Role of Iron in Inducing Oxidative Stress in Thalassemia

Can It Be Prevented by Inhibition of Absorption and by Antioxidants?

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ABSTRACT: The pathophysiology of thalassemia is, to a certain extent, associated with the generation of labile iron in the pathological red blood cell (RBC). The appearance of such forms of iron at the inner and outer cell surfaces exposes the cell to conditions whereby the labile metal promotes the formation of reactive oxygen species (ROS) leading to cumulative cell damage. Another source of iron accumulation results from increased absorption due to decreased expression of hepcidin. The presence of labile plasma iron (LPI) was carried out using fluorescent probes in the FACS. RNA expression of hepcidin was measured in two models of thalassemic mice. Hepcidin expression was also measured in human hapatoma HepG2 cells following incubation with thalassemic sera. LPI was identified and could be quantitatively measured and correlated with other parameters of iron overload. Hepcidin expression was downregulated in the livers of thalassemic mice, in major more than in intermedia. Thalassemic sera down regulated hepcidin expression in HepG2 liver cells. A possible way to decrease iron absorption could be by modulating hepcidin expression pharmacologically, by gene therapy or by its administration. Treatment with combination of antioxidants such as *N*-acetylcysteine for proteins and vitamin E for lipids in addition to iron chelators could neutralize the deleterious effects of ROS and monitored by quantitation of LPI.

KEYWORDS: thalassemia; oxidative stress; antioxidant; hepcidin; iron overload

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INTRODUCTION

Thalassemias are a heterogeneous group of inherited anemias resulting from reduced or absent synthesis of α- or β-globin chains of hemoglobin A.[1] Patients with β-thalassemia have partial or complete lack of synthesis of β-chains of hemoglobin.[2] The remaining excess of α-chains are unstable, and they eventually precipitate and disintegrate, causing damage to the red blood cell (RBC) membrane. The affected RBCs are prematurely hemolysed in the bone marrow and spleen, resulting in increased RBC turnover, ineffective erythropoiesis, and severe anemia,[3] which can be corrected only by regular blood transfusions.

One of the major consequences in this genetic disorder is iron overload due to ineffective erythropoiesis and premature hemolysis in the plasma and in major organs such as heart, liver, and endocrine glands.[4] In this review the etiology of iron accumulation will be discussed, particularly the role of hepcidin in regulating increased iron absorption, as well as the pathophysiology resulting from excess of "free iron." Last, but not least, we will explore whether there are ways and means to decrease the iron overload and to neutralize its deleterious effects in the tissues other than iron chelation.

WHY IS IRON TOXIC?

Iron is known to be a catalyst in the formation of reactive oxygen species (ROS), particularly the hydroxyl radical (·OH) serving as a Fenton reagent. Another Fenton reagent can be hemichromes, which are a family of denatured ferric proteins starting from methemoglobin until the complete dissociation of heme from globin.[5] Hydroxyl radical facilitated by membrane-associated iron might be particularly harmful because radical generation would be relatively sequestered from the cell antioxidant capacity and occur directly adjacent to lipid and protein membrane components. The ·OH-induced membrane damage can be related directly to a membrane-associated Fenton reagent.[6]

The circulating forms of iron that are not tightly bound to plasma transferrin have been termed as non–transferrin-bound iron (NTBI).[7] NTBI is detected whenever the capacity of transferrin to incorporate iron derived either from the gastrointestinal tract (GIT) or from reticuloendothelial cells becomes a limiting factor.[8] Both NTBI and a labile plasma iron (LPI) pool appear primarily in heavily transfused patients where the transferrin iron-binding capacity has been surpassed,[8] although this is not always the case.[9] The pathologically relevant fraction of NTBI is seemingly translocated across cell membrane in a nonregular manner. That fraction is referred to as LPI because it refers to cell-penetrating forms of iron that are redox active and susceptible to chelation.[10]

Cell damage associated with iron overload has been attributed to the emergence of excessive levels of cell LPI that promote production of ROS exceeding cellular defense capacities.[11] Its detection was based on the principle of tracing iron chelation by following either the recovery of a fluorescence signal quenched by complexation of iron to a fluorescent metallosensor or the inhibition of labile iron-mediated ROS formation.[12] Therefore, it can serve as an accessible diagnostic marker of iron

overload and cell toxicity and as a parameter that can be used to monitor the efficacy of iron chelation.

In thalassemia major, there is as an outpouring of catabolic iron, a Fenton reagent, that overwhelms the iron-carrying capacity of plasma transferrin and generates redox-active forms that may potentially cause tissue iron overload, damaging vital organs such as heart, liver, and endocrine glands.[4] The most accessible LPI in cells is assumed to be associated with the cytosol, which under normal conditions represents the crossroads of cell iron movement regulated by a mechanism linked to iron sensing by iron-responsive proteins.[13] The cytosol is also the compartment where iron is released from heme by heme oxygenases and from where iron is sequestered into ferritin molecules.[14] The cytosolic LPI has been shown to be composed of transitory iron (II) and (III) forms whose relative ratios are determined by the redox capacity of cells, possibly mediated also by specific cell iron reductases.[10,11] The organellar LPIs comprise forms of iron that subserve different functions: In mitochondria, they serve as sources for the formation of protein iron–sulfur clusters and porphyrins; in endosomes, they provide the source of iron derived from receptor-mediated endocytosis that translocates into the cytosol or possibly also to mitochondria; and in lysosomes they are apparently associated with products of iron protein degradation.[15,16] The cellular LIPs expand in iron overload conditions and pose a threat to cell integrity, a phenomenon that iron chelators are theoretically designed to alleviate by gaining access to those pools and complexing the metal in non–redox-active forms.

