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Anemia, Ineffective Erythropoiesis and Hepcidin: Interacting Factors in Abnormal Iron Metabolism Leading to Iron Overload in β-Thalassemia

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Synopsis

β-thalassemia is a genetic disorder caused by mutations in the β-globin gene and characterized by chronic anemia due to ineffective erythropoiesis, and accompanied by a variety of serious secondary complications such as extramedullary hematopoiesis, splenomegaly, and iron overload. In the past few years, numerous studies have shown that such secondary pathologies have a genetic basis due to the abnormal expression of genes with a role in controlling erythropoiesis and iron metabolism. In this article, the most recent discoveries related to the mechanism(s) responsible for anemia/ineffective erythropoiesis and iron overload will be discussed in detail. Particular attention is paid to the pathway(s) controlling the expression of hepcidin, which is the main regulator of iron metabolism, and the Epo/EpoR/Jak2/Stat5 signaling pathway, which regulates erythropoiesis. Better understanding of how these pathways function and are altered in β-thalassemia has revealed several possibilities for development of new therapeutic approaches to treat of the complications of this disease.

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β-thalassemia

Genetic causes, consequences and pleiotropic effects

As discussed in more detail in the Overview by Sankaran and Nathan at the beginning of this issue, β-thalassemia is an inherited disorder characterized by mutations in the gene encoding β-globin that lead to the quantitative reduction or, in the most severe cases, the total absence of β-globin synthesis in human erythroid cells. As a consequence, α-globin chains accumulate in excess, forming aggregates that impair erythroid cell maturation, which ultimately leads to a chronic hemolytic anemia and ineffective erythropoiesis (Fig. 1). The severity of the clinical manifestations in β-thalassemia varies widely, ranging from patients that are almost asymptomatic to individuals who suffer from severe anemia and require regular blood transfusion to sustain life (1-6). In general, the clinical severity of the disease correlates with the size of the free α-chain pool and the degree of imbalance between the production of α- and β-like globin chains.

The α/β globin chain imbalance is responsible for the hemolysis of red blood cells and for the premature death (apoptosis) of erythroid precursors in the bone marrow and at extramedullary sites (Fig. 1). The α-globin chain aggregates form inclusion bodies responsible for oxidative stress and membrane damage within red blood cells and immature developing erythroblasts. These events are followed by the premature death of many late erythroid precursors in the bone marrow and spleen. Together, these phenomena are designated as ineffective erythropoiesis (IE). The anemia and resulting hypoxia lead to a dramatic increase in serum erythropoietin (Epo) levels in an attempt to compensate for the reduced oxygen carrying capacity. The marked increase in Epo stimulation, if it is not inhibited by proper transfusion therapy, can lead to uncontrolled expansion of erythroid precursors in the marrow as well as in other sites, such as the spleen and liver, leading to extramedullary hematopoiesis (EMH) (Fig. 1).

The defect in hemoglobin synthesis that occurs in β-thalassemia ultimately leads to the development of pleiotropic effects on different body compartments. One of the consequences of IE and EMH, for example, is splenomegaly (Fig. 1). The abnormal and damaged red blood cells that are produced in β-thalassemia are sequestered by the reticuloendothelial system in the spleen, causing its enlargement. This enlargement leads to increased sequestration of RBCs, including transfused RBCs, in the spleen, with worsening of the anemia and an increase in transfusion requirement. Increased Epo synthesis is also reflected in marrow expansion, leading to bone marrow hyperplasia, bone deformities and osteopenia (Fig. 1), which contribute to increased morbidity as the disease progresses (7).

Iron overload, however, is the principal and multifaceted complication of β-thalassemia. Physiologically, it is caused by an increased absorption of iron from the gastrointestinal (GI) tract as a consequence of ineffective erythropoiesis., and is greatly aggravated by chronic transfusion therapy. Thus, transfusion-independent individuals with thalassemia intermedia have a slower progression of iron overload and generally develop complications later in life compared to patients with thalassemia major who are chronically transfused (Fig. 1). A remarkable variability of tissue iron distribution has been observed in β-thalassemia with liver, heart and endocrine glands being the organs most severely affected (5). Accumulation of iron at these sites (siderosis) leads to oxidative damage due to the generation of reactive oxygen species (ROS). Death from cardiac failure is the most common clinical consequence of iron excess. Pathological analysis of cardiac tissue has shown that this occurs initially by hypertrophy and dilatation followed by degeneration of myocardial fibers and fibrosis. Endocrine problems are also related to iron overload (Fig. 1). These include hypogonadism, which leads to disturbances of growth and sexual maturation, as well as hypothyroidism, hypoparathyroidism and diabetes mellitus that are seen in variable numbers of patients.

