D, F, H and I were prescribed a daily dosage of 20 mg/kg (= 1,000 mg/day) of HU for seven days a week. Patients A and E were respectively given 15 and 17.5 mg/kg/day (= 1,000 mg/day) of HU for four days a week. Patients F and H admitted that they did not take their medication on a regular basis during the second treatment phase. Therefore, we did not include their results obtained during this period.

Hematologic and Biochemical Responses (Tables VII.1 and VII.2)

All patients, except for patient F and H, revealed an increase in MCV and total Hb level during both treatment periods. The lack of compliance by patients F and H during the second treatment phase was confirmed by the absence of increases in their MCV values and HbF levels. In most patients reticulocytes decreased during first and second treatment phases, which was more likely due to HU- induced cytotoxicity than lessened hemolysis, for LDH levels showed no consistent pattern of change. No thrombocytopenia or neutropenia occurred. Patient B developed abnormal liver tests during the first treatment period as a consequence of a liver sequestration crisis. None of the other patients showed abnormalities in kidney or liver function. During the first trial, the levels of HbF increased in all patients 2- to 5- fold and returned to base line between trials. The increase in HbF levels during the second treatment period appeared earlier in most individuals, while the values in patients A, B and E were higher than in the first trial.

Patient E had a $^c\gamma$ level that was twice that of the other patients which can be explained by her carrying # 19 and # 3 β^s haplotype. The other patients displayed some inter-individual difference in the $^c\gamma$ values (from ~ 32 to 44%) which remained remarkably constant, implying that no preferential activation of the $^c\gamma$ genes occurred (Table VII.1).

Table VII.2 Laboratory data for 7 SS patients who participated in the second phase of the HU therapy

Patient sex-age	Months of R _x	HU ¹ mg/kg/day	Hb g/dl	MCV fl	HbF %
A	0	0	5.1	91	6.2
M-58	5	15	6.9	124	26.3
B	0	0	8.2	84	2.5
F-33	5	20	9.0	113	17.1
D	0	0	8.3	95	12.4
F-37	5	20	8.9	103	14.5
E	0	0	8.3	108	12.9
F-48	5	17.5	9.5	130	29.5
F	0	0	9.7	95	6.8
M-20	5	20	7.8	94	7.5
H	0	0	7.9	79	1.5
M-35	5	20	8.0	84	1.4
I	0	0	5.2	93	4.3
F-31	5	20	7.2	131	25.2

¹ Patient A and B were treated with HU for 4 days/week, others for 7 days/week.

Clinical responses

Patients A, C and D, who were known to have relative mild SS disease, did not show any change in their clinical condition during treatment. Patient B was admitted to the hospital twice during the first four months of treatment because of painful crises. In the sixth month, she experienced a third painful episode which was complicated by a liver sequestration crisis (Figure VII.1).

Figure VII.1 Response to HU therapy in Patient B experiencing a life-threatening liver sequestration crisis.

Because of this life-threatening situation she was transfused with two units of packed cells, and intravenous heparin was administered, followed by oral acenocoumarol. She recovered completely. In between trials she was seen twice because of pain crises in the extremities not responding to non-narcotic analgesics. She also developed complains of dyspnea on exertion which was attributed to mild high output failure; she was treated with diuretics and digoxin. As before, she continued to complain about regular cramping pains in her abdomen; these symptoms were treated symptomatically. During the second treatment phase she only complained about occasional dysmenorrhea. Patient E maintained a mild course without painful crises or other vaso-occlusive complications but experienced recurrent epistaxis four months into the first trial. The epistaxis was believed to be HU related and possibly caused by local hyperemia. Examination of the nasal passage revealed no other abnormalities. After interruption and reintroduction of the HU treatment at a lower dose, epistaxis did not reoccur. Patient F did not have any painful crisis or other specific symptoms during the 10 months of HU treatment. Patients G, H and I experienced some improvement in their endurance, probably due to the increase in their total Hb level. Patient H resumed full-time employment, and again complained of lack of energy in the year following the first trial. Also, one of the chronic ulcers seen in patient I was healed and another became considerably smaller during HU treatment although topical ointments remained unchanged. We did not detect spleen activity on isotope scanning which suggests that the lost splenic function in adult SS patients was not significantly restored during five months of HU treatment.

None of our patients complained about adverse symptoms related to the ingestion of HU, nor did any of them show a pre-malignant or malignant disorder. However, it should be noted that the course of treatment was still relatively short.

Discussion

Earlier studies as well as the one reported here show that some patients do benefit from treatment with HU because of a reduction in painful crises. Several patients expressed improvement of endurance and were able to keep up their job requirements. It remains impossible to predict which patient will respond favorably to HU. The MCV value is useful in determining proper compliance but, although in most instances it correlates with the HbF level, the magnitude of the HbF response cannot be estimated from the increase in MCV value. Increase in MCV as well as HbF levels does not lead to significant clinical amelioration in all patients. Patients above 37 years of age who participated in our study appear not to benefit substantially from this therapy. Major vaso-occlusive complications, except for the chest syndrome, are not prevented and, in some cases may even have been

provoked by HU¹¹. Individuals carrying the Asian and Senegal β^S -globin haplotypes¹²⁻¹⁴ as well as SS patients with an additional α thalassemia^{15,16} are known to exhibit clinically milder disease, have a better prognosis, and can be expected to benefit less from HU therapy.

These facts should caution against non-selective administration of HU to SS patients. Furthermore, although HU therapy has not revealed an increase in secondary malignancies¹⁷, data after long- term use of this drug are not yet available. Not much is known about it's possible teratogenic effect and hence prescribing HU to patients of reproductive age still demands appropriate precautions.

In addition to the effects on bonemarrow and cellular hydration, hydroxyurea has been reported to have significant effects on vascular endothelial cells in vitro¹⁸. This indicates that the ultimate clinical effect of HU is certainly not solely dependent on changes in hematologic profile and HbF and F - cell response, but also on factors involved in the mechanisms of adhesion of bloodcells to vascular endothelium. Recent studies have shown that cytokines, endothelium, and soluble adhesion molecules aswell as their membrane-integrated counterparts on blood cells play an important role in the mechanism of vaso-occlusive crisis¹⁹⁻²³. Changes in the activity of specific adhesive processes may therefore be an additional measure of the clinical response to HU (and other therapeutic compounds). We conceive that carefull measurement of the activity of steady state adhesive processes aswell as taking into concern patient's age, β^S -globin haplotype or presence of concomittant α -thalassemia could enable selection of SS individuals who will truly benefit from therapy.

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Chapter VIII

Cytokines and soluble adhesion molecules in sickle cell anemia patients during hydroxyurea therapy

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Abstract

Hydroxyurea (HU) induces HbF production and can reduce painful crises in some patients with sickle cell anemia (SS). However, HbF induction alone cannot explain the beneficial effect of HU treatment as some patients experience clinical improvement while showing only minor increases in HbF. Other actions of HU, in particular its effects on vascular endothelium, adhesion molecule expression and cytokine production may also play a role in the final therapeutic outcome. In order to analyse these effects we have studied the levels of interleukin-3, interleukin-6, granulocyte-macrophage colony stimulating factor, erythropoietin, stem cell factor, soluble (s) vascular adhesion molecule-1, s-intercellular adhesion molecule-1, sE-Selectin and sP-Selectin in 7 SS patients before and during five months of HU treatment. Use of HU seems to have no detectable effect on soluble adhesion molecules. Our results are in agreement with earlier data showing an increase of steady state sVCAM-1 compared to other adhesion molecules. Of the cytokines studied, only IL-3 showed an increase during therapy suggesting HU may induce early erythroid progenitors capable of producing HbF by a direct or indirect effect on IL-3 production. Remarkably, the steady state SCF levels in sickle cell patients seemed to be decreased compared to healthy controls.

Introduction

Epidemiological observations on large sickle cell populations show that patients with high HbF levels usually exhibit clinically milder disease and have a better prognosis^{1,2}. This forms the basis for treatment with HU, which has been shown to induce HbF production³⁻⁶. As recently revealed by a multicenter trial in the United States, HU reduces the frequency of vaso-occlusive manifestations in some patients with sickle cell disease⁷. Most of the beneficial effect has been attributed to the increase in HbF content of red blood cells resulting in less HbS polymerization. The mechanism of action by which HU induces HbF production is not fully elucidated. It is perhaps due to a cytotoxic action on cycling erythroblasts with preferential survival of early non-cycling erythroid progenitors with enhanced capability for γ -globin synthesis, which may undergo accelerated differentiation and maturation. However, some patients on HU therapy exhibit minor increases in HbF but do improve clinically, while others with higher levels of HbF do not. This suggests that other factors are involved which may be equally important^{8,9}. In fact, in addition to HbF induction HU improves red blood cell hydration and deformability¹⁰. In vitro studies also show that hydroxyurea provokes significant changes in vascular endothelial cells¹¹ and thereby may alter the adherence of blood cells to the endothelium. Vaso-active substances, adhesion molecules and cytokines are important mediators in the process of adherence and hematopoiesis¹²⁻¹⁵. Fluctuations in the levels of these molecules may have profound influence on hematopoietic activity, the adhesion of blood cells (among them sickle cells) to vascular endothelium and eventually also clinical manifestations of disease. Recently, in vitro research showed sickle cells to be dependent on vascular cell adhesion molecule-1 (VCAM-1) for binding to endothelium¹⁶. Soluble VCAM-1 levels in sickle cell anemia (SS) patients appear to be significantly increased compared to normal controls suggesting a continuously activated endothelium state^{17,18}. In addition, the important inflammatory cytokines (tumor necrosis factor- α , interleukin-1 (IL-1), interleukin-6 (IL-6)) and hematopoiesis related cytokines (interleukin-3 (IL-3), granulocyte-macrophage-colony stimulating factor (GM-CSF)) have all been found elevated in sickle cell patients^{14,19}, which is most probably the result of endothelial activation as well as tissue damage by sickling and vasoocclusion in the microvasculature. Altered cytokine levels may also depend on white blood cell (WBC) activity. Low WBC counts have been associated with longer survival and fewer crises²⁰.

