IN TRANSLATION

Molecular Mechanisms of Hepcidin Regulation: Implications for the Anemia of CKD

Jodie L. Babitt, MD, and Herbert Y. Lin, MD, PhD

Anemia is prevalent in patients with chronic kidney disease (CKD) and is associated with lower quality of life and higher risk of adverse outcomes, including cardiovascular disease and death. Anemia management in patients with CKD currently revolves around the use of erythropoiesis-stimulating agents and supplemental iron. However, many patients do not respond adequately and/or require high doses of these medications. Furthermore, recent clinical trials have shown that targeting higher hemoglobin levels with conventional therapies leads to increased cardiovascular morbidity and mortality, particularly when higher doses of erythropoiesis-stimulating agents are used and in patients who are poorly responsive to therapy. One explanation for the poor response to conventional therapies in some patients is that these treatments do not fully address the underlying cause of the anemia. In many patients with CKD, as with patients with other chronic inflammatory diseases, poor absorption of dietary iron and the inability to use the body's iron stores contribute to the anemia. Recent research suggests that these abnormalities in iron balance may be caused by increased levels of the key iron regulatory hormone hepcidin. This article reviews the pathogenesis of anemia in CKD, the role and regulation of hepcidin in systemic iron homeostasis and the anemia of CKD, and the potential diagnostic and therapeutic implications of these findings.
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INDEX WORDS: Anemia; chronic kidney disease; dialysis; inflammation; hepcidin.

BACKGROUND

Iron is required for hemoglobin synthesis in the production of red blood cells. Iron also is a constituent of several proteins that carry out essential housekeeping functions and thus is critical for cell growth and survival. However, excess iron can generate free radicals that damage lipid membranes, proteins, and nucleic acids, leading to cell death. As a result, iron levels must be regulated tightly both on a cellular level and systemically. Hepcidin now is recognized to be a key mediator of systemic iron homeostasis. A role for hepcidin in the pathogenesis of the anemia of chronic kidney disease (CKD) increasingly is being elucidated.

From the Program in Membrane Biology, Division of Nephrology, Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA.

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Address correspondence to Jodie L. Babitt, MD, Program in Membrane Biology, Division of Nephrology, Center for Systems Biology, Massachusetts General Hospital, 185 Cambridge St, CPZN-8218, Boston, MA 02114. E-mail: babitt.jodie@mgh.harvard.edu

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CASE VIGNETTE

A 55-year-old woman with hemodialysis-dependent end-stage renal disease (ESRD) secondary to diabetic nephropathy had persistent anemia despite escalating erythropoiesis-stimulating agent (ESA) dosing. Serum hemoglobin level was 7.5 g/dL (reference range, 12-16 g/dL), and hematocrit was 23.4% (reference range, 36%-46%). Serum iron level was 22 μg/dL (reference range, 30-160 μg/dL), total iron-binding capacity was 188 μg/dL (reference range, 230-404 μg/dL), and serum transferrin saturation was 11.7%, consistent with low circulating levels of iron. However, serum ferritin level was increased at 1,315 ng/mL (reference range, 10-200 ng/mL). The patient had a failed arteriovenous fistula and arteriovenous graft and now has a tunneled catheter for hemodialysis access. She has a history of coronary artery disease, severe peripheral vascular disease, calciphylaxis, multiple episodes of skin and catheter infections, and toe amputation for a nonhealing ulcer. This scenario of anemia, ESA resistance, hypoferremia, and high serum ferritin level is not uncommon in the CKD/ESRD population, and anemia management in these patients presently is problematic. It recently has been hypothesized that increased hepcidin levels may contribute to functional iron deficiency, anemia, and ESA resistance in this setting.

PATHOGENESIS

Anemia of CKD

Anemia is prevalent in patients with CKD and contributes to lower quality of life.[1] Anemia in

patients with CKD also is associated with numerous adverse outcomes, including hospitalization, cardiovascular disease, cognitive impairment, and mortality.[1] Inadequate production of erythropoietin commonly is believed to be the most important factor in the pathogenesis of anemia in these patients, and many patients are treated with ESAs. However, approximately 10%-20% of patients are poorly responsive to ESA therapy.[1] Prospective randomized controlled clinical trials, including the US Normal Hematocrit Study and the CHOIR (Correction of Hemoglobin and Outcomes in Renal Insufficiency) Study, raised concerns about the safety of ESAs when dosed to target higher hemoglobin levels, particularly when using higher doses and in patients who are poorly responsive to therapy.[2-4] This has resulted in a US Food and Drug Administration black box warning on the product labeling of ESAs and significant controversy about the management of anemia in patients with CKD.

The cause of anemia in patients with CKD is multifactorial.[5,6] In addition to relative erythropoietin deficiency, shortened erythrocyte survival[6] and the erythropoiesis-inhibitory effects of accumulating uremic toxins also contribute to the anemia of CKD.[5,6] Importantly, patients with CKD also have several abnormalities in systemic homeostasis of iron, an essential component in the production of red blood cells.[5,6] First, hemodialysis patients in particular typically are in negative iron balance, losing approximately 1-3 g of iron per year, caused in part by blood trapping in the dialysis apparatus and repeated phlebotomy.[6] Second, many patients are on ESA therapy to manage their anemia, which depletes iron stores by driving increased production of red blood cells.[6] Third, it has been recognized that patients with CKD also have impaired absorption of dietary iron. Randomized controlled trials have shown that oral iron is no better than placebo to treat iron deficiency in patients on hemodialysis therapy.[1,7-9] These abnormalities of iron metabolism in patients with CKD may result in true iron deficiency, manifest as low serum transferrin saturation and ferritin level, which can be treated with supplemental iron. However, many patients also have a functional iron deficiency or reticuloendothelial cell iron blockade, characterized by low levels of circulating iron that limit erythropoiesis, even in the face of adequate or increased body iron stores. Management of these patients is less clear.[1] This reticuloendothelial cell iron sequestration is characteristic of anemia of inflammation (also known as anemia of chronic disease), seen not only in patients with CKD, but also in patients with many other chronic diseases, including autoimmune disorders, chronic infections, and malignancy.[5] Many patients with CKD have a chronic inflammatory state, which may be caused by an increased incidence of infections and/or induction of inflammatory cytokines by the hemodialysis procedure.[5,6,10] Recent research suggests that the impaired intestinal iron absorption and impaired release of iron from body stores in patients with CKD, as in other patients with anemia of inflammation, may be caused by an excess of the key iron regulatory hormone hepcidin.[11-13]

