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REGULATION OF IRON METABOLISM BY HEPCIDIN

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■ **Abstract** Hepcidin, a peptide hormone made in the liver, is the principal regulator of systemic iron homeostasis. Hepcidin controls plasma iron concentration and tissue distribution of iron by inhibiting intestinal iron absorption, iron recycling by macrophages, and iron mobilization from hepatic stores. Hepcidin acts by inhibiting cellular iron efflux through binding to and inducing the degradation of ferroportin, the sole known cellular iron exporter. Synthesis of hepcidin is homeostatically increased by iron loading and decreased by anemia and hypoxia. Hepcidin is also elevated during infections and inflammation, causing a decrease in serum iron levels and contributing to the development of anemia of inflammation, probably as a host defense mechanism to limit the availability of iron to invading microorganisms. At the opposite side of the spectrum, hepcidin deficiency appears to be the ultimate cause of most forms of hemochromatosis, either due to mutations in the hepcidin gene itself or due to mutations in the regulators of hepcidin synthesis. The emergence of hepcidin as the pathogenic factor in most systemic iron disorders should provide important opportunities for improving their diagnosis and treatment.

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INTRODUCTION

Clinicians have known for many decades that the imbalance between iron absorption and iron loss underlies diseases ranging from iron deficiency anemia to hereditary hemochromatosis (iron overload). However, the molecular circuits that achieve iron balance under most conditions have only recently begun to be characterized. This review focuses on the biochemistry, normal physiology, and pathophysiology of hepcidin, a peptide hormone that is the principal regulator of the absorption and systemic distribution of iron.

IRON HOMEOSTASIS

Iron is an essential element that functions as a component of oxygen-carrying proteins (hemoglobin, myoglobin) and of numerous redox enzymes in cellular metabolism. The average adult human contains 2–4 g of iron. Most of the iron is in hemoglobin of blood erythrocytes, and prolonged systemic iron deficiency results in decreased hemoglobin production and anemia. The supply of iron to erythrocyte precursors in the bone marrow and to other tissues is largely maintained by daily recycling of about 20 mg of iron from senescent erythrocytes. Only 1–2 mg a day needs to be absorbed from the diet to replace ordinary iron losses. Iron absorption takes place in the proximal duodenum, and in humans, both elemental iron and heme are taken up by duodenal enterocytes. Iron absorption must be closely regulated to maintain iron balance because humans cannot excrete surplus iron other than by hemorrhaging. When these regulatory mechanisms fail or are circumvented by parenteral administration of iron or blood, excessive iron is deposited in tissues and promotes the generation of reactive oxygen species, which can cause tissue injury and organ failure. Under normal circumstances, the iron concentration in plasma and extracellular fluid remains in a relatively narrow range despite fluctuating iron supply and demand. This is achieved by tight regulation of iron transport in and out of the cells involved in iron absorption, storage, and recycling, but the molecular pathways of regulation have only recently begun to be elucidated.

The tissues and cells that generate major iron flows into the plasma compartment include the duodenal enterocytes involved in dietary iron acquisition, hepatocytes

that are the main site of iron storage, macrophages that recycle iron from senescent red cells, and the placental syncytiotrophoblast involved in iron transfer from mother to fetus during pregnancy (4, 27). These different cell types use several distinct pathways for iron uptake, but a single pathway for iron export into plasma. In duodenal enterocytes, the uptake of ferric iron is mediated by a combination of a ferric reductase (duodenal cytochrome B) and a divalent metal transporter 1 (DMT1). Heme is also absorbed from the diet, and while the process is less well understood, the candidate heme transporters have been described (80). Macrophages that recycle iron from senescent erythrocytes take up iron indirectly by phagocytosing the erythrocytes and extracting iron from their hemoglobin. Hepatocytes, placental cells, and most other cells in the organism (including the developing erythrocytes) import the transferrin-bound iron from circulation using transferrin receptors. In contrast, cellular iron efflux involves the sole known iron exporter ferroportin, highly expressed in duodenal cells, hepatocytes, macrophages, and placental cells (16). Before loading onto plasma transferrin, exported ferrous iron is oxidized to the ferric form by a ferroxidase (hephaestin or ceruloplasmin).

REGULATION OF CELLULAR AND EXTRACELLULAR IRON CONCENTRATIONS

Iron concentrations and fluxes are regulated on both the cellular and the systemic level. Intracellularly, iron concentrations are sensed by two iron regulatory proteins (IRP1 and IRP2) (27). When cytoplasmic iron is low, IRPs bind to iron regulatory element (IRE) sequences in mRNAs of iron-regulated proteins. Depending on the position of IRE (5′ or 3′ untranslated region), IRP binding has opposite effects on the synthesis of iron-regulated proteins: binding of IRPs to 3′IREs stabilizes mRNA, resulting in increased protein synthesis, whereas binding to 5′IREs prevents the translation of mRNA, resulting in decreased protein synthesis. The IRE/IRP-regulated mRNAs include transferrin receptor, ferritin, one isoform of DMT-1, and ferroportin. Thus, as long as the extracellular iron concentrations are in the normal range, cellular iron homeostasis is maintained by the IRP/IRE system modifying the production of proteins involved in cellular iron uptake, storage, and release, according to the cytoplasmic iron concentrations.