The level of bone marrow activity varies among SS patients. SS patients with a high level of bone marrow activity typically carry low levels of HbF (<9%, LFSS), have increased numbers of circulating BFU-E and elevated plasma levels of GM-CSF. Patients with low bone marrow activity have high levels of HbF (HFSS), normal numbers of circulating BFU-E and low levels of GM-CSF. In contrast,

IL-3 levels are increased in HFSS and low in LFSS¹⁹. These findings suggest that GM-CSF and IL-3 are involved in the regulation of SS circulating early erythroid progenitor cells and possibly play a role in the HbF induction. Furthermore, erythropoietin (EPO), IL-3, GM-CSF and stem cell factor (SCF) are all able to stimulate HbF production in cultures of erythroid progenitor cells²¹⁻²³. In order to examine the contribution of cytokines and adhesion molecules to the therapeutic effect of HU as well as the role of cytokines in HbF induction we studied the levels of several cytokines and soluble adhesion molecules in SS patients before and during HU treatment.

Methods

Patients

Criteria for patient selection were age between 20 and 60 years, carrier of β 19 haplotype (Benin-type), normal liver and renal function, normal folic acid, vitamin B-12, serum iron and ferritin levels, and no transfusions in the 6 months prior to the trial or vasoocclusive crisis within 2 weeks from the start of the study. Pregnancy was ruled out before the start of the study. Seven patients, 2 males and 5 females and ranging in age from 31 to 58 years (mean 40 years) participated in the study. Each patient had four α -globin genes ($\alpha\alpha/\alpha\alpha$) and all patients, except for patient D and F, carried haplotype 19 on each chromosome (19/19 or Benin/Benin). Patient D was found to have a combination of haplotypes 19 and 3 (19/3 or Benin/Senegal) and patient F was 19/20 (Benin/Bantu). None of the patients, except for patient F, experienced a painful crisis in the 3 months prior to the trial. Patient F recovered from a severe airway infection with pain crisis 2 weeks before the start of HU. Patient B was on acenocoumarol because of a liver sequestration crisis two years prior to the trial. She developed a mild high output failure one year before the start of this study and was treated with diuretics and digoxin. Patient G suffered from chronic ulcers on both legs for over 1 year prior to the HU treatment. Patients A,C,D and E did not experience any clinical symptoms or complications in the 3 months prior to the study nor did they use any medication on a regular basis other than folic acid. Each patient received a daily supplement of 5 mg of folic acid. Compliance during the study was evaluated by pill count and by interpretation of the value of the mean corpuscular volume (MCV). The trial was conducted with the approval of the ethical board of the St. Elisabeth Hospital, Curaçao, Netherlands Antilles and all participants gave written informed consent. Ten normal HbAA individuals served as controls.

HbF, cytokines & adhesion molecules

HbF was quantitated by cation exchange high performance liquid chromatography (HPLC)²⁴ and cytokines by Elisa technique according to the manufacturer's guidelines (IL-6: CLB, Netherlands; IL-3, GM-CSF and soluble intercellular adhesion molecule-1 (sICAM-1): Medgenix, Belgium; sVCAM-1, sE-Selectin, sP-Selectin, Epo and SCF: R&D, UK).

Therapy

HU was administered over a five month period in a dose ranging from 15 to 20 mg/kg/day (Table VIII.1). The ideal dose was determined in a study one year earlier⁹. Before and during HU treatment bloodcounts were performed twice a month. HbF, IL-3, IL-6, GM-CSF, sVCAM-1, sICAM-1, sE-Selectin, sP-Selectin, SCF and Epo were determined before start of treatment and at one and five months. Patients were ambulatory and visited the policlinic twice a month at which time compliance was evaluated and symptoms and complaints were registered.

Statistical analysis was performed by Wilcoxon signed rank test.

Table VIII.1 Levels of sVCAM-1, Epo, IL-3, SCF, GM-CSF and HbF in SS patients before and during HU therapy.

Patient (sex-age)	β S-globin haplotype*	Months of Rx	HbF %	IL-3 pg/ml	SCF pg/ml	Epo mIU/ml	sVCAM-1 ng/ml	GM-CSF
A (M-58)	19/19	0	6.2	7.3	559	403	1.910	13.8
		1	-	13.3	785	1.674	2.273	-
		5	26.3	11.2	841	606	1.899	10.0
B (F-33)	19/19	0	2.5	22.4	565	85	1.204	10.6
		1	4.3	16.1	363	43	1.306	-
		5	17.1	19.5	446	88	1.320	8.1
C (F-37)	19/19	0	11.4	22.4	527	68	924	-
		1	-	-	536	49	1.059	-
		5	14.5	44.3	520	128	870	8.3
D (F-48)	19/3	0	12.9	9.2	702	81	2.420	8.7
		1	17.1	16.3	757	59	2.295	-
		5	29.5	32.0	737	57	1.735	7.4
E (F-41)	19/19	0	7.8	4.3	649	113	1.370	8.1
		1	13.5	11.5	435	89	777	-
		5	10.4	19.9	625	144	963	10.6
F (M-35)	19/19	0	1.4	4.0	545	89	4.086	7.1
		1	2.2	3.8	480	32	2.001	-
		5	1.5	7.6	320	83	1.725	5.6
G (F-31)	19/19	0	4.3	12.5	1.055	141	3.814	13.8
		1	10.9	29.3	1.066	138	2.164	-
		5	25.2	21.3	1.059	43	2.195	10.0
Controls**			-	30.2 $\pm$ 16	1.133 $\pm$ 303	10 $\pm$ 4.9	596 $\pm$ 177	0

Patient A was treated with 15 mg/kg/day and patient B with 20 mg/kg/day for 4 days per week; patient C was treated with 17.5 mg/kg/day for 7 days/week; all other patients were treated with 20 mg/kg/day for 7 days/week. Rx = * all patients carry 4 α -globin genes; ** adult HbAA individuals with normal hematology.

Results

Like in previous studies, HbF increased in the majority of patients who took HU (Tables VIII.1 and VIII.2). The HbF level increased 2 to 5 fold in all patients except in patient C and F (Figure VIII.1). Patient C showed a minor increase in HbF. Patient F only had a short-lived increment in his HbF which was explained by proper compliance during the first 2 months of treatment (as confirmed by increase of MCV from 79 to 87 fl and correct pill count) but failing compliance thereafter. A significant decrease in reticulocyte counts was observed suggesting a reduction in the rate of hemolysis (Table VIII.2). WBC counts showed a slight but not significant decrease. No bone marrow toxicity was observed. Except for patient G, who showed healing of one leg ulcer, no significant changes were observed in the clinical condition of the patients during HU treatment.

Table VIII.2 Mean laboratory values before starting HU (initial) and after 5 months of treatment with HU (final).

	Initial value	Final value	p value
Total Hb, g/dl	7.1 ± 1.5	8.1 ± 1.0	n.s.
Leukocytes, x 10 ³ /μl	10.8 ± 2.1	9.0 ± 1.7	n.s.
Lymphocytes x 10 ³ /μl	3.4 ± 1.0	3.2 ± 0.7	n.s.
MCV, fl	94 ± 11	115 ± 17	<0.05
Absolute reticulocytes, x 10 ³ /μl	259 ± 95	98 ± 60	<0.05
HbF, %	6.6 ± 4.3	17.8 ± 10.0	<0.05
Epo, mIU/ml	140 ± 118	164 ± 198	n.s.
SCF, pg/ml	657 ± 186	650 ± 251	n.s.
IL-3, pg/ml	11.7 ± 7.8	22.3 ± 12.5	0.08
GM-CSF, pg/ml	17.8 ± 21.8	8.1 ± 1.4	n.s.
IL-6, pg/ml	7.4 ± 16.4	2.2 ± 3.5	n.s.
sICAM-1, ng/ml	127 ± 95	177 ± 104	n.s.
sVCAM-1, ng/ml	2.247 ± 1.264	1.530 ± 493	n.s.

Values represent mean ± SD. p value of <0.05 considered to be significant; n.s. = non significant

Compared to sVCAM-1 levels in a normal population, baseline levels of s-VCAM were all enhanced ($p < 0.05$, Table VIII.1). However, the baseline sVCAM -1 in patient G should be interpreted with caution since he experienced an infection and crisis only a few weeks prior to the start of this study. During the HU treatment the mean s-VCAM level showed no consistent pattern of change. The sP-Selectin, sE-Selectin and sICAM levels of the patients were comparable to healthy controls and were not influenced by HU treatment (data not shown).

As expected the SS patients revealed increased EPO levels ($p < 0.01$). However, SCF levels were lower in the SS patients compared to healthy controls ($p = 0.001$, Table VIII.1, Figure VIII.2). There existed no correlation between HbF and

baseline levels of EPO or SCF (Table VIII.1); both levels remained unchanged during the HU treatment.

Steady state levels of IL-3 were comparable to healthy controls. No correlation was observed between baseline HbF and IL-3 or GM-CSF levels. After the start of the HU therapy all patients, except patient B, showed an increase in IL-3 (Table VIII.1 and Figure VIII.1). The mean IL-3 level increased but did not reach statistical significance (Table VIII.2).

GM-CSF (Table VIII.1) and Il-6 levels (data not shown) varied considerably but appeared elevated in some patients compared with healthy controls. No specific correlation with any other parameter was observed. The levels did not change during HU therapy (Table VIII.2).

Figure VIII.1 Response of IL-3 and HbF to HU treatment in SS patients.

Discussion

Although HU therapy induces HbF production, this effect has not proven to be the only explanation for the beneficial effect of HU in SS patients. Recent studies have established that cytokines and adhesion molecules play a central role in the interaction between endothelium and blood cells. In order to examine the contribution of cytokines and adhesion molecules to the therapeutic effect of HU as well as the role of cytokines in HbF induction we studied the levels of several cytokines and soluble adhesion molecules in SS patients before and during HU treatment.

Figure VIII.2 Plasma steady state levels of SCF in SS patients compared to normal controls.