Systemic Iron Homeostasis and Hepcidin

Iron enters the body by absorption from dietary sources in the duodenum. Iron circulates bound to transferrin and is delivered primarily to the bone marrow for erythropoiesis. Senescent erythrocytes are phagocytosed by reticuloendothelial macrophages to recycle iron back into the circulation. Iron storage and release also occur in hepatocytes. Sloughing of enterocytes and bleeding are the only significant means for removing iron from the body (reviewed in[14-16]; Fig 1). On average, approximately 1-2 mg of iron is provided on a daily basis by intestinal absorption, and this is balanced by an equal amount of iron loss by epithelial shedding in the gastrointestinal tract and blood loss in menstruating women. Most of the iron required for erythropoiesis, approximately 20-25 mg/d, is provided by iron recycling from senescent erythrocytes. The circulating pool of transferrin-bound iron is much smaller, approximately 3 mg, and therefore must be turned over every few hours to ensure an adequate supply of iron for erythropoiesis. Iron excretion is not a regulated process, and there is no physiologic mechanism for removing larger amounts of iron, even in conditions of severe iron overload. Systemic iron balance therefore is maintained by tight regulation of iron absorption from the diet and iron release from reticuloendothelial and hepatocyte stores.[14-16]

It now is well established that hepcidin is a central mediator of systemic iron homeosta-

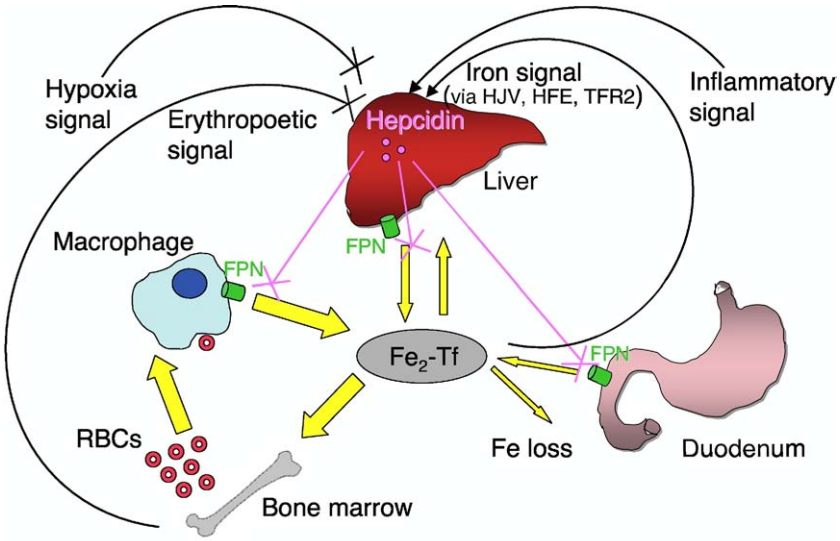

Figure 1. Hepcidin is a central regulator of systemic iron homeostasis. Serum iron levels are determined by the balance of iron entry from intestinal absorption, macrophage iron recycling, and mobilization of hepatocyte stores versus iron utilization, primarily by erythroid cells in the bone marrow. A peptide hormone secreted by the liver, hepcidin controls iron release into the plasma by downregulating cell-surface expression of the iron export protein ferroportin (FPN) on absorptive enterocytes, macrophages, and hepatocytes. Hepcidin production is inhibited by erythropoietic drive and hypoxia to ensure iron availability for erythropoiesis. Hepcidin production is stimulated by iron (through the hemochromatosis proteins HFE, hemojuvelin [HJV], and transferrin receptor 2 [TFR2]) as a negative feedback loop to maintain steady-state iron levels. Hepcidin production also is stimulated by inflammation, thereby sequestering iron from invading pathogens in the setting of infection, but also causing the hypoferremia of anemia of chronic disease. Abbreviation: RBC, red blood cell.

sis.[15,16] A small peptide hormone of 25 amino acids, hepcidin is produced and secreted predominantly by hepatocytes, circulates in the bloodstream, and is excreted by the kidneys.[17-19] Hepcidin regulates systemic iron balance by binding and inducing internalization and degradation of ferroportin, an iron channel on the surface of enterocytes, macrophages, and hepatocytes, which is important in iron export into the plasma.[20,21] Hepcidin thereby decreases both intestinal iron absorption and iron release from reticuloendothelial and hepatocyte stores (Fig 1). The central importance of hepcidin in systemic iron homeostasis has been established by animal models and human patients with abnormalities in hepcidin expression. Hepcidin-null mice[22,23] and humans with mutations in the hepcidin gene develop severe iron overload,[24] whereas transgenic mice overexpressing hepcidin and humans with hepcidin-secreting adenomas have profound iron deficiency anemia.[25,26]

Regulation of Hepcidin Expression

Consistent with its role as a central regulator of systemic iron homeostasis, hepcidin expression is regulated in response to iron, erythropoietic demand, hypoxia, and inflammatory signals (Fig 1). Iron administration increases hepcidin expression, thus providing a feedback mechanism to limit further iron absorption,[18,27,28] whereas anemia and hypoxia inhibit hepcidin expression, thus increasing iron availability for erythropoiesis.[27] Hepcidin expression also is induced by inflammation,[18,27-29] which is believed to be part of the host defense mechanism to fight infection and cancer by limiting iron availability. However, in chronic inflammatory states, this leads to a deficiency of iron available for erythropoiesis, and this is believed to be the mechanism underlying the reticuloendothelial iron sequestration, intestinal iron absorption impairment, and low circulating iron levels characteristic of patients with anemia of chronic disease.[5,15,16] The signaling pathways by which iron, erythropoietic demand, hypoxia, and inflammation affect hepcidin expression increasingly are being elucidated.

