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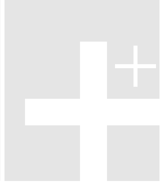

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In this Issue...*Genetically-based Iron Deficient Erythropoiesis*

In human physiology, iron is not a trace element; the normal adult has four to five grams of iron in his or her body. Over two thirds of this metal is found in erythroid tissues as hemoglobin. Erythrocytes are being produced at an astonishing rate of about two million per second, requiring approximately 25 mg of Fe per day. Thus, the cells of the bone marrow must efficiently acquire and process plasma iron, which is almost entirely bound to transferrin, to keep pace with erythropoietic demand.

It is well known that iron deficiency is the most prevalent cause of anemia, affecting more than half a billion people worldwide. However, iron-deficient erythropoiesis can be caused not only by iron deficiency but also by intrinsic defects in developing red blood cells; these defects are genetically based and have been identified in both humans and other vertebrates. These conditions are of considerable importance, since the elucidation of molecular defects responsible for some hypochromic anemias in rodents or zebrafish were of considerable assistance in defining previously unknown steps in the erythroid iron pathway. Moreover, genetically-based hypochromic anemias in experimental animals have suggested that similar conditions may exist in humans. Indeed, the first human mutation of a membrane transporter for iron, DMT1, whose defects were previously shown to cause hypochromic anemia in mice and rats, was recently reported and subsequently confirmed in two additional patients.

In this issue we review this finding, and analyze other recently reported genetic defects responsible for iron-deficient erythropoiesis in mice.

Commentary & Reviews by:
Prem Ponka, MD, PhD.

Guest Editor of the Month:

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Commentary & Reviews

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Our guest editor opinion
+ The Gatekeeper's Message Must Be Stabilized to Support Normal Hemoglobinization
+ Ednosomes Must Pinwheel to Unload Their Cargo
+ Iron Won't Move On, If Not Reduced
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Guest Faculty Disclosures

Prem Ponka, MD, PhD.
Faculty Disclosure: Dr. Ponka has indicated a financial relationship with the Canadian Institutes of Health Research.

Unlabelled/ Unapproved Uses
No faculty member has indicated that their presentation will include information on off label products.

Commentary

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Hemoglobin synthesis occurs via three independent but stringently coordinated pathways: globin synthesis, which is erythroid specific, and heme synthesis which requires both protoporphyrin IX synthesis and the supply of iron from plasma transferrin to mitochondrial ferrochelatase, with the two latter ubiquitous pathways dramatically upregulated in developing red blood cells. A decrease in any of these three pathways leads to anemia characterized by erythrocytes that are smaller than normal (microcytic) and contain reduced amounts of hemoglobin (hypochromic). Hence, hypochromic microcytic anemia can be caused by inadequate production of globin (thalassemias) or protoporphyrin IX (e.g., sideroblastic anemia due to defects in erythroid-specific 5-aminolevulinate synthase [ALA-S2]) or by insufficient supply of iron to ferrochelatase, which inserts ferrous ions into protoporphyrin IX. Although iron deficiency is by far the most frequent cause of the last condition, there is increasing evidence that numerous intrinsic defects in erythroid cells can interfere with the delivery of Fe^{2+} to ferrochelatase. Hence, a brief overview of the path of iron from plasma to hemoglobin is warranted.

From a physiological perspective, with some notable exceptions (e.g., enterocytes, macrophages), virtually all the cells in the organism, including erythroid precursors, take up iron from diferric transferrin. The strict dependency of hemoglobin synthesis on transferrin iron is demonstrated by the observations that both humans and mice with hereditary atransferrinemia have severe hypochromic microcytic anemias; importantly, atransferrinemias are associated with generalized iron overload[1]. Delivery of iron to cells occurs following the binding of transferrin to the transferrin receptor 1 (TfR) on the cell membrane. The transferrin-TfR complexes are then internalized by endocytosis, and iron is released from transferrin by a process involving endosomal acidification. Iron is then transported across the endosomal membrane by DMT1[2]. Because the substrate for DMT1 is Fe^{2+}, reduction of Fe^{3+} must occur in endosomes, but until recently nothing was known about this