No effects of HU on soluble adhesion molecules, sVCAM-1, sICAM-1, sP-Selectin and sE-Selectin, were observed. The finding of increased sVCAM -1, levels before treatment, compared to other adhesion molecules, is in support of previous studies^{17,18,25} and might be due to the effects of VCAM-1 specific cytokines like IL-13²⁶. Furthermore, viral infections known for their ability to trigger vaso-occlusive episodes, can induce VCAM-1 expression on endothelial cells²⁷. sVCAM-1 as well as IL-6 levels have also been shown to increase significantly in the event of a vaso-occlusive crisis^{17,28}. It could be postulated that the increase in sVCAM-1 reflects a continuously activated endothelium state which renders patients more sensitive to crisis - triggering factors. Therefore it could be expected that if HU would have beneficial effects on the endothelium activity state, this should be reflected as lowered sVCAM-1 levels. However, sVCAM-1 levels showed no consistent change. Still, what should be considered is the fact that sVCAM-1 may fluctuate strongly depending on the clinical condition of the patient. In our study patient G had two legulcers and patient F had experienced a pain crisis and airway-infection two weeks prior to the start of the HU therapy. Patient B was on a coumarine derivative which has been shown to inhibit VCAM-1 expression^{25,29}. Obviously, these factors may complicate the interpretation of sVCAM-1 levels in a small group of participants such as ours.

Of the cytokines analysed only IL-3 seemed to be enhanced by HU therapy, with the exception of 1 patient. However, this patient was taking several drugs which could have interfered with IL-3 determination or biochemical activity. From studies

in vitro and in primates it is known that IL-3 can induce formation of early erythroid progenitors and F reticulocytes^{19,21} and may be produced by various celltypes, including bone marrow stromal cells³⁰. The increase of IL-3 observed in most patients after start of HU therapy is perhaps explained by induction of bone marrow stromal cells in response to the cytotoxic action of hydroxyurea on hematopoietic cells. Alternatively, a direct effect of hydroxyurea on bone marrow stromal cells or blood cells leading to production of IL-3 cannot be excluded. Nevertheless, the increment of IL-3 after the start of HU therapy may be responsible for the increase of HbF by stimulating proliferation of erythroid progenitors that carry enhanced capability for HbF synthesis. This is also supported by the correlation between steady state HbF and IL-3 reported by Croizat¹⁹. The correlation between steady state HbF and IL-3 could not be confirmed by us possibly due to the small sample size and discrepancies between studies in the phenotypical background of SS patients.

In addition to IL-3, SCF is also active during early hematopoiesis. We show for the first time a lowered steady state SCF level in SS patients compared to normal controls. Epo levels in SS patients have been reported to be increased, but relatively low for the degree of anemia³¹. It has been shown that SS- circulating progenitors express an increased sensitivity to Epo, which is the result of the interaction between these cells and their environment³². Increased activity of other factors, in particular cytokines which induce proliferation and differentiation of early erythroid progenitors in stress situations, has been postulated to explain the relatively low Epo levels in SS patients. Perhaps, a similar mechanism could also be of influence on circulating SCF levels.

In conclusion, we have found no evidence of a significant effect of HU at the level of vascular endothelium. However, as soluble adhesion molecules tend to fluctuate depending on external factors and the clinical condition of the patient, proper interpretation of HU effects on adhesion molecules will probably require a longer duration of study and a larger group of patients. Cytokine analysis showed a tendency of IL-3 levels to increase during HU therapy, suggesting IL-3 to be involved in the induction of erythroid progenitors capable of producing HbF. Also, a specific decrease of steady state SCF levels was shown. Future studies should further clarify the role and use of these cytokines in the treatment of SS patients.

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Chapter IX

**Levels of endothelial, neutrophil
and platelet specific
factors in sickle cell anemia
patients during hydroxyurea
therapy**

AW Saleh, HFP Hillen, AJ Duits

Abstract

It has been shown that the clinical course of sickle cell (SS) patients can be ameliorated by administration of hydroxyurea (HU). Induction of HbF is thought to be the mechanism responsible for clinical improvement in some patients. However, HU has a variable effect on HbF production and there exists no good correlation between the extend of HbF increase and clinical response. On the other hand the degree of adherence of sickle cells to vascular endothelium and neutrophil counts correlate well with clinical severity. Being a cytotoxic drug, used in myeloproliferative diseases, HU may alter proliferation among various cell lines. Moreover, HU has been reported to reduce RBC adhesion receptor expression in young SS individuals and induces changes in endothelial cells in vitro. It should be conceived that, in addition to its effects on HbF production, HU may change the clinical symptoms of SS patients by affecting the degree of adherence of different blood cells, by influencing the activity of endothelium as well as the activity of WBC and platelets. To analyze whether several of the determinants of adhesion are modulated by HU treatment we studied the levels of endothelial activity [soluble vascular adhesion molecule-1(sVCAM-1), IL-8, fibronectin], neutrophil activity [sL-Selectin, sIL-6 receptor- α , myeloperoxidase] and platelet activity [von Willebrand Factor] in relation to clinical symptoms, hematological data and HbF levels in 8 SS patients before and during 5 months of HU therapy. Steady state sVCAM-1 levels are increased compared to normal controls and a significant decrease is noted during HU treatment, suggesting a decrease in the interactions between RBC and vascular endothelium. The IL-8 levels are comparable to those in normal controls and remain unaffected by HU therapy. Intercurrent infection and crises reveal striking increases in IL-8 which are accompanied by leucocytosis, but otherwise the IL-8 levels do not correlate with hematological data. HU has no demonstrable effect on fibronectin or soluble neutrophil adhesion molecules, but the levels of myeloperoxidase decrease significantly while WBC counts do not, implying a reduction in neutrophil activity which may help attenuate the propagation phase of a vasoocclusive crisis.

Introduction

Hydroxyurea (HU) has been used in sickle cell (SS) anemia with the purpose of inducing HbF production and improving the clinical condition of patients. Following treatment with HU, SS patients are known to exhibit different levels of HbF increase and some patients show clinical improvement measured as a reduction in the frequency of painful crises¹. However, clinical amelioration does not always parallel HbF increment. Some SS patients with considerable HbF increase have been reported to fare worse and others, with very little HbF increase, experience clinical improvement^{2,3}. This discrepancy suggests that factors other than the HbF level may be important to the clinical outcome in response to HU. The factor that is responsible for much of the pathophysiology of sickle cell anemia is adhesion of blood cells resulting in microvascular occlusion. The adhesion of young deformable sickle cells to endothelium, through receptors such as integrin VLA-4 and CD36 on red blood cells and vascular adhesion molecule-1 (VCAM-1) on endothelial cells, has been shown to play an important role in the initiation of the vaso-occlusive crisis⁴⁻⁶. Furthermore, effects of polymorphonuclear leukocytes and several plasma factors, among which cytokines, procoagulant and vaso-active substances also influence the adhesive processes⁷⁻¹⁰. White blood cell (WBC) counts are often increased in sickle cell patients and WBC counts of more than 15,000 cells/ml are in fact associated with an increased risk of early death¹¹. Plasma proteins that have been implicated in promoting the adhesion between sickle cells, WBC (predominantly neutrophils) and endothelium include fibronectin, fibrinogen, von Willebrand factor and thrombospondin¹²⁻¹⁵. Several cytokines can induce multiple activation states of the endothelium and activate leucocytes^{5,16,17}. Interleukin-8, in particular when present in high concentration, may also directly activate the adhesion specific integrin VLA-4 on sickle cells¹⁷. On the other hand, red cell absorption of IL-8, with trapping in the RES system, may be a mechanism of limiting a systemic inflammatory process¹⁸. Among all these determinants of adhesion, the vascular endothelium itself, is also considered to be an important factor in the initiation of the vaso-occlusion^{19,20}. In addition to HbF induction, HU treatment has already been shown to be responsible for changes such as macrocytosis, improved red blood cell (RBC) deformability and hydration status^{4,21}. Moreover, a recent study has shown decrease of VLA-4 and CD36 on reticulocytes in pediatric SS patients treated with HU²². Still, in view of the multifactorial etiology of sickle cell vaso-occlusion many other actions of HU in sickle patients, should be considered. Here we report the effects of hydroxyurea on endothelial and neutrophil activity as well as on IL-8 and von Willebrand factor (vWF) in relation to the clinical symptoms, hematological data and HbF levels in SS patients.

Materials and methods

Patients

Criteria for patient selection were age between 18 and 65 years, carrier of β haplotype 19 (Benin type) and four α -globin genes, normal liver and kidney function and normal iron levels. Eight patients, four males and four females and ranging in age from 18 to 60 years (mean 39 yrs), participated in the study. Six patients carried haplotype #19 on each chromosome (19/19 or Benin/Benin). One patient (patient D) had a combination of haplotype #19 and #3 (Benin/Senegal) and the other (patient F) had haplotype 19/20 (Benin/Bantu). None of the patients had received a transfusion within six months prior to the start of the study, nor had they experienced a painful crisis in the six months prior to the trial. Patient B was on acenocoumarol because of a liver sequestration crisis four years prior to the trial. She recently complained about pain in her left knee which was believed to be a consequence of sickle cell complications at the level of the cartilage. Patient D started to experience backaches one year prior to the study at a frequency of approximately once a month. This symptom was diagnosed as osteoarthritis of the spine rather than a direct sickle cell related complication. She used acetaminophen as analgesic. Patients A,C,E,F,G and H did not experience any clinical symptoms or complications in the six months prior to the study nor did they use any medication on a regular basis other than folic acid. Each patient received a daily supplement of 5 mg of folic acid. Compliance during the study was evaluated by pill count and by interpretation of the value of the mean corpuscular volume (MCV). Clinical parameters to be monitored during the study included episodes of painful crises or any other objective vaso-occlusive event. Painful crises were defined as pain in extremities, chest or back not relieved by acetaminophen or requiring admittance to the hospital. The trial was conducted with the approval of the Ethical Board of the St. Elisabeth Hospital, Curaçao, Netherlands Antilles and all participants gave written informed consent. Ten normal HbAA individuals served as controls.

Therapy

HU was administered over a five month period. Patients received a dose of 1000 mg/day or 1500 mg/day depending on their bodyweight (Table IX.1).

Sampling

To establish baseline values, blood counts, HbF, IL-8, sVCAM- sIL-6 and sL-Selectin were determined at 3 different times during a three week period prior to the start of HU therapy. During HU treatment bloodsamples were taken once a month.