Hepcidin Deficiency and Iron Overload Disorders

Recent progress toward understanding the mechanisms that regulate hepcidin expression

has emerged from studies of the genetic iron overload disorder hereditary hemochromatosis (reviewed in[30]). This heterogeneous disorder is characterized by failure to prevent excess iron from entering the bloodstream, leading to progressive tissue iron deposition and subsequent multiorgan damage. In addition to mutations in the gene encoding hepcidin (*HAMP*), mutations in the genes encoding the hemochromatosis protein HFE,[31] transferrin receptor 2 (*TFR2*),[32] and hemojuvelin (HJV; encoded by the *HFE2* gene)[33] also cause hereditary hemochromatosis. Among other tissues, these proteins are all expressed in the liver,[31,33,34] where hepcidin is produced. All patients and animal models with hemochromatosis caused by mutations in these genes have inappropriately low levels of hepcidin expression,[33,35-42] suggesting the following: (1) hepcidin deficiency and consequent unregulated ferroportin activity are the common pathogenic mechanisms for iron overload in these diseases, and (2) HFE, TFR2, and HJV are involved in the regulation of hepcidin expression by iron (Fig 1).

RECENT ADVANCES

Molecular Mechanisms of Hepcidin Regulation: Hepcidin Activators

Hepcidin Regulation by the BMP6-HJV-SMAD Signaling Pathway

Mutations in the HJV gene are the most common cause of the more severe juvenile-onset form of hereditary hemochromatosis and result in a phenotype similar to mutations in the gene encoding hepcidin itself.[24,33] A link between the bone morphogenetic protein (BMP) signaling pathway and iron metabolism was discovered when HJV was shown to be a BMP coreceptor[43] and BMP signals were shown to regulate hepcidin expression (Fig 2).[43,44]

BMPs are members of the TGF-β (transforming growth factor β) superfamily of signaling molecules. BMP ligands bind to cell-surface type I and type II serine threonine kinase receptors. Upon formation of the complex, type I receptors phosphorylate type II receptors, which then phosphorylate intracellular SMAD1, SMAD5, and SMAD8 proteins. These SMAD proteins form a

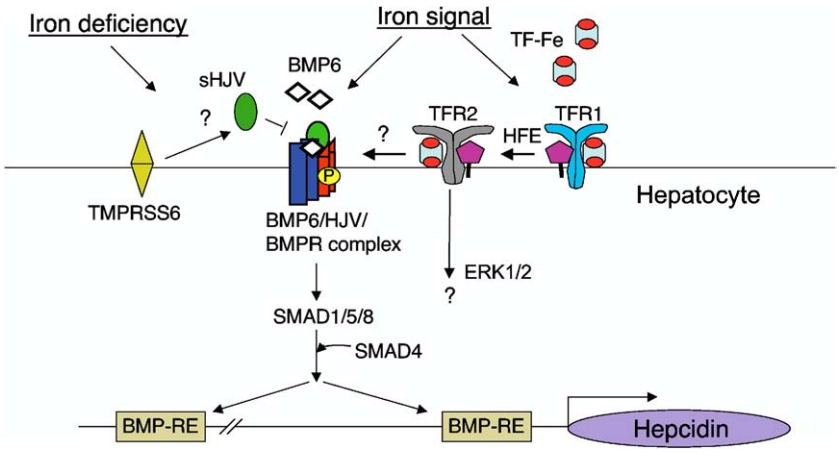

Figure 2. Schematic diagram shows the proposed role of the bone morphogenetic protein (BMP) signaling pathway, HJV, HFE, and TFR2 in iron sensing and hepcidin regulation in the liver. In response to iron, bone morphogenetic protein 6 (BMP6) binds to the BMP coreceptor hemojuvelin (HJV) as part of the BMP6/HJV/BMPR complex on the hepatocyte membrane to activate the SMAD1/5/8 pathway. Activated SMAD complexes bind directly to BMP-responsive elements (BMP-REs) on the hepcidin promoter to induce hepcidin transcription. Holotransferrin (TF-Fe) competes for HFE binding to transferrin receptor 1 (TFR1), causing HFE displacement to form a complex with TFR2 and TF-Fe to induce hepcidin expression, possibly through an interaction with the BMP6-HJV-SMAD signaling pathway and/or alternative signaling pathways, such as the ERK1/2 pathway. In the setting of iron deficiency, the serine protease TMPRSS6 inhibits hepcidin expression by cleaving membrane-bound HJV to form soluble HJV, thereby inhibiting downstream SMAD signaling by loss of membrane-bound HJV and sequestration of BMP6 ligand. Abbreviations: BMPR, BMP receptor; TF, transferrin.

complex with common mediator SMAD4, and the SMAD complex translocates to the nucleus to modulate gene transcription (reviewed in[45]).

