Clin. Lab. Haem.
2006, **28,** 75–83

REVIEW

Hepcidin: an important new regulator of iron homeostasis

A. HUGMAN *Institute of Haematology, Royal Prince Alfred Hospital, Sydney, NSW, Australia*

Summary Hepcidin is an important and recently discovered regulator of iron homeostasis. There is strong evidence in support of an important role for hepcidin dysregulation in the pathogenesis of iron overload disorders, and possibly in the aetiology of the anaemia of chronic disease. Further research is needed into the physiology of hepcidin to elucidate the relative contributions of the liver and kidney to its production and metabolism. The study of the differential roles of prohepcidin and its metabolites as well as the significance of their serum and urine levels will enhance our understanding of their role in iron metabolism.

Keywords Hepcidin, iron, iron metabolism, anaemia, iron overload

Introduction

Our understanding of the molecular control of iron metabolism has increased dramatically over the past 5 years due to the discovery of the iron regulatory hormone hepcidin. Park *et al.* (2001) isolated two small cysteine-rich peptides from human urine, which were encoded by hepatic mRNA. The 20 and 25 amino acid peptides were named hepcidin due to their hepatic origin and because they were found to be active against bacteria and fungi. The major source of hepcidin expression is the liver (Park *et al.*, 2001; Pigeon *et al.*, 2001); however, hepcidin expression has also been documented in the human, mouse and rat kidney (Kulaksiz *et al.*, 2004). The role of hepcidin in iron metabolism was first suggested by Pigeon *et al.* (2001), who in a search for iron upregulated genes, discovered that murine hepcidin mRNA expression was increased by iron overload and decreased by iron depletion.

Genetic defects leading to hepcidin deficiency cause iron overload similar to haemochromatosis in USF-2 knockout (KO) mice (Nicolas *et al.*, 2001). Inappropriately low levels of hepcidin relative to body iron stores are associated with the abnormal iron homeostasis characteristic of hereditary haemochromatosis (HH) in HFE KO mice (Ahmad *et al.*, 2002). Conversely, overexpression of hepcidin leads to severe iron deficiency and a fatal anaemia in transgenic mice (Nicolas *et al.*, 2002a). In humans, patients with large hepatic adenomas found to overexpress hepcidin, had a severe iron refractory microcytic anaemia, which was corrected by removal of the adenoma (Weinstein *et al.*, 2002).

Substantial progress has been made recently into elucidating the mechanism of action of hepcidin. Hepcidin inhibits ferroportin (FPN), which transports iron out of cells and is strongly expressed by enterocytes and liver macrophages (McKie *et al.*, 2000). As body iron increases, hepcidin is induced, which then inhibits the efflux of iron through FPN. This blocks intestinal iron absorption and iron recycling from erythrocytes by macrophages (Nemeth *et al.*, 2004b). Hepcidin has been shown to be an acute phase reactant, increased by interleukin (IL)-6 and markedly induced by infection and inflammation (Nemeth *et al.*, 2003, 2004a; Nicolas *et al.*, 2002b; Shike *et al.*, 2002). The resulting decrease in plasma iron and sequestration by macrophages induced by hepcidin leads to iron-limited erythropoiesis and is postulated to result in anaemia of chronic disease (ACD). This may reflect an evolutionary defence mechanism against invading organisms and tumour cells for which iron is an essential nutrient needed for proliferation (Weinberg, 1999).

The evidence in humans linking hepcidin with inflammation is plentiful (Nicolas *et al.*, 2002b; Shike *et al.*,

Received 20 October 2005; accepted for publication 9 January 2006
Correspondence: Dr Amanda Hugman, 75 Wairakei Road, Wamberal, NSW 2260, Australia. E-mail: adamsa@ausdoctors.net

2002; Nemeth *et al.*, 2003); however, the evidence revealing a direct correlation between hepcidin and ACD is lacking. This may reflect an imprecise definition of ACD, as well as the array of different techniques currently used to assay the peptide. There is good evidence to suggest that an assay for hepcidin would have a place in routine diagnostic pathology laboratories. The best assay methodology and sample type is still to be determined and will be a focus of this review, in addition to highlighting recent advances in the mechanism of action of hepcidin.

Hepcidin structure and localization

Hepcidin mRNA was initially detected by Northern blot analysis in fetal and adult liver. The corresponding cDNA on chromosome 19 encodes an 84 amino acid propeptide. The processed peptides detected in urine, contain two predominant forms, 20 and 25 amino acids each (Park *et al.*, 2001). The molecule is a distorted β-sheet shape with a hairpin loop. This is stabilized by disulphide pairing of cysteine residues and hydrogen bonding between the two strands, leading to amphipathic spatial separation, characteristic of antimicrobial peptides (Hunter *et al.*, 2002).

Work by Kulaksiz *et al.* (2004), on hepatic hepcidin localization indicates concentration highest in the periportal region of hepatocytes, which decreases towards the central veins and sinusoids. Given the documented action of hepcidin at distant sites, this is consistent with hormonal-type regulation. Further localization studies suggest a renal role in hepcidin regulation (Kulaksiz *et al.*, 2005). Prohepcidin expression was detected in the thick ascending limb of the cortex, and in the collecting ducts of the medulla and papilla. In renal epithelial cells, hepcidin was localized to the apical pole and was detected in urine, suggesting apical intraluminal release. From these studies the authors concluded that hepcidin is an intrinsically produced renal peptide with a probable iron regulatory role in the renal tubular system.