Table IX.1 Hematological and clinical data in SS patients before and during HU therapy

Patient* (Sex-Age)	Months of HU therapy	PCV Ll	HbF %	Retic $10^3/\mu\text{l}$	WBC $10^3/\mu\text{l}$	Platelets $10^3/\mu\text{l}$	Clinical Data
A (M-60)	0 2 5	0.143 0.190 0.179	4.8 26.2 15.6	203 103 119	11.8 9.7 9.9	159 213 165	no complications
B (F-36)	0 2 5	0.208 0.212 0.230	3.6 6.8 10.2	361 127 153	8.1 6.3 7.1	361 261 272	no complications
C (M-39)	0 2 5	0.275 0.305 0.339	9.8 20.9 16.9	614 191 439	12.6 6.1 11.7	372 475 380	month 4 Acute Chest Syndrome Exchange Transfusion
D (F-50)	0 2 5	0.220 0.264 0.277	13.5 25.4 27.9	392 60 207	10.1 5.2 6.6	267 195 328	no complications
E (F-43)	0 2 5	0.180 0.192 0.190	4.0 5.0 13.0	155 202 58	8.8 7.1 6.9	340 312 282	no complications
F (M-37)	0 2 5	0.209 0.254 0.255	1.2 5.6 2.8	328 188 341	10.1 10.7 12.4	202 240 232	month 3 Perigium excision right eye
G (M-37)	0 2 5	0.168 0.195 0.231	10.8 15.6 20.9	254 198 227	15.4 13.2 13.9	220 285 314	month 4 Gastroenteritis**
H (M-18)	0 2 5	0.276 0.235 0.340	7.6 12.8 19.2	471 300 373	12.0 9.0 8.7	366 326 302	month 3 and 5 Minor Painful Crisis Phlebotomy (500 ml)

* All patients have four α -globin genes and, except for patient D and F, all carry the Beibib (19/19) haplotype; patient D is a 19/3 hybrid (Benin/Senegal) and patient F is a 19/20 (Benin/Bantu) type.

** Leucocytosis of $19.2 \times 10^3/\mu\text{l}$.

Table IX.2 Levels of sL-Selectin, sIL-6 receptor- α , Myeloperoxidase (MPO), IL-8, sVCAM-1, Fibronectin and von Willebrand Factor (vWF) in SS patients before and during HU therapy.

Patient* (Sex-Age)	Months of HU therapy	sL-Selectin ng/ml	sIL-6 receptor- α ng/ml	MPO ng/ml	MPO/WBC ng/10 ⁶ cells	IL-8 pg/ml	sVCAM-1 ng/ml	Fibronectin μ g/ml	vWF % of normal
A (M-60)	0 2 5	592 560 625	19.6 24.1 21.4	12.8 13.5 3.1	1.11 1.39 0.31	78.6 25.0 59.8	2253 1162 1502	269 ND 294	247 ND 197
B (f-36)	0 2 5	806 878 753	20.0 19.6 18.5	6.0 8.4 2.5	0.74 1.33 0.35	8.4 5.7 8.7	543 549 505	210 277 297	148 ND 139
C (M-39)	0 2 5	950 885 948	19.5 22.5 33.6	17.9 9.2 3.8	1.48 1.51 0.33	18.0 23.4 11.2	680 633 630	292 279 454	260 ND 198
D (F-50)	0 2 5	763 860 717	22.3 23.7 22.0	20.7 25.3 3.6	2.01 4.87 0.55	48.9 36.6 39.2	1326 1032 894	343 382 571	291 ND 221
E (F-43)	0 2 5	677 777 597	16.8 12.5 28.2	14.1 9.1 8.3	1.60 1.05 0.33	7.4 6.1 4.6	560 523 624	224 105 191	167 ND 188
F (M-37)	0 2 5	858 981 829	11.6 19.4 15.7	110.5 8.3 4.9	1.02 0.78 0.40	128.4 162.7 253.7	893 743 812	449 208 291	256 ND 242
G (M-35)	0 2 5	946 990 922	15.6 17.9 11.3	11.8 23.6 4.4	0.83 1.79 0.32	144.7 161.3 80.7***	2255 1234 1231	164 164 215	286 ND 260
H (M-18)	0 2 5	1404 1604 1133	13.4 10.9 7.8	3.5 2.4 3.9	0.29 0.27 0.45	18.2 8.9 112.9	778 751 615	363 302 285	227 ND 203
Controls**		1493 $\pm$ 290	35.7 $\pm$ 23.0	33.5 $\pm$ 28.0	-	6.0 $\pm$ 6.0	596 $\pm$ 177	443 $\pm$ 92	125 $\pm$ 75

* All patients have four α -globin genes and, except for patient D and F, all carry the Benin (19/19) haplotype; Patient D has Benin/Senegal haplotype (19/3) and patient F is 19/20 (Benin/Bantu).

** Adult HbAA individuals with normal hematology.

*** 4th month: Increase of IL-8 to 282 pg/ml (due to gastroenteritis). ND = Not determined.

Patients were ambulatory and visited the polyclinic twice a month at which time compliance was evaluated and symptoms and complaints were registered. HbF, myeloperoxidase (MPO), cellular fibronectin (FN), IL-8, soluble adhesion molecules & vWF.

HbF was quantitated by cation exchange high performance liquid chromatography (HPLC)²³. The level of neutrophil activity was assessed by determining neutrophil-specific soluble adhesion molecules, sL-Selectin (CD62L) and the sIL-6 receptor (CD64 or FcγRI) as well as plasma myeloperoxidase which is released from neutrophils. Endothelial activity was determined by measuring levels of s-VCAM, FN and IL-8. Soluble adhesion molecules, cytokines, cellular FN and MPO levels were detected by Elisa technique according to the manufacturer's guidelines [IL-8: CLB, Netherlands; sVCAM-1, sIL-6, sL-Selectin, MPO and FN: R&D, England]. Von Willebrand factor was determined by adding STA LIA vWF reagent to plasma samples. The reagent consists of latex particles coated with vWF-specific polyclonal antibodies that agglutinate in the presence of vWF. Agglutination results in increase of the optical density, which is directly proportional to the vWF concentration, and can be measured using a STA analyzer (Boehringer Mannheim Diagnostica, Germany).

Statistical analysis

Data were analysed by the Wilcoxon signed rank test. A p value of <0.05 was considered significant.

Results

Hematologic and clinical responses (Tables IX.1 and IX.3)

In most patients the hematological profile improved and hemolytic rate decreased in response to HU. HbF levels increased in all patients but showed a considerable interindividual variability. In order to avoid viscosity related vaso-occlusive complications due to increasing packed cell volumes (PCV), the protocol of the study recommended a phlebotomy at PCV values between 30 and 35 l/l or higher. Patient C developed a high PCV and unfortunately experienced a severe acute chest syndrome in the 3th month of HU therapy before phlebotomy. He recovered after an exchange transfusion was performed. Patient H developed a relatively high PCV in the 5th month of HU treatment but this was reduced by a phlebotomy of 500 ml. This patient also experienced two minor episodes of painful crises, one during the third month, and one in the 5th month of HU therapy. These crises were managed at home with analgesics. Patient A showed a decrease of his hemoglobin level by the 2th month which could have been caused by a cytotoxic effect of HU. The HU

therapy was interrupted for one week and restarted at 10 mg/kg/day (instead of the usual 15 mg/kg/day). Consequently his HbF level also decreased to some extent. The knee complaints of patient B improved subjectively. The complaints of backache of patient D remained unchanged during the study. Patient F underwent a minor surgical procedure on his eye at the start of the 3th month of HU therapy. He showed less proper compliance around this time which resulted in a decrease of his HbF level. Patient E experienced a infectious gastroenteritis at the beginning of the 4th month and accordingly revealed a leucocytosis.

Table IX.3 Mean laboratory values before starting HU (initial and after 5 months of treatment with HU (final).

	Initial value	Final value	p value
Total Hb, g/dl	7.3 $\pm$ 1.6	8.7 $\pm$ 1.9	n.s.
WBC $\times 10^3/\mu\text{l}$	11.5 $\pm$ 2.5	10.1 $\pm$ 3.7	n.s.
Platelets $\times 10^3/\mu\text{l}$	281 $\pm$ 100	287 $\pm$ 103	n.s.
MVC, fl	95 $\pm$ 9	111 $\pm$ 14	<0.05
Absolute retics $\times 10^3/\mu\text{l}$	365 $\pm$ 154	220 $\pm$ 220	n.s.
HbF, %	7.2 $\pm$ 4.1	15.2 $\pm$ 7.8	<0.05
LDH, U/l	1486 $\pm$ 522	1080 $\pm$ 407	<0.05
sIL-6 receptor- α , pg/ml	17.9 $\pm$ 6.2	19.1 $\pm$ 7.2	n.s.
sL-Selectin, ng/ml	892 $\pm$ 239	809 $\pm$ 282	n.s.
MPO*, ng/ml	12.0 $\pm$ 9.0	4.3 $\pm$ 1.7	<0.05
MPO/WBC, ng/ 10^6 cells	1.1 $\pm$ 0.8	0.5 $\pm$ 0.4	<0.05
s-VCAM-1, ng/ml	1216 $\pm$ 860	811 $\pm$ 293	<0.05
Fibronectin, $\mu\text{g/ml}$	289 $\pm$ 135	322 $\pm$ 99	n.s.
IL-8, pg/ml	61 $\pm$ 93	79 $\pm$ 104	n.s.
vWF*, % of normal	235 $\pm$ 52	206 $\pm$ 37	n.s.

Values represent mean $\pm$ SD; p value of <0.05 considered to be significant;

n.s.- Nonsignificant. * MPO=Plasma Myeloperoxidase, vWF= von Willebrand factor

Endothelial activity and RBC adhesion: sVCAM-1, FN and IL-8 (Tables IX.2 and IX.3)

Like in our previous studies^{20,24} sVCAM-1 levels were increased in steady state compared to normal controls (p <0.05). Following HU treatment the levels decreased significantly (p <0.05). The steady state FN levels of the patients were similar compared to normal controls and remained unchanged during 5 months of

HU therapy. Steady state IL-8 levels were increased in 4 patients A,D,F, and G. The steady state IL-8 levels correlated with the WBC number but not with the HbF levels. Following HU treatment the IL-8 levels did not change consistently. However, Patients G and H did show an increase of IL-8 at the time of their specific complaints. In the case of patient C no enhanced IL-8 level was registered despite the fact that he experienced an acute chest syndrome. However, blood sampling as prescribed by the protocol of this study, was performed only once a month and in fact occurred 4 days before the vasoocclusive complication.

Neutrophil activity: sL-Selectin, sIL-6 receptor- α and MPO (Table IX.2)

The baseline sL-Selectin, sIL-6 receptor- α and MPO levels of the patients were comparable to normal healthy controls. The sL-Selectin and sIL-6 receptor- α levels were not influenced by HU but MPO levels decreased. Correction of the MPO levels for the number of circulating WBC also confirmed the decrease of MPO.