As a coreceptor, HJV binds to BMP ligands and BMP receptors and enhances intracellular SMAD signals in response to low levels of BMP ligands.[43] HJV is selective for its interaction with BMP ligands and receptors. HJV binds to BMP2, BMP4, BMP5, and BMP6, but not BMP7 or BMP9.[43,46,47] HJV signals through all 3 BMP type I receptors (ALK2, ALK3, and ALK6), but only 2 of the 3 BMP type II receptors (ActRIIA and BMPRII).[48] Notably, HJV can alter type II receptor use by BMP ligands, allowing BMP ligands that normally signal through BMPRII to signal through ActRIIA.[48] This may be one mechanism by which HJV enhances SMAD signaling in the liver, where ActRIIA is the predominant BMP type II receptor expressed.[48]

In liver-derived cell cultures, BMP-SMAD signals increase hepcidin expression at the transcriptional level.[43,44,46,49] HJV enhances hepcidin induction in response to BMPs,[43] whereas HJV mutants associated with hemochromatosis have impaired ability to generate BMP signals and induce hepcidin expression, and livers from *Hjv*-null mice have evidence of decreased BMP signaling in the liver.[43] These data suggest that mutations in HJV trigger iron overload by causing impaired hepatic BMP-SMAD signaling, decreased hepcidin expression, and consequent unregulated ferroportin activity.[43]

Several additional lines of evidence support the importance of the BMP-HJV-SMAD signaling pathway in regulating hepcidin expression and iron homeostasis in vivo. Targeted disruption of the common BMP/TGF-β signaling mediator *Smad4* in the mouse liver results in low hepcidin levels and iron overload, similar to the phenotype seen in hemochromatosis.[44] Promoter mutational analysis has identified 2 specific motifs on the hepcidin promoter, which appear to mediate hepcidin induction to BMP-SMAD signals.[50-53] A mutation in one of these BMP responsive elements on the hepcidin promoter has been associated with a significantly more severe iron-overload phenotype in a patient with hemochromatosis caused by the most common *HFE* mutation, C282Y (a cysteine to tyrosine change at amino acid 282).[54] In mice, BMP administration increases hepatic hepcidin expression and decreases serum iron levels, whereas administration of BMP inhibitors decreases hepcidin expression, mobilizes reticuloendothelial cell iron stores, and increases serum iron levels.[46,47,55]

More recent data have identified BMP6 as a ligand for HJV and a key endogenous regulator of hepcidin expression and iron metabolism in vivo.[47,56] Genome-wide transcriptional profiling of livers from mice fed on diets of variable iron content identified *Bmp6* as one of the mRNAs and the only BMP ligand that was decreased by a low iron diet and increased by a high iron diet, concordantly with hepcidin.[57] BMP6 binds to HJV, and data from a bioinhibition assay suggest that HJV may have higher binding affinity for BMP6 compared with other BMP ligands, although this remains to be shown directly.[46,47] Administration of specific BMP6 inhibitors in vivo decreases hepcidin expression and increases serum iron levels.[47] Importantly, *Bmp6*-null mice have an iron-overload phenotype that resembles juvenile hemochromatosis due to HJV mutations with decreased hepcidin expression, increased ferroportin expression, increased serum iron levels, and tissue iron overload.[47,56] Taken together, these data support the central role of the BMP6-HJV-SMAD signaling pathway in regulating hepcidin expression and systemic iron balance in vivo (Fig 2).

Hepcidin Regulation by Iron

Iron stimulates hepcidin expression, providing an important feedback mechanism to maintain steady-state iron levels. A principal mechanism by which iron stimulates hepcidin expression is through activation of the BMP6-HJV-SMAD signaling pathway (Fig 2). As discussed, long-term increases in dietary iron increase liver *Bmp6* mRNA expression concordantly with hepcidin expression, and hepatic *Bmp6* mRNA levels are significantly positively correlated with liver iron concentrations in mice.[57,58] Additionally, acute iron administration to mice increases phosphorylation of SMAD1/5/8 in the liver within 1 hour.[55] The ability of iron-saturated transferrin (holotransferrin) to increase hepcidin expression in primary hepatocyte cultures is dependent on BMP-SMAD signaling because it is blocked by BMP signaling pathway inhibitors.[59] Similarly, the ability of iron to induce hepcidin expression in zebra fish is blocked by a BMP inhibitor.[55]

Thus, the BMP6-SMAD signaling pathway in the liver is activated by iron, and the ability of iron to induce hepcidin expression is dependent on BMP-SMAD signaling. The mechanisms by which iron levels are sensed to increase in BMP6-HJV-SMAD signaling are still not fully understood. It has been postulated that HFE, TFR2, and transferrin receptor 1 (TFR1) may be involved in this process.[60,61]

Mutations in *HFE*, encoding an atypical class I major histocompatibility complex (MHC) molecule, are the most common cause of hereditary hemochromatosis and result in a less severe adult-onset phenotype compared with HJV or *HAMP* mutations.[30,31] HFE has been shown to bind to both TFR1 and TFR2.[62,63] TFR1 is a ubiquitously expressed cell-surface receptor that binds transferrin and mediates transferrin-dependent iron uptake into cells (reviewed in[14]). The binding site for HFE on TFR1 overlaps with the binding site for transferrin, and holotransferrin competes for HFE binding to TFR1.[62, 64-67] TFR2 is a TFR1 homologue that has more restricted expression, predominantly in the liver,[34] and mutations in TFR2 are a much more rare cause of adult-onset hereditary hemochromatosis.[31,32] The central importance of the liver expression of HFE and TFR2 for the regulation of iron metabolism is supported because hepatocyte-specific conditional knockout of either *Hfe* or *Tfr2* in mice recapitulates the iron-overload phenotype seen in global *Hfe* or *Tfr2* knockout mice.[68,69] One model proposes that when circulating iron levels increase, holotransferrin binds to TFR1 in the liver, displacing HFE, which then is able to upregulate hepcidin through an interaction with TFR2 and transferrin (Fig 2).[60,61] However, it is notable that humans with mutations in both alleles of *HFE* and *TFR2* present with a more severe juvenile-onset form of hemochromatosis compared with the adult-onset hemochromatosis typical of either mutation alone,[70] and combined *Hfe*-null/*Tfr2*-null mice have a more severe hepcidin deficiency iron-overload phenotype compared with either single *Hfe*-null or *Tfr2*-null mice.[71] These data suggest that the mechanisms by which HFE and TFR2 regulate hepcidin expression and iron homeostasis are not entirely overlapping.