Systemic iron regulation maintains a stable concentration of iron-transferrin in plasma and extracellular fluid (25). This is achieved by regulating the major iron flows into the plasma: release of iron from macrophages recycling senescent red blood cells, release of stored iron from hepatocytes, absorption of dietary iron by duodenal enterocytes, and during fetal development, transfer of iron from mother to fetus across placenta. Recent studies indicate that iron is released from all these tissues into plasma through the membrane iron exporter ferroportin (16), which is posttranslationally regulated by hepcidin, a circulating peptide hormone (56). Hepcidin causes the degradation of ferroportin, thereby blocking iron flow into plasma. The plasma levels of hepcidin are in turn regulated by iron and

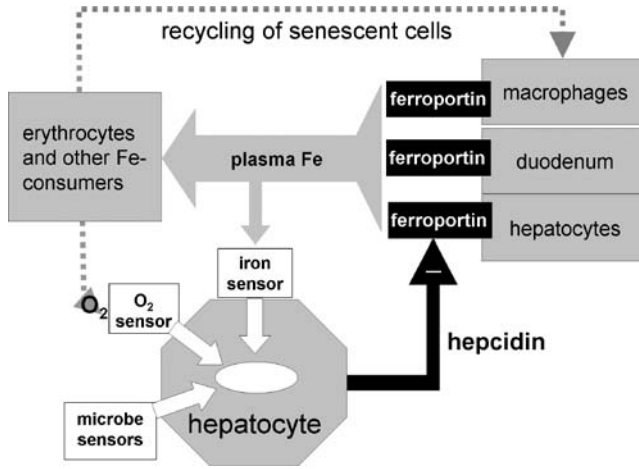

Figure 1 A model of homeostatic regulation of plasma iron. For simplicity, the sensing of iron, oxygen, and microbes is shown taking place in a hepatocyte. It is likely that other cell types sense these stimuli and generate second messengers that converge on the hepatocyte. For inflammation, IL-6 serves as a second messenger of microbial infection or equivalent inflammatory stimuli.

anemia/hypoxia, thus completing the homeostatic loop controlling systemic iron concentrations (Figure 1).

Dysregulation of hepcidin or its receptor ferroportin results in a spectrum of iron disorders (25). On one hand, in inflammatory disorders and infections, cytokine-induced hepcidin excess contributes to development of anemia of inflammation, characterized by hypoferremia and anemia despite adequate iron stores. On the other hand, inappropriately low hepcidin production due to mutations in the hepcidin gene or its putative regulators appears to be the cause of most types of hereditary hemochromatosis, the iron overload disease characterized by excessive dietary iron uptake and iron deposition in vital organs. The remaining types of hereditary iron overload disorders are caused by mutations in ferroportin that either render the protein nonfunctional (not exporting iron, resulting in "ferroportin disease") or unresponsive to hepcidin (excessively exporting iron, resulting in more typical hemochromatosis) (12, 17, 79).

IRON AND HOST DEFENSE

During infections, microbial invaders must obtain iron for their own metabolic needs, including the synthesis of such essential molecules as respiratory cytochromes. Vertebrate hosts have evolved multiple mechanisms to starve microbes of iron during infections. In some cases, specialized homologues of molecules involved in iron transport and distribution are used to keep iron from microbes. White cells and epithelia produce lactoferrin, an iron-sequestering

homolog of the plasma iron transport protein, transferrin. The phagocytic vacuoles of macrophages contain a divalent metal transporter, Nramp1, a homolog of the duodenal iron transporter DMT1 (Nramp2). Nramp1 is thought to deplete iron from phagocytic vacuoles that contain ingested or parasitic microbes (20). More recently, lipocalin (siderocalin), an abundant product of blood neutrophils and some epithelia, was shown to bind bacterial siderophores, small organic molecules essential for iron uptake in some bacteria (19).

In addition to these localized mechanisms, vertebrates respond to infection systemically by rapidly lowering plasma iron concentrations (hypoferremia of inflammation). It is generally believed that this response has a role in host defense, a concept supported by observations that patients with hemochromatosis are more susceptible to certain types of infections (24, 81, 85). However, the specific impact of hypoferremia on microbes remains to be demonstrated. Recent evidence suggests that inflammatory hypoferremia is mediated by a cytokine-driven increase in hepcidin production (54, 57, 60) that causes the internalization and degradation of macrophage and duodenal ferroportin. Consequently, the release of recycled iron from macrophages and absorbed iron from duodenal enterocytes into plasma is blocked, and iron remains sequestered in the cytoplasm of macrophages and enterocytes. Continued utilization of plasma iron for erythrocyte production then rapidly depletes the relatively small (~3 mg) transferrin-bound iron compartment in blood. When prolonged, the restriction of plasma iron limits hemoglobin synthesis and causes anemia of inflammation (also called anemia of chronic disease), discussed later in this chapter.

HEPCIDIN STRUCTURE AND ACTIVITY

Hepcidin is predominantly synthesized in the liver (40, 68). The hepcidin gene contains three exons that encode an 84-amino-acid preprohepcidin with a signal peptide and a characteristic furin cleavage site immediately N-terminal to the mature bioactive peptide (Figure 2). The bioactive human hepcidin is a 25-amino-acid peptide first identified in human urine (68) and plasma (40). In addition to the 25-amino-acid form, the urine also contains minor 20- and 22-amino-acid forms truncated at the N-terminus. The truncated peptides display much reduced iron-regulatory activity (53, 73) and are probably the products of degradation of the 25-amino-acid form.