The molecular regulation of iron

Iron is an essential element necessary for oxygen transport and storage. It is also important in many enzymes in the body, including those involved in redox reactions required for energy production and host defence (Ganz, 2003). Total body iron in men is 3000–4000 mg (Ganz, 2003), of which more than half is contained in haemoglobin. The daily requirement for erythropoiesis is about 20–25 mg, obtained largely from recycled iron returned to the circulation by macrophages, which have phagocytosed erythrocytes (Andrews, 2005). If required, iron stores can be utilized from the liver, which stores iron as ferritin. The loss of iron from the body each day is only 1–2 mg, mainly from gut epithelial and skin cells, and small losses in the urine. Thus the major regulation of iron is through the duodenum where absorption is limited to daily needs, approximately 0.5–2 mg/day. Hepatic storage capacity is limited and eventually if there is unregulated iron absorption, free iron will be deposited in parenchymal tissues causing organ damage due to free radical formation (Andrews, 2005).

The mechanism of action of hepcidin

Dietary iron is transported through the apical plasma membrane by divalent metal transporter 1 (DMT1), stored as ferritin or released into the plasma by FPN, a basolateral surface membrane iron exporter (Viatte *et al.*, 2005). In a mouse cell line, hepcidin incubation caused internalization of the FPN molecule from the surface to intracellular vesicles, and so reduction of surface expression (Nemeth *et al.*, 2004b). Hepcidin forms part of a homeostatic negative feedback loop. An increase in serum iron will result in hepatic production of hepcidin, which interacts with FPN on the enterocyte membrane to cause internalization of the complex. Iron is trapped intracellularly until the enterocyte is shed into the intestine in 2 days. As iron continues to be utilized, serum iron will fall which will lead to suppression of hepcidin secretion, with resultant strong expression of FPN and iron transfer across the enterocyte basolateral membrane restoring serum iron levels. FPN is also expressed on macrophages, and hepatocytes (Nemeth *et al.*, 2004b; Ganz, 2005) and hepcidin-induced FPN downregulation, may explain the trapping of iron in macrophages and hepatic stores, in situations associated with increased hepcidin, such as in inflammation or infection.

A pharmacodynamic study of the effects of a radiolabelled hepcidin injection in mice, showed that a single 50 μg dose resulted in 80% drop in serum iron within 1 h which did not return to normal until 96 h (Rivera *et al.*, 2005b). This time course is consistent with the blockage of recycled iron from macrophages and previous reports of the rapid hepcidin response to IL-6 administration (Nemeth *et al.*, 2004a). The rapid disappearance of plasma iron was followed by a delayed recovery, possibly due to the slow resynthesis of membrane FPN. Tissue concentrations revealed that hepcidin preferentially accumulates in the proximal duodenum and spleen, reflecting the high expression of FPN in these areas.

Hepcidin may also mediate the regulation of other iron transport proteins. Duodenal cytochrome *b* (Dcytb) reduces ferric iron to its ferrous form at the brush border to allow uptake by DMT1 (Ganz, 2003). In hepcidin-deficient mice, enterocyte DMT1 and Dcytb at the apical membrane, and FPN at the basolateral membrane were all dramatically increased, with consequent increased iron absorption and iron overload (Viatte *et al.*, 2005). It is not known whether the increased expression of DMT1 and Dcytb is a direct effect of hepcidin deficiency or due to relative enterocyte iron deficiency secondary to FPN-mediated iron export.

Hepcidin and iron storage

Iron deficiency

Two patients with large hepatic adenomas and severe iron refractory anaemia, were found to have inappropriately high expression of hepcidin mRNA in the adenoma and on resection of the tumours, the anaemia in both cases completely reversed (Weinstein *et al.*, 2002). Transgenic mice, engineered to express hepcidin constitutively, developed severe iron deficiency anaemia (IDA) and the majority of animals died shortly after birth (Nicolas *et al.*, 2002a). In these examples, the high hepcidin levels presumably blocked intestinal iron absorption and materno-fetal iron transport respectively. In patients with IDA, hepcidin expression would be expected to be low or absent, to enable intestinal iron transfer. A recent report compared urine hepcidin levels in patients with IDA to healthy volunteers. Patients with IDA did show significantly reduced hepcidin excretion compared with normal subjects consistent with its role as a negative regulator of iron absorption (Kemna *et al.*, 2005b).