Whether HFE and/or TFR2 induce hepcidin expression through an interaction of the BMP6-HJV-SMAD signaling pathway and/or a separate as yet uncharacterized signaling pathway is still not fully understood (Fig 2). Notably, *Hfe*-null mice have appropriately increased hepatic *Bmp6* mRNA levels relative to their increased body iron burden, but inappropriately low levels of phosphorylated SMAD1/5/8 protein and *Id1* mRNA, a target gene upregulated by BMP6-SMAD signaling.[58,72] Furthermore, BMP6 induction of hepcidin is decreased in primary hepatocyte cultures from *Hfe*-null mice versus wild-type mice.[58] This suggests that HFE regulates hepcidin expression through an interaction with the BMP6-SMAD signaling pathway.[58]

Some studies have suggested that TfR2 can activate the mitogen-activated protein kinase (MAPK) pathway, including ERK1/ERK2 and p38 MAP kinases.[73,74] Although 1 study suggested that ERK1/ERK2 pathway activation may be involved in iron-saturated transferrin induction of hepcidin expression in conjunction with the BMP-SMAD pathway in primary hepatocyte cultures,[74] a direct demonstration of the relevance of the ERK/MAPK signaling pathway in hepcidin regulation and iron homeostasis in vivo is lacking.

Hepcidin Regulation by Inflammation

Hepcidin excess is believed to be the mechanism underlying the low circulating iron levels and reticuloendothelial cell iron sequestration that is a hallmark of anemia of inflammation.[5,16] Inflammation is a potent inducer of hepcidin expression.[18,27-29] The most well-characterized mechanism is direct transcriptional activation of hepatic hepcidin expression by interleukin 6 (IL-6) binding to its receptor complex containing gp130 to activate janus kinase (JAK) and activator of transcription 3 (STAT3), which binds to a conserved DNA element in the proximal hepcidin promoter (Fig 3).[75-77] Other proinflammatory cytokines, such as IL-1, also may have a role in hepcidin induction.[78] Notably, hepcidin induction by IL-6 appears to require an intact BMP-SMAD signaling pathway because hepatocyte-specific loss of the common mediator *Smad4*,[44] administration of soluble HJV,[46] or administration of a small-molecule inhibitor of BMP type I receptor kinase activity[55] all impair IL-6 induction of hepcidin expression. The cross-talk between these pathways appears to occur in part at

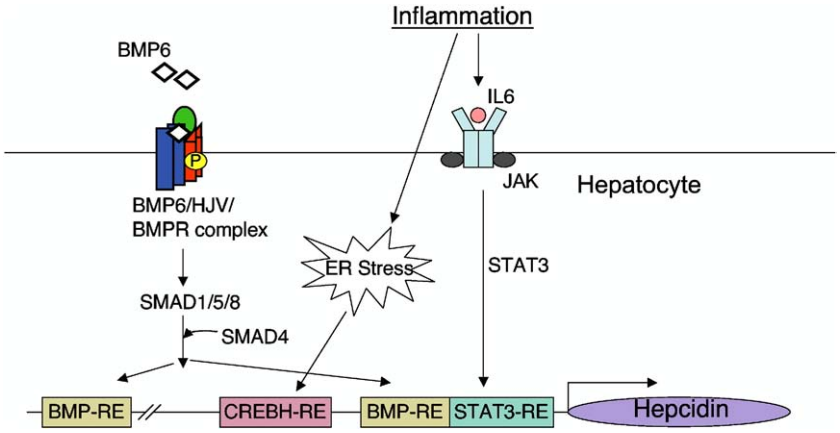

Figure 3. Schematic diagram shows the molecular mechanisms by which inflammation activates hepcidin transcription in the liver. Inflammation mediated by cytokines (eg, IL-6) leads to activation of the JAK/STAT3 pathway. Activated STAT3 binds directly to a STAT3-responsive element (STAT3-RE) on the hepcidin promoter to induce hepcidin transcription. The JAK/STAT3 pathway depends on an intact BMP responsive element (BMP-RE) that is adjacent to the STAT3-RE for full activity. Inflammation also may activate the endoplasmic reticulum (ER) stress pathway using a CREBH-responsive element (CREBH-RE) on the hepcidin promoter to induce hepcidin. Abbreviations: BMP, bone morphogenetic protein; CREBH, cyclic AMP response element-binding protein H; JAK, janus kinase; STAT3, activator of transcription 3.

the level of the hepcidin promoter, where the proximal BMP-responsive element and the STAT3-binding element are in close proximity, because mutations in the proximal BMP-responsive element severely impair hepcidin induction by IL-6 (Fig 3).[52]

More recently, a second mechanism has been characterized by which these proinflammatory cytokines and bacterial lipopolysaccharide (LPS) may induce hepatic hepcidin expression. Proinflammatory cytokines and LPS activate endoplasmic reticulum (ER) stress and the unfolded protein response and increase expression and cleavage of CREBH (cyclic AMP response element-binding protein H),[79] which activates transcription of acute-phase response genes in the liver.[79] ER stress also increases hepcidin expression through CREBH binding and transactivation of the hepcidin promoter (Fig 3).[80] ER stress also has been suggested to transcriptionally regulate hepcidin expression through CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) and C/EBPα, although this has not been linked directly to inflammation.[81]