Mass spectrometry and circular dichroism spectroscopy indicated that the 25-amino-acid hepcidin peptide contains four disulfide bonds and is rich in beta-sheet (68). The nuclear magnetic resonance spectroscopy (Figure 3) showed that hepcidin forms a simple hairpin, with three disulfide bonds stabilizing the antiparallel strands and a fourth bond linking adjacent Cys in the turn (29). The high degree of disulfide bonding confers hepcidin stability in circulation, but the removal of individual bonds did not have a significant impact on hepcidin iron-regulatory function in vitro (53).

Similar to antimicrobial and antifungal peptides, hepcidin has an amphipathic structure, with hydrophobic residues distributed on the convex side and positively charged residues on the concave side (Figure 3). The significance of this has not been established, but may be responsible for hepcidin's modest antimicrobial properties in vitro (68). The antimicrobial activity was apparent only at very high (10–30 μM) concentrations unlikely to be reached in urine, where hepcidin concentrations are normally in the 3–30 nM range and rise to as high as a few μM during severe inflammation. However, plasma and tissue concentrations of hepcidin have not yet been reliably measured, and the physiologic relevance of its direct antimicrobial activity remains to be established.

The iron-regulatory activity of hepcidin, on the other hand, is readily demonstrated at 100-fold lower concentrations than those required for the antimicrobial activity (56, 73). Additionally, in contrast to the antimicrobial activity of both the full-length (25 aa) and the N-terminally truncated hepcidin (20 aa), the iron-regulatory activity is exhibited only by the full-length peptide. Serial deletion of the 5 N-terminal residues leads to a progressive decrease in iron-regulatory activity, so that the 20 aa peptide is completely inactive both in vitro and in vivo (53).

Also unlike antimicrobial peptides whose sequences vary even between closely related species, hepcidin is highly conserved across vertebrate species, from fish to humans (68) (Figure 4). Particularly conserved are the cysteines forming disulfide bonds and the N-terminal region. Moreover, zebrafish hepcidin was fully active when tested as a regulator of mouse ferroportin (53). Interestingly, genomes of some species contain two (mouse) or more (certain fish species) hepcidin genes, a finding of unknown relevance. In mice, hepcidin-2 appears to have arisen by duplication of the region encompassing hepcidin and part of the neighboring gene USF-2 (30). Although the two mouse hepcidins are similarly regulated by iron, hepcidin-2 does not appear to have a role in iron metabolism. Unlike the transgenic overexpression of mouse hepcidin-1, which caused severe iron-refractory

Figure 4 Amino acid sequences of vertebrate hepcidins. The mammalian species are h = human, p = pig, r = rat, m = mouse hepcidin-1 (the functional homolog of human hepcidin), and d = dog. Zebrafish hepcidin-1 (zHep) is shown as a representative of the many fish hepcidins reported. The conserved cysteines are boxed.

anemia, transgenic overexpression of hepcidin-2 had no detectable effect on iron metabolism (47).

IRON-REGULATORY ACTIVITY OF HEPCIDIN

The essential role of hepcidin in iron metabolism was established in mouse models with hepcidin deficiency or excessive hepcidin production. Hepcidin deficiency was first identified as an unexpected by-product of knocking out an adjacent gene, USF2 (58). The mice developed massive iron overload with iron deposition in the liver and pancreas and sparing of the macrophage-rich spleen. These findings were similar to human hereditary hemochromatosis and suggested that hepcidin is an inhibitory regulator of intestinal iron uptake and the release of iron from macrophages. An independent USF2 knockout line, which expressed normal levels of hepcidin mRNA, showed no aberrations in iron metabolism, confirming that the iron overload phenotype was due to hepcidin deficiency (59). In contrast to hepcidin deficiency, mice that overexpressed hepcidin-1 under the control of a liver-specific promoter developed severe iron-deficiency anemia and most died at birth, which suggests that hepcidin also inhibited placental transport of iron (59). Hepcidin also appears to negatively regulate the export of iron from hepatocytes, as indicated by hepatic iron accumulation in mice carrying hepcidin-overproducing tumors (72).

The essential role of hepcidin in iron homeostasis has been confirmed in humans. Inactivating hepcidin mutations have been found in several patients with juvenile hemochromatosis, the most severe form of hereditary hemochromatosis (75). Conversely, patients with rare glycogen storage disease who developed liver tumors with autonomous hepcidin overexpression suffered from severe iron-refractory anemia, which resolved only after tumor resection or liver transplantation (84).

The bioactive form of hepcidin is the 25-amino-acid peptide, and it acts by causing hypoferremia, due to inhibition of iron recycling by macrophages, iron release from hepatic stores, and iron absorption in the intestine (Figure 1). Indeed, injection of a single dose of synthetic 25 aa hepcidin (but not the 20 aa N-terminally truncated peptide), induces a profound hypoferremia in mice within just 1 hour, and this effect lasts for up to 72 hours (73).

REGULATION OF HEPCIDIN SYNTHESIS

Regulation by Iron

In mice, oral or parenteral iron loading increases hepatic hepcidin mRNA expression (54, 69). In humans, even a single dose of oral iron (65 mg as FeSO4) increases urinary hepcidin excretion within several hours (54). Hepcidin induction by iron is homeostatic because increased plasma hepcidin would act to inhibit further intestinal iron absorption and iron release from stores. However, the mechanism of hepcidin regulation by iron is turning out to be unexpectedly complex.

