Novel tools for the evaluation of iron metabolism

Léon Kautz and Elizabeta Nemeth

Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA, USA

E-mail: enemeth@mednet.ucla.edu *doi:10.3324/haematol.2010.033993*

(Related Original Article on page 2031)

Iron disorders, including various anemias and iron overload conditions, are common and affect millions of people worldwide. Advances in iron biology over the last decade have prompted a fundamental revision of our understanding of iron homeostasis, and opened the door to a new era of diagnostic and therapeutic developments for iron disorders.

During normal homeostasis, iron is conserved. On a daily basis, less than 0.1% of total body iron is lost by shedding of various cells and about the same amount is absorbed from the diet. The bone marrow is the main consumer of circulating iron, which is used for the synthesis of hemoglobin for new red blood cells. Erythrocyte hemoglobin contains most (about two-thirds) of the total body iron. As red blood cells complete their life-span of about 120 days, their iron is recycled by specialized macrophages, and made available for the synthesis of new red blood cells. Restricting iron availability to the bone marrow, as occurs in iron deficiency or in various inflammatory disorders, limits hemoglobin synthesis and results in the development of anemia. At the other extreme, excessive iron absorption or iron loading by multiple blood transfusions leads to iron overload (primarily of the liver, heart and endocrine glands) which can cause organ toxicity due to iron's ability to catalyze generation of highly reactive oxygen species.

Iron absorption and recycling, and thus the availability of iron for hemoglobin synthesis are regulated by the peptide hormone hepcidin.[1] Hepcidin acts by controlling the number of iron channels through which cellular iron is delivered into plasma. In turn, hepcidin concentration is regulated by a variety of influences. Iron loading increases hepcidin production (to block further iron absorption and maintain homeostasis), and so does inflammation (to decrease extracellular iron concentration and its accessibility to microorganisms). On the other hand, iron deficiency and increased erythropoietic activity suppress hepcidin (to increase iron absorption and the release of recycled iron). As would be expected, dysregulation of hepcidin production, whether genetic or acquired, causes iron disorders: a lack of hepcidin leads to iron overload, whereas its excess leads to iron restriction and anemia. Considering hepcidin responsiveness to iron, erythropoiesis and inflammation, as well as its causative role in the pathogenesis of different iron disorders, hepcidin assays may become a useful clinical test for the diagnosis or the monitoring of treatment in various diseases (Table 1).

In this issue of the journal, Brasse-Lagnel *et al.* present a new tool in the arsenal of tests for the evaluation of iron metabolism.[2] They describe the development and validation of the first enzyme-linked immunosorbent assay (ELISA) for soluble hemojuvelin in human serum.

Hemojuvelin is an important regulator of hepcidin production. Mutations in hemojuvelin lead to severe hepcidin deficiency and juvenile hemochromatosis, a particularly severe form of genetic iron overload.[3] Hemojuvelin, which is a glycophosphatidylinositol-linked membrane protein, is a member of the repulsive guidance molecule family. Like other members of this family, it is a co-receptor for bone morphogenetic proteins (BMP) and potentiates BMP pathway signaling.[4] The BMP signaling cascade is initiated by BMP binding to a complex composed of two type I and two type II BMP receptors, and hemojuvelin appears to increase the utilization of specific combinations of type I and type II receptors.[5] Upon ligand binding, BMP-type II receptor phosphorylates BMP-type I receptor, and the activated complex phosphorylates receptor-activated Smad proteins. R-Smad proteins form heteromeric complexes with the common mediator Smad4 and translocate to the nucleus to activate the transcription of target genes (e.g. hepcidin) (Figure 1).

Hemojuvelin is highly expressed in skeletal muscle and the liver. The biological role of this protein in the muscle is unclear, but in the liver, hemojuvelin appears to be an essential component of the mechanism by which iron regulates hepcidin expression.[6-8] However, the specifics of hemojuvelin involvement in the sensing of extracellular

Table 1. Potential applications of a hepcidin assay.

Clinical applications	Pharmacological development
• Differential diagnosis of anemias (AI, IDA, mixed AI/IDA, IRIDA, bone marrow destruction)	• Erythropoiesis-stimulating agents
• Screening assay for iron deficiency	• Prolyl hydroxylase inhibitors
• Monitoring of anemia therapy (for AI, IDA, CKD, AoC)	• Iron chelators
• Clinical monitoring of inflammatory states	• Iron supplementation therapy
• Selection of patients for specific therapy	• Anti-inflammatory therapies
• Screening assay for hereditary hemochromatosis	• Hepcidin agonists
• Stratification of hereditary hemochromatosis	• Hepcidin antagonists
• Monitoring of treatment in iron overload states	

AI: anemia of inflammation, IDA: iron deficiency anemia, IRIDA: iron-refractory iron deficiency anemia, CKD: chronic kidney disease, AoC: anemia of cancer.