Although the role of liver-derived hepcidin in systemic iron homeostasis has been characterized best, hepcidin also is produced in monocytes/macrophages and is induced in these cells by LPS and certain bacterial pathogens through Toll-like receptors and possibly also the IL-6/STAT3 pathway.[82-86] In contrast to the liver, hepcidin expression in macrophages is not induced by iron.[82] Hepcidin production stimulated by bacterial pathogens or LPS in monocytes/macrophages decreases ferroportin mRNA expression and ferroportin protein cell-surface expression in an autocrine fashion.[82,83,86] Although the amount of hepcidin production in macrophages is much less than in the liver,[83] hepcidin induction in macrophages in the setting of infection may contribute to the host defense by acting locally to limit iron availability to invading pathogens.[82-84,86]

Molecular Mechanisms of Hepcidin Regulation: Hepcidin Inhibitors

Soluble HJV

HJV is attached to the plasma membrane by a GPI (glycosylphosphatidylinositol) anchor. In addition to its cell-associated form, soluble forms of HJV have been detected in the conditioned media of transfected cells and the blood of humans and other animals.[87-93] Mechanisms for the release of soluble HJV from the cell appear to involve cleavage by the proprotein convertase furin or the transmembrane serine protease 6 TMPRSS6 (see hepcidin regulation by iron deficiency next).[91-94] Notably, administration of exogenous soluble HJV has been shown to act as an inhibitor of BMP signaling and hepcidin expression, presumably by binding to BMP ligands and

preventing their interaction with cell-surface signaling receptors.[46,59,87] The quantity and physiologic relevance of endogenous forms of soluble HJV in vivo are still poorly understood, although some data suggest that soluble HJV may be decreased by iron loading and increased by iron deficiency and hypoxia.[87,89,90,92] It has been hypothesized that although GPI-anchored membrane HJV acts as a coreceptor to enhance BMP-SMAD signaling and hepcidin expression, generation of soluble HJV inhibits the BMP-SMAD signaling and hepcidin expression, either exclusively by removing the enhancing effects of the membrane form of HJV or with the additive effect of sequestering BMP ligands (Fig 2).[43,46,59] Interestingly, HJV is expressed not only in the liver, but also in skeletal and cardiac muscle,[33] where its function currently is unknown. It has been proposed that HJV expression in these other tissues may serve as a source of soluble HJV.[87,95]

Hepcidin Regulation by Anemia

Anemia and ESA administration are potent inhibitors of hepcidin expression, allowing greater iron availability for erythropoiesis.[27,96] The dominance of the inhibitory effect of anemia and erythropoietic drive over the stimulatory effects of iron on hepcidin regulation is evidenced in β-thalassemias. In this disease, hepcidin levels remain low because of very high erythropoietic activity, even in the face of increasing serum iron and tissue iron deposition that ultimately lead to fatal iron overload.[97,98] Although ESAs have been shown to inhibit hepcidin transcription in isolated liver cells in vitro through erythropoietin receptor signaling and inhibition of C/EBPα binding to the hepcidin promoter,[99] 2 studies suggested that in vivo, the inhibitory effects of anemia or ESAs on hepcidin expression require erythropoietic activity.[100,101] These studies showed that inhibition of erythropoiesis by chemotherapy, an ESA-blocking antibody, or irradiation prevented hepcidin suppression by either ESAs or anemia.[100,101] It has been suggested that proliferating erythrocyte precursors may secrete a substance that circulates to the liver to inhibit hepcidin expression. Recently, 2 modulators of the TGF-β/BMP superfamily signaling pathway, growth and differentiation factor 15 (GDF15) and twisted gastrulation (TWSG1), were reported to be secreted by erythroblast precursors and have a role in hepcidin suppression in thalassemia.[102,103] GDF15 is a TGF-β superfamily ligand that activates SMAD2 and SMAD3, and TWSG1 can function as a BMP agonist or antagonist.[104-107] Notably, an iron phenotype was not described in mice with GDF15 dysregulation, and sera from patients with sickle cell anemia or myelodysplastic syndrome did not have increased GDF15 levels,[102,108,109] suggesting that the role of these proteins in hepcidin suppression may be limited to anemias such as β-thalassemia with ineffective erythropoiesis.

Hepcidin Regulation by Hypoxia

Hypoxia also is a potent inhibitor of hepcidin expression, even in the absence of anemia.[27] The mechanism for this is incompletely understood, but seems to be related to the hypoxia-inducing factor (HIF) pathway, which also mediates expression of erythropoietin and many other hypoxia-induced genes.[110] HIFs are heterodimeric transcription factors consisting of an alpha regulatory subunit (HIF-1α, HIF-2α, or HIF-3α) and a ubiquitously expressed beta subunit (HIF-1β, also known as ARNT). Under normoxic iron-sufficient conditions, the HIF alpha subunit is hydroxylated by oxygen and iron-dependent 2-oxoglutarate–dependent oxygenases, ubiquitinated by von Hippel–Lindau (VHL) protein, and degraded. Under hypoxic or iron-depleted conditions, hydroxylase activity is inhibited and the HIF alpha subunit accumulates, translocates to the nucleus, heterodimerizes with ARNT, and binds to specific hypoxia-responsive promoter elements (HREs) of target genes to modulate gene transcription (reviewed in[110]). Consistent with a role for the HIF pathway in regulating hepcidin expression in vivo, mice with a liver-specific deletion of *Vhl*, the absence of which increases HIF activity, have decreased hepcidin levels, whereas a double deletion of *Arnt* in combination with *Vhl* in the liver restored normal hepcidin levels, showing the specificity of the effect of the *Vhl* deletion on HIF activity.[111] However, mice with the liver-specific deletion of *Vhl* also had increased erythropoietin levels and polycythemia,[111] suggesting that another factor related to erythropoietic drive could have contributed to hepcidin suppression. Although 1 report suggests that HIF-1α binds directly to HREs in

the hepcidin promoter, and HIF binding suppresses hepcidin gene transcription,[111] conflicting data have been reported in other studies.[112,113] Alternative pathways suggested by other studies include other HIF-1–independent 2-oxoglutarate–dependent pathways[113] or pathways involving reactive oxygen species.[112] Interestingly, furin, which cleaves HJV, and TFR1 also are encoded by HIF target genes.[92,114,115] Thus, HIF could suppress hepcidin expression indirectly by decreasing BMP-HJV-SMAD–mediated hepcidin induction and/or HFE/TFR2-mediated hepcidin induction.[92,114-116]