process. As discussed in this issue, at least one plausible candidate for endosomal ferrireductase is Steap3, whose defect is responsible for impaired iron uptake by erythroid cells from *nml054* mouse mutants[3]. Following its escape from endosomes, iron is transported to intracellular sites of use and/or storage in ferritin, but this aspect of iron metabolism, including the nature of the elusive intermediary pool of iron and its cellular trafficking, remains enigmatic. Only in erythroid cells does some evidence exist for specific targeting of iron toward mitochondria, the sites of heme production, where ferrochelatase resides. This targeting is demonstrated in hemoglobin-synthesizing cells in which iron acquired from transferrin continues to flow into mitochondria even when the synthesis of protoporphyrin IX is suppressed either experimentally[4] or in sideroblastic anemia[5]. Moreover, the absence of a cytoplasmic chelatable iron source, in addition to the requirement for endosomal movement for Fe incorporated into heme from ^{59}Fe-labeled endosomes[6], suggests that in erythroid cells a transient mitochondrion-endosome interaction may be involved in iron translocation to its final destination[7].

It has recently been proposed that Sec15l1, whose mutation is responsible for anemia in *hbd* mice, may play a role in iron targeting to mitochondria[8]. Mechanisms and regulation of iron transport in mitochondria are poorly understood, but a compelling candidate, mitoferrin, which seems to be involved in iron transport towards ferrochelatase, has recently been identified[9]. Importantly, zebrafish with mutated mitoferrin (so-called *“frascati”* mutant) have profound hypochromic anemia due to defects in mitochondrial iron uptake[9].

The articles reviewed in this issue provide convincing evidence that reduced TfR expression or altered trafficking of endosomes or their impaired capacity to reduce iron, all based on genetic defects, can cause microcytic anemias in mice. It is tempting to speculate that equivalent genetic defects in humans will cause phenotypes similar to those seen in mice. However, there is a need for caution and a clear lesson comes from mutations of DMT1, the Fe^{2+} transporter, which is involved not only in the egress of iron from endosomes, but also in the absorption of inorganic iron in the duodenum. Since DMT1 mutations cause microcytic anemias as well as iron deficiency in mice and rats, it was initially predicted that human patients with similar phenotypes[10,11] would have DMT1 defects; however, no DMT1 mutations have ever been found in such patients[12]. In fact, all patients with DMT1 mutations thus far described have hypochromic microcytic anemia associated with iron overload — proving that, despite a number of physiologic and metabolic similarities, humans are not rodents.

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THE GATEKEEPER'S MESSAGE MUST BE STABILIZED TO SUPPORT NORMAL HEMOGLOBINIZATION

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Two independent groups reported[1,2] that mice with ablated iron regulatory protein 2 suffer from microcytic anemia that likely develops as a consequence of decreased stability of mRNA for transferrin receptors (TfR). The iron regulatory proteins (IRP1 and IRP2) play an important role in the post-transcriptional control of the expression of TfR, ferritin, erythroid-specific 5-aminolevulinic synthase (ALA-S2, the first enzyme of heme biosynthesis) and some other proteins whose mRNAs contain so-called iron-responsive elements (IREs). Binding of IRPs to IREs found in the 5' untranslated regions (UTRs) of mRNAs (e.g., ferritin, ALA-S2) inhibits translation of these transcripts, whereas binding to IREs in the 3' UTR of TfR mRNA stabilizes this mRNA. In contrast, the lack of binding of IRPs to IREs results in efficient translation of mRNAs for ALA-S2 (in erythroid cells) and ferritin and rapid degradation of mRNAs for the TfR.

