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New strategies to target iron metabolism for the treatment of beta thalassemia

Paraskevi Rea Oikonomidou[1], **Carla Casu**[1], and **Stefano Rivella**[1,2]

[1]Department of Pediatrics, Division of Hematology, Children's Hospital of Philadelphia (CHOP), Philadelphia, Pennsylvania

[2]Cell and Molecular Biology Graduate Group (CAMB), University of Pennsylvania, Philadelphia, Pennsylvania

Abstract

Iron is one of the most abundant elements in the Earth and a fundamental component of enzymes and other proteins that participate in a wide range of biological processes. As the human body has no mechanisms to eliminate excessive iron, its metabolism needs to be tightly controlled in order to avoid all the sequelae associated with high iron levels. Iron overload is the main cause of morbidity and mortality in beta thalassemia. The master regulator of iron homeostasis, hepcidin, is chronically repressed in this disorder, leading to increased intestinal iron absorption and consequent iron overload. Many groups have focused on obtaining a better understanding of the pathways involved in iron regulation. New molecules have recently been synthesized and used in animal models of dysregulated iron metabolism, demonstrating their ability to target and reduce iron load. Antisense oligonucleotides (ASOs), as well as lipid nanoparticle (LNP)–formulated siRNAs and minihepcidins peptides, are novel agents that have already proved to be efficient in modulating iron metabolism in mouse models and are therefore promising candidates for the treatment of patients affected by iron disorders.

Keywords

iron metabolism; beta thalassemia; iron overload; red blood cells

Introduction

Beta thalassemias are monogenic disorders originating from more than 300 mutations in the beta globin gene or its promoter (http://globin.cse.psu.edu/) that lead to reduced or absent production of the βglobin chain, with consequent impaired hemoglobin A synthesis.[1,2] The mutations can be inherited in homozygosity or compound heterozygosity, giving rise to

Address for correspondence: Stefano Rivella, Ph.D., Kwame Ohene-Frempong Chair on Sickle Cell Anemia, Professor of Pediatrics, Children's Hospital of Philadelphia (CHOP), Cell and Molecular Biology Graduate Group (CAMB), University of Pennsylvania, 3615 Civic Center Blvd, room 316B, Abramson Research Center, Philadelphia, PA 19104. rivellas@email.chop.edu.

Conflicts of interest

S.R. has restricted stocks in Merganser Biotech. S.R. is a consultant for Novartis Pharmaceuticals, Bayer Healthcare, and Keryx Pharmaceuticals. S.R. is a member of scientific advisory boards of Merganser Biotech and Isis Pharmaceuticals.
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different degrees of clinical severity. The main features of beta thalassemia are anemia, iron overload, ineffective erythropoiesis (IE), and extramedullary hematopoiesis (EMH) in the liver and spleen. Patients affected by the most severe form of the disease (thalassemia major or transfusion-dependent thalassemia (TDT)) develop life-threatening anemia within the first two years of life and require lifelong blood transfusions for survival, in combination with adequate chelation therapy to prevent or reduce progressive iron overload. In the milder form of the disease (thalassemia intermedia or non–transfusion-dependent thalassemia (NTDT)) there is no need for regular blood transfusions; however, iron overload eventually develops owing to abnormally increased intestinal iron absorption.[3,4]

Normal and ineffective erythropoiesis

Normal erythropoiesis is a dynamic multistep process by which erythroid progenitors multiply and differentiate, giving rise to mature enucleated red blood cells (RBCs).[5–7] This complex process is controlled by several different pathways. The master regulator of erythropoiesis is erythropoietin (EPO), a hormone primarily produced by fibroblasts in the kidney.[8] When EPO is released into circulation, it binds to its receptor (EPO-R), which is predominantly expressed on the surface of erythroid cells. The interaction of EPO with EPO-R induces rapid phosphorylation of Janus kinase 2 (JAK2), which subsequently activates additional critical targets for erythropoiesis, including the signal transducer and activator of transcription 5 (STAT5).[9,10] Activated STAT5 translocates to the nucleus, where it controls the expression of genes involved in proliferation, differentiation, and survival of erythroid progenitors.[11–13] The expression of EPO is low at steady state, while in conditions where increased tissue oxygenation is required, such as anemia, it is upregulated to ensure the production of sufficient numbers of RBCs.[8] The response to low levels of oxygen is mediated by activation of hypoxia-inducible transcription factors (HIFs). During this complex signaling cascade, HIF-2α binds to the hypoxia response element of the EPO gene and induces its expression, resulting in increased erythropoiesis.[14–16]