Although hepatocytes are the main sources of hepcidin, and the simplest model would place the iron sensor there (Figure 1), it is not certain that iron sensing takes place in hepatocytes. In vitro iron loading of primary mouse or human hepatocytes, or human hepatic cell lines, does not increase hepcidin mRNA, regardless of whether hepatocytes are loaded with iron-transferrin or other forms of iron (26, 57, 69). The hepcidin mRNA lacks any stem-loop structures containing the consensus IRE motif for binding of iron-regulatory proteins. Perhaps the best clue about hepcidin regulation by iron comes from the studies of genes involved in hereditary hemochromatosis. Despite iron overload, hepcidin was found to be deficient in patients or mice with homozygous mutations in HFE, transferrin receptor 2 (TfR2), and hemojuvelin (HJV) (3, 7, 28, 35, 52, 55, 63, 66), which suggests that these molecules regulate hepcidin synthesis in response to iron. The detailed implications of these studies for hepcidin regulation by iron are discussed later in this chapter.

Regulation by Oxygen and Anemia

Hepcidin production is suppressed by anemia and hypoxemia. Anemia due to bleeding or PHZ-induced hemolysis in mice caused a decrease in hepcidin mRNA levels (6, 21, 41, 60). Hepcidin mRNA was also suppressed within 2 days in mice housed in hypobaric chambers (60) and in rats exposed to 10% oxygen for 30 days (44). Blood loss and hypoxia stimulate erythropoietin release, which increases the production of erythrocytes. The simultaneous decrease in hepcidin levels is adaptive because it allows increased iron absorption from the diet and iron mobilization from macrophages and hepatocytes, making more iron available for erythrocyte production.

Unlike regulation by iron, hepcidin response to hypoxia appears to be readily reproduced in HepG2 and Hep3B hepatoma cell lines (60) when these were grown at less than 2% O_2 for 24 hours. However, the molecular pathways that regulate hepcidin in response to hypoxia are not known. Hypoxia-inducible factor (HIF) is a master transcriptional regulator of genes that promote adaptation to hypoxia (33). Although the human hepcidin promoter contains several consensus binding sites for HIF, these are not conserved in other mammals, and their role, if any, has not yet been experimentally tested. Hepcidin regulation by hypoxia may be very important for the understanding of iron accumulation and maldistribution in chronic congenital anemias, which are discussed later in the chapter.

Regulation by Inflammation

Hepcidin synthesis is markedly induced by infection and inflammation (37, 57, 60, 69). These effects are mediated by inflammatory cytokines, predominantly IL-6. In human volunteers infused with IL-6, urinary hepcidin excretion was increased an average of 7.5-fold within two hours after infusion, whereas IL-6 knockout mice (unlike control mice) failed to induce hepcidin in response to turpentine (54). In vitro treatment of primary hepatocytes with (*a*) IL-6 directly, (*b*) LPS or

peptidoglycan, or (*c*) supernatants of LPS- or peptidoglycan-stimulated macrophages, increased hepcidin mRNA expression (54), and this induction was blocked by treatment with anti-IL-6 antibodies. IL-1 also increased hepcidin mRNA expression in vitro, but in human primary hepatocytes this was blocked by anti-IL-6 antibodies, whereas in mouse hepatocytes, IL-1 effect was independent of IL-6 (43). This finding suggests that there may be species-specific differences in cytokine involvement between mice and humans. TNF-α, on the other hand, was shown to suppress hepcidin mRNA expression in human hepatic cells in vitro (54).

It is unknown if cross talk exists between different pathways regulating hepcidin production. Although it has been suggested that the HFE molecule participates in both the inflammatory and iron-related pathways, the evidence is still controversial (23, 42, 77).

MECHANISM OF HEPCIDIN ACTION

Ferroportin

The hepcidin receptor, ferroportin (1, 15, 51), is the only known cellular iron exporter in vertebrates. Studies in zebrafish (15) and mouse (16) demonstrated that the complete loss of ferroportin expression is embryonic lethal due to the inability to transfer iron from the mother to the embryo. In addition to placental trophoblast, ferroportin is found in all other tissues where major iron flows are regulated (duodenal enterocytes, macrophages, and hepatocytes). Indeed, in the selective ferroportin knockout that preserves ferroportin at the maternal-fetal interface (16), the newborn mice rapidly developed severe iron-deficiency due to deficient dietary iron absorption. Additionally, the release of iron from hepatic storage was defective, as was the release of iron from macrophages that recycled red cells. This study indicates that ferroportin is the sole significant exporter of iron in tissues involved in iron absorption, recycling, and storage.

