HEPCIDIN AS THE NEW PLAYER IN INFLAMMATORY "FUNCTIONAL IRON DEFICIENCY ANEMIA" – KEY ROLES, (DE) REGULATION AND THERAPEUTIC IMPLICATIONS

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ABSTRACT

Anemia of chronic kidney disease is responsible for a significant increase in morbidity and mortality among patients, as well for a poor life quality. Impairment of iron homeostasis and inflammation underlie the development of the anemia, and hepcidin is the pivotal player in this process. Ferroportin is an iron exporter present in the surface of cells that permits the absorption of iron through enterocytes and the mobilization of iron from macrophages and hepatocytes. When ferroportin is degraded by hepcidin, iron absorption is reduced as well as the mobilizion of iron for erythropoiesis, favoring the development of anemia. Many factors influence hepcidin expression and activity, including iron status, inflammatory state, tissue oxygen tension and the erythropoietic activity. The molecular mechanisms behind the regulation of hepcidin synthesis and activity became clearer during the last years due to a deep experimental and clinical investigation on this area of knowledge. In this review, we summarize the role of hepcidin in iron homeostasis, in inflammation and in the physiopathology of functional iron deficiency anemia; the molecular mechanisms behind the regulation of its expression, as well as the possibility of using hepcidin as a therapeutic target or as a diagnosis tool in functional iron deficiency anemia.

Keywords: Hepcidin; functional iron deficiency anemia; inflammation.

INTRODUCTION

The anemia that accompanies chronic renal disease (CKD) is associated with precocious mortality and morbidity rates, as well as with a decrease in life quality of patients[1-3]. The existing treatments include hemodialysis, together with the administration of iron and erythropoiesis-stimulating agents (ESA)[4]. However, about 25% of the patients require

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high doses of ESA, with potential adverse effects[5,6]. Furthermore, approximately 10% of patients develop resistance to therapy[7], complicating the treatment and the prognosis.

It is established that inflammation plays a central role in this type of anemia, as it limits erythropoiesis[8], suppresses the production of erythropoietin (EPO)[9] and, mainly, changes iron homeostasis. Hepcidin, a protein produced in hepatocytes and stimulated by inflammation, especially interleukin-6 (IL-6), plays a central role in this process since it promotes retention of iron in tissues[10]. Hepcidin degrades ferroportin, which leads to the accumulation of iron in macrophages and hepatocytes, and to a reduced flow from duodenal enterocytes to the circulation[11-13]. Thus, although there are adequate deposits of iron (thereafter normal or elevated ferritin), the circulating and available amount for hemoglobin synthesis is reduced (low serum iron, transferrin saturation and reticulocyte counts). This condition is defined as "functional iron deficiency anemia". In recent years, some authors have suggested that changes in the expression and/or activity of hepcidin may be related to resistance to ESA treatment, a process which appears to be mainly caused by disturbances in iron homeostasis and by a pro-inflammatory state[14,15]. In addition, hepcidin has been increasingly viewed as a putative therapeutic target and/or diagnosis tool in this condition of functional iron deficiency anemia.

ROLE OF HEPCIDIN IN IRON HOMEOSTASIS

Iron, an essential element for life, is involved in the transport of oxygen, as a component of heme (in hemoglobin and myoglobin) and is present in numerous enzymes essential for cellular respiration (e.g. redox mechanisms)[16], survival and growth[4]. In fact, iron is necessary for a normal hemoglobin synthesis and erythropoiesis. However, free iron is toxic[17], in a way that organisms had to develop intricate mechanisms to transport iron attached to molecules, deliver iron where it is strictly needed and store it in proteins, when in excess. There is a low iron bioavailability from human diet; iron released from senescent erythrocytes is recycled and losses are reduced to the minimum (they represent <0.1% of the 3-4 g of total iron and have to be replaced by diet)[16]. There isn't a proper pathway to excrete iron, even in the presence of high iron storages, which may lead to iron overload.