Platelet activity: vWF (Tables IX.2 and IX.3)

Steady state vWF levels were increased in 6 out of 8 patients. No correlation could be detected between the vWF levels and the number of platelets, HbF, fibronectin or the IL-8 levels. During HU therapy the levels decreased slightly in the majority of patients, but the change was not significant.

Discussion

The clinical course of SS patients is characterized by symptoms due to hemolytic anemia and complications arising from microvascular occlusion. The vaso-occlusion and tissue damage is the end result of a process that involves many steps. Adhesion of young, deformable RBCs to postcapillary, venular endothelium is believed to initiate vaso-occlusion. Several other factors, such as plasma proteins, cytokines, procoagulant and circulatory factors, neutrophil activity and endothelial abnormalities, may also be involved in the initiation of vaso-occlusion or may enhance adhesion and tissue damage^{7,10,19,20}. Propagation of the vaso-occlusive crisis is thought to involve trapping of dense and irreversibly sickled cells in the microvasculature with consequent total obstruction and tissue damage. The mechanisms that determine the resolution phase of vaso-occlusion or reconvalescence remain largely unspecified. When treating SS patients with HU, improvement of their hematological values is evident and is attributed to HbF induction. However, there is evidence that suggests that clinical improvement during HU therapy is not only the result of increase of HbF, but also dependent on several factors that modulate adherence of blood cells to vascular endothelium.

In addition to the amelioration of the hematological data and increase of HbF during HU treatment of SS patients in this study, the majority of patients showed a significant decrease of sVCAM-1 levels. The decrease of sVCAM-1, being a soluble receptor specific for RBC adhesion to endothelium, most likely reflects reduced adhesion of immature RBCs to endothelium during HU treatment. This is also in agreement with the study of Styles et al.²² that showed a decrease of VLA-4 and CD36 on reticulocytes in SS patients treated with HU. Baseline IL-8 levels seemed increased in half of the patients, but remained unchanged during HU therapy. However, the IL-8 level and number of WBC correlated well with clinical events. Interpretation of FN levels, detected with commercial kits as specified in this study, is obscured for these tests do not provide a distinction between the 2 known forms of FN (cellular/endothelial FN and acute-phase reactant FN).

No effects of HU on soluble -granulocyte specific- adhesion molecules, sL-Selectin and sIL-6 receptor- α , were observed. However, the plasma MPO levels decreased significantly. MPO, a lysosomal protease produced by neutrophils, is synthesized during the very early stages of neutrophil development²⁵. It is involved in the mechanisms that cause endothelial injury and damage of the interstium after adhesion of the neutrophil to endothelium has occurred²⁶. The decrease of MPO levels may be explained by special effects of HU on granulocyte proliferation or MPO production. The findings suggest that not only a reduction in neutrophil count, but also a decrease of MPO, rather than a reduction in individual neutrophil adhesion, may help limit the extend of tissue destruction and the severity of pain in HU treated patients.

The enhanced steady state plasma vWF levels in most of the SS patients probably reflect the presence of activated platelets and their interaction with vascular endothelium which is in agreement with earlier studies⁷. Although HU therapy produced a decrease of the vWF level in some patients, the changes did not reach statistical significance. This could be explained either by the small sample size or a lack of direct effect of HU on platelets.

We conclude that in sickle cell anemia the activated endothelium state and increased adherence of young RBCs set the stage for vaso-occlusion, while many other factors can influence the adherence of cells as well as the process of microvascular obstruction. Our results showed a decrease of sVCAM-1 during HU therapy reflecting less endothelial-RBC interaction. Also the MPO levels, but not the neutrophil specific soluble adhesion molecules, decreased in response to HU treatment, suggesting a specific effect of HU on neutrophil proliferation or MPO production. This finding may represent a new clinical important action of HU in sickle cell anemia

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Chapter X

**Summary and conclusive
remarks**

Summary and conclusive remarks

Sickle cell anemia, being the most common hereditary hemolytic anemia in the world, still presents a difficult medical problem. The cytotoxic agent hydroxyurea has recently shown to cause clinical amelioration in some sickle cell patients but it remains difficult to predict which patients will benefit from therapy with this drug. The beneficial effect of hydroxyurea has been mainly attributed to the induction of fetal hemoglobin (HbF). In this thesis it was investigated if the clinical effects of hydroxyurea in sickle cell (SS) patients were due to HbF induction only, or whether other mechanisms were also involved. Moreover, patients treated with hydroxyurea were studied in order to find some criteria that would adequately predict which patients would benefit from this treatment. Also, the effects on HbF production of isobutyramide, a non-cytotoxic alternative to hydroxyurea, were studied.

In Chapter I a discussion on the pathophysiology, diagnosis and treatment of sickle cell anemia is presented. Standing at the basis of the disease is a point mutation in the β globin gene which results in a substitution of the amino acid valine for glutamine at position 6 in the β chain of hemoglobin giving rise to the abnormal hemoglobin S. Hemoglobin S will polymerize in deoxygenated state and thus may form large strands of polymeres which cause the red blood cell to deform (typically a sickle shape) and interrupt red cell membrane function. Abnormal adherence, sludging and microvascular occlusion by sickle cells may cause ischemic damage which is clinically expressed as pain crises or specific organ vaso-occlusion. Hemolysis, another important clinical feature, results from increased breakdown of sickled cells. However, the clinical severity of disease of the SS patients reveals marked differences and may be influenced by many factors such as those that affect the HbF level and coexistent α -thalassemia, factors determining the level of total hemoglobin and other hematological parameters, plasma components, vascular and environmental factors. The severity of sickle cell disease also correlates very well with the leucocyte count and the number of young deformable sickle cells which are known for their tendency to adhere to vascular endothelium. This adherence may be significantly modulated by the degree of imbalance in substances like nitric oxide, prostacyclins, platelet derived growth factor, endothelin, free oxygen radicals, proagulant factors, ligand proteins and cytokines that all interact with endothelium. The other factors that play a role in the clinical expression of disease are the extend of prior organ damage, changes in properties of the red blood cell itself and environmental factors. Treatment of the patient with sickle cell disease is still mainly supportive and symptomatic. Improved nutrition, better living standards, treatment and prevention of infections (vaccinations and antibiotic treatment) are responsible for the increase in life expectancy of SS patients during

the last decades. Rehydration, oxygen delivery, analgesia and in some situations antibiotics and exchange transfusions, help to relieve vaso-occlusive crises. Pharmacological HbF stimulation by hydroxyurea has resulted in improvement of symptoms in some patients. In the near future, butyrates and hematopoietic growth factors that also induce HbF synthesis could perhaps be useful either as combination treatment with hydroxyurea or as an alternative to hydroxyurea. Other chemical products that have been tried to ameliorate or prevent symptoms of the disease are drugs that have an antisickling effect, agents that increase oxygen affinity or promote capillary flow and anticoagulation. The benefit of most of these agents could not be confirmed in controlled trials and for some the data from ongoing trials are still unavailable. Experience with allogenic bone marrow transplantation for sickle cell disease has increased considerably, but transplantation still carries a mortality of 10%, as well as a risk of infertility. Moreover, there are still no data on chemotherapy-induced malignancies. Gene therapy, still the subject of intensive investigate research at the laboratory level, is considered to become the treatment of choice in the future. Meanwhile, with the growing knowledge of mechanisms of cellular adhesion, therapies that intervene at the level of cell adhesion to vascular endothelium should be explored.

Chapter II focuses on epidemiological and clinical aspects of SS disease in Curaçao using laboratory data, information on β^S haplotypes, α -gene number and clinical features of a local group of 18 SS patients. Based on the findings in this group, the majority of the estimated 100 local SS patients are expected to carry haplotype 19/19 or the Benin type. The combination of HbS or HbC with α -thalassemia carries an incidence of 4,1 %. No attempt has been made so far to detect compound heterozygotes for HbS and β -thalassemia. The painful crises and chronic hemolysis are the most common clinical presentations. Legulcers also have a high incidence and often reoccur repetitively in the same individual. The acute chest syndrome has a lower incidence but may be lifethreatening and is the most common cause of death in sickle cell patients in Curaçao. Before the sixties the average life expectancy of local SS patients was reported to be 20 years but this figure has undoubtedly improved in view of the advances in local health care over the past three decades. Environmental and psychosocial aspects as well as local management are briefly discussed.

Chapter III provides a review on the regulation and role of the HbF level as well as on the factors that influence the HbF levels. The clinical significance of HbF and the rationale of HbF induction in sickle cell anemia are discussed. The generally accepted fact that increased HbF levels have an inhibitory effect on sickling and ameliorate the clinical course of sickle cell disease has triggered research of pharmacological compounds that stimulate HbF synthesis. Agents such as

hydroxyurea, butyrate and its derivatives, erythropoietin and other hematopoietic growth factors have been shown to induce HbF production. The mechanism of action and clinical effects of these agents are reviewed.

The main objectives of the studies (chapter IV) described in this thesis were: A) to analyse the effects of hydroxyurea and the oral butyrate derivative, isobutyramide, on HbF production in sickle cell anemia. B) To determine which factors may predict the HbF as well as the clinical response of SS patients, and C) to study the effects of hydroxyurea on cytokines and soluble adhesion molecules, specifically involved in adherence of red cells and neutrophils to vascular endothelium, as well as hydroxyurea effects on endothelial and neutrophil activity and the procoagulant von Willebrand Factor.

Chapter V offers a short description on the methods that were used for detection and quantification of the various parameters that were studied in this thesis.

Chapter VI. The effects of isobutyramide on HbF synthesis were analysed as a treatment alternative to hydroxyurea, which is theoretically assumed to have a carcinogenic potential. Isobutyramide, an oral derivative of butyrate with a long half life, was administered to SS patients over a period of three months. The dosage was started at 50 mg/kg/day and increased in increments of 25 mg/kg up to 150 mg/kg/day. The maximum dose was maintained for three weeks. No significant change in HbF levels occurred but at the maximum dose of isobutyramide a relative increase of $\alpha\gamma$ levels (one of the two globin chains of HbF) was observed. While in three patients the rate of hemolysis remained stable during treatment, one patient revealed an increase in lactate dehydrogenase. None of them experienced painful crises or other vaso-occlusive events. All patients developed temporary epigastric discomfort related to ingestion of isobutyramide. The trial was terminated when the epigastric discomfort turned to persistent intolerable dyspepsia in one patient. In view of these findings treatment with isobutyramide can not be recommended as a addition or alternative to hydroxyurea therapy.