Therefore, effective iron chelation therapy is a critical component in the management of thalassemia (8-10). For more than three decades, deferoxamine (DFO, Desferal®) was the chelator of choice (11). While this drug is capable of placing all patients into net negative iron balance, it suffers from the fact that it must be infused subcutaneously over 8 to 12 hours, 5 to 7 days a week for the duration of life. Not surprisingly, adherence to such a regimen is generally poor. More recently, two orally effective iron chelators, Exjade® (DFX, deferasirox) and deferiprone (DFP, Ferriprox®), have been developed to overcome this limitation (11). The topics of iron overload and chelation therapy are discussed in greater detail elsewhere in this issue in the article by Porter and Shah.

Currently, the only definitive cure for thalassemia is the transplant of hematopoietic stem cells (HSC) from cord blood or the bone marrow. This topic is discussed in detail elsewhere in this issue in the article by Gaziev and Lucarelli, as well as that by Kanathezhath and Walters. Splenectomy is another facet in the management of β-thalassemia. However, removal of the spleen can lead to further problems such as an increased risk of infections, pulmonary hypertension and thrombosis (12-14), and is usually considered only in the most severe cases of splenomegaly. New approaches to treat/cure the disease are being developed, such as gene therapy, discussed in the article by Bank elsewhere in this issue, or the use of induced pluripotent stem cells as alternatives to the use of HSC (15,16).

Mouse models of β-thalassemia

The use of mouse models of β-thalassemia has been of fundamental importance in clarifying the molecular mechanisms responsible for disease. The two available mouse models of β-thalassemia intermedia are *th1*/*th1* and *th3*/+. In the *th1*/*th1* mouse, the deletion removes the β^{major} gene in the homozygous state (17), producing hemoglobin levels in the range of 9 to 10 g/dL (17-19). In the *th3*/+ mouse model, the deletion removes both the β^{minor} and β^{major} genes in the heterozygous state (20,21). These mice have hemoglobin levels in the range of 8 to 9 g/dL (19-21). Both models have a degree of disease severity (hepatosplenomegaly, anemia, aberrant erythrocyte morphology) comparable to that of patients affected by transfusion-independent β-thalassemia intermedia. Their ineffective erythropoiesis is characterized by a modest reduction in red blood cells and an increase in reticulocytes (22). Homozygous (*th3*/*th3*) mice that completely lack any adult β-globin chain synthesis die late in gestation (20), precluding their use as a model for β-thalassemia major. To overcome this limitation, *th3*/*th3* mice are generated by transplantation of hematopoietic fetal liver cells (HFLCs) into lethally irradiated syngeneic adult recipients, the HFLCs being harvested from *th3*/*th3* embryos at 14.5 days of gestation (22). These mice exhibit severe anemia (3-4 g/dL) 6-8 weeks post-transplant as well as very low red blood cell (RBC) levels and reticulocyte counts, together with massive splenomegaly and extensive EMH (22).

Ineffective erythropoiesis

The Epo/EpoR/Jak2/Stat5 pathway and its potential effect(s) on iron intake in erythroid cells

The hallmark of β-thalassemia is IE that stems from a lack of or reduced synthesis of β-globin, which leads to an excess of α-globin chains that aggregate and precipitate, adhering to the membrane of erythroid precursors. These α-globin aggregates cause cellular and membrane damage, apoptosis of the erythroid precursors in the bone marrow and generation of mature red cells that are abnormal and accumulate in very limited numbers. Moreover, the production of red cells, EMH and the anemia can change significantly over time. Therefore, β-thalassemia serves as a good example of the dynamic balance between expansion of the erythroid pool and production of red cells, and of the many factors that can alter this relationship with critical effects. When pathological levels of damaged

erythrocytes are trapped in the spleen they cause splenomegaly, anemia and hypoxia. Anemia and hypoxia, in turn, stimulate Epo synthesis, which increases the number of erythroid precursors and abnormal mature red cells in circulation. This exacerbates the trapping of erythrocytes in the spleen thereby worsening the splenomegaly. Moreover, increased erythropoiesis augments iron absorption. Iron overload can increase the formation of ROS causing damage to many organs and further aggravating the anemia. Blood transfusion is a very effective method for ameliorating the anemia and the consequences of IE. But good adherence to iron chelation therapy is necessary to prevent the detrimental effects of iron overload.

