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REVIEW ARTICLE

The genetics of blood disorders: hereditary hemoglobinopathies

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Abstract

Objective: To summarize recently published data on the pathophysiology, diagnosis and treatment of sickle cell diseases and β-Thalassemias, the most relevant hereditary hemoglobinopathies in the global population.

Sources: Searches were run on the MEDLINE and SCIELO databases, limited to the period from 2003 to May 2008, using the terms hereditary hemoglobinopathies, sickle cell diseases and β-thalassemia. Two books and two chapters were also included.

Summary of the findings: More than 2,000 articles were identified; those providing the most important information and broadest views were selected.

Conclusions: Morbidity and mortality rates from sickle cell diseases and β-thalassemia are still very high and represent an important challenge. Increased understanding of pathophysiological aspects has lead to significant improvements in treatment and prevention of these diseases.

J Pediatr (Rio J). 2008;84(4 Suppl):S40-51: Hereditary hemoglobinopathies, sickle cell diseases, β-thalassemia.

Introduction

Anemia is a common finding during the neonatal period and early childhood, and one which can lead to significant morbidity and mortality.[1,2] The causes are multifactorial, but some of the most common diseases are intrinsic to the erythrocytes, especially the hemoglobinopathies.[1-3] These are the most common forms of hereditary hemolytic anemia. They are a group of autosomal abnormalities, the majority of which are recessive and which are characterized by the production of structurally abnormal hemoglobin (Hb) variants or by an imbalance in the rate of synthesis of the globin chains (thalassemias); less frequently, both phenotypes may be present in the same individual (concomitant reduced synthesis and structural variation).[3-6]

The hemoglobinopathies are among the most common monogenic diseases, with more than 1,000 different mutant alleles having been identified on the molecular level.[7] The most significant of these from a clinical point of view are the sickle cell diseases (SCD) and β-thalassemia, which affect populations with origins in Africa, the Mediterranean region, Southeast Asia, the Middle East and the Far East.[8] Around 1-2% of the global population are heterozygous for Hb S and 3% are heterozygous for β-thalassemia.[9]

Historically, the majority of children who were carriers of these diseases died during their first 10 years of life from complications. However, recent important advances have extended the average life of patients and significantly improved their quality of life. Improved understanding of the etiology and mechanisms of anemia, earlier diagnosis, new therapeutic approaches and better management of transfusion iron overload have dramatically improved the clinical picture.[10] This article summarizes the data that have most

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recently been made available in the literature on pathophysiologic aspects, diagnosis and treatment of the SCD and β-thalassemia.

Hereditary hemoglobinopathies

Human Hb is a globular tetramer formed by the combination of two "type α" (α or ζ) polypeptide (globin chains) with two "type β" (β, δ, ^{G}γ, ^{A}γ or ε) chains. Each chain has its own prosthetic heme group, which forms a reversible bond with the oxygen molecule (O_2), thereby fulfilling the primary function of Hb, which is to transport O_2 from the lungs to peripheral tissues.[11-13]

Synthesis of each of the globins is controlled by distinct genes, which are arranged in two clusters; the genes that code for the α and ζ chains (α clusters) are located in the telomeric region of the short arm of chromosome 16 (16p 13.3), whereas the genes that code the β, δ, γ and ε chains (β *cluster*) are on the short arm of chromosome 11 (11p 15.5). During the embryonic period, the embryonic Hb variants Gower I ($\zeta_2\varepsilon_2$), Gower II ($\alpha_2\varepsilon_2$) and Portland I ($\zeta_2\gamma_2$) are produced; during the fetal period these are substituted by fetal Hb or Hb F ($\alpha_2\gamma_2$), which then cedes its place to Hb A ($\alpha_2\beta_2$) and A_2 ($\alpha_2\delta_2$) in adulthood. Six months after birth, Hb A predominates absolutely, making up more than 95% of total cellular Hb, while Hb A_2 levels are around 2-3%, and Hb F levels are 0-2%.[11-13]

Hemoglobinopathies are the result of mutations that affect the globin genes and can be classified into two major groups: structural alterations, which form anomalous Hb variants, and synthesis alterations (thalassemias), where one or more types of globin chain are partially or completely suppressed; less frequently the two phenotypes can occur in combination.[11-13]