Balance between erythroid progenitor production, erythroid differentiation, and apoptosis of unneeded RBC progenitors ensures that appropriate numbers of RBCs are produced for adequate tissue oxygenation.[17,18] In conditions characterized by IE, such as beta thalassemia, this balance cannot be maintained. Huff *et al.,* followed by Finch and his group, were the first to introduce the concept of IE in thalassemia, by observing that the massive production of erythroid progenitors in the bone marrow (BM) of thalassemic patients did not reflect the limited number of mature RBCs in circulation.[19–21] Early on, the main mechanism recognized to be responsible for the noted unbalance was apoptosis of erythroid progenitors.[22,23] However, further studies introduced the notion that reduced differentiation of progenitors, which ultimately fail to become mature RBCs, may be an additional pathogenic process.[24]

Oxidative stress

In beta thalassemia, impaired β-globin synthesis results in a proportional excess in α-globin chains. Free α chains aggregate, leading to the formation of unstable and insoluble hemichromes, which precipitate into the cells, triggering cell damage and death.[25,26]

Senescent or damaged erythroid cells, including aged RBCs derived from blood transfusions, are then phagocytosed by macrophages of the reticuloendothelial system in the spleen, liver, and BM.[27,28] Within the phagocytic cells, red cell components are digested, and free iron is released into the cytosol. When storage capacity of the macrophages is achieved, the excess iron is released into the circulation, where it binds to its transporter transferrin (TF) and is transferred either to the erythron for usage or to storage sites (i.e., hepatocytes) in the liver. Under normal conditions, the majority of the iron present in the body results from recycling after erythrophagocytosis.[28] In thalassemia, increased erythroid destruction and excessive intestinal iron absorption contribute to the development of high iron levels.[3,29,30] As a result, over time, the transferrin-binding capacity of hepatocytes, as well as the iron-storage capacity, becomes saturated. Subsequently, non–transferrin-bound iron appears in the plasma.[31,32] This form of iron is extremely reactive and catalyzes the formation of dangerous reactive oxygen species (ROS) that lead to oxidation of membrane proteins, structural membrane changes, and alteration of signaling pathways. Moreover, this process results in exposure of senescence antigens on erythroid cells that induce premature death of both erythroid progenitors in the BM (IE) and circulating RBCs (hemolysis).[26,33,34]

Iron overload

Iron is among the most abundant elements on Earth and is essential for living organisms. It is a fundamental component of hemoglobin and other proteins that participate in important biological reactions. However, in excess it can lead to formation of dangerous ROS that have deleterious effects, as previously described. Iron overload can be a severe complication of several diseases, including beta thalassemia, where it represents the main cause of morbidity and mortality.[1,35,36]

These adverse consequences highlight the need for tight regulation of iron metabolism. In fact, body iron balance is controlled by the 25–amino acid peptide hormone hepcidin (HAMP), which is produced by the liver in response to plasma and intracellular iron levels.[35,37] In normal erythropoiesis, hepatocytes respond to elevated iron levels by increasing hepcidin production. Hepcidin is then released into the circulation and subsequently binds to its target ferroportin (FPN-1), inducing its internalization and degradation within lysosomes.[38] FPN-1 is the only known iron exporter and is expressed on the surface of cells that are involved in iron absorption, storage, and recycling (i.e., duodenal enterocytes, hepatocytes, and macrophages respectively). As a result of hepcidin binding to FPN-1, iron flow into the plasma decreases.[38,39]