Epo is a 34-kDa renal glycoprotein that functions as the main regulator of erythropoiesis, both under basal and stress conditions. Epo binds to its specific receptor, the erythropoietin receptor (EpoR), at the surface of erythroid cells. EpoR is expressed by the earliest erythroid progenitors at the burst forming unit-erythroid (BFU-E) stage (23-25). The Epo/EpoR signaling pathway begins with dimerization of the receptor and activation of the tyrosine-Janus kinase 2 (Jak2) (26-29), which preassembles at a conserved site in the cytoplasmic domain of the EpoR. Jak2 catalyzes transfer of the gamma-phosphate group of adenosine triphosphate to the hydroxyl groups of specific tyrosine residues in signal transduction molecules and mediates signaling downstream of cytokine receptors after ligand-induced autophosphorylation of itself and of tyrosine residues of the receptor. In particular, Jak2 mediates the phosphorylation of tyrosine residues localized in a conserved cytoplasmic domain of the EpoR. The resulting phosphorylated tyrosine residues of the EpoR function as a ‘scaffold’ or docking sites for the assembly of signal transduction factors containing Src-homology 2 (SH2) domains. The main downstream effectors of Jak2 are a family of transcription factors known as signal transducers and activators of transcription (Stat). In erythroid cells, the main target is Stat5 which, upon phosphorylation, translocates to the nucleus as a dimer to drive expression of target genes. The ultimate effect of Jak2 and Stat5 activation is to induce multiple signaling pathways designed to regulate erythroid proliferation and differentiation, and to protect the cells from apoptosis (28,30,31).

Polycythemia vera (PV), essential thombocytosis (ET) and primary myelofibrosis (PMF) are classified as myeloproliferative disorders (MPD), a subgroup of myeloid malignancies (32). These are clonal stem cell diseases characterized by an expansion of morphologically mature cells of the granulocyte, erythroid, megakaryocyte, or monocyte lineage. Interestingly, several studies have described a close association between an activating JAK2 mutation (Val 617 to Phe; JAK2V617F) and these disorders (33). This mutation is thought to prevent the pseudokinase domain from inhibiting the kinase domain, resulting in a constitutively active state of the protein. Thus, JAK2V617F confers constitutive kinase activation. In erythroid cells this leads to STAT5 phosphorylation and Epo-independent erythroid colony formation (34).

Mice lacking Stat5 expression ($Stat5^{-/-}$) have shown early lethality associated with microcytic anemia and enhanced apoptosis of early erythroblasts (35-37). While the anemia in these mice correlates with loss of expression of the antiapoptotic *Bcl-X_L* gene and enhanced apoptosis (38), additional analyses indicate that $Stat5^{-/-}$ mice have a significant decrease in expression of the iron transporter transferrin receptor-1 (Tfr1) (38,39). Therefore, it is possible that erythroid cells might express higher levels of Tfr1 under conditions of constitutive activation or up regulation of Jak2. Potential consequences of Jak2 modulation of iron intake in β-thalassemic red cells will be discussed in the next section.

New studies on Jak2 and IE in β-thalassemia

Original ferrokinetic studies and analysis of erythroid precursors in β-thalassemia indicated that many of these cells die in the marrow and extramedullary sites (40-43), and suggested

that the relative excess of α chains triggering apoptosis was responsible for IE. However, several recent observations suggest that the balance between proliferation and differentiation in some of the erythroid precursors might be different under normal conditions and those of IE (44). These results and those of new studies in mice (discussed later) suggest that the mechanism(s) and various factors associated with the process known as IE have not yet been completely elucidated.

The splenomegaly exhibited by *th3*/+ mice(44) is very similar to that observed in patients affected by β-thalassemia intermedia (transfusion independent). Studies on *th3*/+ mice have shown that their spleen fills with erythroid precursors and sequesters abnormal/damaged erythrocytes, likely contributing to lowering of the hemoglobin level over time (44). Similar observations have been confirmed in splenic specimens from patients with thalassemia intermedia (15,44,45). However, analysis of the erythroid precursors in both the bone marrow and spleen in these animals indicated that a large number of these cells were actively proliferating while the proportion of cells undergoing apoptosis, although higher than in normal mice, was relatively modest (44). Compared to wild-type animals, *th3*/+ mice have a higher number of erythroid cells associated with the expression of cell cycle-promoting genes such as *EpoR*, *Jak2* (Fig. 1), *Cyclin-A*, *Cdk2* and *Ki-67*, together with increased levels of the Bcl-X_L protein (44). These cells also differentiate less than the corresponding normal cells *in vitro*. Based on this observation, we can speculate that thalassemic patients may increase the number of erythroid precursors in their spleen and liver over time, this being one factor leading to hepatosplenomegaly. In turn, this would also exacerbate the anemia in β-thalassemia (15,44,45).