Mice with targeted deletion of IRP1 have no obvious hematological phenotype, with their iron metabolism misregulated only in the kidney and brown fat, two tissues in which the endogenous expression levels of IRP1 greatly exceed those of IRP2[3]. In contrast, $IRP2^{-/-}$ mice were found to have microcytic anemia that develops in spite of normal or even slight increase in serum iron levels and transferrin saturation[1,2]. Since the TfR, the gatekeeper for iron entry into cells, is well known to play a crucial role in hemoglobinization, Roualt's[1] as well as Hentze's[2] group examined TfR expression in $IRP2^{-/-}$ mice. Cooperman et al[1] found that TfR protein levels were markedly deceased in erythroid cells isolated from $IRP2^{-/-}$ and IRP $1^{+/-}$ $IRP2^{-/-}$ mice as compared to those from wild-type mice. In accordance with this finding, Galy et al[2] showed that both TfR mRNA and protein levels were significantly reduced in bone marrow cells of $IRP2^{-/-}$ animals. Hence, the most plausible explanation for microcytic anemia in $IRP2^{-/-}$ animals, put forward by both groups, is as follows: The lack of IRP2 binding to TfR mRNA decreases the stability of this transcript and, consequently, the expression of TfR in erythroid cells that likely take up iron with lower efficiency than those from wild-type mice.

However, there are some intriguing differences between the experimental results reported by these two groups. Cooperman et al described that IRP2-deficient animals overexpressed ALA-S2, which was associated with markedly increased levels of free and zinc protoporphyrin; these levels are typically increased when the supply of Fe^{2+} for ferrochelatase is inadequate. This is not a surprising finding, since (as noted above) the loss of IRP activity would be expected to increase the translation of ALA-S2 mRNA, which has an IRE at the 5' end. Moreover, Rouault's laboratory reported earlier[4] that this particular $IRP2^{-/-}$ mouse line develops adult-onset progressive neurodegeneration, resulting from misregulated iron metabolism in the central nervous system. In contrast, $IRP2^{-/-}$ mice generated in Hentze's laboratory have no increase in ALA-S2 protein levels and no signs of neurodegeneration[2]. It is possible that these discordant results may be caused by either age differences at which mice were examined or the distinct targeting strategies used to knockout IRP2[2]. However, more solid explanations are needed, since a clear phenotype accompanying IRP2 deficiency is of potential clinical significance.

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ENDOSOMES MUST PINWHEEL TO UNLOAD THEIR CARGO

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Recently, two laboratories independently identified[1,2] a defective gene responsible for the hemoglobin-deficient mouse mutant (*hbd*), that had previously been mapped to chromosome 19[3]. The mutant is characterized by hypochromic microcytic anemia (inherited in an autosomal recessive manner), reticulocytosis, hyperferremia, and increased red cell protoporphyrin levels. The defect appears to be specific for erythroid tissues. Reticulocytes from *hbd* mice have decreased iron uptake from transferrin although transferrin receptor levels are normal[4-6].

Lim et al[1] and White et al[2] almost simultaneously reported that a compelling candidate gene for *hbd* is *Sec15l1*, which is a homologue of yeast *Sec15*. Both groups are in full accord as to the nature of the defect and showed that the *hbd* defect is an in-frame mutation of exon 5 that is expected to remove 23 aminoacids from the Sec15l1 protein. Both groups have also demonstrated that *Sec15l1* is expressed not only in erythroid cells but also in numerous non-erythroid tissues. Lin et al[1] showed that *hbd* mice express lower levels of *Sec15l1*. Moreover, these investigators transduced *hbd* bone marrow cells with a retrovirus expressing wild-type *Sec15l1* and transplanted them into *hbd* mice. They found the expression of the wild-type protein partially corrected the anemia[1]. Interestingly, as White et al have pointed out, the deleted portion of *hbd* Sec15l1 protein contains a cysteine and a proline that may be important for tertiary structure of the protein.