Hepcidin Regulates Cellular Iron Export

Hepcidin acts by directly binding to ferroportin, causing ferroportin to be internalized and degraded in lysosomes (Figure 5*A*) (13, 56). The loss of ferroportin from cell membrane consequently ablates cellular iron export (39, 56). Considering the tissue distribution of ferroportin, the hepcidin-ferroportin interaction explains the efferent arm of iron homeostasis because it can control the iron absorption in the duodenum, as well as the release of iron from macrophages and hepatocytes. When iron stores are adequate or high, the liver produces hepcidin, which circulates to the small intestine. By targeting ferroportin on the basolateral membrane of absorptive enterocytes, hepcidin blocks the delivery of dietary iron to plasma transferrin. Within a day or two, the short-lived enterocytes are shed into the intestine, removing the iron from the body. When iron stores are low,

hepcidin production is suppressed, ferroportin molecules are displayed on basolateral membranes of enterocytes, and iron is transported to plasma transferrin (Figure 5*B*). Similarly, in macrophages recycling aged red blood cells, hepcidin-induced degradation of ferroportin results in the trapping of iron in macrophages. High levels of hepcidin in inflammatory states would therefore account for the characteristic finding of iron-containing macrophages in spite of low plasma iron. In addition to the posttranslational regulation of ferroportin density on cell membranes by hepcidin, iron homeostasis could also be affected by altering the rate of ferroportin resynthesis. Indeed, iron and inflammation have both been reported to suppress ferroportin mRNA expression independently of hepcidin (14, 22, 46, 48, 51).

Hepcidin would be expected to have an indirect effect on the cellular IRE/IRP system. By blocking cellular iron efflux, hepcidin causes a rise in intracellular iron levels, which would affect expression of the IRE-containing DMT1, the transferrin receptor, possibly other less characterized uptake mechanisms (heme, nontransferrin iron), as well as ferroportin itself, but the extent of interaction between the hepcidin-ferroportin and the IRE/IRP system remains to be characterized in detail.

Hepcidin Causes Hypoferremia

Injection of a single dose (50 (μg) of hepcidin in mice causes a rapid drop in serum iron within just 1 hour (73). Marked hypoferremia is also caused by turpentine injections in mice, almost certainly due to increase in hepcidin mRNA expression (54, 60), since the response is completely ablated in hepcidin-deficient mice (60). In humans, the hepcidin increase elicited by IL-6 infusion is accompanied by a 30% decrease in serum iron and in transferrin saturation within hours after infusion (54), and similar response is seen after LPS injection in human volunteers (37). It therefore appears that, at least acutely, hepcidin is the main mediator of hypoferremia of inflammation and that the IL-6-hepcidin axis is critically important for this response. This does not preclude the involvement of other cytokines in hepcidin regulation, but the specific alternative cytokine pathways remain to be characterized and could differ between the human and the mouse (43).

How does hypoferremia develop so rapidly? The plasma transferrin compartment contains only about 3 mg of iron. Through this transit compartment flows about 20 mg of iron each day, mostly iron recycled from senescent erythrocytes. Therefore, the entire content of iron in plasma turns over every 3–4 hours. If hepcidin could completely block iron recycling, in one hour plasma iron would drop by at least 25%, consistent with the degree of hypoferremia observed in humans infused with IL-6. Regarding the significance of hypoferremia in inflammation, it has long been postulated that it is a host defense mechanism that limits pathogen survival by depriving them of iron (32, 83). However, only circumstantial evidence has shown that hypoferremia is beneficial in infection, and direct contribution of hepcidin to host defense remains to be experimentally confirmed.

IRON DISORDERS AND THE REGULATION OF HEPCIDIN

Genes Involved in Hereditary Hemochromatosis Encode Regulators of Hepcidin

Hereditary hemochromatosis caused by homozygous disruption of HFE, TfR2, and HJV is characterized by hepcidin deficiency in spite of massive iron overload, indicating that all these molecules act as direct or indirect regulators of hepcidin synthesis. Of these, HJV appears to have the greatest impact on hepcidin regulation because its disruption phenotypically completely mimics homozygous disruption of the hepcidin gene itself, and both result in the most severe form of hereditary hemochromatosis, juvenile hemochromatosis (66, 76). We measured urinary hepcidin in HJV hemochromatosis and found that hepcidin is undetectable in this disorder (66). Untreated patients with juvenile hemochromatosis usually develop iron overload and damage to the liver, endocrine glands, heart, and other organs in their teens and twenties, and both genders are affected more or less equally.

Humans and mice with homozygous HFE disruption have hepatic hepcidin mRNA levels that are inappropriately low for the degree of iron loading (3, 7, 52). However, our measurements of urinary hepcidin in untreated HFE patients (manuscript submitted) indicate that hepcidin is not as severely decreased in HFE hemochromatosis as it is in hemochromatosis due to defects in HJV or TfR2 (55). The HFE mutations indeed cause a milder and less penetrant form of hemochromatosis, with a later onset and less severe presentation in women. Therefore, HFE is likely a modulator of signaling from the iron sensor to hepcidin but is not essential for the function of this pathway. In contrast, the hemochromatosis due to homozygous disruption of TfR2 is somewhat more severe (9), and urinary hepcidin levels are very low (55). TfR2 is predominantly expressed in the liver and its levels are regulated by transferrin saturation (31, 74), making TfR2 a strong candidate for an iron sensor.