Hepcidin Regulation by Iron Deficiency

Iron deficiency also inhibits hepcidin expression. The HIF pathway described may be one mechanism by which this occurs because the 2-oxoglutarate–dependent dioxygenases that hydroxylate HIF alpha subunits are dependent on iron as well as oxygen.[110] In mouse studies, Hif-1α is increased in the liver by an iron-deficient diet.[111] However, deletion of *Hif1a* in the liver in 1 study had no effect,[117] and in a second study, rescued only a small percentage of the hepcidin decrease induced by an iron-deficient diet.[111] Similarly, a liver-specific deletion of *Arnt*, which inactivates all Hif-α isoforms (Hif-1α, Hif-2α, and Hif-3α), only slightly rescued the hepcidin suppression induced by an iron-deficient diet and had no effect on serum iron or red blood cell parameters.[117] Notably, Hif-2α appears to have an important role in iron homeostasis in the intestine by increasing the expression of proteins important for iron uptake by duodenal enterocytes.[117,118]

TMPRSS6 also appears to have an important role in hepcidin suppression by iron deficiency.[119-121] Mutations in *TMPRSS6* in humans and mice lead to iron-refractory iron deficiency anemia (IRIDA), a congenital form of iron deficiency anemia that is unresponsive to oral iron therapy and only partially responsive to parenteral iron treatment.[119-121] Patients and mice with mutations in *TMRPSS6* have inappropriately high hepcidin expression relative to their iron deficiency and anemia, suggesting that hepcidin excess is the mechanism causing the IRIDA, and that TMPRSS6 normally functions as a hepcidin suppressor in response to iron deficiency.[119-121] The mechanism by which TMPRSS6 inhibits hepcidin expression is incompletely understood. TMPRSS6 is expressed predominantly in the liver.[122] In vitro studies in which TMPRSS6 is overexpressed suggest that TMPRSS6 can cleave HJV and inhibit hepcidin induction by HJV, BMPs, and IL-6 (presumably because of loss of cell-surface HJV and/or the presence of soluble HJV, as described previously; Fig 2).[94,119] Whether HJV cleavage occurs at physiologic levels of TMPRSS6 expression in vivo remains to be determined. Notably, combined *Tmprss6/Hjv*-mutant mice have an iron-overload phenotype that resembles *Hjv*-null mice, consistent with a genetic interaction between TMPRSS6 and the BMP-HJV-SMAD pathway.[123]

A recent study also suggests that GDF15 levels may be increased by cellular iron deficiency in vitro, and GDF15 levels are modestly increased in human sera in the setting of iron deficiency or iron chelator administration.[124] However, whether this has a physiologic role in hepcidin suppression by iron deficiency in vivo is still unknown.

Hepcidin Assays

Hepcidin excess has been postulated to have a role in the anemia of patients with CKD/ESRD because of decreased renal clearance and induction by inflammatory stimuli, particularly in patients who are ESA resistant. However, it is only recently that hepcidin assays have been developed to start to investigate these hypotheses.

Hepcidin is synthesized in the liver as an 84–amino acid prepropeptide and processed by peptidase cleavage to a 60–amino acid propeptide (prohepcidin), followed by furin and related proprotein convertase cleavage to yield the mature carboxy terminal 25–amino acid hepcidin (hepcidin-25).[125] Hepcidin-25 is a cationic peptide that forms a hairpin loop stabilized by 4 disulfide bonds.[126,127] In addition to prohepcidin and hepcidin-25, 22 and 20 carboxy terminal amino acid forms of hepcidin are found in the circulation and/or urine, most likely because of N-terminal truncation of hepcidin-25, although the mechanism by which hepcidin-22 and hepcidin-20 are generated is still poorly understood.[19,128,129] Hepcidin-25 is the bioactive form of hepcidin, whereas the other forms of hepcidin have little or no biological activity.[130-132] Fractional excretion of hepcidin is reported to be

approximately 3%-5% because it either is not freely filtered or it is reabsorbed, similar to other small peptides that are reabsorbed and degraded in proximal tubules.[11,133] Hepcidin recently was reported to bind specifically to α_2-macroglobulin, and it has been estimated that approximately 89% of circulating hepcidin is protein bound.[134]

The development of immunochemical methods to detect mature hepcidin has been complicated by hepcidin's small size and its conservation in animal species.[133] The first described hepcidin assay was an immunodot assay to measure urinary hepcidin.[28,29] However, this assay is semiquantitative, laborious, and not suitable for serum hepcidin measurements.[11,133] A commercially available immunoassay was developed to detect serum prohepcidin, but prohepcidin levels do not correlate with biological activity, iron status, or inflammation.[135,136] Others have developed mass spectroscopic techniques to measure mature hepcidin in serum and urine. Although this technique has the potential advantage of being able to distinguish among hepcidin-25, hepcidin-22, and hepcidin-20, these assays depend on expensive equipment that is not widely available, and most are semiquantitative, although more recent refinements are improving the quantitative ability.[128,133,137-141] In the last year, immunoassays to quantitate mature serum hepcidin have been developed, as well as an assay based on competition against hepcidin-25 labeled with iodine 125 binding to a peptide identical to the ferroportin hepcidin-binding site.[11,12,142-144] A recently published round robin study comparing these various mass spectrometry and immunochemical-based methods to quantify urinary and plasma mature hepcidin has shown that absolute hepcidin concentrations differ widely between methods, but Spearman correlations between individual sample mean hepcidin values obtained using most methods generally were high for 7 of 8 methods tested.[145] Analytical variance generally is low and similar for all methods, indicating the potential suitability of all methods to distinguish hepcidin levels of different samples.[145] It was hypothesized that differences in absolute hepcidin levels between methods may be caused by the use of different calibrators, hepcidin aggregation, hepcidin protein binding, and/or the existence of hepcidin-25, hepcidin-22, and hepcidin-20 isoforms that may be detected to some extent by immunochemical methods, depending on the antibody used.[145] This report called for efforts to further harmonize the various hepcidin assays.[145]