Intracellular and extracellular iron concentrations play major roles in hepcidin regulation. In the serum, iron binds to its transporter transferrin, which shuttles it to either the erythron or peripheral tissues via the transferrin receptor–mediated endocytosis pathway.[40] Increased serum iron levels are sensed by the HFE/TFR1 complex and TFR2, which ultimately trigger hepicidin synthesis.[39,41,42] Several intracellular pathways, such as the BMP/SMAD pathway, are also involved in hepcidin regulation. High levels of intracellular iron in the liver stimulate the expression of bone morphogenetic protein 6 (BMP6), which interacts with BMP receptors type I/II and the co-receptor hemojuvelin (HJV), which is required to fully activate the SMAD pathway.[43–47] This interaction leads to phosphorylation and activation of

the SMAD1/5/8–SMAD4 complex. Subsequently, the activated complex translocates to the nucleus and induces hepcidin expression.[44,48–52] On the contrary, *HAMP* expression can be negatively modulated by transmembrane-serine protease TMPRSS6 (or matriptase-2), which cleaves HJV and therefore acts by reducing phosphorylation of the SMAD complex and, consequently, hepcidin production.[53,54]

Hepcidin production is regulated by various different factors, including increased erythropoiesis, elevated erythropoietin levels, and inflammation.[55–58] Several studies have demonstrated that hepcidin is chronically suppressed in thalassemia.[4,59–61] In systemic hypoxia, hepcidin expression is reduced in order to increase iron delivery to the expanding erythron. Inappropriately low levels of hepcidin lead to progressive iron overload, as seen in beta thalassemia intermedia and other disorders characterized by ineffective erythropoiesis.[4,59,60,62,63] Extensive studies over the past decade have been focused on identifying the molecules responsible for this suppression. To this end, several candidates, namely GDF15 and TWGF1, both members of the bone morphogenetic superfamily, have been proposed; however, their role has still not been well clarified.[64–69] Recently, studies in a mouse model of thalassemia intermedia highlighted the role of erythroferrone (Erfe), a new potential erythroid factor produced by erythroblasts. These studies reported that, in beta thalassemia, high levels of EPO induce *Erfe* expression, which contributes to reduced hepcidin synthesis in the liver.[69,70]

Mouse models of beta thalassemia intermedia

The development of mouse models of beta thalassemia has been pivotal to our better understanding of the pathophysiology of the disease. In the murine genome, the β-globin chains are encoded by a multigene cluster on chromosome 7. Adult mice express two different β-globin genes, named β^{major} and β^{minor}.[71] Two widely used murine models of thalassemia intermedia are the $Hbb^{th1/th1}$ and $Hbb^{th3/+}$, further referred as *th1/th1* and *th3*/+, respectively.The *th1/th1* mouse was the first model generated and carries a homozygous naturally occuring deletion of the β^{major} gene.[72] The *th3*/+ mouse has an artificial deletion of both β^{major} and β^{minor} genes in heterozygosity.[73] When this deletion is homozygous, the model recapitulates the most severe form of thalassemia major and is lethal *in utero*. Both the *th1/th1* and *th3*/+ mice present with a clinical phenotype similar to human thalassemia intermedia, which includes anemia, reticulocytosis, hepatosplenomegaly, and iron overload. Therefore, these models are ideal for studies on iron metabolism.[73,74]

Novel treatments targeting iron metabolism

The intertwined relationship between iron and erythropoiesis highlights the importance of regulating iron metabolism in thalassemia. Moreover, iron overload can be a devastating complication of the disease that significantly affects patient quality of life. For this reason, several novel therapeutic approaches that target iron metabolism have been developed and are currently under investigation.

The idea that in thalassemia the restriction of iron availability to the expanded erythron would reduce heme synthesis and the formation of hemichromes supports the hypothesis

that treatment with transferrin could be beneficial for this population. In fact, administration of apotransferrin (apo-TF) in the *th1/th1* model of thalassemia intermedia improved the anemia and ineffective erythropoiesis. Treatment altered the maturation and survival of erythroid precursors, an effect indicated by a decrease in the proportion of immature erythroid precursors versus mature RBCs and a lower degree of apoptosis of mature erythroid precursors in the bone marrow and spleen.[75] Furthermore, additional therapeutic effects were noted, including a reduction in α-globin precipitation on RBC membranes, and normalization of labile plasma iron concentrations and iron content in the liver, heart and kidney, together with decreased expression of the hormone erythroferrone and increased levels of hepcidin.