Iron is absorbed by enterocytes (approximately 1 to 2 mg per day[18]) of the duodenum and exported[19] by ferroportin to the circulation, where it binds to transferrin, that transports most of the iron to the bone narrow, for erythropoiesis. Red cell hemoglobin contains most of the iron of our body (2.5 g of iron). Reticuloendothelial macrophages from spleen phagocytize senescent erythrocytes, degrade hemoglobin and recycle iron[16]. The excess is stored as ferritin and hemosiderin (responsible for one-third of the body's iron stores) in liver and reticuloendothelial macrophages. Every time iron is needed, it is exported from iron storages through ferroportin channels into circulation[10,13].

Hepcidin is an acute phase protein that regulates iron absorption and its distribution[10]. It is produced mainly in the liver and is encoded as a pre-

propeptide, cleaved to a propeptide of 60 amino acids, and it's only secreted in its mature form, a 25 amino acid protein (convertases, like furin, PACE4, PC5/6 and PC7/LPC process hepcidin *in vitro??*)[20]. Its peptide resembles a bent hairpin held together by four disulfide bonds[20]. Its production occurs in response to a number of stimuli, like inflammation and high iron blood levels. It is excreted by the kidneys, although only 5% of the hepcidin from plasma appears intact in urine[21]. Probably, it is not freely filtered or it is reabsorbed and degraded by proximal tubular cells.

Hepcidin binds to ferroportin and degrades it[22]. Ferroportin is a membrane iron export protein present in cells that export iron, such as, duodenal enterocytes, splenic and hepatic macrophages and hepatocytes[23], and has a fundamental role in the iron metabolism. Animal models without ferroportin expression die in the embryonic stage because they can't mobilize iron[23]. Patients with thiol residue Cys326 mutations in ferrportin (probably the binding local to hepcidin) have a form of early-onset iron overload disorder[24]. Nevertheless, it seems that hepcidin is not the only ferroportin regulator, as cellular iron can, independently, regulate ferroportin expression[25].

When attached to hepcidin, the proper degradation mechanism involves ferroportin conformational changes and endocytosis of the ferroportin-hepcidin complex, which will be degraded in the lysosomes (this is another way, apart from the renal one, to clear hepcidin)[16,22]. By doing so, hepcidin inhibits iron absorption from enterocytes and iron deliver from its reserves (e.g. liver, reticuloendothelial macrophages) to erythroid cells[11,13,22]. In fact, an injection of hepcidin in mice caused a prolonged (more than 48 hours) hypoferremia, although hepcidin was cleared in a few hours by kidneys[26]. This may be due to the fact that organism takes a longer time to resynthetize degraded ferroportin[16]. Thus, the clinical effects will be a normocytic normochromic anemia, low serum iron, low reticulocyte count, low transferrin saturation and, paradoxically, higher levels of ferritin (in contrast to iron deficiency anemia where it's low)[27,28].

This type of anemia (with high levels of hepcidin) occurs in many situations, such as in chronic infections, cancer, rheumatoid arthritis and chronic kidney disease, and is called "anemia of inflammation" or "functional iron deficiency anemia"[29]. It's hypothesized that our body developed this iron sequester mechanism to protect us against invading pathogens, many of which require iron to their growth[30,31]. In fact, patients with hereditary hemochromatosis, a disease where hepcidin levels are low, have susceptibility to infections by unusual microorganisms, like *Vibrio, Yersinia* and *Listeria*[20].

Moreover, experiments in mice with hepcidin overexpression showed that they had the classical features of functional iron deficiency anemia[32]. A report from Weinstein *et al*. (2002) showed that an hepatic adenoma resection, secreting high hepcidin levels, in a patient with a severe anemia, resolved the patient's condition[33]. In contrast, patients with hepcidin deficiency, due to hereditary or acquired causes, have a great propensity to develop iron overload[16].