Hemojuvelin

HJV is a recently discovered glycosylphosphatidyl inositol (GPI)-linked protein (66) that belongs to the family of repulsive guidance molecules (RGMs) involved in neuronal differentiation, migration, and apoptosis (8, 50, 64, 78). However, unlike other RGMs that are expressed in neural tissue, HJV is expressed predominantly in skeletal muscle, the liver, and the heart. One form of HJV is GPI-linked to the membrane (86), and this cell-associated form was shown to undergo cleavage in cell culture, resulting in the release of the soluble HJV into the medium (45, 86). The process also occurs in vivo, as the soluble form was detected in human sera (45). Importantly, the cleavage of HJV in vitro is inhibited by iron: increasing concentrations of Fe_2-Tf or ferric ammonium citrate progressively suppressed the release of soluble HJV from cells into the medium, a finding that suggests that HJV could be a part of the iron sensor complex (45).

Hemojuvelin is a Cellular Regulator of Hepcidin

HJV directly regulates hepcidin expression in vitro (45). Knockdown of HJV expression by siRNA in Hep3B cells resulted in a decrease of hepcidin mRNA expression, showing that cellular HJV positively regulated hepcidin mRNA expression. In contrast, recombinant soluble HJV suppressed hepcidin mRNA in primary human hepatocytes in a log-linear dose-dependent manner, suggesting binding competition between soluble and cell-associated HJV. Examination of the global effect of soluble HJV on gene expression in primary hepatocytes indicated that the effect on hepcidin was highly selective, as hepcidin mRNA showed the largest change of any transcript. We propose that soluble and cell-associated HJV reciprocally regulate hepcidin expression in response to changes in extracellular iron concentration (45) (Figure 6).

The mechanism of HJV action, however, is still unclear. It likely involves interactions with other proteins, since HJV lacks a cytoplasmic tail for direct signaling to the cell interior and other members of the RGM family (RGMa and RGMb) function as receptor ligands. Both RGMa and RGMb are coreceptors for BMP signaling (5); RGMa was also shown to bind to neogenin, an N-CAM receptor, to regulate neuronal survival (49, 71). Zhang et al. (86) reported that HJV also interacts with neogenin in HEK293 cells overexpressing HJV, and coexpression of the two proteins in HEK293 led to increased cellular iron retention. It remains to be determined whether HJV-neogenin interaction regulates hepcidin expression, and if it affects iron homeostasis in vivo.

Hepcidin in Other Diseases of Iron Overload

Patients with chronic anemias with hemolysis or dyserythropoiesis, such as thalassemia syndromes, congenital dyserythropoietic anemias, and sideroblastic anemias suffer from iron overload and its maldistribution. Measurements of urinary hepcidin in these patients indicated that hepcidin levels were severely decreased, despite systemic iron overload reflected by the patients' elevated serum ferritin levels (36, 67). Even in regularly transfused thalassemia patients, hepcidin levels were inappropriately low given the patients' iron load, as indicated by the decreased ratio of urinary hepcidin to serum ferritin, used as an index of appropriateness of hepcidin response to iron load (36, 55, 67). A mouse model of thalassemia also indicated that hepcidin mRNA expression was decreased (2). Similarly, injections of iron-dextran in mice which led to increase in liver and serum iron failed to counteract the suppressive effects of anemia/hypoxia due to PHZ-induced hemolysis (60). It therefore appears that the effects of anemia, especially when associated with increased erythropoiesis, outweigh the effects of iron overload on hepcidin regulation and result in a net inhibition of hepcidin synthesis. The low hepcidin levels in hereditary anemias may be responsible for increased iron absorption and maldistribution of iron in these patients, contributing to systemic iron overload and related organ damage.

Anemia of Inflammation

Anemia of inflammation (AI), also known as anemia of chronic disease, occurs in acute and chronic infections and in a variety of inflammatory disorders, including rheumatologic diseases, inflammatory bowel disease, multiple myeloma, and other malignancies (11, 32, 38). AI is characterized by decreased iron and iron-binding capacity (transferrin), increased ferritin, and the presence of iron in bone-marrow macrophages, indicating impaired mobilization of iron from stores (10). As discussed in a previous section, inflammatory hypoferremia and the development of AI is thought to be a result of host defense responses that evolved to deprive bacteria of iron (32, 83).

Recent studies indicate that inflammation causes hypoferremia and eventually AI through the cytokine-mediated increase in hepcidin production (25). Patients with anemia of inflammation have elevated urinary excretion of hepcidin compared with healthy controls (57). Mice or rats with inflammation induced by turpentine, LPS, or Freund's adjuvant rapidly increased hepatic hepcidin-1 mRNA expression (23, 54, 60, 69). The effects of hepcidin excess mirror the hematological changes seen in AI. Mice administered synthetic hepcidin develop hypoferremia within hours of a single intraperitoneal injection (73). Mice with tumors engineered to overexpress hepcidin develop more severe anemia and hypoferremia despite having increased liver iron stores when compared with mice with control tumors (72). Similarly, in humans, patients with hepatic adenomas secreting large amounts of hepcidin were reported to have severe iron-refractory anemia resembling AI that resolved only when the tumor was resected or after liver transplantation (84).

As pointed out above, hypoferremia results from hepcidin-mediated inhibition of iron export into plasma from macrophages involved in iron recycling, hepatocytes that store iron, and enterocytes absorbing dietary iron. Because most of the iron in plasma is destined for the bone marrow, hypoferremia diminishes the amount of iron available for hemoglobin synthesis and erythrocyte production. Therefore, AI develops as a side effect of the hypoferremic response to infection and inflammation. IL-6 excess is also commonly associated with anemia, such as that seen in patients on experimental IL-6 treatment or transgenic mice overexpressing IL-6 (34, 65, 82), as well as in Castleman's disease or multiple myeloma, diseases associated with elevated IL-6. Thus, development of anemia of inflammation likely involves the pathogenic cascade from IL-6 to hepcidin to hypoferremia, and eventually anemia.