Structural hemoglobinopathies are generally caused by simple substitutions, small insertions or deletions of bases that affect coding regions of the genes and lead to amino acids in the protein chain being substituted.[11-13] Notable among these is Hb S ($\alpha_2\beta^{S}_2$), a variant that affects position 6 on the β chain, substituting glutamic acid with valine ($\beta^{6\ Glu\rightarrow Val}$), i.e. where the normal form has glutamic acid the variant has valine. It was described in 1949 by Linus Pauling et al. as a form of Hb found in the red blood cells of patients with sickle-cell anemia (SCA), with electrophoretic migration that differentiated them from normal individuals. When Hb S is in its deoxygenated state (deoxy-Hb S) and in elevated concentrations, it polymerizes, resulting in abnormally rigid and inflexible red blood cells (sickled red blood cells). These in turn lead to chronic hemolysis and vasoocclusion, which are the pathophysiologic bases of the disease.[5,14]

The thalassemia are the result of a reduction, or an absence of production, of one or more globin chain types, leading to a buildup of another type, the synthesis of which is unaffected. The excess chains are unstable and precipitate, leading to changes to the erythrocyte membrane and early destruction of the cell. Furthermore, the deficient hemoglobinization of erythrocytes results in hypochromia and microcytosis, which are characteristic abnormalities of this group of diseases.[6,11,13,15-18] Thalassemias are classified as α, β, γ, δ, δβ or γδβ, depending on the type of chain whose production is affected. Thalassemias α and β are the most common, and while the majority of the first are caused by deletions that remove α genes, the β-thalassemias are generally the result of substitutions of bases on the exons, introns and promoter regions of β genes.[15-18] Hemoglobinopathies are among a group of genetic polymorphisms that are selected for positively by malaria.[19,20] Because they alter the structure and/or function of erythrocytes, they confer increased resistance to *Plasmodium falciparum* infection on heterozygotes and, as a result, increased survival, particularly of children, in areas where malaria is endemic. In these regions hemoglobinopathic genes reach extremely elevated frequencies. Migration movements, followed by miscegenation have carried these genes to other regions and other populations, as is the case of populations in American countries and the north of Europe.[7,9]

Sickle cell diseases

Homozygosis of the β^{S} gene (20 GAG→GTG) results in SCA, which was the first "molecular disease" to be described.[21] A single base substitution in the β globin gene results in a collection of cellular, tissues and organic alterations known as the pleiotropic effects of the β^{S} gene.

In the Hb A molecule, the external residues are polar, making them soluble and preventing intermolecular interactions, whereas the internal ones are apolar, creating an environment in which O_2 can bind without oxidizing the heme. Individual tetramers within a single blood cell do not interact with each other. Red blood cells can suffer deformation, which allows them to pass through the circulation and carry O_2 to all of the tissues in the body. When Hb S is in its deoxygenated form, the polar valine, rather than the usual glutamic acid, is exposed on the surface of the β^{S} chain. This allows hydrophobic intermolecular interactions and polymerization of Hb. The red blood cells that contain polymerized Hb S are rigid and inflexible, which contribute to the process of microvascular occlusion which subjects tissues to ischemia and causes organ dysfunction.[5,14]

The clinical complications of SCA include chronic hemolytic anemia, of moderate or severe intensity, painful and intermittent episodes of vasoocclusion, permanent risk of infections as a result of autosplenectomy, acute chest syndrome, cerebral vascular accidents (CVA), priapism, retinopathy and cumulative damage to multiple organs.[5,22-30] Pulmonary hypertension has also been recognized as a serious complication particularly in adults.[31-36] Inflammation, endothelial activation, abnormalities of the erythrocyte membrane, leukocyte adhesion, platelet aggregation and activation, coagulation activation and abnormal bioavailability of several

vasoactive factors all play important roles in the vasoocclusive phenomena.[6] Patients with SCA are apparently in a chronic state of inflammation. Children are at elevated risk of infarction in the major cerebral arteries, resulting in a vascular process involving the major arteries of the Circle of Willis.[25]

Combinations of Hb S with other structural variants and with β-thalassemia result in SCD, SC, SD and S-β-thalassemia, respectively. The SCD SC, SD and S-β^+ thalassemia are conditions that may have more benign presentation than SCA, with hemolytic anemia of lesser intensity and, occasionally, splenomegaly. The combination of Hb S withβ^0 thalassemia leads to clinical manifestations that are very similar to those of SCA.[37,38]

Heterozygotes for Hb S (AS) are, as a rule, asymptomatic and protected from malaria infection.[19,20,30] In some endemic regions of Africa, the frequency of the β^S gene can pass 40%.[38] In Brazil, the regions of greatest prevalence are the Southeast and the Northeast, with around 8% of individuals with African ancestry being heterozygotes.[22,39] This is, however, an average estimate, since in certain populations, such as the population of Salvador, in the state of Bahia, the incidence of carriers of the sickle cell trait can be greater than 10%.[39]