Many factors can influence hepcidin production, like iron status, inflam-

mation, hypoxia, erythropoietic activity, EPO levels and anemia[4,16,18]. Iron affects hepcidin production in a feedback loop. When in excess, it can enhance its production and, when in deficiency, it can suppress it. Patients with various types of hemochromatosis have low levels of hepcidin[18]. Inflammation is a great promoter of hepcidin production. It was already tested in humans that injection of lipopolysaccharide, which increases IL-6 levels, leads to an elevation in hepcidin levels, followed by the hypoferremia[34]. Moreover, in monkeys with collagen-induced arthritis, anti-IL-6 receptor antibodies caused a diminution in hepcidin and C-reactive protein (CRP) levels in a week, with improvement of anemia parameters in 4 weeks[35]. Anemia with preserved erythropoietin production and enhanced erythropoietic activity are potent suppressors of hepcidin production, through the formation of hepcidin inhibitor proteins by erythroid precursors[36,37]. Indeed, increased erythrocyte production requires higher iron consumption and iron availability. These responses are evident in ineffective erythropoiesis, where there is an erythroid hyperplasia, although the reticulocyte count is low because of apoptosis in later stages of erythropoiesis. A perfect example is β-thalassemia, where the levels of hepcidin are very low, even in the presence of high iron serum levels and iron overload[38,39]. Hypoxia also suppresses hepcidin expression[14,15,18].

REGULATION OF HEPCIDIN EXPRESSION

Great advances in understanding the molecular mechanisms of hepcidin have been made by studying hereditary hemochromatosis[40]. These patients have mutations in hemochromatosis protein gene (HFE), in transferrin receptor 2 gene (TFR2) or in hemojuvelin gene (HFE2) and present low hepcidin levels, high iron blood and ferritin levels[4,18]. Very rare mutations in hepcidin itself cause a severe early-onset form of hemochromatosis[41].

Hepcidin is encoded by the HAMP gene[18] and it is known that its transcription is enhanced by iron "sufficiency" and inflammation and suppressed by hypoxia, anemia and iron deficiency.

Iron "sufficiency": With sufficient blood iron, transferrin-bound iron binds to transferrin receptor 1 (TfR1), displacing hemochromatosis protein (HFE), which then binds to transferrin receptor 2 (TfR2)[20]. HFE and TfR2 induce hepcidin expression through the morphogenic protein receptor complex BPM/SMAD signaling pathway[42]. The morphogenic protein receptor complex consists of BPM-6 (it's expression seems to be regulated by iron[43]), BMP receptor (BMP type I and type II serine threonine kinase receptors[4]) and hemojuvelin (HJV)[42], which, all together, activate the SMAD signaling cascade. HJV enhances the SMAD signaling and, thus, stimulates hepcidin expression[42]. This pathway leads to sequential protein activation that translocate to the the nucleus where hepcidin expression is induced through BMP responsive elements (BMP-RE) located on the promoter region of hepcidin gene[4,18]. Deletion in any of the genes that express BPM/SMAD signaling pathway molecules result in hepcidin deficiency[4,18].

Inflammation: Inflammation also plays a central role in hepcidin gene

expression. The proinflammatory cytokine IL-6 activates the JAK/STAT3 signaling pathway (binding to IL-6 receptor) and promotes hepcidin expression through STAT3 responsive element (STAT3- RE) on the promoter region of hepcidin gene[44-46]. Other proinflammatory cytokines, such as interleukin-1 (IL-1) may play a similar role[47], as shown by the increased expression of hepcidin mRNA independently of IL-6 in mouse hepatocytes and in IL-6 knockout mice with chronic inflammation[20]. Zhang *et al.* (2006) and Vecchi *et al.* (2009) reported another possible pathway to induce hepcidin expression, in inflammation. Proinflammatory cytokines and bacterial lipopolysaccharide (LPS) are thought to cause endoplasmic reticulum (ER) stress and, thus, activate CREBH (cyclic AMP response element-binding protein H) which, in turn, activates numerous acute phase genes and induces hepcidin production, by binding to the promoter gene[48,49]. The available data suggests there's an interaction between JAK/STAT3 and BPM/SMAD signaling pathways, as they cooperate to promote hepcidin expression. Inhibition or abolishment of BMP/SMAD pathway resulted in blunted responses to hepcidin transcription by the IL-6 pathway[44].