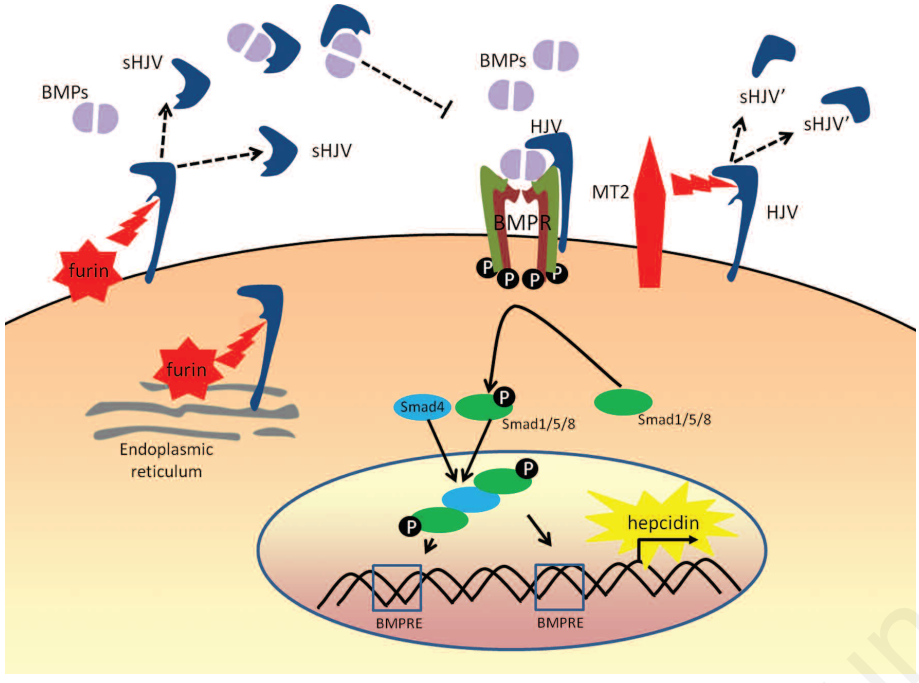

Figure 1. Generation of soluble hemojuvelin (sHJV) forms and their proposed interactions. Cell surface hemojuvelin (HJV) is a BMP co-receptor. Although the precise binding/interaction configuration of the BMP receptor (BMPR), BMP and HJV is unknown, cell surface HJV binds BMP molecules and stabilizes the BMP ligand-BMP receptor interaction. Once activated, the heteromeric complex activates a subset of Smad proteins that translocate to the nucleus to stimulate hepcidin gene transcription. Under the influence of different proteolytic activities, soluble forms of hemojuvelin are generated (sHJV, sHJV'). Furin-like proprotein convertase cleaves HJV to release a form of sHJV that can sequester BMP, thereby inhibiting the signal transduction by decreasing the effective concentrations of BMP and membrane HJV. Matriptase 2 (MT2) binds and cleaves cell surface HJV, decreasing the association of cell surface HJV with the BMPR complex, and thereby decreasing signaling. The small sHJV isoform generated by MT2, sHJV', may not sequester BMP. However, the physiological role of these soluble products remains uncertain.

and intracellular iron and subsequent signal transduction are still being delineated.

In addition to the glycophosphatidylinositol-linked membrane form which stimulates hepcidin production, hemojuvelin also exists in a soluble form, which inhibits hepcidin production.[9] There are at least two different mechanisms by which soluble hemojuvelin is generated and, depending on the mechanism, soluble hemojuvelin may have an indirect or direct inhibitory effect on hepcidin expression.[10] The indirect effect may be due to the removal of the BMP receptor-interacting membrane hemojuvelin and consequent decrease in BMP signaling, while the direct effect may be due to soluble hemojuvelin binding various BMP and sequestering them from BMP receptors and membrane hemojuvelin. At least two proteases can cleave membrane hemojuvelin, furin and matriptase 2, and each releases a different isoform of soluble hemojuvelin.[10]

The ubiquitously expressed furin-like proprotein convertase acts in the endoplasmic reticulum and/or cell surface and cleaves hemojuvelin at the conserved cleavage site (residues 332–335 in humans) to release an approximately 42 kDa fragment.[11,12] This fragment actively suppresses hepcidin expression when added to primary hepatocytes *in vitro*,[9] or when injected into mice as an Fc-conjugate.[13] Furin-dependent cleavage of hemojuvelin was shown to be increased by hypoxia or iron deficiency *in vitro*, due to hypoxia inducible factor-1α-mediated up-regulation of furin.[12] This was proposed as a possible mechanism to suppress hepcidin expression during muscle differentiation or physical activity, to increase iron supply for myoglobin synthesis.[12] However, in mice *in vivo*, there was no effect of repeated bleeding on muscle and liver furin mRNA content.[14] Thus, it remains to be determined whether furin regulation by iron and oxygen, and consequent generation of soluble hemojuvelin has a relevant physiological or pathological role in iron homeostasis.