During the first study with hydroxyurea in Curaçao (chapter VII) nine SS patients (eight with haplotype 19/19 and one with haplotype 19/3) have been treated during a 10 month period. Six of these patients were again treated with the drug during five months after an intermission of one year. Their individual hematological and clinical responses to this treatment are discussed. The hematological and biochemical values, the HbF level and the number of painful crises improve during therapy with hydroxyurea. However, although some patients experience clinical amelioration, the older patients as well as the patient carrying haplotype 19/3 (or

Benin/Senegal type) did not observe any changes in their condition. Also, one patient experienced a life-threatening liver sequestration crisis during treatment. It is concluded that the selection of patients who may benefit from hydroxyurea needs further evaluation. Furthermore, effects of hydroxyurea that may influence the clinical outcome other than by HbF induction, in particular effects on cellular adhesion, cytokines, vascular endothelium, neutrophil and platelet activity as well as procoagulant factors should be analysed.

Chapter VIII highlights the effects of hydroxyurea on soluble adhesion molecules (plasma representatives of specific membrane integrated adhesion molecules) and cytokine behaviour in seven SS patients. Baseline values of interleukin-3, interleukin-6, erythropoietin, granulocyte-macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF) and soluble adhesion molecules (sVCAM-1, sICAM-1, sE-Selectin and sP-Selectin) in SS patients are compared with normal adult controls and the cytokines and soluble adhesion molecules have been determined twice during a five month treatment with hydroxyurea. Results show no detectable effect on soluble adhesion molecules during hydroxyurea treatment, but the baseline levels of sVCAM-1 are enhanced in SS patients compared to normal controls. The sVCAM-1 levels during hydroxyurea therapy appear difficult to interpret due to fluctuation caused by clinical events. Baseline SCF levels in the SS patients are significantly reduced compared to normal adult controls. The explanation for this finding could be similar to the mechanism that has been postulated by others to clarify the relatively low erythropoietin in SS patients. The mechanism involves increased activity of other plasma factors which render circulating red progenitor cells much more sensitive to erythropoietin. Baseline levels of GM-CSF, interleukin-3 and interleukin-6 interleukin-3 in SS patients were equivalent to normal adult controls. Except for interleukin-3, the cytokines remained stable or revealed inconsistent changes during hydroxyurea therapy. Interleukin-3 showed an increase which paralleled the HbF increase in six patients during hydroxyurea therapy, suggesting that hydroxyurea may induce early erythroid progenitors capable of producing HbF production by a direct or indirect effect on interleukin-3 production.

Chapter IX discusses the effects of hydroxyurea on endothelial factors (such as IL-8, fibronectin and sVCAM-1), neutrophil soluble adhesion molecules (sL-Selectin, sIL-6 receptor- α), and neutrophil activity (myeloperoxidase) as well as the procoagulant von Willebrand Factor.

These determinants of adhesion and vasooclusion are compared with hematological and clinical data in a group of eight SS patients before and during five months of hydroxyurea treatment. Steady state sVCAM-1 levels are increased in all SS patients and a significant decrease is noted during hydroxyurea treatment implying

a decrease in the red cell- endothelial interaction. IL-8 levels are comparable to those in normal controls and remain unaffected by hydroxyurea therapy. Intercurrent infection and crises reveal significant increases in IL-8 which are accompanied by leucocytosis, but otherwise the IL-8 levels do not correlate with hematological data. Hydroxyurea does not affect levels of fibronectin or soluble neutrophil adhesion molecules. However, the levels of myeloperoxidase decrease significantly while leucocyte counts do not, suggesting a reduction in neutrophil activity which may help limit the extend of tissue destruction in the event of a vasoocclusive crisis.

Conclusive remarks

The data presented in this thesis as well as other studies show that most sickle cell patients respond to hydroxyurea therapy with increase of HbF and a reduction in the rate of hemolysis. The consequent increase in hemoglobin diminishes transfusion requirements which may benefit patients that suffer of very low hemoglobin levels. Moreover, some patients experience a reduction in their frequency of painful crises. Still, the therapeutic effects of hydroxyurea in a sickle cell patient cannot be adequately predicted and the evaluation of significant clinical effect by laboratory means also has its shortcomings. The HbF increase in response to hydroxyurea differs widely between SS individuals. New information from a study by Steinberg (Semin Hematol 1997; 34: 8-14) reveals that increases in HbF are greatest in patients who have the highest baseline counts of reticulocytes and leucocytes, have one or more episodes of myelotoxicity and absence of the Bantu haplotype. Also, HbF levels during hydroxyurea therapy vary in time with only half of the patients showing a sustained HbF increase after 2 years. These SS individuals apparently have sufficient bone marrow reserve to continue to respond to myelotoxicity with proliferation of HbF producing erythroblasts. Probably reticulocyte and neutrophil counts are a measure of this bone marrow reserve. Patients older than 40 years should be expected not to gain much from hydroxyurea therapy since they have less frequent pain crises and are more prone to myelotoxicity because of diminished bone marrow reserve. Moreover, it is doubtful if patients with a less severe phenotypical expression of disease, like the Senegal or Saudi Arabian-Indian haplotype, will experience significant clinical benefit from hydroxyurea since they already carry high HbF levels. The identification of the SS individuals that qualify for hydroxyurea treatment is even more important due to the fact that hydroxyurea still poses a theoretical threat of carcinogenicity and teratogenicity. In addition to the influence of HbF on the clinical presentation of sickle cell anemia, the enhanced tendency of young deformable sickle cells to adhere to endothelium is also of critical importance to clinical course of the disease. The interpretation of clinical response to drug therapy should therefore include practical methods that determine the intensity of blood cell-endothelium

interaction. Circulating activated endothelial cells, enhanced steady state levels of sVCAM-1 in sickle cell patients compared to normal controls and increase of sVCAM-1 at the start of the vaso-occlusive crisis suggest the presence of a chronic activated endothelium state with increased sensitivity to certain crisis-precipitating factors as well as specific involvement of sVCAM-1 in red cell -endothelium interaction. The decrease of sVCAM-1, VLA-4/CD36 reticulocytes and myeloperoxidase levels following hydroxyurea therapy, are proof of beneficial actions of hydroxyurea other than its effects on HbF production. A combination of these parameters should be followed during future treatment of SS patients with hydroxyurea or other compounds in order to adequately predict and confirm clinical responses.

Regarding the follow up of SS patients during treatment with hydroxyurea, it is important to indicate that regular control should not only include careful monitoring of blood counts, but also of the packed cell volume. A High packed cell volume - in the case of the SS patient usually in excess of 0.30 l/l - results in a significant higher blood viscosity which may cause serious vaso-occlusive complications.

Oral derivatives of butyrate as well as certain hematopoietic growth factors could perhaps become alternatives to therapy with hydroxyurea particularly because they lack the myelotoxic effects and do not pose a risk of carcinogenicity. Isobutyramide, the drug tested in SS patients on Curaçao, did not qualify as effective therapy. There exists a narrow therapeutic margin which also holds true for butyrate as shown in other studies. Perhaps other oral butyrate analogues now under investigation may prove to possess better pharmacological qualities as well as being more potent HbF inducers. Combination of high dose erythropoietin with hydroxyurea and iron supplements has shown superior HbF increases but effects on clinical symptoms have not been adequately analysed and the financial costs involved with this treatment are extremely high. Due to these high costs it is doubted that this regimen will ever be of benefit to sickle cell patients in general. Furthermore, other hematopoietic growth factors should be evaluated with extreme caution since parenteral administration poses a serious risk of initiating a vasoocclusive crisis.

Finally, it should be stated that treatment with hydroxyurea or other agents does not replace invaluable supportive measures such as hygiene, proper nutrition, antibiotic treatment and regular counseling. However, in the next decade the HbF inducing agents and new therapies that are directed at specific mechanisms of adherence, may offer the possibility of clinical improvement to some sickle cell patients until - through gene manipulation - the addition of normal genes to the genome of the sickle cell patient becomes reality.

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Chapter XI

Samenvatting

Samenvatting

De behandeling van sikkelcelanemie, de meest voorkomende erfelijke hemolytische anemie in de wereld, vormt nog steeds een moeilijk medisch probleem. Hydroxyureum blijkt bij sommige patiënten verbetering van klinische symptomen te geven maar de klinische uitwerking is in het algemeen moeilijk te voorspellen. Het effect van deze behandeling is tot nu toe voornamelijk toegeschreven aan inductie van foetaal hemoglobine (HbF). Dit proefschrift heeft onderzocht of de klinische effecten van hydroxyureum alleen berusten op deze HbF inductie of dat er ook andere werkings-mechanismen van belang zijn. Bovendien is aan de hand van verschillende parameters gekeken of een eventueel gunstig klinisch effect van hydroxyureum therapie voorspeld kon worden. Tot slot zijn de effecten op de HbF synthese onderzocht van isobutyramide, een niet-cytotoxisch alternatief voor hydroxyureum.