Iron deficiency: In contrast, iron deficiency, is linked to low hepcidin levels because it seems to activate TMPRSS6 or matriptase-2, a hepcidin suppressor[50]. TMPRSS6 is a liver transmembrane serine protease that cleaves membrane-bound HJV into soluble HJV. This may result from inhibition of hepcidin induction by HJV in the BMP complex or by the action of soluble HJV that, presumably, binds competitively to the BMP receptor complex, inhibiting the signaling pathway and impairing hepcidin expression[50-52].

Anemia with preserved EPO production: hepcidin production may be also inhibited in the presence of anemia with preservation of EPO production[36,37]. The proteins mainly involved in these mechanism are growth differentiation factor 15 (GDF15) and twisted gastrulation protein (TWSG1), produced by erythroid precursors[36,37]. The intact erythropoiesis activity (with normal EPO levels) is essential for hepcidin suppression, as shown by some *in vivo* experiments, in which cytotoxic agents or irradiation suppressed erythropoiesis, leading to normal hepcidin levels, even in the presence of anemia[53]. GDF15 interferes with the BPM/SMAD signaling pathway, by an unknown mechanism, thus inhibiting hepcidin expression. Curiously, GDF15 knockout mice didn't have iron homeostasis impairment, suggesting that its action may be limited to anemias (like β-thalassemia) with ineffective erythropoiesis, where there is GDF15 overproduction[20,36]. TWSG1, *per se*, is thought to interfere with BMP protein, disrupting BPM/SMAD signaling pathway[18,20].

Hypoxia: Hypoxia is a potent suppressor of hepcidin. The complete physiological regulation is still incomplete but it seems to be related with the hypoxia-inducible factor (HIF) pathway[54]. In normoxic conditions, HIF pathway proteins are degraded by oxygen hydroxylase and von Hippel-Lindau protein oxygenases, but, in case of hypoxia, those enzymes are inactivated and HIF accumulates. Evidence suggests that activated HIF pathway proteins bind to hepcidin pro-

moters impairing their expression[54]. In fact, mice with deletion of von Hippel–Lindau protein (thus, simulating hypoxia conditions) had, consistently, low levels of hepcidin, but when added a new deletion to a gene encoding HIF, hepcidin levels where restored to normal (the inhibitory effect of HIF ended)[55]. Interestingly, furin (which cleaves HJV) and TFR1 are encoded by HIF target genes[56,57], suggesting that HIF could indirectly lower hepcidin through inhibition of BPM/SMAD signaling pathway[56,57]. Finally, hypoxia stimulates EPO production, which, as referred above, inhibits hepcidin, and may act as a bias factor in understanding HIF-induced hepcidin suppression.

CONCLUSIONS: HEPCIDIN AS A THERAPEUTIC TARGET OR DIAGNOSIS TOOL IN FUNCTIONAL IRON DEFICIENCY ANEMIA?

Functional iron deficiency anemia is very common among patients with inflammatory conditions (such as CKD, cancer, chronic infection and autoimmune diseases)[29] and is a predictor of poor prognosis (longer hospitalization, cognitive impairment, heart failure, and increased morbidity)[1-3]. The recent discover of new molecular pathways regulating hepcidin expression allowed the development of hepcidin targeting drugs. Treatments that attempt to lower the levels of hepcidin or its activity (inhibitors of synthesis, antagonists of their action, stabilizers of ferroportin and soluble hemojuveline[4,14]) or inhibitors of inflammation (IL-6 antagonists, statins and others[4,14,15]) are becoming very promising options to manage and/or treat the "functional iron deficiency anemia" and the resistance to ESA therapy. Although some of them have shown good potential in preliminary pre-clinical or clinical trials, more studies concerning their efficacy and adverse effects, namely in humans, need to be done.

The recent advances in this field have also brought the light on hepcidin measurement as a diagnostic and follow-up tool. Although the urgent need of standardization of the methods to measure hepcidin, most of the used assays correlated very well and have reported higher hepcidin levels in CKD patients compared with healthy ones, suggesting that, if further confirmed, could be viewed as good tools to improve diagnosis and/or to a better follow-up on the efficacy of treatments. In any case, new perspectives have been rising with the improved knowledge on this area and we may expect further developments in a short-time.

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