Matriptase-2, also known as TMPRSS6, is a transmembrane serine protease produced by the liver, and a key repressor of hepcidin expression. Loss of matriptase-2 function in either humans or mice leads to inappropriately elevated hepcidin, resulting in iron-refractory iron deficiency anemia.[15] Matriptase-2 binds and cleaves cell surface hemojuvelin,[16] and Maxson *et al.* recently showed that the site of cleavage is the hemojuvelin residue R288, generating one major soluble form of hemojuvelin (~36 kDa).[10] Importantly, unlike the larger furin-cleaved form, soluble hemojuvelin generated by matriptase-2 cleavage has a decreased ability to bind BMP6 and does not suppress BMP6-induced hepcidin expression.[10] Thus matriptase-2 seems to function as the terminator of the cell surface hemojuvelin signal. Neogenin has also been reported to alter soluble hemojuvelin generation, but its exact role remains to be clarified.[17,18]

The new soluble hemojuvelin assay reported by Brasse-Lagnel *et al.*[2] is a competitive ELISA based on an anti-hemojuvelin polyclonal antibody. The antibody was raised against the recombinant protein comprised of hemojuvelin residues 226-400. Thus, the assay may detect both the furin- and the matriptase-2-cleaved forms of soluble hemojuvelin. Considering that patients with iron-refractory iron deficiency anemia caused by the loss-of-function TMPRSS6 mutations had lower soluble hemojuvelin levels in this assay, the matriptase-2-cleaved form of soluble hemojuvelin may account for a significant portion of the total soluble hemojuvelin in serum. It remains to be determined whether a differential measurement of the two forms may be more informative than measuring the total amount of soluble hemojuvelin.

What are the potential uses of the soluble hemojuvelin assay? This is difficult to answer given that very little is known about soluble hemojuvelin biology. Based on the studies in hemojuvelin knock-out mice, hemojuvelin plays an important role in hepcidin regulation by iron,[6,8] and not in hepcidin regulation by erythroid activity,[19] but the function of *soluble* hemojuvelin *in vivo* is unknown. Hemojuvelin mRNA does not change with iron loading or phlebotomy,[20] but *in vitro* treatment of cells with increasing concentrations of iron leads to decreased release of soluble hemojuvelin[9] suggesting that soluble hemojuvelin may reflect the body iron load. Hemojuvelin does not appear to be involved in the increase of hepcidin occurring in inflammation, as hemojuvelin knock-out mice preserve hepcidin mRNA inducibility in response to inflammation.[6] Furthermore, hemojuvelin mRNA in the liver (but not in skeletal muscle) is markedly suppressed by inflammatory cytokines or lipopolysaccharide. However, Brasse-Lagnel *et al.* surprisingly found that soluble hemojuvelin was increased in the serum of patients with anemia of chronic disease (patients in intensive care unit, C-reactive protein >50 mg/L, hemoglobin <10 g/dL), and soluble hemojuvelin concentrations correlated positively with C-reactive protein levels. Liver hemojuvelin would be expected to be decreased in these patients because of the potent effect of inflammation on hemojuvelin mRNA, but their soluble hemojuvelin could have been derived from the muscle. It is possible that elevated soluble hemojuvelin levels in inflammation are a consequence of decreased serum iron concentrations, given that *in vitro* treatment of cells with lower iron concentrations resulted in greater release of soluble hemojuvelin.[9] Indeed, the concentration of soluble hemojuvelin in patients with anemia of chronic disease was inversely correlated with serum iron concentration. Do elevated soluble hemojuvelin concentrations in patients with anemia of chronic disease have a biological function? If the soluble hemojuvelin originated from the muscle where matriptase-2 expression is very low, it would likely be the furin-cleaved form which would be expected to suppress hepcidin production, opposing the effect of inflammation. However, in the subset of patients in whom serum hepcidin was measured (n=28 out of 36), the concentration of soluble hemojuvelin did not correlate with the hepcidin concentration. Although this could mean that soluble hemojuvelin has no effect on hepcidin production, it may also result from inflammation outweighing any effect of soluble hemojuvelin on hepcidin expression.