We already discussed the increase of Epo levels in response to anemia and hypoxia. Based on the observations above, it is reasonable to expect that increased Epo levels could also activate the EpoR/Jak2 pathway, leading to a “physiological” gain of function of Jak2. Under these circumstances, the persistent phosphorylation of Jak2 might lead to an increased number of erythroid progenitor cells. Therefore, suppression of Jak2 activity might modulate IE. Based on this model, we showed that use of a Jak2 inhibitor has a beneficial effect in limiting IE, splenomegaly and the number of erythroid progenitor cells in β-thalassemia (44). In particular, limiting the number of erythroid progenitors might have a beneficial effect on iron metabolism, since it has been suggested that these cells are the most plausible source of the erythroid factor, whose function is to increase iron absorption by repressing hepcidin expression, as shown in Fig. 1 and 2 and further discussed below. Therefore, future studies will need to address whether the use of Jak2 inhibitors could also limit iron absorption in β-thalassemia.

Activation of the Epo/EpoR/Jak2 pathway is not likely to be the only cause for the limited erythroid differentiation observed in β-thalassemia. For example, in the absence of other erythroid defects, mutations responsible for the constitutive activation of Jak2 lead to the development of *polycythemia vera* rather than IE (46). Therefore, it is possible to predict that other factors and/or abnormal physiological conditions present in β-thalassemia interfere with erythroid cell differentiation. Among the possible factors acting together with Jak2, iron overload, ROS, the unbalanced synthesis of globin chains and/or heme can be considered (45). Iron is essential for all cells but is toxic in excess. We recently focused on the potential role of iron and heme in modulating IE. Our hypothesis was that thalassemic erythroid cells accumulate an excess of toxic heme associated with free α chains, leading to the formation of ROS, which has been associated with red cell hemolysis and altered differentiation (47,48). Our preliminary data suggest that this is the case and that if the hemoglobin content per cell (MCH) and the overall amount of heme is reduced in thalassemic erythroid cells, both ROS and the accumulation of α-chains on red cell plasma membranes are reduced, ameliorating not only the quality and lifespan of the RBCs, but also

the anemia and IE (Gardenghi et al, unpublished data). The amelioration of IE was associated with decreased splenomegaly and a better balance between erythroid precursors and mature RBCs. Based on these preliminary observations, we speculate that ROS play a role in modulating the balance between proliferation and differentiation of the erythroid cells. Additional studies point to the role of ROS in cell differentiation in erythropoiesis, stem cells and cancer, supporting this hypothesis (49,50).

Serum iron is bound to transferrin and enters erythroid cells primarily via receptor-mediated endocytosis of the transferrin/transferrin receptor 1 (Tfr1) complex. Tfr1 is essential for developing erythrocytes. Reduced Tfr1 expression is associated with anemia. *Stat5-null* mice are severely anemic and die perinatally. Two studies have associated Stat5 with iron homeostasis (38,39), showing that ablation of Stat5 leads to a dramatic reduction in both the mRNA and protein levels of iron regulatory protein 2 (IRP-2) and *Tfr1*. Both genes have been shown to be direct transcriptional targets of Stat5, establishing a clear link between EpoR/Jak2/Stat5 signaling and iron metabolism. As we proposed previously, reduced iron intake in erythroid cells limits the formation of toxic α-chain/heme aggregates and ROS formation, having a beneficial effect on IE. This has been supported indirectly by the work of Ginzburg and colleagues (51). In their studies, iron delivery to erythroid cells in mice affected by β-thalassemia intermedia was modulated by administration of apo-transferrin. This was associated with reduction of splenomegaly and IE, improvement in hemoglobin levels and an increased number of RBCs. The hemoglobin content per cell (MCH) was also decreased, as was the formation of membrane bound α-chain aggregates. These data reinforce the notion that decreasing iron availability, either by decreasing iron absorption or transferrin saturation, is beneficial to abnormal erythroid cells. Therefore, based on the association between Jak2 and Tfr1, Jak2 inhibitors might also ameliorate IE by decreasing expression of Tfr1, iron intake, formation of toxic α-chain/heme aggregates and ROS. In conclusion, use of Jak2 inhibitors in β-thalassemia might also be beneficial due to their role in controlling iron metabolism in erythroid cells.

Iron metabolism

Hepcidin and regulation of iron absorption

Erythropoiesis and iron metabolism are closely interconnected. The iron utilized by the body is obtained by recycling that present in senescent red blood cells or absorbed from the diet at the level of the proximal intestine. More than two-thirds of the body's iron content is incorporated into hemoglobin in developing erythroid precursors and mature red blood cells (52). Hepcidin (HAMP/Hamp) (53,54), a cysteine-rich 25-amino acid peptide synthesized in the liver from an 84-amino acid prepropeptide plays a major role in iron homeostasis. Hepcidin was originally isolated from blood ultra filtrate and urine (53,54). Its target is ferroportin, which is the only known iron exporter on enterocytes, hepatocytes and macrophages. Hepcidin binds ferroportin promoting is internalization and degradation (55,56), thereby negatively regulating iron absorption and iron recycling within the body. Hepcidin is up regulated in response to iron overload (57) and inflammation (58-61), and down regulated by erythropoietic stimuli such as anemia, hypoxia or EPO synthesis/ administration (58,62,63). In all these scenarios, hepcidin acts primarily on ferroportin controlling iron egress from enterocytes and macrophages, and modulating dietary iron absorption as well as erythropoiesis.