In hoofdstuk I worden de pathofysiologie, diagnose en behandeling van sikkelcelanemie beschreven. De cruciale afwijking, te weten een puntmutatie in het β globine gen, is verantwoordelijk voor het ontstaan van het abnormale hemoglobine S, wat wordt gekarakteriseerd door een substitutie van het aminozuur valine in plaats van glutamine op positie no.6 in de β keten van het hemoglobine molecuul. In gedeoxygeneerde toestand polymeriseert het hemoglobine S en vormt dan multiple strengen van polymeren welke de erythrocyt -karakteristiek sikkelvormig-deformereren, waardoor de erythrocyt membraan functie verstoord wordt. Abnormale adhesie, vertraging van bloeddorstroming en microvasculaire occlusie door sikkeldcellen, kan ischemische beschadiging tot gevolg hebben, welke zich klinisch uit als pijn crises en/of ischemische beschadiging van specifieke organen. Een ander belangrijk gevolg is hemolyse, wat wordt veroorzaakt door de verhoogde afbraak van sikkeldcellen. De ernst van de klinische symptomatologie van HbSS patiënten verschilt per individu en wordt beïnvloed door verschillende factoren, zoals bijkomende α -thalassemie en factoren, die een effect hebben op het HbF gehalte, het totale hemoglobine gehalte en andere hematologische parameters, alsmede verscheidene plasma componenten, vasculaire factoren en milieu invloeden. De ernst van sikkeldcelziekte blijkt niet alleen goed te correleren met het aantal leucocyten, maar ook met het aantal jonge, reversibel, deformeerbare sikkeldcellen, welke bekend zijn om hun verhoogde neiging tot adhesie aan de vaatwand. Adhesie van sikkeldcellen aan endotheel wordt op haar beurt beïnvloed door wanverhoudingen tussen stoffen zoals stikstofoxide, prostacyclines, endotheliale groeifactoren (o.a. PDGF), endotheline, vrije zuurstofradicalen, procoagulantia, ligand eiwitten, en cytokines welke allen de functie van het endotheel beïnvloeden. Tot slot spelen de eerder opgelopen orgaanschade en specifieke afwijkingen in membraan en cytoplasma van de erythrocyt ook een rol

bij de klinische expressie van sikkelcelanemie. Behandeling van de patiënt met sikkelcelanemie is nog steeds voornamelijk ondersteunend en symptomatisch van aard. Adequate voeding, verbeterde leefomstandigheden en vroege behandeling en preventie van infecties (vaccinaties en tijdige antibiotische behandeling) hebben de afgelopen decennia een significante verbetering van de overleving van HbSS patiënten met zich meegebracht. Rehydratie, zuurstof toediening, pijnstilling, en in specifieke situaties, antibiotica en wisseltransfusies, dragen bij tot de verlichting van sikkelcelcrisen. HbF stimulatie door hydroxyureum, veroorzaakt bij sommige patiënten een verbetering van de symptomatologie. Wellicht kunnen in de nabije toekomst nieuwe boterzuurderivaten en hematopoietische groeifactoren, welke ook HbF inductie veroorzaken, van nut zijn als alternatieve therapie of als combinatie behandeling met hydroxyureum. Andere chemische producten welke zijn uitgetest om de symptomatologie van de ziekte te verbeteren zijn stoffen die het sikkelen van cellen tegen zouden gaan, middelen die de zuurstofaffiniteit verhogen of de capillaire flow bevorderen en anticoagulantia. Bij de meerderheid van deze studies kon echter geen gunstige respons vastgesteld worden. Sommige trials zijn nog niet afgerond en resultaten zijn nog niet voorhanden. Ervaringen met allogene beenmerg transplantatie bij sikkelcel anemie zijn positief, maar de procedure heeft een mortaliteit van 10% en een risico op infertiliteit. Bovendien bestaan er nog onvoldoende gegevens over de kans op het ontstaan van een maligniteit als gevolg van de chemotherapie die bij de transplantatie gebruikt wordt. Gentherapie, momenteel nog steeds een onderwerp van intensieve research op laboratorium niveau, zal in de toekomst ongetwijfeld de behandeling van keuze worden. Tussentijds is er, gezien de groeiende kennis van de mechanismen van cellulaire adhesie, zeker plaats voor het ontwikkelen van behandelingen welke ingrijpen op het niveau van adhesie van bloedcellen aan vasculaire endotheel.

In hoofdstuk II wordt ingegaan op epidemiologische en klinische aspecten van sikkelcelziekte op Curaçao. Hierbij is gebruik gemaakt van laboratorium gegevens, β^S haplotypering, het aantal α -genen en de klinische gegevens van 18 lokale HbSS patiënten. Op grond van deze gegevens kan worden aangenomen dat de meerderheid van de geschatte 100 lokale HbSS patiënten het haplotype 19/19 oftewel Benin type dragen. Voorts blijkt uit de literatuur dat lokaal de combinatie van HbS of HbC met α -thalassemie een incidentie heeft van 4,1%. Er bestaat tot dusver geen informatie over het vóórkomen van combinaties van HbS met andere Hb varianten en β -thalassemie. De pijncrisen en chronische hemolyse vormen de meest voorkomende klinische presentatie. Recidiverende ulcera aan de benen hebben eveneens een relatief hoge incidentie. Het "chest syndroom" heeft een lage incidentie maar is levensbedreigend en vormt de belangrijkste doodsoorzaak van sikkelcelpatiënten op Curaçao. Veertig jaar geleden was de levensverwachting van de lokale sikkelcelpatiënten 20 jaar. Dit is sindsdien sterk verbeterd als gevolg van

de vooruitgang in de lokale gezondheidszorg. Invloeden van milieu, psychosociale aspecten en lokale aanpak van sikkelanemie worden kort besproken.

Hoofdstuk III biedt een overzicht van de regulatie van het HbF gehalte en de factoren welke het HbF gehalte beïnvloeden. De klinische betekenis van HbF en het nut van HbF inductie bij sikkelcelpatiënten worden besproken. Het feit dat een hoog HbF gehalte een remmend effect heeft op het sikkelen en vaak samengaat met een klinisch milder beloop van sikkelcel anemie heeft aanleiding gegeven tot onderzoek naar farmacologische stoffen, die de HbF productie stimuleren. Verscheidene stoffen zoals hydroxyureum, boterzuur, boterzuurderivaten, erythropoietine en andere hematopoietische groeifactoren zijn in staat om HbF productie te stimuleren. Het werkingsmechanisme en de klinische uitwerking van deze stoffen worden besproken.

De studies in dit proefschrift (hoofdstuk IV) hebben tot doel gehad: A) het bestuderen van het effect van hydroxyureum evenals het orale boterzuur derivaat, isobutyramide, op de productie van foetaal hemoglobine (HbF) bij patiënten met homozygote sikkelcel- anemie (HbSS). B) het bepalen van specifieke factoren bij homozygote sikkelcel-patiënten welke zowel de respons van het HbF-gehalte als de klinische symptomen op hydroxyureum therapie kunnen voorspellen, en C) het bestuderen van effecten van hydroxyureum op cytokines and oplosbare adhesie moleculen, welke specifiek betrokken zijn bij de adhesie van erythrocyten en neutrofiele granulocyten aan de vaatwand, evenals de invloed van hydroxyurcum op de activiteit van endotheel, de activiteit van neutrofiele granulocyten en de procoagulant von Willebrand Factor.

In hoofdstuk V worden de methoden, welke gebruikt zijn bij de bepaling van diverse parameters in de studies, toegelicht.

In hoofdstuk VI wordt het effect van isobutyramide op de HbF synthese bestudeerd. Isobutyramide, een oraal derivaat van boterzuur met een lange halfwaardetijd, wordt verondersteld, in tegenstelling tot hydroxyureum, geen carcinogene werking te hebben. Het werd toegediend aan vier HbSS patiënten over een periode van drie maanden. De start dosis bedroeg 50 mg/kg/dag en werd geleidelijk opgehoogd met 25 mg/kg/dag tot een maximum van 150 mg/kg/dag. De maximum dosis werd gedurende drie weken gehandhaafd. Er werden geen significante veranderingen in het HbF gehalte vastgesteld, maar bij de maximum dosis van isobutyramide ontstond een relatieve toename van G_{γ} (één van de twee soorten eiwitketens van het foetale hemoglobine). Drie patiënten toonden geen verandering van hemolyse parameters. Bij één patiënt echter, werd een toename van het lactaat dehydrogenase waargenomen. Geen enkele patiënt ontwikkelde een

pijncrise of een andere orgaancrisis tijdens therapie. Alle vier patiënten ontwikkelden een branderig gevoel in epigastrio aansluitend aan de ingestie van isobutyramide. De trial werd gestaakt als gevolg van het ontwikkelen van onverdragelijke dyspepsie bij één van de patiënten. Gezien deze bevindingen werd geconcludeerd dat isobutyramide niet beschouwd kan worden als een waardevolle aanvulling op, of alternatief voor, de hydroxyureum behandeling.

In hoofdstuk VII wordt de eerste studie met hydroxyureum in Curaçao besproken. Er zijn negen HbSS patiënten, acht met haplotype 19/19 en één met haplotype 19/3, behandeld gedurende een periode van 10 maanden. Na een pauze van één jaar, zijn zes van deze patiënten opnieuw behandeld met hydroxyureum gedurende vijf maanden. Van elk van de patiënten wordt de hematologische en klinische respons besproken. Gedurende hydroxyureumtherapie verbeterden de hematologische en biochemische parameters, het HbF-gehalte en het aantal pijncrisen. Desondanks ondervonden de oudere patiënten, alsmede de patiënt met haplotype 19/3, geen verandering in hun conditie. Bovendien ontwikkelde één van de patiënten een levensbedreigende leversequestratie-crisis gedurende de behandeling. Er wordt geconcludeerd dat er meer criteria gedefinieerd dienen te worden teneinde te kunnen komen tot selectie van die patiënten, die klinisch goed reageren op hydroxyureum. Bovendien is het zinvol, naast de effecten op de HbF synthese, ook de effecten van hydroxyureum op cellulaire adhesie, cytokines, endotheel, activiteit van granulocyten en thrombocyten alsmede de uitwerking op procoagulantia te onderzoeken.

In hoofdstuk VIII wordt ingegaan op de invloeden van hydroxyureum op oplosbare adhesiemoleculen (plasmavertegenwoordigers van specifieke membraangeïntegreerde adhesiemoleculen) en gedrag van cytokines tijdens behandeling met hydroxyureum van zeven HbSS patiënten. Uitgangswaarden van interleukine-3, interleukine-6, erythropoietine, granulocyt-macrofaag kolonie stimulerende factor (GM-CSF), stamcelfactor (SCF) en oplosbare adhesiemoleculen (sVCAM-1, sICAM-1, sE-Selectine en sP-Selectine) van HbSS patiënten werden vergeleken met normale volwassen controle personen. Tijdens de vijf maanden durende behandeling zijn cytokines en oplosbare adhesiemoleculen twee maal bepaald. De resultaten tonen geen significant effect van hydroxyureum op oplosbare adhesiemoleculen. De uitgangswaarden van sVCAM-1 zijn significant verhoogd bij alle patiënten vergeleken met normale controles. De sVCAM spiegels tijdens behandeling blijken moeilijk te interpreteren. Dit wordt verklaard door de mate van fluctuatie van sVCAM-1 als gevolg van klinische effecten c.q. complicaties. De uitgangswaarden van SCF zijn significant verlaagd in vergelijking met normale controles. Mogelijk dat deze bevinding verklaard kan worden door hetzelfde mechanisme, dat verantwoordelijk is voor de relatief lage erythropoietine spiegels

bij HbSS patiënten. Dit mechanisme behelst een toegenomen activiteit van diverse plasmafactoren welke de circulerende voorlopers van de erythrocyt veel gevoeliger maken voor de effecten van erythropoietine. Uitgangswaarden van GM-CSF, interleukine-3 en interleukine-6 van HbSS patiënten waren vergelijkbaar met normale volwassen controle personen. Met uitzondering van interleukine-3 bleven de cytokines stabiel gedurende behandeling. Bij zes van de zeven HbSS patiënten werd een stijging van interleukine-3 parallel aan de HbF toename waargenomen tijdens hydroxyureumtherapie. Hieruit zou geconcludeerd kunnen worden dat hydroxyureum, via een direct dan wel indirect effect op de interleukine-3 productie, vroege voorlopers van de erythroïde reeks induceert, met name die welke in staat zijn tot HbF-productie.

