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Ineffective Erythropoiesis, Anemia and Iron Overload

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Abstract

Stress erythropoiesis (SE) is characterized by an imbalance in erythroid proliferation and differentiation under increased demands of erythrocyte generation and tissue oxygenation. β -thalassemia represents a chronic state of SE, called ineffective erythropoiesis (IE), exhibiting an expansion of erythroid-progenitor pool and deposition of alpha chains on erythrocyte membranes, causing cell death and anemia. Concurrently, there is a decrease in hepcidin expression and a subsequent state of iron overload. There are substantial investigative efforts to target increased iron absorption under IE, by genetic and pharmacological agents. There are also avenues for targeting cell-contact and signaling within erythroblastic-islands under SE, for therapeutic benefits.

Keywords

Erythroblastic island; Hepcidin and iron homeostasis; Stress and ineffective erythropoiesis; Iron overload

Erythrocytes are the primary carrier of oxygen in vertebrate systems. Oxygen carrying capacity of erythrocytes stems from their key constituent heme, capable of binding to iron and delivering oxygen to tissue through circulating red blood cells (RBC). As a result, erythropoiesis and iron levels are tightly linked in mammalian systems in order to maintain a healthy balance of iron utilization for generation of new erythroblasts and iron recycling from senescent erythrocytes.

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Conflict of Interest statement

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Iron has unique properties that make it crucial to the functioning of the mammalian physiological system. One such property is its interconversion from ferric (Fe^{3+}) to ferrous (Fe^{2+}) form¹. While this makes iron an essential component of oxygen carrying proteins like hemoglobin and myoglobin, as well as many redox enzymes, it also allows iron to generate harmful oxidative radicals². This dichotomous nature of iron functioning requires this element to be tightly regulated. Loss of this regulation is the underlying cause of many disorders, with symptoms ranging from anemia to hemochromatosis (iron overload). Although many of these disorders have been identified and some characterized, their underlying molecular mechanisms have only recently begun to be elucidated.

Erythropoiesis and the erythroblastic island

Erythropoiesis occurs in specialized niches within the bone marrow and the spleen consisting of a central nursing macrophage surrounded by erythroid cells in different stages of differentiation. Although the first erythroblastic island was observed by Bessis *et al.* through electron micrographs, long term liquid cultures of bone marrow cells have also been used to generate erythroblastic islands in vitro³. Erythroblastic islands have also been isolated and cultured from spleens of phlebotomized mice, where the central stromal macrophage extended cytoplasmic processes to surrounding erythroblasts with the latter also exhibiting differentiation⁴. Moreover, cell to cell adhesion has been shown to be a key factor regulating erythroid differentiation in an erythroblastic island. This is not only mediated by the erythroblast macrophage protein as demonstrated extensively using knock-out mice of this protein that are embryonic lethal, but also by the intracellular adhesion molecule-4 (Icam-4) such that mice lacking Icam-4 show significantly reduced erythroblastic islands⁵. Furthermore, blocking the interaction between Icam-4 on erythroid cells and alpha V integrin on macrophages results in a marked reduction of erythroblastic islands⁶. Interaction between VCAM-1 and Beta-1 integrin has also been implicated in erythroid macrophage contact within the erythroblastic island⁷(Figure 1).

Erythropoiesis within erythroblastic islands consists of multiple developmental stages. Erythroid progenitor proliferation begins as multipotent HSCs hematopoietic stem cells (HSC) proliferate and differentiate into burst forming unit-erythroid (BFU-E) stage which in turn gives rise to colony forming unit-erythroid (CFU-E). Terminal erythroid differentiation begins at the pro-erythroblast stage. This stage undergoes three consecutive mitosis to generate basophilic erythroblast followed by polychromatic erythroblast and then orthochromatic erythroblasts. The orthochromatic cells expel their nuclei to generate reticulocytes which undergo further changes to give rise to erythrocytes or RBCs⁸. The different stages of terminal erythroid differentiation have been elucidated both by sequencing of RNA and by morphology⁹.