In the last few years, many studies have characterized proteins that contribute to hepcidin regulation. Fig. 2 is a non-exhaustive representation of the proteins and pathways involved in hepcidin regulation. Hepatic cells can take up holo transferrin (holoTf) through Tfr1 by receptor-mediated endocytosis. The same region of Tfr1 that binds holoTf is also recognized by Hfe (Fig. 2) (64,65). HFE is an atypical HLA class I protein. Mutations in *HFE* cause

hemochromatosis, which is a disorder characterized by excess total body iron due to hyper absorption from the diet (66). When the concentration of holoTf in the serum increases, both holoTf and Hfe bind to Tfr2, which is a protein homologous to Tfr1, although the relative affinity of Tfr2 for holoTf and Hfe is reduced compared to that of Tfr1 (67-69). The Hfe/Tfr2 complex then interacts with a second protein complex to signal up-regulation of hepcidin. This second complex of proteins involves the association of bone morphogenetic protein-6 (Bmp6) ligand with a complex of type I and type II serine threonine kinase receptors and the hemojuvelin (Hjv) co-receptor yielding the "hepcidin signaling" or "iron sensor complex" (Fig. 2) (68,70-74). The resulting complex propagates the signal through phosphorylation of cytoplasmic effectors Smad1, Smad5 and Smad8. Once phosphorylated, Smad1/5/8 form heteromeric complexes with the common mediator Smad4 and translocate to the nucleus where they modulate transcription of target genes, including hepcidin (73,75). Moreover, it has been shown that Tfr2 can be activated by its ligand holoTf leading to stimulation of the extracellular signal regulated kinase (Erk)/mitogen activated protein kinase (MapK) pathway, and induction of hepcidin (76,77). Even in this case, Erk activation by holoTf provokes increased levels of phospho-Smad1/5/8.

Mutations in some of these proteins (Hamp, Hfe, Hjv, Tfr2, Bmp6) have clearly been associated with conditions that lead to iron overload due to the fact that they impair hepcidin expression, either directly or indirectly (78,79). In contrast, mutations in TMPRSS6, a type II transmembrane serine protease, are associated with a condition termed iron-refractory iron-deficiency anemia (IRIDA), in which hepcidin expression is increased and both patients and mice suffer from iron deficiency and severe anemia (80,81). Several studies indicate that Tmprss6 targets and degrades Hjv, preventing its assembly in the "iron sensor complex" profoundly impairing the Bmp6/Smad pathway (Fig. 2) (82). A soluble form(s) of Hjv (sHjv) might also act as a decoy for some of the proteins in the complex, limiting its assembly (83). Moreover, it has been shown that mice deficient in Tmprss6 have decreased iron stores and decreased Bmp6 mRNA, but markedly increased mRNA for Id1, another target gene of Bmp6 signaling. Id1, whose promoter is strongly activated by Bmp6 in a Smad-dependent manner, encodes a negative inhibitor of basic helix-loop-helix (bHLH) proteins. Mice deficient in both Tmprss6 and Hjv showed decreased hepatic levels of hepcidin and Id1 mRNA whereas Bmp6 mRNA was markedly increased (84). These mice suffer from systemic iron overload similar to mice deficient in Hjv alone (85). Such findings suggest that regulation of hepcidin expression and maintenance of systemic iron homeostasis by Tmprss6 requires down regulation of Bmp6/Smad signaling and expression of Id1.