ROLE OF HEPCIDIN IN REGULATION OF IRON ABSOPTION

Besides the iron overload resulting from hemolysis and blood transfusions, and counter to all expectations, in thalassemia more iron is absorbed from the GIT.[4] Hepcidin, a key mediator in the regulation of iron absorption, was identified in the urine as an antimicrobial peptide. This small, cysteine-rich peptide has three forms: 20, 22, or 25 amino acids long. It is cleaved from an 84-amino-acid propeptide and is secreted from the liver.[17,18] Hepcidin inhibits duodenal iron absorption, iron release from the reticulo-endothelial system macrophages, and iron transport across the placenta.[19]

Hepcidin binds to ferroprotein, also known as Iregl, an iron exporter on the surface of absorptive enterocytes, macrophages, hepatocytes, and placenta. As a consequence, export of cellular iron from these cells, such as liver cells, is decreased.[20]

Hepcidin was also identified in mice, and its expression is regulated by iron status. Iron loading of mice increased hepcidin expression,[21] while deprivation of dietary iron resulted in decreased hepcidin level in the urine followed by enhanced intestinal iron absorption. Hepcidin is also playing a significant role in the etiology of anemia of inflammation.[22]

Following the former observations, an obvious question was whether hepcidin expression has any role in the pathogenesis of increased iron absorption in thalassemia, despite the presence of severe iron overload from other etiologies. Moreover, it has been shown that erythropoietic stimuli caused by hemolysis, bleeding, hypoxia, and following injections of erythropoietin, were associated with decreased hepcidin expression in mice livers.[23]

We have studied hepcidin mRNA levels in the livers of two models of mice mimicking human thalassemia intermedia and major: $Hb^{th3/+}$ and $Hb^{th3/th3-}$, respectively.[24] The results showed that hepcidin expression in the β-thalassemia major mouse model, obtained following bone marrow transplantation,[24] is downregulated much more than in the model β-thalassemia intermedia.[25,26] Thus, the severity of the anemia in the thalassemic mouse models might be directly related to the downregulation of hepcidin expression.

To check whether there exists a serum factor originating from the bone marrow, as a result of ineffective erythropoiesis, we tested the influence of thalassemic sera on the expression of endogenous hepcidin in human hepatic cell line HepG2. The results showed that hepcidin expression was dramatically decreased in the presence of thalassemic sera compared with that in sera from healthy individuals.[27] On the other hand, when sera from patients with hemochromatosis, who also suffer from iron overload, were incubated with the HepG2 cells, hepcidin expression was increased as might be expected, to decrease iron absorption. Hepcidin expression was also related to the degree of transferrin saturation in these patients, while no such correlation was found in the presence of thalassemic sera. There was no correlation between Hb, ferritin, and LPI levels in thalassemic sera and hepcidin expression.[27] It is also of interest that a slight decrease in hepcidin liver mRNA expression was found in mice treated with phenylhydrazine, a potent hemolysis-inducing compound.[28] The increased erythropoietic activity following hemolysis increases the demand for iron, and consequently more iron must be absorbed from the GIT. The similarity between this model and the thalassemic mouse model adds additional support to the concept that ineffective erythropoiesis influences the regulation of hepcidin expression through factors present in the serum, a condition that contributes to the development of iron overload in thalassemia.

The ultimate conclusion from these results is that administration of hepcidin might be indicated whenever iron overload is present in association with low hepcidin expression.

POTENTIAL ROLE OF ANTIOXIDANTS

The fact that iron plays a major role in the generation of ROS implies that iron chelators can also serve as antioxidants. Obviously, chelation of iron is one of the major therapeutic goals in thalassemia. Consequently, the orally administered iron chelator deferiprone (L1), which has an enhanced capacity to permeate cell membranes, was able to remove free iron from β-thalassemic red cell membranes in a dose-related fashion, both *in vivo* and *in vitro*. Deferiprone alleviated membrane damage possibly mediated by catalytic iron, such as lipid peroxidation, measured by increased levels of malonyldialdehyde, a breakdown product of lipid membrane peroxidation, and hemichrome formation, and also reduced the KCl cotransporter activity.[29,30] In a few patients with Hb E/β thalassemia in Thailand, following administration of deferiprone alone for an average of 50 weeks, Hb levels increased concomitant with a decrease in transfusion requirements.[31] One possible explanation for this finding is that deferiprone acted like an antioxidant by removing excess free iron from the cells and, as a result, ROS generation was decreased. However, the

antioxidant effect of this iron chelator by itself was not sufficient to neutralize the damage induced by ROS, since only in a few patients were Hb levels increased.

Another link between the presence of NTBI and oxidation of lipid membranes was demonstrated by the correlation between NTBI levels and high levels of malonyldialdehide.[32] Moreover, oral administration of vitamin E, which is a lipid antioxidant, normalized increased levels of ROS and exhibited improvement in oxidant–antioxidant balance in the plasma.[33] On the other hand, prolonged administration of vitamin E did not result in any significant changes in Hb levels in patients with β-thalassemia intermedia, and only in three patients was RBC survival prolonged.[34] Therefore, vitamin E by itself is probably insufficient to induce major changes in the rate of RBC hemolysis and prolong their survival, resulting in increased Hb levels. Another antioxidant that acts primarily on proteins is *N*-acetylcysteine, which improved certain parameters resulting from oxidative damage to sickle RBCs.[35]

The ultimate purpose of all these observations is to try to design a combination of antioxidants consisting of an iron chelator, such as deferiprone, vitamin E for the lipids, and *N*-acetylcysteine for the proteins. This approach is a subject of a clinical trial that will begin shortly.

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