Pathophysiology

In addition to the abnormal properties of Hb S, the adhesion of sickled cells to the endothelium and alterations to ion transport mechanisms also contribute to the pathophysiology of SCD.[40]

The erythrocyte membrane has several ion transport pathways that maintain cell hydration. The two most important are the K-Cl cotransporter system and the Gardos channel. The first, when activated, allows K^+ and Cl^- to leave the cell, followed by water, which results in dehydration. This pathway is abnormally active in SCA, which leads to an increased intracellular concentration of Hb S and encourages it to polymerize. The Gardos channel is a K^+ efflux pump activated by the intracellular increase of Ca^{++} resulting from deoxygenation and sickling of red blood cells. In common with the K-Cl system, when K^+ leaves the cell it is followed by efflux of water, cellular dehydration and increased internal concentration of Hb S. Endothelin (a vasoactive compound that is elevated in SCA patients), prostaglandin E2 and other cytochemokines alter Gardos channel kinetics, provoking dehydration and polymerization of Hb S.[41,42]

Sickled red blood cells are more adherent to the vascular endothelium and extracellular matrix proteins than normal red blood cells. Endothelial adhesion is mediated by several erythrocyte surface receptors, including the proteins BCAM/Lu (CD239), CD47, CD147, ICAM-4 and phosphatidylserine, which are restricted to the internal surface of the bilipid membrane in normal cells. In addition to mature red blood cells, reticulocytes, which are present in elevated numbers in SCD patients, overexpress the antigens CD36 and VLA-4, which can also significantly increase endothelial adhesion.[5,40,43]

Everything points to endothelial cell inflammation and activation playing a central role in the vasoocclusion observed in SCD. Monocyte activation, with secretion of proinflammatory cytokines (IL-1 and TNF-α), leads the leukocytes, which are always present in large numbers, to adhere to the inflamed endothelium and interact with the sickled red blood cells.[44-49] Neutrophils and eosinophils also appear to have greater adhesion to the endothelium in SCD.[44,45,47,49] Studies with transgenic mice have demonstrated that the damage caused by ischemia followed by reperfusion appears to have an influence on the genesis of inflammation in SCA, through increased production of oxidants and proinflammatory cytokines and due to the increased adhesion of leukocytes to the endothelium and exfiltration from the vasculature to adjacent tissues.[50] More recently, in vitro experiments have led to the hypothesis that the increased erythroblast production that results from chronic hemolysis leads to elevated levels of placental growth factor (PIGF), which, in turn, activates monocytes, which activate the endothelium and contribute to vasoocclusion.[51]

The endothelium itself is also abnormal in SCD. The circulating endothelial cells of patients with SCD have increased expression of ICAM-1 intercellular adhesion molecules, VCAM-1 vascular adhesion molecules and thromboplastin. These increases are the result of elevated concentrations of inflammatory cytokines in plasma. Adhesion proteins such as E-selectin, P-selectin, laminin, fibronectin and integrin $\alpha V\beta 3$ interact with adhesion receptors expressed by sickled erythrocytes and leukocytes, provoking vasoocclusion.[5,40,52]

Several different studies have suggested that the bioavailability of nitric oxide (NO) is reduced in SCD, in common with other hemolytic anemias.[53] Nitrous oxide is a signal gas, with a half-life of seconds, which is produced in the endothelium from the amino acid L-arginine by the action of the NO synthetase enzyme (NOS). Its primary function is to regulate vasodilation and systemic and pulmonary vascular tone. Nitrous oxide is also an important inhibitor of the expression of adhesion molecules in endothelial cells and of leukocyte activation. It is consumed by oxyhemoglobin in reactions that generate methemoglobin and nitrate, and by deoxyhemoglobin, producing Fe-nitrosyl-hemoglobin. The low levels observed in SCD are thought to be the result of the intravascular hemolysis process, of increased consumption by the excess reactive oxygen species (ROS), which are produced in plasma and endothelium, and by reaction with free Hb in plasma that is released during hemolysis. Reduced NO bioavailability results in vasoconstriction, with increased platelet activation and expression of adhesion molecules in leukocytes and endothelial cells.[53,54] The loss of response to NO and, consequently, of vascular regulation, was demonstrated using transgenic mice, known as Berkeley (BERK), in

which the murine genes for the α and β globins were knocked-out and the human α and β^S genes expressed as a transgene.[55]