Modulation of hepcidin has proven to be critical in controlling body iron levels. In beta thalassemia, which is characterized by inappropriately low levels of Hamp and systemic iron overload, regulating hepcidin could be therapeutic. In fact, it has been already demonstrated that *th3*/+ thalassemic mice moderately overexpressing hepcidin show reduced iron levels in the serum, liver, spleen, and kidney and improvement of IE, RBC survival, and morphology. These effects are combined with a concomitant reduction of anemia and splenomegaly and decreased hemicrome and ROS formation.[76] Therefore, strategies that target the master regulator of iron homeostasis will limit iron availability to the erythron and may be beneficial in improving the anemia and IE.

Several molecules play an important role in *Hamp* regulation and can be used as therapeutic targets. Studies in *th3*/+ mice lacking *Tmprss6* (*Tmprss6*$^{-/-}$ *Hbb*$^{th3/+}$), one of the major suppressors of hepcidin expression, demonstrated that the absence of this protein results in limited iron overload coupled with improved anemia, splenomegaly, and ineffective erythropoiesis.[77] On the basis of this information, it was postulated that pharmacological reduction of *Tmprss6* using antisense technology could be beneficial for the treatment of diseases like beta thalassemia. These antisense oligonucleotides utilize an RNaseH mechanism to degrade the *Tmprss6* RNA species.[78,79] In fact, by reducing *Tmprss6* liver expression, ASOs increased synthesis of *Hamp* to therapeutic levels in the *th3*/+ mouse model.[78] Treated mice showed reduction of ROS, hemichrome formation, and apoptosis, along with improvement of ineffective erythropoiesis, splenomegaly, RBC survival, and, consequently, anemia.[78] This concept was also proved by a parallel study that used lipid nanoparticle (LNP)–formulated small interfering RNAs (siRNAs) in *th3*/+ mice.[80] Recent studies using Tmprss6 inhibitors in combination with iron chelators demonstrated a more powerful effect of the combined therapy, compared to each single agent alone, on iron restriction, and as a result on reduction of IE.[81,82]

The powerful therapeutic effect of these compounds was also demonstrated in a mouse model of hereditary hemochromatosis (*Hfe*$^{-/-}$). In humans, this genetic disease is caused from mutations in the *HFE* gene that has a critical role in controlling hepcidin expression. Hereditary hemochromatosis (HH) is characterized by excessive dietary iron absorption and disproportionally low levels of hepcidin synthesis. With no intervention, patients suffer from severe complications due to iron accumulation in parenchymal organs. Reduced synthesis of *Tmprss6*, by administration of *Tmprss6* ASO- or LNP-formulated siRNAs, in the *Hfe*$^{-/-}$ mouse model increased hepcidin expression and decreased iron levels.[78,80] These results

suggest that this novel approach could be used in iron-overload disorders associated with low hepcidin levels.

Other molecules called minihepcidins are currently under development for the treatment of beta thalassemia intermedia. These are short peptide mimetics of hepcidin that reproduce its iron-restrictive effect.[83,84] It has been shown that administration of these molecules in a mouse model of hereditary hemochromatosis results in reduced iron absorption and increased iron retention in splenic macrophages.[84] Minihepcidins also reduced iron overload and improved the anemia in a mouse model of beta thalassemia intermedia.[85] Treated mice had reduced total iron levels in the spleen, liver, and kidney coupled with decreased hemichrome and ROS formation.

All of these studies demonstrate that, in mouse models of beta thalassemia, therapeutic strategies aiming to modulate iron metabolism are beneficial in reducing ineffective erythropoiesis and oxidative stress, as well as in improving the anemia. On the basis of these recent findings, all of these new molecules that target iron metabolism are promising candidates for the treatment of patients affected by beta thalassemia or other iron-related disorders, such as hereditary hemochromatosis.

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