However, these two groups have not reached a consensus as to a possible function of the Sec15l1 protein that can be, at least in part, explained by an incomplete understanding of an iron metabolism defect in the erythroid cells of *hbd* mice. Andrews' laboratory stressed[1] that Sec15l1 is "linked to the transferrin cycle through its interaction with Rab11, a GTPase involved in vesicular trafficking". These investigators hypothesized that *hbd* mutation enhances the recycling of transferrin receptor-containing vesicles. Such a more rapid return of endosomes to the cell surface would be expected to decrease the layover time of iron in acidified endosomes, causing a decrease in erythroid iron uptake. However, this proposal is in conflict with recent observations from this author's laboratory[7] indicating that transferrin cycling is significantly decreased in *hbd* reticulocytes. Hence, it is more likely that the consequences of *Sec15l1* mutation involve a reduction in the efficiency of vesicular trafficking, docking, fusing, and/or cargo delivery, as White et al put forward[2].

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IRON WON'T MOVE ON, IF NOT REDUCED

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Ohgami et al[1] recently analyzed a previously undescribed mouse mutant, designated as *nml054*, that is characterized by a hypochromic microcytic anemia, male infertility, hydrocephalus, and abnormal fur. Anemia in *nml054* mutant mice is congenital, moderately severe, and marked by elevated red cell zinc protoporphyrin levels that are characteristically increased when erythropoiesis is iron deficient. However, serum and tissue iron analyses revealed that the *nml054* mutant mice are not systematically iron deficient[2]. These observations have strongly suggested that *nml054* anemia is due to an intrinsic defect in red blood cell precursor iron uptake. Ohgami et al[1] went on to show that a mutation of a gene, known as *Steap3* (six transmembrane epithelial antigen of the prostate[3]), is responsible for the iron-deficient erythropoiesis in the *nml054* mouse. Moreover, the authors provided compelling evidence that *Steap3* gene encodes an endosomal ferrireductase that is required for efficient transferrin-dependent iron uptake by erythroid cells.

To identify the gene underlying the *nml054* anemia, the authors employed positional cloning[1]. This strategy revealed that *nml054* is a large genomic deletion on chromosome 1, encompassing all or part of six genes. Transgenic bacterial artificial chromosome (BAC) complementation then identified a single candidate gene (*Steap3*) which, when deleted by gene targeting, was allelic with the anemia but not the other phenotypes. Steap3 protein was then shown to be highly expressed in hematopoietic tissues and to be present in endosomes, where it colocalizes with transferrin, its receptor and DMT1. The expression of Steap3 protein rescued the anemia, as indicated by increases in the mean cell volume and hemoglobin content of erythrocytes and the decrease in zinc protoporhyrin/heme ratio in these cells. Homozygozity with respect to the *Steap3* null allele recapitulated the homozygous *nml054* phenotype, and *Steap3*$^{-/-}$ reticulocytes, like *nm1054* reticulocytes, had a profound defect in transferrin-mediated iron uptake. Steap3 contains a predicted oxido-reductase domain with a flavin-NAD(P)H binding structure homologous to those found in archea and bacteria as well as a domain (containing a putative heme binding site) that shares a homology with the yeast FRE family of metalloreductases. Importantly, overexpression of *Steap3* stimulates the reduction of iron, whereas reticulocytes from mice lacking *Steap3* have a significant decrease in ferrireductase activity.

Transferrin binds the ferric, oxidized form of iron, and the only substrate for DMT1 that exports iron from endosomes is ferrous iron. Hence, the identification of an endosomal ferric reductase by Ohgami et al[1] is an important advance in our understanding of transferrin-mediated iron uptake by developing red blood cells. However, some intriguing new questions have arisen. Since homozygosity for a *Steap3* defect is not associated with a lethal phenotype, erythroid cell endosomes must contain an additional ferrireductase. The authors propose that such a role could be played by *Steap2* or *Steap4*, but other putative ferrireductases[3] could be involved. Moreover, it would be of considerable interest to examine *Steap3* expression during erythroid differentiation and explain high *Steap3* mRNA levels in the liver and pancreas of humans.