The severity of SCD is modulated by a variety of genetic factors. Production of Hb F and α thalassemia has a positive effect on certain clinical features.[56] *In vitro*, $\alpha 2\gamma\beta^S$ hybrids do not polymerize; *in vivo*, patients with more elevated levels of Hb F tend to have a better clinical course and increased survival rates.[56,57] In α thalassemia, the reduced availability of α chains reduces their incorporation into Hb S molecules, resulting in a reduction in their concentration. These patients have a lower proportion of hemolysis, higher Hb levels and better life expectancy compared with patients who are not thalassemic. Their red blood cells are less dehydrated and more flexible.[56]

The β cluster haplotypes have also been associated with the clinical course of this disease. The CAR haplotype is associated with increased severity, while the Camaroon and India haplotypes are related to milder forms of the disease.[5,58]

There are many polymorphisms of the genes related to HLA system antigens that can predispose carriers to vaso-occlusive phenomena.[59,60] The HLA-DRB1*03 allele appears to be associated with an increased susceptibility to CVA, whereas HLA-DRB1*02 can offer protection against these complications.[59] The human platelet antigen HPA-5b also appears to be a strong indicator of risk of vascular complications in SCD: in a study carried out with Brazilian patients, Castro et al. observed a significantly greater frequency of this allele in patients with SCA who had vaso-occlusive complications.[61]

More recently, polymorphisms in genes involved in inflammation, in intercellular interactions or directly in NO biology have also been investigated as possible modulators related to the subphenotypes exhibited by patients with SCD.[62] The interactions between genes and their single nucleotide polymorphisms (SNP) were recently studied by Sebastiani et al. in order to develop a prognostic model for CVA in patients with SCA.[60] The authors analyzed 108 SNP in 39 candidate genes, from 1,398 patients with SCA, and found that 31 SNP, in 12 genes, interact with Hb F modulating the risk of CVA.

Diagnosis

Laboratory diagnosis of SCD is very simple and is primarily based on the electrical charge of the different variants. The methods most often used to separate and identify them are electrophoresis, isoelectric focusing and cation exchange high performance liquid chromatography (HPLC). In the case of Hb S, tests for sickling and solubility of deoxy-Hb S in a high molarity phosphate buffer can be used for confirmation and/or screening of carriers. It is worth pointing out that irrespective of the method chosen, a family study is always of fundamental importance to establishing a diagnosis.[13,63,64] The importance of neonatal screening should also be pointed out, since early detection of the SCD is of fundamental importance to reducing their morbidity and mortality. Therefore, this procedure should be carried out wherever there is an elevated frequency of the Hb S gene. The National Neonatal Screening Program run by the Brazilian National Health Service (SUS - Sistema Único de Saúde), tests for SCD (and other hemoglobinopathies) together with three other genetic diseases– phenylketonuria, congenital hypothyroidism and cystic fibrosis –using the Guthrie test, which is employed in the majority of the country's maternity units. Once patients have been identified, it is essential that they receive adequate clinical follow-up regularly.[11,22]

Molecular techniques are generally reserved for prenatal diagnosis of the SCA, from DNA samples taken from fetal cells in amniotic fluid, from the chorionic villi or maternal plasma. Several strategies have been proposed, all of them simple and all of them based on amplifying the β globin gene using polymerase chain reaction (PCR), followed by restriction analysis.[13]

A number of methods using microarray platforms to detect hemoglobinopathies are also already available and may, gradually, substitute conventional methods to the extent that their widespread use reduces their cost, which remains high.[65,66]

Treatment

The classical features of treatment for the SCD, including management of acute episodes (sickling crises) and of infections, have already been extensively described in previously published reviews.[3,5,11,22] It is worth emphasizing, however, the importance of the prophylactic use of penicillin and vaccination against pneumococcus and *Haemophilus influenzae* type b, both of which are encapsulated microorganisms to which children with SCD are much more susceptible than healthy children.[11,22] The introduction of this treatment dramatically reduced the infant morbidity and mortality of these patients. We will now deal with the drugs used to treat SCD and with new prospects for treatment.

The discovery that SCA patients with hereditary Hb F persistence tend to be asymptomatic has demonstrated the potential that increasing Hb F levels has to improve clinical status in SCD. Several drugs, such as 5-azacitidina, sodium butyrate and hydroxyurea (HU), have the capacity, through different mechanisms, to reactivate the γ genes and increase Hb F^5 production. However, the toxicity of these drugs limits their use. Hydroxyurea is the only Hb F inductor that has been approved for use with SCD patients, while the remaining drugs are still in the investigative phases. Multicenter studies have demonstrated that it is highly effective at reducing painful crises, acute chest syndrome and transfusion requirements, reducing mortality by around 40%.[67,68] Its exact mechanism of action has not yet been fully elucidated. Since HU is a myelosuppressive drug, some authors have attributed its beneficial