Hepcidin and iron homeostasis

Hepcidin (HAMP) is considered a key regulatory molecule of systemic iron homeostasis, produced primarily by hepatocytes^{10, 11}. HAMP acts as a negative regulator of iron availability by preventing export of iron from duodenal enterocytes, hepatocytes, macrophages and placental trophoblasts. High levels of HAMP trigger hypoferremia, as

evidenced by studies where a single dose of 50 ug of HAMP in mice caused a rapid drop in serum iron in 1 hour. HAMP carries out this negative regulation of iron availability by directly binding to and degrading the iron exporter FPN^{12,13}. In a feedback loop, HAMP in itself is also regulated by the element it controls: iron. This happens such that when iron levels are plentiful, HAMP expression is upregulated. Conversely, under conditions of iron deficiency, HAMP expression is downregulated, releasing iron into circulation thereby increasing TF saturation and plasma iron levels^{14, 15, 16, 17}.

One pathway controlling HAMP expression involves iron availability and the presence of holo-TF. This is mediated by the membrane protein HFE. Under conditions of high serum iron levels and TF-saturation or increased concentrations of holo-transferrin, the membrane protein HFE, generally sequestered away by binding to TFR1 is displaced to transferrin receptor 2 (TFR2), thereby forming an Fe-TF, HFE, TFR2 complex. This complex has been implicated in upregulating transcription of HAMP^{18, 19, 20} (Figure 1). The key pathway in inducing expression of HAMP involves BMP6, binding to the BMP receptor I and II complex including the BMP coreceptor Hemojuvelin (HJV), which confers sensitivity of binding. Activation of the BMP receptor leads to phosphorylation of SMAD1/5/8 in the cytosol and the latter then goes on to interact and form a complex with SMAD 4 which translocates to the nucleus and induces expression of HAMP^{21, 22, 23, 24}. A serine protease Matriptase -2 encoded by the TMPRSS6 gene is capable of cleaving the BMP coreceptor HJV (Figure 1). Thus, it negatively regulates expression of HAMP such that TMPRSS6^{-/-} mice have severe iron deficiency anemia^{25, 26, 27, 28, 29}.

Stress and ineffective erythropoiesis

Erythropoiesis is driven by the kidney hormone erythropoietin (EPO) (Figure 1). EPO binding to the EPO-receptor triggers activation of JAK2 and phosphorylated JAK2 can bind to STAT5 wherein the JAK2-STAT5 complex translocates to the nucleus and induces expression of target genes³⁰ including but not limited to IREB2 (Iron responsive element binding protein 2) and anti-apoptotic factors such as BCL-XL³¹. Under conditions of acute blood loss or demands for increased oxygenation, the existing cellular hypoxia controls EPO expression through Hypoxia Inducible factor alpha 2 (HIF2 alpha). Upregulated EPO expression elicits an increased erythropoietic response, referred to as stress erythropoiesis. Stress erythropoiesis is characterized by an imbalance of erythroid proliferation and differentiation axis, resulting in an expansion of the erythroid progenitor pool to meet the demands of increased RBC generation and oxygenation. Erythropoiesis extends to extramedullary sites such as the liver and the spleen, and indeed splenomegaly is another hallmark of stress erythropoiesis. Under these conditions increased iron absorption is facilitated by the erythroid factor erythroferrone (ERFE) produced by erythroid progenitors which acts on the liver by an as yet unknown mechanism to suppress HAMP expression^{30, 32}. This, with a concurrent upregulation of EPO, works to increase generation of RBC, iron availability and oxygenation of tissues under conditions of stress erythropoiesis³⁰.