As mentioned previously, hepcidin synthesis is down regulated by erythropoietic stimuli such as anemia, hypoxia and Epo synthesis/administration (58,62,63), thereby increasing the GI absorption of iron and its release from stores. It has been postulated that an "erythroid regulator" modulates these responses by acting, directly or indirectly, on the synthesis of hepcidin (Fig. 2). The existence of an erythroid factor is supported by studies in which the serum of patients affected by β-thalassemia or Hfe-related hemochromatosis were compared in terms of their ability to induce the expression of hepcidin and other genes related to iron metabolism in hepatic cells. Sera from β-thalassemia major and intermedia patients down regulated hepcidin expression, while that from those affected by hemochromatosis had no effect on hepcidin (86). While it has been suggested that hypoxia and Epo suppress hepcidin expression, there is considerable evidence indicating that the erythropoietic regulator must involve a soluble factor from the hematopoietic bone marrow. For instance, inhibitors of erythropoiesis can be administered after phlebotomy to disassociate the effects of anemia, hypoxia, and Epo from those of increased erythropoiesis. Phlebotomized mice develop anemia, tissue hypoxia, increased levels of Epo and erythropoiesis and decreased levels of hepatic hepcidin mRNA. When erythropoietic inhibitors were administered, hepcidin mRNA rose dramatically, even though the mice were anemic, hypoxic and exhibited

elevated Epo levels (87). If this scenario is correct, it is reasonable to predict that the mediator is a factor secreted by erythroid cells, most likely immature and proliferating erythroid cells. Therefore, we propose that this factor should be more abundant under conditions in which the number of erythroid progenitors is increased, such as after recovery from acute anemia or when IE is present (**Fig.** 1 and 2). Since erythropoiesis is the most important process utilizing iron in the body, we also speculate that the "erythroid regulator" operates very efficiently in controlling iron metabolism. In other words, the function of the erythroid regulator should be to maintain production of erythrocytes irrespective of the body's iron balance. However, as we do not know yet how this factor works, we can only speculate that it might operate by targeting one or more components of the "iron sensor complex", the mediators activated by this complex or the hepcidin promoter, as shown in Fig.2.

The role of hepcidin in β-thalassemia

Studies have shown that the rate of iron absorption from the GI tract in patients affected by β-thalassemia is approximately 3-4 times greater than that in healthy individuals (88). But how does the increased iron absorption affect tissue iron distribution and ultimately erythropoiesis in β-thalassemia? Ferrokinetic studies showed that when donor serum labeled with ^{59}Fe was injected into healthy subjects, 75% to 90% of the iron was incorporated into newly formed red cells within 7 to 10 days. However, when ^{59}Fe was injected into thalassemic patients, only 15% to 20% of it was found in circulating erythrocytes (40). It was hypothesized that the remaining iron was sequestered in those organs where erythroid precursors are subject to premature destruction, such as the bone marrow in humans, and the bone marrow and spleen in mice.

Numerous studies have demonstrated that altered hepcidin expression is responsible for the increased iron absorption observed in β-thalassemia (89-91). In particular, the correlation between IE, iron distribution, and the expression of hepcidin have been investigated in mice affected by thalassemia intermedia and major, models characterized by different degrees of anemia and IE (19,89). The major conclusion is that the pattern of iron distribution in β-thalassemia is dictated by the degree of IE. Where severe anemia exists, hepcidin levels are extremely low, and iron overload occurs rapidly involving predominantly liver parenchymal cells. On the other hand, when the anemia is milder, iron accumulates progressively in splenic macrophages and Kupffer cells in the liver. When thalassemia major mice were transfused, their anemia and IE improved, while iron deposition in the liver was reduced. Analyses using human specimens indicated that urinary hepcidin levels were lower in β-thalassemic patients than those which would be predicted by their iron burden, while transfusion therapy led to an increase in the hepcidin levels(92-96).

In the case of mice affected by β-thalassemia intermedia, it has been observed that they exhibit very low hepcidin levels during the first months of life (16,89,91,97-99**).** As these animals age, the level of hepcidin increases, resulting in increased organ iron concentrations (16,89,98). This indicates that hepcidin is still partially responsive to iron overload when IE is relatively low. In contrast, the extreme degree of IE in mice affected by thalassemia major limited hepcidin from sensing the iron burden and kept its expression very low. Altogether these observations indicate that the relative levels of IE and iron overload mediate the synthesis of hepcidin in β-thalassemia.

Two erythroid regulators have been proposed called growth differentiation factor 15 (GDF15) and twisted gastrulation protein homolog 1 (TWSG1) (100,101). They are members of the TGF-β super family of proteins known to control proliferation, differentiation, and apoptosis in numerous cell types. Both GDF15 and TWSG1 are elevated in the serum of β-thalassemic patients and suppress hepcidin expression *in vitro* (101).

Comparing normal and thalassemic erythroid cells differentiating in vitro, GDF15 was isolated during the final stages of erythroid differentiation (101). This suggests that GDF15 is secreted by erythroid precursors undergoing cell death. In fact, GDF15 is not elevated after stem cell transplantation in patients with hematopoietic malignancies or in patients with iron deficiency secondary to blood donation, conditions in which apoptosis of erythroid cells is not observed (102,103). These findings confirm that GDF15 is not produced by proliferating erythroid precursors but rather by apoptotic erythroid cells, as in β-thalassemia or refractory anemia with ring-sideroblasts (104). Therefore, GDF15 may limit hepcidin synthesis when erythroid precursors undergo cell death (44). In contrast to GDF15, the highest levels of TWSG1 were detected at early stages of erythroblast differentiation, before hemoglobinization of the cells (100). Future studies will determine whether TWSG1 is an erythroid factor present only in sera of thalassemic patients or whether it is associated with many other conditions characterized by increased erythropoiesis and hepcidin suppression.