Anemia of inflammation is also characterized by blunted erythropoietin response and a decrease in red blood cell survival, and it remains to be experimentally determined whether hepcidin also contributes to these phenomena.

CLINICAL POTENTIAL (DIAGNOSTIC, THERAPEUTIC)

In principle, hepcidin administration would be expected to prevent (and possibly reverse) the manifestations of most types of hereditary hemochromatosis with the possible exception of those due to insensitivity to hepcidin. Indeed, transgenic mice

with HFE hemochromatosis did not develop iron overload if they also expressed a hepcidin transgene (62), providing a proof of concept for the use of hepcidin to prevent hemochromatosis. Acute administration of synthetic hepcidin to mice (73) rapidly lowered serum iron, and the effect lasted at least 48 hours, indicating that therapeutic hepcidin would not have to be administered very frequently. However, current treatment of hemochromatosis by the removal of iron through bleeding is inexpensive and generally well tolerated, setting a high benchmark for any new treatment. In thalassemias and other hereditary anemias accompanied by iron overload, bleeding is not an option, and hepcidin could be very useful in controlling the component of iron overload due to increased intestinal absorption of iron. This component is significant, as indicated by the development of severe iron overload in patients with thalassemia intermedia who do not receive transfusions (70). It is less certain whether hepcidin could also influence the distribution of iron delivered by frequent transfusions, possibly by sequestering iron in macrophages where it is less toxic than in hepatocytes and other tissues.

At the other end of the spectrum, a hepcidin antagonist should be useful in treating anemia of inflammation, probably the most common form of anemia in the United States and other economically advanced countries. Future studies should lead to the development of suitable antagonists and the demonstration of their efficacy in vivo.

The measurement of hepcidin concentrations in biological fluids would be expected to help in the differential diagnosis of anemia of inflammation (elevated hepcidin concentrations) and iron deficiency anemia (low hepcidin concentrations). It could also be useful in classifying hereditary hemochromatosis as either due to hepcidin deficiency (common) or resistance to hepcidin (rare) with potential future therapeutic implications. Recent research studies have employed urinary hepcidin measurements, but a serum or plasma assay may also be feasible and perhaps less burdensome for the patient.

CHALLENGES/PERSPECTIVE/SUMMARY

It has been less than five years since the appearance of the first publications on hepcidin (40, 68, 69) and the first indication that hepcidin could be involved in iron metabolism (69). Building on older studies that defined the phenomenology of the regulation of iron absorption and recycling, and more recent work that identified the key iron transporters and their associated ferroxidases, the identification of hepcidin as the long sought iron-regulatory hormone greatly simplified our understanding of systemic iron metabolism. It now appears that a single molecule, hepcidin, is the "stores" regulator, the "erythropoietic" regulator, and the "inflammation" regulator of iron absorption and recycling (18, 61), and that hepcidin acts principally or perhaps solely by causing the internalization and degradation of its receptor/iron channel ferroportin (56). The delineation of the molecular determinants of the hepcidin-ferroportin interaction is a worthwhile long-term goal that could facilitate the development of hepcidin agonists and antagonists. However,

the many membrane-spanning segments that comprise much of the ferroportin molecule make it unlikely that its structure will be solved very soon.

Although we have learned much about how hepcidin regulates iron transport (the efferent arc of the homeostatic loop), we know very little about the afferent arc: how hepcidin production and release are regulated by iron and oxygen. The genetics of hereditary hemochromatosis points to TfR2, HJV, and HFE as components of the regulatory pathways, but it is by no means clear how these (and likely other) components fit together. Neither is it certain whether all of the hepcidin-regulating circuitry is contained in the liver or whether remote oxygen or iron sensors and circulating signals (perhaps soluble HJV) contribute to hepcidin regulation.

In assigning the appropriate role for hepcidin in the pathophysiology of human diseases, the evidence that hepcidin deficiency underlies most types of hemochromatosis is fairly strong. However, at the opposite pole of iron disorders, it is not yet clear whether excessive hepcidin is a necessary and nonredundant factor in anemia of inflammation or merely one of several pathways by which inflammation can cause anemia.

The remarkable progress made in the past ten years in understanding systemic and cellular iron metabolism is a tribute to the many workers in the field who have labored largely outside the spotlight of fashionable science. We anticipate that in the next ten years we will learn the answers to some of the remaining puzzles, and that these will lead to diagnostic and therapeutic advances that will improve the well-being of the many patients with iron disorders.

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Figure 2 The sequence of the 84-amino-acid hepcidin prepropeptide. Arrows denote the three processed forms isolated from human urine.