effects to reduced leukocyte counts and suppression of inflammation, in addition to the increase in Hb F levels itself.[68] There is also evidence that HU affects hydration of red blood cells, adhesion to the endothelium and NO production.[68-74] Possibly also as a result of the suppressor effect, patients who take HU have lower numbers of circulating reticulocytes and less dense cells.[68,70] The safety and efficacy of HU in the majority of adult patients are well established.[70] Some questions remain with respect to its long-term effects,[75] and particularly with respect to its carcinogenic potential[67] in relation to the pediatric population. Children treated with HU exhibit reductions in anemia, increases in the mean cell volume of red blood cells and increases in Hb F levels, with a concurrent reduction in leukocyte counts, but the impact of chronic use over longer periods is not yet known.[5]

Multicenter studies have revealed that allogeneic transplantation of hematopoietic cells obtained from related and compatible donors results in survival rates of more than 90% and event-free survival of 85%. This is an option that can cure the disease for patients who have a compatible donor in the family,[10] particularly patients aged less than 16 years, who have not yet accumulated the organ dysfunctions that lead to unsuccessful transplants in older patients.[76] Unfortunately, the majority of patients do not have related donors. Transplants from compatible but not related or haploidentical donors still result in very high, unacceptable, mortality rates. Non-myeloablative transplants have not been successful in these cases. Transplantation of hematopoietic cells from umbilical cord blood from related donors has been proposed as a promising alternative.[77]

Other anti-sickling treatments are still being trialed. Poloxamer 188 is a co-polymeric non-ionic surfactant which increases the solubility of Hb S, reducing the viscosity of blood and the duration and frequency of vaso-occlusive episodes.[78] Anti-adhesion treatments have also been explored, including general anti-inflammatories and others targeted specifically at the adhesion molecules. Anionic polysaccharides, IgG and statins fall into this category.[79]

Avoiding cellular dehydration may also minimize the symptoms of SCD. In transgenic mice, oral supplementation of magnesium, an inhibitor of K-Cl cotransporter system cellular dehydration, improved red blood cell hydration and increased levels of Hb. Similar results have been achieved in animal models using clotrimazole, which blocks the Gardos channel. Other compounds are being studied, with the objective of reducing cell density and reticulocyte counts.[5,80]

Certain pharmaceutical options available for the treatment of SCD can be classified as possible NO donors. In addition to HU, already mentioned above, L-arginine (oral route) or sodium nitrate (inhaled) may be effective, but remain difficult to manage.[81] Certain agents that reduce production of ROS,[81] such as xanthine oxidase, or others that reduce cell-endothelium adhesion, may be of benefit. Sildenafil amplifies the response of vascular musculature to NO and is being used in clinical trials for the treatment of pulmonary hypertension in SCA,[82] but the greatest concern related to the use of this type of medication is the possibility of male patients developing priapism. Recently, Canalli et al. demonstrated that *in vitro* adhesion of neutrophils to fibronectin and the ICAM-1 protein, which is increased in SCD, is significantly reduced in the presence of pharmacological NO donation agents, such us sodium nitroprusside and diethylamine NONOate (DEANO).[83] These results indicate that the drug is able to donate or increase bioavailability of NO and may represent a promising treatment for reducing vasoocclusion in patients with SCD.

With relation to gene therapy, retroviral vectors which could correct the mutation or its effects and could integrate permanently with the host genome have been investigated in animal models, to be transferred in hematopoietic stem cells.[84] Among the main obstacles are the instability of the vectors and the difficulty in integrating them into this type of cell. Other approaches, such as silencing the β^S gene using interference RNA (iRNA),[85] or homologous recombination to substitute the β^S gene with the β^A gene,[86,87] are promising, but improvements are still needed to the gene transfer methods and efficacy must be demonstrated in animal models.

β-thalassemia

This type of thalassemia results from mutations to the β globin genes that lead to reduced or absent synthesis of the Hb β chains, microcytic and hypochromic anemia and a range of syndromic presentations caused by the allelesβ^0 (absent expression) and β^+ (reduced expression).[4,6,88] The degree of β^+ allele expression is highly variable, depending on the region of the gene affected by the mutation: some result in a small reduction in the rate of β chain synthesis, while others result in almost complete absence of synthesis.[88]