Nongenetic iron overload

In mice, hepcidin expression increases during dietary or parenteral iron overload (Pigeon *et al.*, 2001). In humans, hepatic iron overload has been shown to correlate with hepcidin mRNA expression in a study of liver samples from 36 patients undergoing hepatic surgery (Detivaud *et al.*, 2005) and liver biopsies on 25 patients with chronic hepatitis C (Aoki *et al.*, 2005). Kemna *et al.* (2005a) quantified urinary hepcidin in an attempt to separate different disorders of iron metabolism on the basis of hepcidin levels. They found that patients with transfusion-induced iron overload, had relatively increased but greatly varying hepcidin levels and that hepcidin was unable to separate patients with secondary iron overload from normal levels. Iron-induced hepatic hepcidin production is not likely a direct effect. Direct iron loading of both human (Viatte *et al.*, 2005) and murine (Pigeon *et al.*, 2001) hepatocytes, results in either no change to, or suppression of, hepcidin expression. This suggests complex indirect regulation of hepcidin production in response to body iron fluctuation.

Genetic iron overload

Hereditary haemochromatosis is due to increased gastrointestinal iron absorption resulting in iron overload and tissue damage in the liver, endocrine organs and heart (Pietrangelo, 2004). Haemochromatosis has been broadly classified into four groups: type 1 caused by mutations in the HFE gene; type 2 or juvenile haemochromatosis (JH); type 3, associated with a mutation of a low affinity transferrin receptor (TFR2); type 4 associated with the iron exporter FPN. The clinical iron overload phenotype in type 1 HH is secondary to a mutation of the HFE gene, C282Y, a point mutation causing substitution of tyrosine for cysteine at position 282 of the HFE protein (Feder *et al.*, 1996). The exact role of HFE is unknown but it may be involved in iron sensing and has been localized largely to the digestive tract and macrophages, especially Kupffer cells (Parkkila *et al.*, 2000).

There is evidence from a number of studies that abnormalities of hepcidin production and regulation are involved in the pathogenesis of HH. Hepcidin mRNA increases in response to iron loading in wild-type mice, whereas this response is absent in HFE KO mice (Ahmad *et al.*, 2002). In individuals with heterozygous and homozygous HFE C282Y mutations, the iron overload is more severe if there is a co-existent heterozygous HAMP mutation. The severity of the HAMP mutation, which disrupts the normal function of hepcidin, corresponds to the severity of the iron overload. (Merryweather-Clarke *et al.*, 2003). The exacerbating effect of hepcidin deficiency on iron accumulation in those with HFE mutations is further supported by a study in mice which intercrossed $HFE^{-/-}$ mice with $Usf2^{+/-}$ mice, which carry only one allele for hepcidin. They found that liver iron accumulation in the $HFE^{-/-}/Usf2^{+/-}$ mice was greater than in mice lacking HFE alone at 8 weeks (Nicolas *et al.*, 2004). Thus differences in hepcidin expression may contribute to the marked phenotypic variability seen in type 1 (HFE) HH.

Juvenile haemochromatosis is a rare form of haemochromatosis (type 2) causing parenchymal iron overload in young adults, and is linked to chromosome 1q and mutations in the haemojuvelin (HJV) gene. The function of the HJV gene is unknown, however, hepcidin levels

have been shown to be low in patients with HJV mutations (Papanikolaou *et al.*, 2004) and there are case reports linking hepcidin mutations with JH in three families (Roetto *et al.*, 2003; Matthes *et al.*, 2004) The hepcidin gene was sequenced in patients from 21 families regardless of linkage with chromosome 1q and only one new hepcidin mutation (C70R) was identified (Roetto *et al.*, 2004). Thus hepcidin mutations do lead to JH, but are rare with the majority caused by mutations in the HJV gene.

A common association in the HH types 1, 2 and 3 is the finding of low hepcidin levels (Merryweather-Clarke *et al.*, 2003; Papanikolaou *et al.*, 2004, 2005). Hepcidin deficiency does not contribute to the pathophysiology of type 4 HH. Conversely, hepcidin has been found to be markedly high in two patients with FPN disease (Papanikolaou *et al.*, 2005). FPN disease is a recently recognized rare disease caused by heterozygous mutations in the SLC40A1 gene, which encodes FPN, an iron export protein (McKie *et al.*, 2000). The phenotype varies depending on the type of FPN mutant and its susceptibility to inhibition by hepcidin (Drakesmith *et al.*, 2005). FPN mutations have been detected which have lost iron export ability *in vitro*. Thus iron is trapped intracellularly leading to Kupffer cell iron loading and a high serum ferritin with normal transferrin saturation (Drakesmith *et al.*, 2005). The second set of FPM mutants described by the same group, maintained full iron efflux ability; however, FPN was unable to be inhibited by hepcidin resulting in increased dietary iron uptake with more iron recycling by macrophages. *In vitro* phenotype correlations, in patients with hepcidin-resistant mutations confirm markedly high transferrin saturation with a lower serum ferritin. The high hepcidin levels in two patients with the 'loss of function' mutants, is presumably induced by the iron locked in macrophages or the high serum ferritin and will be discussed further.