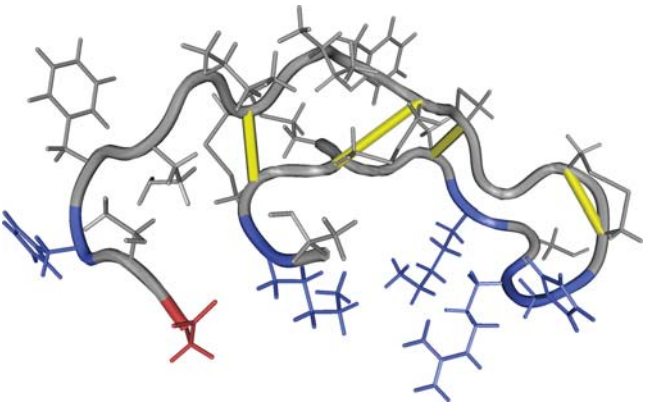

Figure 3 Nuclear magnetic resonance structure of hepcidin. The backbone and side chains are shown, with positively charged residues in blue, negatively charged in red, and disulfide bonds in yellow. The model clearly shows the spatial segregation of charged residues in the peptide and the unusual vicinal disulfide bond in the turn.

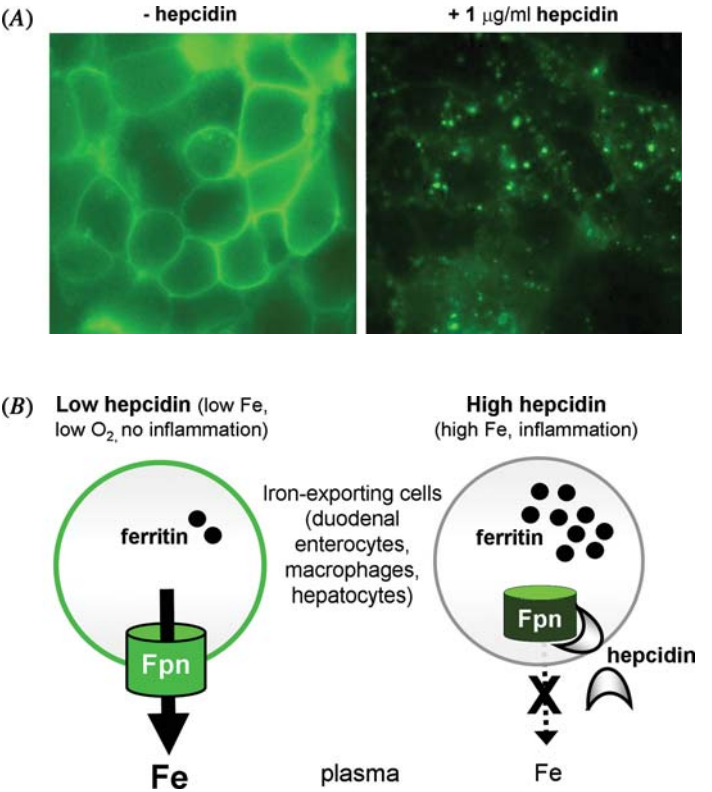

Figure 5 Hepcidin inhibits iron efflux by inducing the internalization and degradation of ferroportin. (*A*) HEK293 cell line was stably transfected with ponasterone-inducible construct encoding ferroportin-GFP fusion protein. Addition of hepcidin (1 μg/ml) caused rapid internalization of ferroportin. Ferroportin was subsequently degraded, resulting in the inhibition of cellular iron efflux. (*B*) A cartoon representation of the effect of hepcidin on iron-exporting cells.

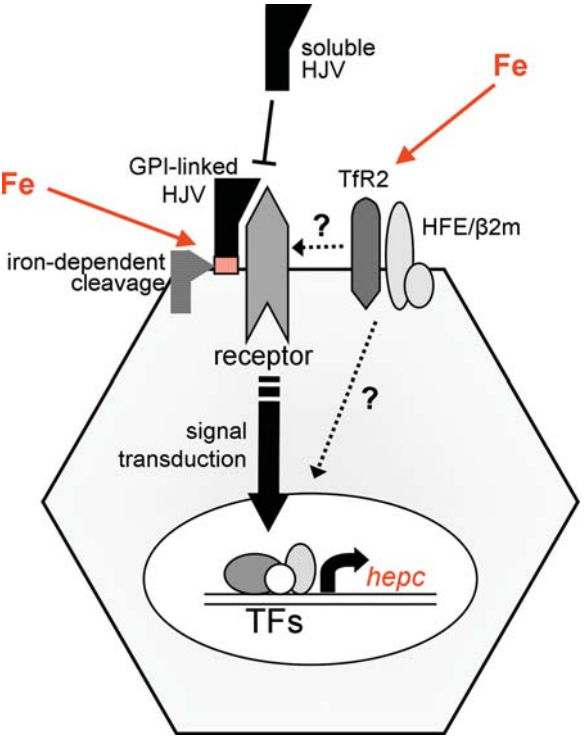

Figure 6 A hypothetical model of hepcidin regulation by iron. Soluble and glycosylphosphatidyl inositol (GPI)-anchored hemojuvelin (HJV) compete for interaction with a transmembrane receptor (possibly neogenin). Hepcidin is induced only when the GPI-anchored HJV is bound to the receptor. Conversion from GPI-linked to soluble form is regulated by iron concentrations. Transferrin receptor 2 (TfR2) is stabilized by Fe-Tf and may interact with the HJV-receptor complex or transmit a signal to the nucleus independently. HFE also appears to modulate iron sensing and/or signal transduction by an unknown mechanism. For simplicity, all events are shown as taking place on a hepatocyte, but it is possible that other cell types generate soluble HJV or other signals that modulate hepcidin production by hepatocytes.

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ERRATA

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