Tot slot worden in hoofdstuk IX de effecten van hydroxyureum op endotheliale factoren (zoals interleukin-8, fibronectine en sVCAM-1) bestudeerd, evenals de effecten op oplosbare adhesiemoleculen van de neutrofiele granulocyt (sL-Selectine, sll-6 receptor- α), de activiteit van de neutrofiele granulocyt (myeloperoxidase) en de procoagulant von Willebrand Factor. Deze determinanten van adhesie en vaso-occlusie zijn bij acht HbSS patiënten bekeken en tegelijkertijd bestudeerd in relatie tot de hematologische en klinische gegevens vóór en gedurende vijf maanden van therapie met hydroxyureum. Uitgangswaarden van sVCAM-1 waren wederom verhoogd in HbSS patiënten. Tijdens hydroxyureum therapie werd ditmaal een significante daling van sVCAM-1 waargenomen hetgeen een afname impliceert in de erythrocyt-endothelium interactie. Interleukine-8 waarden waren vergelijkbaar met die van normale controles en veranderden niet tijdens hydroxyureumtherapie. Echter, intercurrente infectie en crises werden gekarakteriseerd door een duidelijke stijging van het interleukine-8 gehalte. Behoudens leucocytose tijdens infectie of crise werd er geen duidelijke correlatie tussen IL-8 en hematologische parameters waargenomen. Hydroxyureum bleek geen invloed uit te oefenen op fibronectine of oplosbare adhesie moleculen van de neutrofiele granulocyt. Daarentegen namen de myeloperoxidase-spiegels significant af, terwijl het aantal leucocyten vrijwel onveranderd bleef. De afname van de activiteit van neutrofiele granulocyten welke hierdoor gesuggereerd wordt, kan in geval van een sikkelcel-vaso-occlusie de uitbreiding van weefseldestructie helpen beperken.

Conclusies

De resultaten welke gepresenteerd worden in dit proefschrift evenals in andere studies tonen dat de meeste sikkelcelpatiënten reageren op hydroxyureum met een stijging van HbF en een afname van hemolyse. De daaruit voorkomende stijging van het hemoglobine gehalte veroorzaakt een reductie in de transfusie behoefte hetgeen een klinische verbetering betekent voor patiënten die een erg laag

hemoglobine gehalte hebben. Een aantal patiënten ervaart een vermindering van hun pijncrisen. Echter, de therapeutische effecten van hydroxyureum bij de sikkelcelpatiënt kunnen nog niet goed voorspeld worden en de evaluatie van een significant klinisch effect door middel van laboratoriumonderzoeken kent ook tekortkomingen. De toename van HbF tijdens hydroxyureum therapie laat belangrijke interindividuele verschillen zien. Volgens een zeer recente studie van Steinberg (Semin Hematol 1997; 34: 8-14) wordt de sterkste stijging van HbF waargenomen bij patiënten met de hoogste aantallen reticulocyten en leucocyten, bij patiënten die één of meer episoden hebben van myelotoxiciteit en bij afwezigheid van het Bantu haplotype. Ook blijkt het HbF gehalte te variëren in de tijd en slaagt slechts de helft van de patiënten erin om het niveau van HbF toename te handhaven gedurende een twee jaar durende therapie. Deze HbSS patiënten worden gekenmerkt door een goede beenmergreserve welke steeds weer instaat is om te reageren op myelotoxiciteit met proliferatie van HbF-producerende erythroblasten. Waarschijnlijk zijn reticulocyt en leucocyt aantallen een maat voor deze beenmergreserve. Patiënten boven de 40 jaar zullen minder baat hebben bij de hydroxyureumtherapie aangezien zij over het algemeen minder pijncrisen hebben en gevoeliger zijn voor myelotoxiciteit als gevolg van geringere beenmergreserve. Bovendien kan betwijfeld worden of patiënten met een minder ernstige klinische expressie van sikkelcelanemie, zoals patiënten met het Senegal of Saudi Arabische-Indiase haplotype, een significante verbetering zullen ervaren bij hydroxyureumtherapie, gelet op het feit dat zij in het algemeen reeds een verhoogd HbF gehalte hebben. Ook het feit dat hydroxyureum therapie nog steeds een theoretisch risico op carcinogenese en teratogenese draagt, maakt het des te belangrijker HbSS patiënten, welke baat zullen hebben bij hydroxyureum therapie, correct te identificeren. Naast de uitwerking van HbF inductie op de klinische symptomen, speelt ook de neiging tot adhesie van jonge sikkelcellen aan de vaatwand een kritieke rol. Met betrekking tot de interpretatie van de klinische respons op farmacologische behandeling is het derhalve noodzakelijk dat deze verbeterd en vereenvoudigd kan worden door middel van laboratoriumbepalingen die de intensiteit van bloedcel-endotheel interactie weergeven. Circulerende geactiveerde endotheliale cellen, toegenomen uitgangswaarden van sVCAM-1 in sikkelcelpatiënten ten op zichte van normale controle personen en toename van sVCAM-1 bij aanvang van de sikkelcelcrise pleiten voor een chronisch geactiveerd endotheel met verhoogde gevoeligheid voor crisis-uitlokkende factoren evenals een specifieke rol van sVCAM-1 bij de erythrocyt-endotheel interactie. Afname van sVCAM-1, VLA-4/CD36 reticulocyten en myeloperoxidase tijdens hydroxyureumbehandeling is een bewijs van een therapeutische werking van hydroxyureum náást het effect op de HbF productie. Een combinatie van deze parameters zou, bij behandeling van HbSS patiënten met hydroxyureum of andere producten, bepaald dienen te worden teneinde een adequate klinische respons te

kunnen voorspellen.

Ten aanzien van de follow up van HbSS patiënten tijdens behandeling met hydroxyureum moet opgemerkt worden dat men het bloedbeeld niet alleen dient te controleren op tekenen van myelotoxiciteit, maar ook op belangrijke veranderingen in de hematocriet. Een verhoogde hematocriet, in geval van de HbSS patiënt meestal boven 0.30 l/l, veroorzaakt significante stijging van de bloedviscositeit en kan zodoende een ernstige sikkcelcrise luxeren.

Met betrekking tot de behandeling van patiënten met sikkcelanemie, zouden orale derivaten van boterzuur evenals bepaalde hematopoietische groeifactoren een alternatief op hydroxyureum therapie kunnen vormen, met name daar zij geen myelotoxische en mutagene werking hebben. Isobutyramide, het orale boterzuur derivaat waarmee enkele HbSS patiënten in Curaçao in trialverband werden behandeld, bleek geen effectieve therapie te zijn. Er bestond een nauwe therapeutische breedte zoals reeds bij eerdere studies met intraveneuze behandeling met boterzuur was vastgesteld. Wellicht dat andere orale boterzuur derivaten, welke momenteel onderzocht worden, over betere farmacologische kwaliteiten beschikken en een sterker effect uitoefenen op de HbF- productie. Van combinatie van hoge dosis erythropoietine met hydroxyureum en ijzer suppletie is aangetoond dat dit nog sterkere HbF toename veroorzaakt, maar de klinische effecten werden tot dusver niet goed bestudeerd. Daarbij zij vermeld dat de financiële kosten, gemoeid met deze vorm van behandeling, extreem hoog zijn. In verband met deze hoge kosten is het twijfelachtig of deze behandeling ooit op grote schaal bij sikkcelpatiënten zal worden toegepast. Bovendien dient het inschakelen van andere groeifactoren bij de behandeling van sikkcelpatiënten met uiterste voorzichtigheid te geschieden, aangezien parenterale toediening van deze producten als potentieel risico heeft het luxeren van vaso-occlusie.

Tot slot mag gesteld worden dat behandeling met hydroxyureum of andere producten geen vervanging is voor belangrijke ondersteunende maatregelen zoals hygiëne, goede voeding, antibiotische behandeling en educatie. Desalniettemin, kunnen in de komende tien jaar producten, die HbF induceren en producten die op specifieke plaatsen aangrijpen in het mechanisme van adhesie, klinische vooruitgang bieden aan sommige sikkcelpatiënten. Dit alles uiteraard in afwachting van het moment dat genetische manipulatie de incorporatie van normale β genen in het chromosoom tot werkelijkheid maakt.

Dankwoord

Dankwoord

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Curriculum Vitae

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The author of this thesis was born on november 30, 1959 in Curaçao, The Netherlands Antilles. He graduated in 1978 from the Radulphus College (Atheneum B) in Curaçao. From 1978 until 1983 he studied at the medical faculty of the University of Leiden in the Netherlands where he obtained his doctoral degree. In 1983 he transferred to the medical faculty of Groningen which enabled him to do his internships at the St. Elisabeth Hospital in Curaçao. He received his medical degree in 1985 and started his training in internal medicine at the Department of Internal Medicine of the St. Elisabeth Hospital in Curaçao (head Prof dr AEC Saleh). In 1986 he passed the American Foreign Medical Graduate Examination (FMGEMS). From 1987 he worked at the Department of Internal Medicine of the Academic Hospital of Maastricht, The Netherlands (head at that time Prof dr JA Flendrig) where he finished his training in internal medicine. In 1990 he returned to Curaçao where he practices in the St. Elisabeth Hospital with special interest in the field of oncology and gastroenterology.