Regulation of hepcidin by anaemia and hypoxia

In studies of hepcidin gene regulation in mice, hypoxia and anaemia induced by haemolysis or bleeding, both produced a considerable decrease in hepcidin mRNA expression (Nicolas *et al.*, 2002b). Iron loading was unable to counteract the anaemia or hypoxia-induced downregulation. Thalassaemic mice with iron overload have significantly lower hepcidin expression compared with controls, supporting the dominant force of anaemia over hepatic iron in hepcidin induction (Adamsky *et al.*, 2004). Hepcidin inhibition in this scenario can be seen as an inappropriate physiological response, which leads to worsening iron overload. This was supported by a recent study of urinary hepcidin levels in humans with iron overload (Papanikolaou *et al.*, 2005). Very low urinary hepcidin levels (11 patients) or normal hepcidin levels (four patients) were found in 15 patients with thalassaemia, despite high ferritin levels. Hepcidin was undetectable in two patients with congenital dyserythropoietic anaemia type 1, despite iron overload, consistent with dominance of the erythropoietic drive secondary to hypoxia or anaemia on hepcidin expression.

A summary of the four major clinical syndromes with disturbed iron metabolism and the observed correlation with haematinics and hepcidin levels is given in Table 1.

Molecular regulation of hepcidin

The inappropriately low levels of hepcidin production in HFE-associated HH (Ahmad *et al.*, 2002) suggests that HFE is upstream of hepcidin in the molecular regulation of hepcidin production. Similarly, the HJV gene, which is mutated in JH, is associated with low hepcidin levels (Ganz, 2005), suggesting regulation proximal to hepcidin. Type 3 haemochromatosis is due to homozygous mutations in TfR2, a membrane glycoprotein that mediates

Table 1. Correlation between hepcidin and clinical disorders of iron metabolism

Disease	Serum iron	Ferritin	Transferrin saturation	Hepcidin
Anaemia of chronic disease	↓	↑	↓	↑
Iron deficiency	↓	↓	↓	↓
HH: types 1–3	↑	↑	↑	↓
HH: type 4 FPN mutants with absent iron export	±	↑ (high at young age)	Normal (A77D rises with age)	↑
HH: type 4 FPN mutants with hepcidin resistance	±	↑ (lower at young age)	↑↑	?
Nongenetic iron overload (transfusional)	↑	↑	↑	↑

HH, hereditary haemochromatosis; FPN, ferroportin.

cellular iron uptake from transferrin. TfR2 mutant mice have low levels of hepcidin mRNA expression, even after massive intraperitoneal iron loading also suggestive of iron modulation proximal to hepcidin (Kawabata *et al.*, 2005). It is possible that hepcidin is the common pathway modulating iron absorption via HFE, TfR2 and HJV, mutations of which all result in an iron overload phenotype.

It is not known which sites are involved in iron sensing. It may take place in duodenal crypt cells, reticuloendothelial cells, erythroid bone marrow cells or the hepatocyte. In the case of patients with FPN mutations, the signal stimulating hepcidin production is presumably related to enterocyte or reticuloendothelial iron stores rather than from the erythron. FPN mutants with reduced macrophage iron export, presumably will result in iron-deficient erythroid cells, and consequent decreased hepcidin expression, if hepcidin were to act as the bone marrow to intestine, iron regulatory signal (Nemeth *et al.*, 2004b). The increased hepcidin levels observed by Papanikolaou *et al.* (2005), in this group, suggest that this is not the case. Possible hepatic iron sensing pathways include HFE interaction with HJV or TfR2, to sense transferrin-bound iron or ferritin; however, studies in humans of urinary and serum hepcidin have failed to find a consistent relationship between hepcidin and haematological parameters such as transferrin saturation and thus the underlying mechanisms regulating iron sensing and hepcidin expression are still unknown (Dallalio, Fleury & Means, 2003; Kulaksiz *et al.*, 2004; Viatte *et al.*, 2005).

Hepcidin and inflammation

Anaemia of chronic disease is characterized by hypoferraemia with adequate reticuloendothelial iron stores, and affects patients with a wide variety of diseases including infection, malignancy and inflammatory conditions (Means, 2004). The pathogenesis is multifactorial, due to a blunted response to erythropoietin, decreased survival of mature red cells, and reduced iron delivery to erythroid precursors because of reduced absorption and macrophage iron recycling (Weinstein *et al.*, 2002). The molecular basis of the ACD involves cytokines and acute phase proteins that affect the regulation of iron homeostasis, but has not been fully elucidated.

Prior to the recognition of its role in iron regulation, hepcidin was initially reported as the first identified member of a family of small widely distributed disulphide-linked cationic antimicrobial peptides in vertebrates (Park *et al.*, 2001). The peptide, initially detected in plasma ultrafiltrate by Krause *et al.* (2000), and termed liver-expressed antimicrobial peptide (LEAP-1) was later identified in human urine by Park *et al.* (2001) and named hepcidin. Both groups identified the liver as the main site of hepcidin expression and demonstrated its antibacterial and antifungal properties. Cysteine-rich peptides are abundant in animal and plant tissues and are part of the innate immune response of the insect fat body, the functional equivalent of the liver (Park *et al.*, 2001). In humans, the liver is central in the innate immune response and is responsible for the increased synthesis of many secreted proteins involved in host defence, called the 'acute phase response' (APR).