Questions and potential novel therapies

Administration of Jak2 inhibitors, and potential effects following reduced iron intake by erythroid cells

Previously, we discussed how Jak2 might influence IE, splenomegaly and anemia in β-thalassemia. One obvious consequence of these observations has been to investigate whether Jak2 inhibitors might have beneficial effects in reducing/preventing splenomegaly and ameliorating the clinical phenotype of this disease. Our preclinical data obtained by using Jak2 inhibitors in mice affected by β-thalassemia intermedia support the notion that patients might benefit from using such compounds (44). In fact, many patients with thalassemia intermedia develop splenomegaly and the need for transfusion therapy, most of them eventually requiring splenectomy. However, because the V617F mutation is localized in a region away from the adenosine triphosphate (ATP)-binding site of JAK2, the ATP-competitive inhibitors of JAK2 kinase (ATP analogues) presently utilized in clinical trials are not likely to discriminate between the wild-type and mutant enzyme. Therefore, their use could decrease RBC synthesis and worsen anemia. With these facts in mind, we tested the effect of administering a Jak2 inhibitor to thalassemic mice in association with blood transfusion. Our preliminary data indicate that the combined use of a Jak2 inhibitor and blood transfusion is superior to either treatment alone in ameliorating splenomegaly and IE (Melchiori et. al. unpublished data). Thus, Jak2 inhibitors might be used temporarily to reduce the spleen size and, in the presence of blood transfusions, to treat or prevent worsening of the anemia. The ultimate goal would be to prevent or delay the need for splenectomy and indirectly to improve the management of the anemia thereby reducing the need for blood transfusions. Moreover, future studies should investigate whether Jak2 inhibitors, through their potential modulation of Tfr1 synthesis, can also modify iron uptake into forming thalassemic erythroid cells, limiting the formation of α-chain/heme aggregates and ameliorating the phenotype of the erythroid cells.

Administration of hepcidin agonists or activators of hepcidin expression

Due to hepcidin deficiency, patients with β-thalassemia intermedia develop iron overload in a manner similar to those with hereditary hemochromatosis. Accordingly, abnormal iron absorption in these patients might be prevented by administration or up regulation of hepcidin (Gardenghi et al., Ann. N.Y. Acad. Sci., 2010, in press; Gardenghi et al. unpublished data). In β-thalassemia major, transfusions rather than dietary iron absorption are the predominant cause of iron overload. In affected individuals, hepcidin levels are higher because IE is suppressed by transfusion therapy. Moreover, hepcidin production is stimulated by the additional iron load derived from the transfusion of red cells. However, hepcidin concentrations decrease in the intervals between transfusions, as the effect of each

transfusion is lost (92,93,101). Thus, although intestinal iron absorption contributes less to the total iron load in these patients, hepcidin therapy may be effective when endogenous hepcidin falls and intestinal iron uptake increases. The potential benefits of hepcidin therapy are already supported by a few studies using mouse models of hemochromatosis (65,105). Based on these assumptions and observations, we are further investigating whether or not modulation of hepcidin could be beneficial in β-thalassemia. For this purpose we are utilizing mice affected by β-thalassemia intermedia genetically altered to over-express hepcidin.

Our preliminary data indicate that hepcidin-mediated iron restriction may not only ameliorate iron overload in mice affected by β-thalassemia intermedia, but also improve their erythropoiesis. We hypothesize that amelioration of erythropoiesis in these mice is due to decreased α-chain/heme aggregates and ROS formation. α-chain/heme aggregates can precipitate and lodge in red cell plasma membranes, affecting the properties and lifespan of RBCs. Therefore, reduction of these aggregates might improve the phenotype of the thalassemic red cells and increase their lifespan, with positive feedback on IE (Fig. 3). Moreover, these aggregates are also likely to cause the formation of ROS in erythroid cells. In fact, ROS levels are elevated in red cells derived from patients with β-thalassemia. As a result, glutathione (GSH) levels are lower than in normal red cells (106). ROS likely exacerbate the anemia, further decreasing the already shortened survival of mature RBCs in the circulation (47,107). Excessive ROS formation in maturing erythroid cells might also affect the cell cycle and worsen IE. The Forkhead Box O (FoxO) family of transcription factors plays an essential role in the regulation of oxidative stress in hematopoietic stem and erythroid cells (108). In FoxO3 null mice, red cell survival is reduced and is associated with enhanced mitotic arrest of intermediate erythroid progenitor cells, resulting in a decreased rate of erythroid maturation (48). This raises the interesting possibility that ROS levels regulate not only RBC survival, but also the maturation process of erythroid progenitor cells, modulating IE. Therefore, using hepcidin as a tool to reduced iron absorption might be a novel and exciting approach to controlling IE and managing β-thalassemia.