The highest prevalence rates of β-thalassemia are found among populations from the Mediterranean Region and Southeast Asia. Currently, around 200 mutant alleles have been described, and each population has its own spectrum, with one or a few alleles predominating.[6,7,88] In Brazil, the mean frequency of carriers in the Caucasian population is around 1%, with the alleles$\beta^0$39 (C→T), β^+-IVS-I-6 (T→C), β^+-IVS-I-110 (G→A) andβ^0-IVS-I-1 (G→A) being responsible for almost all of the cases in the South and Southeast regions of the country;[13] while in populations in the Northeast Region, in addition to these, the allele β^+-IVS-I-5 (G→C) accounts for 9.3% of β-thalassemic alleles. The$\beta^0$39 mutation reaches frequencies of between 50 and 60% in Southeast Brazil, while in the Northeast it does not reach 5%, and β^+-IVS-I-6 is predominant in that population.[89]

From a clinical point of view, thalassemias are classified as *minor* (heterozygosis for the formsβ^0 or β^+, also known as

the β-thalassemia trait; carriers are generally asymptomatic, but may suffer anemia during situations such as childhood, pregnancy and stress), as *major* (homozygosis$\beta^0\beta^0$ or double heterozygosis$\beta^0\beta^+$, also known as Cooley's anemia; severe anemia, with dependence on regular blood transfusions), or as *intermediate* (homozygosis $\beta^+\beta^+$ or double heterozygosis$\beta^0\beta^+$; comprising the intermediate clinical phenotypes between the thalassemia trait and major thalassemia).[6,11,13,88]

In major thalassemia, patients suffer from the direct consequences of anemia (cachexia, fatigue, congestive heart failure) and from the effects of extramedullary erythropoiesis expansion resulting from the anemia, such as bone abnormalities, bone abnormalities, splenomegaly, compression of the spinal marrow and growth restriction. The intense hemolysis leads to lithiasis, the formation of leg ulcers and pulmonary hypertension. Hypercoagulability is also a complication of this disease. Chronic treatment with blood transfusions leads to a buildup of iron in vital tissues, with cardiac, hepatic and endocrine complications, such as dark, metallic pigmentation of the skin, diabetes, hypopituitarism, hypothyroidism, hypoparathyroidism, hypogonadism, cardiac arrhythmia and cirrhosis and myopathy, the principal causes of death. A proportion of patients may even become carriers of infectious diseases, a possible complication of chronic transfusions.[6,11,88]

Intermediate β-thalassemia covers a wide spectrum of clinical phenotypes associated by a less severe hemolytic anemia than described above, with total Hb levels between 7 and 9 g/dL, and for which chronic transfusion treatment is not normally require. With age, patients may develop complications resulting from bone marrow expansion, including bone abnormalities, growth restriction, infertility and tissue iron overload due to increased gastrointestinal absorption of iron resulting from the anemia and hypercoagulability. Thrombotic complications are more common than among patients with major thalassemia receiving regular transfusions. Some patients develop severe pulmonary hypertension, similar to that which occurs in other chronic hemolytic anemias. Osteoporosis is another important complication which can occur in intermediate thalassemia.[6,11,88,90]

Thalassemia minor is generally an asymptomatic condition, associated with erythrocyte morphology abnormalities, but with discrete hypochromic and microcytic anemia. This becomes more likely to occur during childhood, pregnancy or situations of physiological stress. The main importance of diagnosing these forms is the need for genetic counseling and to prevent the iatrogenic administration of ferrous compounds to heterozygotes.[6,11]

Pathophysiology

In β-thalassemia, deficient production of β globins during erythropoiesis leads to anemia. The excess α chains that do not become incorporated into tetramers form insoluble and unstable compounds which damage the membrane and lead to premature destruction of the cells. This process takes place both in the immature erythroid precursors (ineffective erythropoiesis) and in mature cells (hemolysis), leading to anemia. The ineffective erythropoiesis is mediated by apoptosis: cells undergoing programmed death signal to the macrophages, , probably by exposing phosphatidylserine on the membrane surface, and are phagocytosed.[91,92]

Those red blood cells that do get into the circulation, on the other hand, contain inclusions that cause damage as they pass through the microcirculation, causing extravascular hemolysis, primarily in the spleen. The majority of these inclusions are hemichromes formed by oxidation of α chain subunits, which interact on the membrane with the proteins 4.1, ankyrin and spectrin.[6,11,91-93]

With relation to the membrane, several abnormalities of structure and function have been described. In addition to those mentioned above, there are increases in phospholipids and cholesterol, in cation flow and permeability to calcium. The membranes are more rigid and less stable, probably because oxidized α chains bind to protein 4.1.[6,93]

In β-thalassemia, the cytoplasmatic viscosity of red blood cells is also increased, as a result of cellular dehydration, by a process similar to that which takes place in SCD, involving the systems that control efflux of ions and water, which are abnormally active. Although they have less intracellular Hb content, thalassemic red blood cells may have higher or lower densities than normal red blood cells.[6,94]