Pigeon *et al.* (2001) found that hepcidin expression is increased by lipopolysaccharide (LPS), which induces the APR proteins produced in response to infection or inflammation. The link between hepcidin and the ACD was made by Fleming and Sly (2001) who suggested that hepcidin may mediate the changes in iron haemostasis seen in inflammation. They based this on the evidence outlined by Pigeon *et al.* (2001) demonstrating the link between hepcidin and inflammation, in conjunction with the data from USF-2 KO mice which are iron overloaded due to hepcidin deficiency (Nicolas *et al.*, 2001). They proposed that hepcidin excess would cause reduced intestinal iron absorption, reduced serum iron and increased reticuloendothelial iron (the opposite changes to those seen in the USF-2 KO mice), which is consistent with the abnormalities of iron haemostasis seen in inflammation.

Hepcidin, IL-6 and the APR

The APR was evaluated in hepatocyte cultures exposed to LPS. Hepcidin was induced by IL-6, not IL-1α or α-tumour necrosis factor (TNF-α), consistent with a type II APR protein (Nemeth *et al.*, 2003). In humans injected with LPS, there was an early and transient induction of TNF-α, and interferon-γ, which was boosted by a dramatic increase in IL-6 which peaked at 3–4 h. This correlated with both maximal hepcidin excretion at 6 h, and the onset of hypoferraemia (Kemna *et al.*, 2005b). This time course is consistent with the recently demonstrated mechanism of action of hepcidin, which regulates iron efflux by binding to FPN, and inducing its internalization. Macrophage recycling of iron through the plasma transferrin compartment every few hours, largely supplies the daily iron requirement for erythropoiesis. An IL-6-induced block of this pathway by hepcidin induction, would thus lead to rapid hypoferraemia within hours (Kemna *et al.*, 2005b). This time course of iron restriction is compatible with the role of hepcidin in innate immunity as the first line defence against invading pathogens.

Direct evidence of IL-6-induced anaemia, came from observations in oncology patients treated with recombinant IL-6 as an anti-tumour agent. IL-6 produced a rapid dilutional anaemia in 3 days. At 4 weeks serum iron levels decreased by 65% and there was a slow decrease in MCV, nadir at 6 weeks, which in conjunction with the hypoferraemia, likely represents iron restricted haemopoiesis. The dilutional anaemia and hypoferraemia reversed after cessation of IL-6 (Nieken *et al.*, 1995). In human volunteers, a recombinant IL-6 infusion resulted in a 7.5-fold increase in urine hepcidin levels, and serum iron decreased by 34% within 2 h. IL-6 neutralizing antibodies were used to show that there is an IL-6-dependant, inflammation-mediated, hepcidin regulatory pathway, distinct from regulation by iron, anaemia and hypoxia (Nemeth *et al.*, 2004a).

Hepcidin and the ACD

There is evidence that hepcidin excess causes IDA in transgenic mice (Nicolas *et al.*, 2002a) and in humans with hepatic adenomas expressing excess hepcidin (Weinstein *et al.*, 2002). There is also evidence that IL-6 causes hypoferraemia (Nemeth *et al.*, 2004a) and anaemia (Nieken *et al.*, 1995) and that hepcidin is induced by IL-6 as an inflammatory acute phase protein (Nemeth *et al.*, 2003). However, there is no conclusive evidence yet showing that patients with ACD have elevated hepcidin levels.

There are three main studies looking at the expression of hepcidin in humans. In 37 patients with anaemia undergoing bone marrow examination and 55 specimens submitted for ferritin examination, hepcidin concentration exhibited a statistically significant correlation with serum ferritin, but not with iron status or anaemia. The specimens were unable to be classified into anaemia syndromes on the basis of hepcidin concentration (Dallalio *et al.*, 2003). Nemeth *et al.* (2003) evaluated patients with iron overload, the anaemia of inflammation and infection. They found that hepcidin correlated with serum ferritin levels and that elevated hepcidin appeared to separate patients with ACD from normal controls. A major difference between the two studies was the method of hepcidin determination. Dallalio *et al.* used *serum* specimens to detect the 9 kDa prohepcidin whereas Nemeth *et al.* used *urine* specimens to detect the 2 kDa processed form. Given that hepcidin is produced in the kidney and involved in the regulation of renal iron homeostasis (Kulaksiz *et al.*, 2005) it is difficult to assess the extent to which *urine* hepcidin correlates with liver production and hence pathological conditions.

The third study in humans also detected prohepcidin in *serum*. Prohepcidin was significantly increased in patients with chronic renal insufficiency with normal haemoglobin and significantly decreased in patients with HH. The increased hepcidin in chronic renal disease patients suggest that the kidneys are involved in the metabolism of hepcidin, which accumulates in renal failure. No correlation was found between prohepcidin and serum iron or ferritin (Kulaksiz *et al.*, 2004). At present it appears that the only consistent clinical correlation between hepcidin levels and disease is the finding of inappropriately low hepcidin levels in HH (types 1–3) and that the relationship between hepcidin levels and ACD will require further evaluation.

Measurement of hepcidin

At present there is no consensus on the best assay method for hepcidin. LEAP-1 is analogous to hepcidin-25 and was initially identified in human *blood* using a mass spectrometric assay (Krause *et al.*, 2000). Park *et al.* (2001) then isolated hepcidin-20 and hepcidin-25 from human *urine* by cation exchange chromatography and reversed-phase high-performance liquid chromatography. These findings suggested that prohepcidin originates in the liver (detected via mRNA expression) and after processing, the small hepcidin peptides reach the kidney in plasma, where they are detected as the 20 and 25 amino acid forms in urine.