Thus far, the studies undertaken to evaluate the effect of over expressing hepcidin on iron overload and erythropoiesis have utilized genetic models and did not explore the feasibility of using hepcidin or its agonists as drugs. Development of such compounds will determine their potential to prevent iron overload or reverse its toxic effects based on a dose-response relationships. However, since ferroportin is localized on macrophages as well, the administration or up-regulation of hepcidin in thalassemia may also affect iron recycling and its availability for erythropoiesis, ultimately worsening anemia (Fig. 3). For this reason, preclinical studies must address the effect of these drugs on both iron overload and erythropoiesis. Based on the fact that the levels of hepcidin are low in thalassemia and body iron is in excess, hepcidin therapy may be feasible depending upon the level of hepcidin achieved (Fig. 3). Further study in mouse models of thalassemia followed by rigorous clinical trials in patients will address these issues.

Summary

- Patients with β-thalassemia develop secondary effects such as splenomegaly and iron overload.
- IE is the hallmark of β-thalassemia, characterized by the premature death of erythroid precursors in the bone marrow and extramedullary sites.
- Erythrocytes trapped in the spleen are the cause of splenomegaly, anemia and hypoxia, which leads to increased Epo production.

- Splenomegaly eventually contributes to worsening of the anemia, necessitating splenectomy.
- Because of increased Epo expression, the Epo/EpoR/Jak2/Stat5 pathway, which regulates erythropoiesis, is over-active in β-thalassemia, contributing to EMH and splenomegaly.
- Jak2 inhibitors, used in combination with transfusion therapy, represent an alternative approach aimed at modulating IE and preventing splenomegaly/splenectomy.
- Inhibition of Jak2 activity might also modulate the number of erythroid progenitor cells in β-thalassemia. These cells are the most plausible source of the erythroid factor, the proposed function of which is to increase iron absorption by suppressing hepcidin expression. Therefore, reducing Jak2 activity might have a beneficial effect on iron metabolism as well.
- Currently, the only effective treatment for iron overload is chelation therapy.
- Decreased expression of hepcidin is the cause of increased iron absorption in β-thalassemia.
- Alternative therapies to prevent iron overload include up regulation of hepcidin expression and the direct administration of hepcidin agonists.

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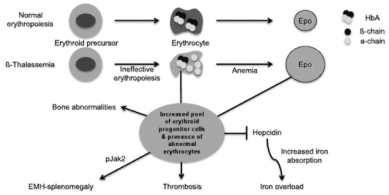

Fig.1. Consequences of ineffective erythropoiesis and abnormal erythrocyte production

Lines ending with an arrow indicate activation. Lines ending with a line indicate repression.

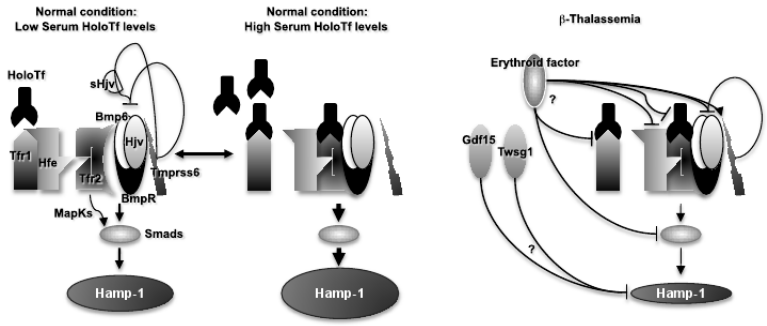

Fig.2. Potential pathways controlling iron absorption in β-thalassemia

Lines ending with an arrow indicate activation. Lines ending with a line indicate repression.

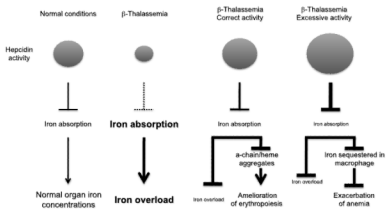

Fig.3. Potential effects of Hepcidin agonists or activators on iron absorption under normal and β-thalassemic conditions

Lines ending with an arrow indicate activation. Lines ending with a line indicate repression.