The interaction between denatured Hb and hemichromes with the band 3 protein, at the membrane, triggers bonding of autologous IgG antibodies, followed by fixation of the complement and consequent removal of erythrocytes from circulation. Reduction of sialic acid, as takes place in normal senescent red blood cells, leads to increased exposure of β-galactosyl residues, which are recognized by IgG antigalactosyl antibodies which promote sequestration of the cells by the reticuloendothelial system.[95,96]

Thalassemic red blood cells have a 10 to 15 times greater rate of destruction than is observed in normal red blood cells. The bone marrow attempts to compensate with accelerated production of erythrocytes, although insufficient to avoid severe anemia. Liberation of heme from lysed cells, increases gastrointestinal iron absorption due to increased erythropoiesis and inadequate suppression of hepcidin, a protein that regulates intestinal iron intake, combined with a regular transfusion regime, lead to the iron overload observed in these patients. The excess iron, which is highly oxidative, causes the formation of toxic free radicals, with lipid peroxidation of membranes, followed by lysing. Transferrin saturation results in increased plasma iron levels, affecting several organs, particularly the heart.[6,11,97-100]

Heart failure secondary to hemochromatosis is responsible for the majority of β-thalassemia deaths. Anemia, iron overload, lung disease, myocarditis and pericarditis are some of the causes of cardiac complications. Several different studies with cardiomyocytes in culture have revealed that the toxicity caused by the iron profoundly modifies the contractility and electrophysiological behavior of these cells, and that these abnormalities are probably associated with elevated peroxidation of cellular membrane lipids.[98,101]

There is also an increased incidence of thromboembolic events in β-thalassemia. This state of hypercoagulability can be caused by the increased exposure of phosphatidylserine on the erythrocyte membrane, by platelet activation, by increased adherence of thalassemic red blood cells to the endothelium, by increased expression of adhesion molecules in endothelial cells of thalassemic patients and by monocyte activation. Furthermore, levels of anticoagulant proteins C, S and antithrombin appear to be reduced in these cases.[102,103]

Chronic anemia and iron overload contribute to the endocrine abnormalities that are observed. The pituitary, gonads, pancreas, and thyroid, parathyroid and adrenal glands are affected; diabetes, hypogonadism, osteopenia and osteoporosis are common. The frequent fractures seen in inadequately treated cases are due to bone marrow expansion to compensate for ineffective erythropoiesis, endocrine dysfunction and complications related to treatment for iron overload.[97]

Several functional pulmonary abnormalities are described in β-thalassemic patients, which appear, in part, to be caused by iron deposits, generation of free hydroxyl radicals, connective tissue and alveolar capillary membrane abnormalities. Hypercoagulability, platelet thromboembolism and possible reductions in NO have also been associated with the pulmonary hypertension observed in thalassemic patients, and which is more pronounced among those who have undergone splenectomy.[84,97,98,101-105]

The type of mutation to the β gene is associated with the clinical severity of the disease.[7] Thus, β^+-IVS-I-6 mutation, known as the Portuguese type, is related with a more benign clinical course, whereas$\beta^0$39 corresponds with a more severe clinical course.[6,11,89] Certain hyperunstable structural Hb variants result in dominant thalassemic phenotypes, i.e., the presence of a single mutant allele results in clinical manifestations corresponding to intermediate thalassemia.[106] As with the SCD, heterozygotes for β-thalassemia appear to be positively selected by malaria in endemic areas, which sustains the elevated frequency of thalassemic alleles in some of these regions.[19,20,95]

Among the genetic modulators of the severity of this disease is coexistence with α thalassemia, which improves patients' clinical course.[107] In contrast, excess α chains in individuals who have triplicate or quadruple α genes worsens their condition, leading β-thalassemia heterozygotes to exhibit clinical manifestations compatible with intermediate thalassemia.[108]

Mutations to γ genes, which lead to increased production of Hb F and reduced quantities of free α chains, also contribute to better clinical progress.[109] Presence of the polymorphism (C→T) at position -158 of the ^{G}γ gene, recognized by the restriction enzyme XmnI, appears to correlate with increased production of ^{G}γ chains and, as a result, also contributes to reducing severity of the disease.[6,11]

Another potential modifier of the clinical expression of β-thalassemia is the alpha-hemoglobin stabilizing protein (AHSP), a chaperone that forms stable complexes with free α chains, preventing them from precipitating. Studies with mice and humans have suggested that the presence of mutations to the HSP genes could exacerbate the thalassemic phenotype.[96,110,111]