Further research has now complicated this scenario. The propeptide has been detected in serum and urine using a specific N terminal prohepcidin antibody, in the ranges 51–153 ng/ml (Kulaksiz *et al.*, 2004). The same group were unable to detect the smaller 20–25 amino acid peptides in serum, in contrast to prior investigators (Park *et al.*, 2001). Dallalio *et al.* (2003) detected serum hepcidin in the range of 0–7 mg/ml. The antibody bound to a complex of 9 kDa and it is probable that they have isolated prohepcidin, given the similar molecular weights.

Two groups studied urinary hepcidin-25 concentrations in mice using an immunodot assay (Papanikolaou *et al.*, 2005; Rivera *et al.*, 2005a). Hepcidin was detected in the range of 4–552 ng/mg creatinine. A recent study in mice assessed the serum concentrations of an exogenously administered radiolabelled ^{125}I hepcidin-25 and studied the kinetics of hepcidin in circulation as well as the tissue distribution of hepcidin. They demonstrated that only the 25 amino acid form induced hypoferraemia, in their opinion, confirming its role as the active form in plasma with the biological role of the prohormone still not elucidated (Rivera *et al.*, 2005b). A new method for the

quantification of hepcidin-25 in urine by surface-enhanced laser desorption/ionization time of flight mass spectrometry was recently reported and was able to discriminate between some relevant clinical iron disorders. In comparison with the difficulties in the development of immunochemical methods, the authors state that the method is simple and fast with the potential for high throughput, which may aid future large clinical studies (Kemna *et al.*, 2005a).

Thus there is controversy as to the nature of the active circulating form of hepcidin. It appears that both pro-hepcidin and the smaller 20–25 amino acid forms are detectable in both serum and urine. Hepcidin-25 has been shown to be biologically active (Krause *et al.*, 2000; Park *et al.*, 2001; Rivera *et al.*, 2005b). There is also evidence that prohepcidin may have biological activity with evidence that it is involved in the regulation of iron metabolism in haemochromatosis (Kulaksiz *et al.*, 2004) and that it is an intrinsic renal peptide, synthesized in the kidney tubular system with a possible renal iron regulatory role (Kulaksiz *et al.*, 2005). This lack of understanding of the physiology of hepcidin both in terms of the relative contributions of the liver and kidney to its production and the biological significance of the circulating forms, may underlie some of the conflicting results obtained to date in regard to the measurement of hepcidin and its correlation with disease states.

Conclusion

The discovery of hepcidin and the role that it plays as a negative regulator of intestinal iron absorption and macrophage iron release has significantly progressed our understanding of iron metabolism. Further research towards understanding hepcidin's molecular control, physiology and pharmacodynamics, is needed prior to the development of a reliable laboratory immunoassay. Such an assay may have potential clinical utility in the future in both the diagnosis and classification of iron storage disorders such as HH, IDA and ACD.

The development of hepcidin analogues may have future therapeutic applications. A hepcidin agonist may be useful in the management of patients with HH, although the majority of these patients are generally well controlled on simple venesection programmes. A hepcidin agonist may also have utility in reducing the iron overload in patients with inappropriately suppressed hepcidin, secondary to a hypoxic or anaemic stimulus, such as patients with thalassaemia. Patients with ACD in whom the underlying condition is not reversible may traditionally not have had anaemia severe enough to warrant a packed cell transfusion, given the risk and inconvenience involved in this treatment. This group of patients may gain significant symptomatic benefit from the development of a hepcidin antagonist and potential reversal of the iron-restricted erythropoiesis contributing to the anaemia.

Acknowledgements

I thank Dr Ross Brown, Institute of Haematology, Royal Prince Alfred Hospital, Camperdown, for his support and assistance in reviewing this manuscript.

References

Adamsky K., Weizer O., Amariglio N., Breda L., Harmelin A., Rivella S., Rachmilewitz E. & Rechavi G. (2004) Decreased hepcidin Mrna expression in thalassemic mice. *British Journal of Haematology* **124,** 123–124.

Ahmad K.A., Ahmann J.R., Migas M.C., Waheed A., Britton R.S., Bacon B.R., Sly W.S. & Fleming R.E. (2002) Decreased liver hepcidin expression in the Hfe knockout mouse. *Blood Cells, Molecules and Diseases* **29,** 361–366.

Andrews N.C. (2005) Molecular control of iron metabolism. *Best Practice and Research. Clinical Haematology* **18,** 159–169.

Aoki C.A., Rossaro L., Ramsamooj R., Brandhagen D., Burritt M.F. & Bowlus C.L. (2005) Liver hepcidin Mrna correlates with iron stores, but not inflammation, in patients with chronic hepatitis C. *Journal of Clinical Gastroenterology* **39,** 71–74.

Dallalio G., Fleury T. & Means R.T. (2003) Serum hepcidin in clinical specimens. *British Journal of Haematology* **122,** 996–1000.