Clearly, not enough is known about soluble hemojuvelin, and questions abound. Measurements of soluble hemojuvelin should have a major impact on our understanding of soluble hemojuvelin biology, including how the production of different proteolytic forms is regulated during different physiological states or diseases, which tissues generate the soluble hemojuvelin isoforms, and what is the biological role of endogenous soluble hemojuvelin *in vivo*. The development of an assay for soluble hemojuvelin is an essential first step in these explorations.

Léon Kautz, PhD, is a postdoctoral fellow at the Center for Iron Disorders, University of California, Los Angeles. Elizabeta Nemeth, PhD, is an Associate Professor of Medicine and co-director of the Center for Iron Disorders, University of California, Los Angeles.

Financial and other disclosures provided by the author using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are available with the full text of this paper at www.haematologica.org.

References

1. Ganz T, Nemeth E. Hepcidin and Disorders of iron metabolism. Annu Rev Med. 2010 Jan 27. [Epub ahead of print]
2. Brasse-Lagnel CG, Poli M, Lesueur C et al. Immunoassay for human serum hemojuvelin. Haematologica. 2010;95(12):2031-7.
3. Papanikolaou G, Samuels ME, Ludwig EH, MacDonald ML, Franchini PL, Dubé MP, et al. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet. 2004; 36(1):77-82.
4. Babitt JL, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, et al. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. Nat Genet. 2006;38(5):531-9.
5. Xia Y, Babitt JL, Sidis Y, Chung RT, Lin HY. Hemojuvelin regulates hepcidin expression via a selective subset of BMP ligands and receptors independently of neogenin. Blood. 2008;111(10):5195-204.
6. Niederkofler V, Salie R, Arber S. Hemojuvelin is essential for dietary iron sensing, and its mutation leads to severe iron overload. J Clin Invest. 2005;115(8):2180-6.
7. Lin L, Valore EV, Nemeth E, Goodnough JB, Gabayan V, Ganz T. Iron-transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4. Blood. 2007;110(6):2182-9.
8. Ramos E, Kautz L, Rodriguez R, et al. Evidence for distinct pathways of hepcidin regulation by extracellular and intracellular iron. Hepatology. In press.
9. Lin L, Goldberg YP, Ganz T. Competitive regulation of hepcidin mRNA by soluble and cell-associated hemojuvelin. Blood. 2005;106(8):2884-9.
10. Maxson J, Chen J, Enns CA, Zhang AS. Matriptase-2 and proprotein convertase cleaved forms of hemojuvelin have different roles in the down-regulation of hepcidin expression. J Biol Chem. 2010 Oct 11. [Epub ahead of print]
11. Lin L, Nemeth E, Goodnough JB, Thapa DR, Gabayan V, Ganz T. Soluble hemojuvelin is released by proprotein convertase-mediated cleavage at a conserved polybasic RNRR site. Blood Cells Mol Dis. 2008;40(1):122-31.
12. Silvestri L, Pagani A, Camaschella C. Furin mediated release of soluble hemojuvelin: a new link between hypoxia and iron homeostasis. Blood. 2008;111(2):924-31.
13. Babitt JL, Huang FW, Xia Y, Sidis Y, Andrews NC, Lin HY. Modulation of bone morphogenetic protein signaling in vivo regulates systemic iron balance. J Clin Invest. 2007;117(7):1933-9.
14. Krijt J, Fujikura Y, Sefc L, Vokurka M, Hlobenová T, Necas E. Hepcidin downregulation by repeated bleeding is not mediated by soluble hemojuvelin. Physiol Res. 2010;59(1):53-9.
15. Lee P. Role of matriptase-2 (TMPRSS6) in iron metabolism. Acta Haematol. 2009;122(2-3):87-96.
16. Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C. The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. Cell Metab. 2008;8(6):502-11.
17. Lee DH, Zhou LJ, Zhou Z, Xie JX, Jung JU, Liu Y, et al. Neogenin inhibits HJV secretion and regulates BMP induced hepcidin expression and iron homeostasis. Blood. 2010;115(15):3136-45.
18. Zhang AS, Yang F, Meyer K, Hernandez C, Chapman-Arvedson T, Bjorkman PJ, Enns CA. Neogenin-mediated hemojuvelin shedding occurs after hemojuvelin traffics to the plasma membrane. J Biol Chem. 2008;283(25):17494-502.
19. Krijt J, Niederkofler V, Salie R, Sefc L, Pelichovská T, Vokurka M, Necas E. Effect of phlebotomy on hepcidin expression in hemojuvelin-mutant mice. Blood Cells Mol Dis. 2007;39(1):92-5.
20. Bondi A, Valentino P, Daraio F, Porporato P, Gramaglia E, Carturan S, et al. Hepatic expression of hemochromatosis genes in two mouse strains after phlebotomy and iron overload. Haematologica. 2005;90(9):1161-7.