Hepcidin Excess and the Anemia of CKD

Using the mentioned immunoassays to measure mature hepcidin, several groups now have confirmed that hepcidin levels are increased in patients with CKD and ESRD[11-13] and inversely correlate with GFR,[12,13] suggesting that decreased renal clearance contributes to the hepcidin increase in this patient population. Hepcidin levels are decreased by dialysis, but return to predialysis levels before the next dialysis session.[146] Interestingly, 1 study using a mass spectrometry–based technique to distinguish among hepcidin-25, hepcidin-20, and hepcidin-22 suggested that although hepcidin-25 was increased in patients with ESRD and total hepcidin (encompassing hepcidin-25, hepcidin-20, and hepcidin-22) correlated inversely with estimated GFR in patients with CKD not requiring dialysis, no significant correlation was found between hepcidin-25 and estimated GFR in patients with CKD not requiring dialysis.[147] These results need to be confirmed in larger studies.

Hepcidin levels correlate with markers of iron burden in the CKD population, as in other patient populations, particularly serum ferritin.[11-13,146-148] In multivariate analyses, ferritin level is the strongest predictor of serum hepcidin level.[12,13,147] Hepcidin levels also are increased by iron administration in the CKD population, as in healthy controls.[11,12] Interestingly, although hepcidin is stimulated by inflammation and has been increased in patients with inflammation, defined as high C-reactive protein (CRP) levels,[11] initial studies of patients with CKD have not shown a consistent or robust correlation between levels of hepcidin inflammatory markers, such as CRP, erythrocyte sedimentation rate (ESR), or IL-6.[12,13,146,148] One explanation for these findings may be related to the patient populations selected for these studies, some of which excluded patients with active illness or infection.

Hepcidin levels are associated with anemia in dialysis patients, consistent with a role for hepcidin excess in the anemia of CKD; however, there is an inverse correlation between ESA dose and hepcidin level, arguing against a diagnostic role

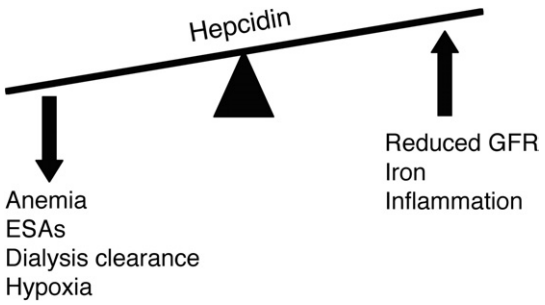

Figure 4. Hepcidin levels in patients with chronic kidney disease and end-stage renal disease. Hepcidin levels are increased in patients with chronic kidney disease and end-stage renal disease and reflect the balance of stimulatory factors: decreased renal clearance (GFR, glomerular filtration rate), inflammation, and iron administration; and inhibitory factors: anemia, erythropoiesis-stimulating agent (ESA) administration, clearance by dialysis, and hypoxia.

for hepcidin as a predictor of ESA resistance.[12,148] The likely explanation for this finding is that ESA administration is an inhibitor of hepcidin expression in the CKD population, as in the general population.[12,146] It has been proposed that the initial decrease in hepcidin levels after starting ESA therapy might be a better indicator of long-term responsiveness to ESAs.[149]

Given the limited number and size of studies to date, the unresolved issues surrounding hepcidin assays themselves, and the numerous factors that can modulate hepcidin levels in the CKD/ESRD population, including iron administration, ESA administration, body iron burden, inflammation, renal clearance, and dialysis (Fig 4), more studies are needed to determine whether hepcidin will have diagnostic utility as a measure of iron status, inflammatory status, and/or ESA responsiveness or resistance.

Hepcidin-Lowering Agents for the Treatment of Anemia of Inflammation

The notion that hepcidin excess may contribute to the dysregulation of iron homeostasis and anemia in patients with CKD raises the possibility that hepcidin-lowering agents may be an effective strategy for ameliorating anemia in this patient population. Although ESAs target the relative erythropoietin deficiency and supplemental iron addresses the true iron deficiency found in this patient population, hepcidin-lowering agents might be able to complement these strategies by improving iron availability from the diet and existing body stores. Some initial small-animal studies have shown that BMP inhibitors, including soluble HJV and a small-molecule BMP inhibitor, can function as hepcidin-lowering agents to mobilize splenic iron stores and increase serum iron levels in vivo.[46,55] It remains to be proved whether BMP inhibitors and/or other hepcidin-lowering strategies will be effective for treating the anemia of CKD. Furthermore, the potential side effects of these strategies are unknown.

SUMMARY

Hepcidin excess increasingly is being identified as a contributing factor to anemia in patients with CKD/ESRD by impairing iron absorption from the diet and iron mobilization from body stores. A multitude of factors can modulate hepcidin levels in the CKD/ESRD population: iron administration, ESA administration, body iron burden, inflammation, renal clearance, and dialysis. More studies are needed to better understand the diagnostic utility of hepcidin in patients with CKD/ESRD as a measure of iron status, inflammatory status, and/or ESA resistance. More studies are needed to investigate whether hepcidin-lowering agents may have a role in treating anemia in patients with CKD/ESRD.

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