Detivaud L., Nemeth E., Boudjema K., Turlin B., Troadec M.B., Leroyer P., Ropert M., Jacquelinet S., Courselaud B., Ganz T., Brissot P. & Loreal O. (2005) Hepcidin levels in humans are correlated with hepatic iron stores, hemoglobin levels and hepatic function. *Blood* **106,** 746–748.

Drakesmith H., Schimanski L.M., Ormerod E., Merryweather-Clarke A.T., Viprakasit V., Edwards J.P., Sweetland E., Bastin J.M., Cowley D., Chinthammitr Y., Robson K.J. & Townsend A.R. (2005) Resistance to hepcidin is conferred by hemochromatosis-associated mutations of ferroportin. *Blood* **106,** 1092–1097.

Feder J.N., Gnirke A., Thomas W., Tsuchihashi Z., Ruddy D.A., Basava A., Dormishian F., Domingo R. Jr, Ellis M.C., Fullan A., Hinton L.M., Jones N.L., Kimmel B.E., Kronmal G.S., Lauer P., Lee V.K., Loeb D.B., Mapa F.A., Mcclelland E., Meyer N.C., Mintier G.A., Moeller N., Moore T., Morikang E., Prass C.E., Quintana L., Starnes S.M., Schatzman R.C., Brunke K.J., Drayna D.T., Risch N.J., Bacon B.R. & Wolff R.K. (1996) A novel Mhc class I-like gene is mutated in patients with hereditary haemochromatosis. *Nature Genetics* **13,** 399–408.

Fleming R.E. & Sly W.S. (2001) Hepcidin: a putative iron-regulatory hormone relevant to hereditary hemochromatosis and the anemia of chronic disease. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 8160–8162.

Ganz T. (2003) Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood* **102,** 783–788.

Ganz T. (2005) Hepcidin – a regulator of intestinal iron absorption and iron recycling by macrophages. *Best Practice and Research. Clinical Haematology* **18,** 171–182.

Hunter H.N., Fulton D.B., Ganz T. & Vogel H.J. (2002) The solution structure of human hepcidin, a peptide hormone with antimicrobial activity that is involved in iron uptake and hereditary hemochromatosis. *Journal of Biological Chemistry* **277,** 37597–37603.

Kawabata H., Fleming R.E., Gui D., Moon S.Y., Saitoh T., O'kelly J., Umehara Y., Wano Y., Said J.W. & Koeffler H.P. (2005) Expression of hepcidin is down-regulated in Tfr2 mutant mice manifesting a phenotype of hereditary hemochromatosis. *Blood* **105,** 376–381.

Kemna E., Tjalsma H., Laarakkers C., Nemeth E., Willems H. & Swinkels D. (2005a) Novel urine hepcidin assay by mass spectrometry. *Blood* **106,** 3268–3270.

Kemna E.H., Pickkers P., Nemeth E., Van Der Hoeven H. & Swinkels D. (2005b) Time-course analysis of hepcidin, serum iron, and plasma cytokine levels in humans injected with LPS. *Blood* **106,** 1864–1866.

Krause A., Neitz S., Magert H.J., Schulz A., Forssmann W.G., Schulz-Knappe P. & Adermann K. (2000) Leap-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. *FEBS Letters* **480,** 147–150.

Kulaksiz H., Gehrke S.G., Janetzko A., Rost D., Bruckner T., Kallinowski B. & Stremmel W. (2004) Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. *Gut* **53,** 735–743.

Kulaksiz H., Theilig F., Bachmann S., Gehrke S.G., Rost D., Janetzko A., Cetin Y. & Stremmel W. (2005) The iron-regulatory peptide hormone hepcidin: expression and cellular localization in the mammalian kidney. *Journal of Endocrinology* **184,** 361–370.

Matthes T., Aguilar-Martinez P., Pizzi-Bosman L., Darbellay R., Rubbia-Brandt L., Giostra E., Michel M., Ganz T. & Beris P. (2004) Severe hemochromatosis in a Portuguese family associated with a new mutation in the 5′-Utr of the hamp gene. *Blood* **104,** 2181–2183.

McKie A.T., Marciani P., Rolfs A., Brennan K., Wehr K., Barrow D., Miret S., Bomford A., Peters T.J., Farzaneh F., Hediger M.A., Hentze M.W. & Simpson R.J. (2000) A novel duodenal iron-regulated transporter, Ireg1, implicated in the basolateral transfer of iron to the circulation. *Molecular Cell* **5,** 299–309.

Means R.T. Jr (2004) Hepcidin and anaemia. *Blood Reviews* **18,** 219–225.

Merryweather-Clarke A.T., Cadet E., Bomford A., Capron D., Viprakasit V., Miller A., Mchugh P.J., Chapman R.W., Pointon J.J., Wimhurst V.L., Livesey K.J., Tanphaichitr V., Rochette J. & Robson K.J. (2003) Digenic inheritance of mutations in hamp and Hfe results in different types of haemochromatosis. *Human Molecular Genetics* **12,** 2241–2247.