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HUMANS ARE NOT BIG MICE

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Divalent metal transporter 1 (DMT1) serves a dual function. Following the internalization of transferrin-receptor complexes, it transports transferrin-derived ferrous iron across the endosomal membrane; in erythroblasts the vast majority of this iron is delivered to mitochondria for heme synthesis. DMT1 is also expressed at the brush border of enterocytes in the proximal duodenum where it is involved in ferrous iron uptake from the gut lumen. Mims et al[1] recently described the first mutation in human *DMT1* in a Czech patient with congenital hypochronic microcytic anemia which was, rather surprisingly, associated with liver

iron overload. Indeed, DMT1 mutation (G185R) in both the *mk/mk* mouse[2] and the Belgrade rat[3] had previously been found to cause hypochromic microcytic anemia as well as iron deficiency caused by defective iron absorption.

The patient analyzed by Mims et al[1] was a female resulting from a consanguineous union, who came to medical attention at the age of 3 months due to severe hypochromic microcytic anemia. Bone marrow exam revealed erythroid hyperplasia with features of abnormal erythroid maturation. Serum iron levels have been consistently elevated but serum ferritin levels have been normal to slightly increased. At the age of 8 years, she developed mild liver function abnormalities and, at age 19, liver biopsy demonstrated significantly increased iron depositions in both Kupffer cells and hepatocytes. The fact that the patient had iron overload, together with the preliminary evidence indicating defective iron utilization by the patient's erythroblasts[4], led to the analysis of the transferrin receptor. No abnormalities were found in the coding regions of the transferrin receptor or ferroportin (iron exporter, whose mutations sometimes cause anemia in combination with iron overload). Upon a decision to analyze DMT1, the patient was shown to carry a homozygous mutation in the DMT1 gene (1285 G > C) that changes Glu 399 to Asp (E399D). This single nucleotide substitution also causes preferential skipping of exon 12 during mRNA processing. As a consequence, there are 2 different DMT1 transcripts present in the patient's cells: a) a full-length transcript containing the point mutation, and b) that missing exon 12 (the latter version comprises ~ 90% of the total DMT1 mRNA). This shorter transcript is also present in low levels in normal control erythroid cells, but it was not detected in the duodenum of control subjects 1. Subsequent analysis has revealed that the E399D substitution has no effect on DMT1 protein expression and function. In contrast, deletion of exon 12 leads to a decreased expression of the protein and disruption of its subcellular localization and iron uptake activity[5].

More recently, two additional non-consanguineous Caucasian patients with DMT1 mutations were reported, one from Italy[6] and the other one from France[7]. Iolascon et al[6] reported a compound heterozygote with the following mutations: a 3-bp deletion in intron 4 (310-3_5del CTT) resulting in a splicing abnormality and a C>T transition at nucleotide 1246 (p.R416C). One of the mutations in the patient reported by Beaumont et al[7] is a GTG deletion in exon 5, leading to the V114 in-frame deletion in transmembrane domain 2, and the second is a G>T substitution in exon 8 leading to the G212V replacement in transmembrane domain 5. All three patients with DMT1 mutations have remarkably similar phenotype, thus defining a new syndrome of congenital hypochronic microcytic anemia associated with liver iron overload but normal or moderately increased serum ferritin levels. As already pointed out, DMT1 defects in *mk* mice[2] and Belgrade rats[3] led to decreased in intestinal iron absorption, whereas all three patients[1,6,7] had iron overload. The most likely explanation of iron overload in patients with DMT1 mutations is an increased absorption of heme-derived iron in response to anemia, as proposed by Priwitzerora et al[4]. *mk* mice and Belgrade rats are unlikely to develop such compensatory mechanisms since rodents absorb heme iron inefficiently and, in laboratories, do not have heme in their diets. This hypothesis may now be testable since a putative intestinal heme transporter was recently reported[8].

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This activity has been developed for Hematologists. There are no fees or prerequisites for this activity.

Learning Objectives

At the conclusion of this activity, participants should be able to:

- Recognize that iron deficient erythropoiesis resulting from a genetic defect may be associated with normal or increased serum iron levels and iron overload;
- Describe the pathway of iron from plasma transferrin to its final destination (heme) in developing red blood cells;
- Identify currently known genetic defects in the above pathway in humans.

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