A chronic state of stress erythropoiesis seen in certain disorders is referred to as ineffective erythropoiesis. Under ineffective erythropoiesis, the imbalance of erythroid proliferation and

differentiation is characterized by an increase in erythroblast proliferation that fails to differentiate and give rise to enucleate RBCs thereby resulting in anemia. The disease model of β -thalassemia has been used to study and characterize ineffective erythropoiesis. In face of a lack of decreased generation of beta globin chains, there is an accumulation of alpha chains that get deposited on erythroid membranes, generating hemichromes, causing substantial oxidative stress and cell death. As result of this, despite an expansion of the erythroid progenitor pool, a continuous state of chronic stress erythropoiesis ensues and the expanded pool of erythroid precursors are unable to generate RBCs. The resulting anemia is also accompanied by a decrease in expression of HAMP, thereby increasing iron availability. Indeed, it has been shown that Erfe expression is upregulated in mouse model of beta thalassemia intermedia (*Hbb^{th3/+}*)³³. Thus, despite the inability to generate RBCs due to apoptosis of erythroid precursors, the ineffective erythropoiesis in beta thalassemia is characterized by increased serum EPO levels and concurrent fall in Hamp levels, leading to increased iron absorption and iron overload.

The pathophysiology of ineffective erythropoiesis has been attributed in part to macrophages, which are a key component of erythroblastic islands within the bone marrow and the spleen. Depletion of macrophages by clodronate encapsulated liposomes or by using CD169 DTR mice substantially ameliorated anemia and splenomegaly seen in a mouse model of beta thalassemia intermedia^{34, 35}. The various proteins and pathways implicated in maintaining contact between the central nursing macrophage and surrounding erythroblasts, including FAK (focal adhesion kinase) signaling downstream of alpha4beta1 integrin potentially contributes to macrophage mediated pathophysiology seen in the ineffective erythropoiesis of beta thalassemia. The underlying mechanism of this remains unknown.

There are also other factors that have also been implicated in anemia associated with ineffective erythropoiesis seen in beta thalassemia, primarily associated with the imbalance of alpha-beta chains. One such factor is HSP 70 (heat shock protein 70) which translocates to the nucleus and protects the erythroid transcription factor GATA1 from cleavage by caspase 3. In in-vitro studies of human BM erythroblasts from beta thalassemic patients it has been shown that HSP 70 is sequestered by alpha chains as a result of which GATA1 is cleaved and erythroid maturation is impaired. Another factor is GDF11, a member of the TGF beta super family that has been shown to be elevated in both beta thalassemia and another disorder exhibiting some hallmarks of ineffective erythropoiesis, MDS (Myelodysplastic syndrome). There is also work to indicate that blocking GDF11 using trap ligands can reduce precipitation of alpha globin chains on erythroid membranes and subsequent oxidative stress, although the complete mechanism has not been completely elucidated³⁶.

In the absence of transfusion therapy, ineffective erythropoiesis in beta thalassemia patients is associated with local bone defects due to marrow expansion and the appearance of extramedullary hematopoietic pseudotumors. It is also associated with several clinical sequelae due to the resulting anemia and iron overload. A hypercoagulable state due premature red cell death has also been described in this patient population, leading to frequent thrombotic and other vascular events^{37,38,39,40,41}

Iron overload and its therapeutic targeting

Downregulated hepcidin and its subsequent impact on iron homeostasis results in increased iron absorption and a state of iron overload and ensuing oxidative stress. Primary disorders of iron overload include hereditary hemochromatosis (HH), the most common cause of which is mutations in the gene HFE. Beta thalassemia is also associated with iron overload due to hepcidin downregulation by ineffective erythropoiesis as stated earlier. Although iron loading from increased intestinal absorption in beta thalassemia is a slower than that secondary to regular transfusion therapy, it can still reach clinically significant thresholds associated with serious morbidity. Another iron overloading anemia that exhibits some hallmarks of ineffective erythropoiesis are autosomal recessive disorders caused by mutations in Codenin-1 and referred to as CDA-1⁵⁰. The factor GDF-15 that has been found to downregulate hepcidin in vitro, is upregulated in patients of both beta thalassemia and CDA-1, indicating that this factor might be an important biomarker of ineffective erythropoiesis and iron overload. The exact role of this factor in ineffective erythropoiesis, if at all, remains debatable^{42,43}.