Nemeth E., Valore E.V., Territo M., Schiller G., Lichtenstein A. & Ganz T. (2003) Hepcidin, a putative mediator of anemia of inflammation, is a type Ii acute-phase protein. *Blood* **101,** 2461–2463.

Nemeth E., Rivera S., Gabayan V., Keller C., Taudorf S., Pedersen B.K. & Ganz T. (2004a) Il-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *Journal of Clinical Investigation* **113,** 1271–1276.

Nemeth E., Tuttle M.S., Powelson J., Vaughn M.B., Donovan A., Ward D.M., Ganz T. & Kaplan J. (2004b) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* **306,** 2090–2093.

Nicolas G., Bennoun M., Devaux I., Beaumont C., Grandchamp B., Kahn A. & Vaulont S. (2001) Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (Usf2) knockout mice. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 8780–8785.

Nicolas G., Bennoun M., Porteu A., Mativet S., Beaumont C., Grandchamp B., Sirito M., Sawadogo M., Kahn A. & Vaulont S. (2002a) Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 4596–4601.

Nicolas G., Chauvet C., Viatte L., Danan J.L., Bigard X., Devaux I., Beaumont C., Kahn A. & Vaulont S. (2002b) The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *Journal of Clinical Investigation* **110,** 1037–1044.

Nicolas G., Andrews N.C., Kahn A. & Vaulont S. (2004) Hepcidin, a candidate modifier of the hemochromatosis phenotype in mice. *Blood* **103,** 2841–2843.

Nieken J., Mulder N.H., Buter J., Vellenga E., Limburg P.C., Piers D.A. & De Vries E.G. (1995) Recombinant human interleukin-6 induces a rapid and reversible anemia in cancer patients. *Blood* **86,** 900–905.

Papanikolaou G., Samuels M.E., Ludwig E.H., Macdonald M.L., Franchini P.L., Dube M.P., Andres L., Macfarlane J., Sakellaropoulos N., Politou M., Nemeth E., Thompson J., Risler J.K., Zaborowska C., Babakaiff R., Radomski C.C., Pape T.D., Davidas O., Christakis J., Brissot P., Lockitch G., Ganz T., Hayden M.R. & Goldberg Y.P. (2004) Mutations in Hfe2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. *Nature Genetics* **36,** 77–82.

Papanikolaou G., Tzilianos M., Christakis J.I., Bogdanos D., Tsimirika K., Macfarlane J., Goldberg Y.P., Sakellaropoulos N., Ganz T. & Nemeth E. (2005) Hepcidin in iron overload disorders. *Blood* **105,** 4103–4105.

Park C.H., Valore E.V., Waring A.J. & Ganz T. (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *Journal of Biological Chemistry* **276,** 7806–7810.

Parkkila S., Parkkila A.K., Waheed A., Britton R.S., Zhou X.Y., Fleming R.E., Tomatsu S., Bacon B.R. & Sly W.S. (2000) Cell surface expression of Hfe protein in epithelial cells, macrophages, and monocytes. *Haematologica* **85,** 340–345.

Pietrangelo A. (2004) Hereditary hemochromatosis – a new look at an old disease. *New England Journal of Medicine* **350,** 2383–2397.

Pigeon C., Ilyin G., Courselaud B., Leroyer P., Turlin B., Brissot P. & Loreal O. (2001) A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *Journal of Biological Chemistry* **276,** 7811–7819.

Rivera S., Liu L., Nemeth E., Gabayan V., Sorensen O.E. & Ganz T. (2005a) Hepcidin excess induces the sequestration of iron and exacerbates tumor-associated anemia. *Blood* **105,** 1797–1802.

Rivera S., Nemeth E., Gabayan V., Lopez M.A., Farshidi D. & Ganz T. (2005b) Synthetic hepcidin causes rapid dose-dependent

hypoferremia and is concentrated in ferroportin-containing organs. *Blood* **106,** 2196–2199.

Roetto A., Papanikolaou G., Politou M., Alberti F., Girelli D., Christakis J., Loukopoulos D. & Camaschella C. (2003) Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. *Nature Genetics* **33,** 21–22.

Roetto A., Daraio F., Porporato P., Caruso R., Cox T.M., Cazzola M., Gasparini P., Piperno A. & Camaschella C. (2004) Screening hepcidin for mutations in juvenile hemochromatosis: identification of a new mutation (C70r). *Blood* **103,** 2407–2409.

Shike H., Lauth X., Westerman M.E., Ostland V.E., Carlberg J.M., Van Olst J.C., Shimizu C., Bulet P. & Burns J.C. (2002) Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. *European Journal of Biochemistry* **269,** 2232–2237.

Viatte L., Lesbordes-Brion J.C., Lou D.Q., Bennoun M., Nicolas G., Kahn A., Canonne-Hergaux F. & Vaulont S. (2005) Deregulation of proteins involved in iron metabolism in hepcidin-deficient mice. *Blood* **105,** 4861–4864.

Weinberg E.D. (1999) Iron loading and disease surveillance. *Emerging Infectious Diseases* **5,** 346–352.

Weinstein D.A., Roy C.N., Fleming M.D., Loda M.F., Wolfsdorf J.I. & Andrews N.C. (2002) Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. *Blood* **100,** 3776–3781.