Diagnosis

Laboratory diagnosis of heterozygotes is based on elevated levels of Hb A_2 followed or not by a small increase in Hb F. Homozygotes for$\beta^0\beta^0$ exhibit only Hb A_2 and F (around 98%), while $\beta^+\beta^+$ homozygotes and$\beta^0\beta^+$ double heterozygotes have a variable proportion of Hb F with relation to Hb A, generally between 40 and 70%. It is worth once again pointing out the fundamental importance of performing a family study.[13,64]

Molecular diagnosis can be made by a variety of techniques, with the most used being direct sequencing of the β genes and restriction analysis when possible. Microarray platforms with probes for the most common mutations have also been used and are tending to substitute conventional techniques to the extent that their prices reduce.[13,65]

Treatment

The treatment currently employed involves regular transfusion therapy, to maintain minimum Hb levels of 9.5-10 g/dL, administration of iron chelators, such as desferrioxamine or other oral chelating agents, and monitoring iron overload by means of serum ferritin assays, T2* magnetic resonance to evaluate excess iron in the heart and endocrine support.[6,11,90,98,101]

Desferrioxamine is the chelating treatment of choice. Since it requires prolonged daily parenteral infusion, new chelating agents have been tested and proposed.[11] Deferiprone is an orally administered drug that penetrates the cell membrane and chelates toxic intracellular iron species. Some adverse effects have been reported, such as leukopenia, neutropenia and arthritis, but a large number of clinical studies indicate that it can be more effective at removing iron from the heart than desferrioxamine.[112,113] More recently, a combination of these two drugs has been tested.[114] A new oral chelator, deferasirox, was recently approved in the United States and in Brazil. Its efficacy appears to be equivalent to

that of desferrioxamine , although its side-effects over the long term are not yet known.[115-117]

Recent discoveries indicate that there is potential for therapeutic intervention in β-thalassemia by means of manipulating iron metabolism.[6] Administration of synthetic hepcidin or of agents that increase its expression, may be beneficial in controlling absorption of this metal.[118] Hepcidin is a small peptide produced by the liver in elevated quantities during infection. It inhabits intestinal iron uptake, thereby depriving infectious agents of the element.[99,100] Hepcidin levels become elevated when iron stores are elevated, but in patients with major or intermediate thalassemia, and in the thalassemic mouse model, levels are reduced, permitting increased iron uptake. In an attempt to understand the cause of this inappropriate reduction, Tanno et al. (2007) demonstrated that inhibition of hepcidin expression in thalassemic syndromes was correlated with an increase in expression of growth differentiation factor GDF15, which is a member of a superfamily of molecules (TGFB) recently identified as regulating hepcidin expression. The serum of thalassemic patients suppresses hepcidin expression in primary human hepatocytes, while GDF15 depletion reverses this suppression. The authors propose that, in thalassemic syndromes, elevated GDF15 expression (and possibly of other proteins with similar roles) originates in an expanded erythroid compartment and contribute to iron overload by means of inhibition of hepcidin expression. This factor would therefore be another potential therapeutic target.[100]

Increasing Hb F production could also be a treatment alternative, but in β-thalassemia there are not yet any inductive agents that have demonstrated efficacy in large numbers of patients, although some cases may respond to HU.[6,11] Since the problem is actually excess α chains, treatments that could suppress expression of α genes could help to reduce clinical severity. To achieve this, the mechanisms involved in regulation of the globin genes need to be better elucidated.[84,85,119-121]

A number of antioxidants have been tested with a view to protecting the cell membrane, including vitamins C and E and certain flavonoids from plants, but the results are not conclusive and the studies need to be amplified in humans.[122,123]

Allogeneic transplant of hematopoietic stem cells from related and HLA-compatible donors is a very promising alternative and is the only that offers the possibility of cure to date. Once more, as with the SCD, the best results are observed with younger patients, who have not yet accumulated the tissue and organ damage seen in older patients. The limitation to achieving greater success with this method is the scarcity of related HLA-compatible donors.[10,124]

Gene therapy is also being investigated with animal models for this reason.[120] Transferring the normal β gene to hematopoietic stem cells could lead to a permanent cure. Several viral vectors that integrate themselves permanently with the host genome have been tested, but the problems are the same as those described above with relation to SCD.[125-127]

Conclusions

Hemoglobinopathies are among the most common monogenic diseases found in populations. The complexity of their pathophysiologic processes and the severity and diversity of their clinical manifestations mean that the SCD and β-thalassemia are an enormous challenge for medicine and science. Increased knowledge about the biological basis of these diseases, although still associated with elevated morbidity and mortality, has offered important advances in therapeutic management and in prevention of new cases and may, in the near future, offer more concrete possibilities of cures.

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