Several avenues have been explored to target the ineffective erythropoiesis and iron overload in mouse models of non-transfusion dependent beta thalassemia (Figure 1). Given the significant role of TMPRSS6 in cleaving HAMP, genetic or pharmacological depletion of TMPRSS6 has been the subject of much study. *Tmprss6*^{-/-} mice crossed with a mouse model of beta thalassemia intermedia (*Hbb*^{th3/+}) resulted in improved splenomegaly, anemia and iron loading⁶⁰. Moreover, targeting *Tmprss6* using siRNA and antisense oligonucleotides (ASO) significantly improved anemia and reduced hemichrome formation in *Hbb*^{th3/+} mice^{44,45}. *Tmprss6* ASOs were also able to decrease serum iron transferrin saturation, liver iron accumulation in *Hfe*^{-/-} mice exhibiting HH⁴⁶. Additionally, minihepcidins which are small molecule agonists mimetic of HAMP activity and are capable of binding to and degrading FPN have also been used in preclinical studies of *Hbb*^{th3/+} mice showing amelioration of anemia and symptoms of ineffective erythropoiesis⁴⁷. The Minihepcidins, *Tmprss6* ASO and *Tmprss6* siRNA therapies have also been used in combination with treatment of the iron chelator deferiprone to target organ iron content under conditions of ameliorating ineffective erythropoiesis^{47, 48,49}. Additional therapies that have also been in use involve administration of Apo transferrin in *Hbb*^{th3/+} as well as *Hbb*^{th1/th1} animals showing substantial fall in iron overload, hemichrome formation, and improvement of anemia^{50,51}. Other preclinical studies encompassing therapies to ameliorate ineffective erythropoiesis include the use of JAK2 inhibitors. Given the role of the JAK2-STAT5 pathway in erythropoietin (EPO) production under hypoxic conditions and stress erythropoiesis, these seems to be an ideal possibility for therapeutic targeting. Indeed, use of JAK2 inhibitor in animals affected by beta-thalassemia show significant reduction of splenomegaly and improvement of anemia⁵². Additionally, ligand traps based on the extracellular domains of activating receptors, such as RAP011 and RAP536 which target GDF11 have been shown to improve the erythroid precursor to mature RBC ratio in both *Hbb*^{th3/+} and *Hbb*^{th1/th1} animals, with amelioration of splenomegaly, anemia, and iron overloading conditions⁵³.

Concluding remarks

Current understanding of iron homeostasis and its dysregulation warrants a two-pronged approach at combating iron overload under conditions of ineffective erythropoiesis. The first one involves a reduction of iron absorption. Both pre-clinical and preliminary clinical studies show promise in reducing the burden of iron excess, with or without the combination of iron chelators, the latter being in clinical use to treat iron overload. However, the second approach points towards the possible iron independent role of macrophages within erythroblastic island under stress erythropoiesis. Macrophage-erythroblast interactions could trigger signaling that promotes erythroid proliferation, tipping the balance of erythroid proliferation versus differentiation, that is characteristic of pathophysiological stress erythropoiesis in beta thalassemia. Understanding the proteins and pathways involved in these interactions within the erythroblastic island might be key to identifying new therapeutic targets for ameliorating the symptoms of ineffective erythropoiesis. There is substantial investigative appreciation for both these approaches, and hopefully, this will help us better understand and treat iron overload and ineffective erythropoiesis in clinical settings.

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Key points

- Stress erythropoiesis (SE) is characterized by an imbalance in erythroid proliferation and differentiation under increased demands of erythrocyte generation and tissue oxygenation.
- β -thalassemia represents a chronic state of SE, called ineffective erythropoiesis (IE), exhibiting an expansion of erythroid-progenitor pool and deposition of alpha chains on erythrocyte membranes, causing cell death and anemia. Concurrently, there is a decrease in hepcidin expression and a subsequent state of iron overload.
- There are substantial investigative efforts to target increased iron absorption under IE, by genetic and pharmacological agents. There are also avenues for targeting cell-contact and signaling within erythroblastic-islands under SE, for therapeutic benefits.

Figure 1.
Ineffective erythropoiesis and its therapeutic targeting.