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Possible roles of the hereditary hemochromatosis protein, HFE, in regulating cellular iron homeostasis

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ABSTRACT

Hereditary hemochromatosis (HH) is the most common inherited disorder in people of Northern European descent. Over 83% of the cases of HH result from a single mutation of a Cys to Tyr in the HH protein, HFE. This mutation causes a recessive disease resulting in an accumulation of iron in selected tissues. Iron overload damages these organs leading to cirrhosis of the liver, diabetes, cardiomyopathy, and arthritis. The mechanism by which HFE influences iron homeostasis in cells and in the body remains elusive. Lack of functional HFE in humans produces the opposite effects in different cell types in the body. In the early stages of the disease, Kupffer cells in the liver and enterocytes in the intestine cells are iron depleted and have low intracellular ferritin levels, whereas hepatocytes in the liver are iron overloaded and have high intracellular iron levels. This review gives the background and a model as to possible mechanisms of how HFE could exert different effects on iron homeostasis in different cell types.

Key terms: hereditary hemochromatosis, HFE, iron overload, iron homeostasis

Hereditary hemochromatosis (HH) is a disease of iron-overload in specific organs including the liver, heart, and pancreas. Early studies demonstrated that iron absorption in the intestine of these patients is abnormally elevated (Powell et al., 1970). Excess iron in affected tissues catalyzes the production of oxidants, resulting in cirrhosis, hepatoma, cardiomyopathy, diabetes, hypogonadotropic hypogonadism, and arthritis (Bothwell et al., 1995). HH type 1 is an autosomal recessive disease due to a mutation in HFE and is the most common inherited disease in Caucasians (reviewed in Cox and Walter, 1996). The carrier frequency in the United States is approximately 1 in 9 for individuals of Northern European heritage in the U.S. The penetrance of the gene is still debated, with estimates ranging from 1: 400 to 1: 10,000 individuals in this population having clinical disease (McCune et al., 2003). Although carrying the HH type 1 mutation could be an advantage with an iron poor diet, it presents a distinct disadvantage when iron is readily available. The frequency and importance of HH type 1 has been appreciated only recently because it is difficult to diagnose. Iron accumulates gradually, and the effects of this disease are not usually apparent until middle age or later. Many of the problems that result from the damaged organs, such as liver failure, adult-onset diabetes, and heart failure, are often attributed to other causes in this older age group.

The gene for HH type 1 was mapped to human chromosome 6 almost 30 years ago (Simon et al., 1975), and a linkage to the HLA locus was observed. Developments within the last eight years provide key insights into the molecular definition of HH type 1. First, β2-microglobulin knockout mice were shown to have iron overload with a phenotype similar to that of HH type 1 patients (Rothenberg and Voland, 1996; Santos et al., 1996). β2-microglobulin is a subunit of all major histocompatibility class (MHC) 1

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molecules. Its association with the a subunit of MHC-1 proteins is required for the +*transport of the complex to the cell surface. The finding that β2-microglobulin knockout mice suffered from iron overload raised the possibility that the HH type 1 gene product might have a structure similar to MHC-1 molecules and a similar requirement for association with β2-microglobulin for proper folding and function. A second important breakthrough was the mapping and cloning of the human HFE by Feder and colleagues in the same year (Feder et al., 1996). The sequence of HFE is closest in amino acid sequence to those of MHC-1 molecules, especially HLA-A2. Thus, the predicted HFE structure was consistent with the iron overload found in the β2-microglobulin knockout mouse (Feder et al., 1996; Santos et al., 1996). The crystal structure of the HFE ectodomain confirmed the predicted similarities in the structures of HFE and MHC-1 proteins (Lebron et al., 1998). Unlike MHC-1 molecules, which present peptides to the T-cell receptor, the pocket formed by the a1 and α2 domains in HFE is too narrow to accept peptides. Analysis of HFE mRNA levels in different tissues indicates that it is abundant in small intestine, liver, pancreas, placenta, kidney, and ovary, while it is present in low concentrations in colon, leukocytes, brain, and lung (Feder et al., 1996). These results are in keeping with the pathogenesis of the disease; iron transport occurs in the duodenum and placenta, and iron accumulation due to a mutation in HFE is seen in the tissues with high HFE mRNA levels. Immunohistochemistry confirms the location of HFE in the intestine and liver (Parkkila et al., 1997b). These initial developments gave insight into the structure and location of HFE.

Although how HFE regulates iron homeostasis is still unknown, the disease appears to result from a loss of HFE function. The mutation in 83% of the cases of HH is a single base change that converts Cys 260 to Tyr (C260Y) (Feder et al., 1996). The mutant HFE fails to associate with β2-microglobulin and is not transported to the plasma membrane (Feder et al., 1997). Other members of the MHC-1-like family include the human neonatal Fc receptor (hFcRn) and HLA-G. They, too, have four highly conserved Cys residues that form two disulfide bonds. The disulfide bonds are critical for the secondary and tertiary structure (Miyazaki et al., 1986). Both HFE knockout mice and β2-microglobulin knockout mice are similarly iron overloaded (Santos et al., 1996; Zhou et al., 1998), confirming that the C260Y HFE produces a decrease in HFE function.

Control of iron metabolism at the cellular level: Proteins involved in iron homeostasis that could be affected by HFE

Almost all cells obtain at least part of their iron requirements by TfR1 Tf-mediated iron-uptake, a process that is exquisitely regulated at the posttranscriptional level (reviewed in Hentze and Kuhn, 1996; Klausner et al., 1993; Theil, 1994). Regulation is achieved via iron regulatory proteins (IRPs) and mRNA stem loop structures known as iron responsive elements (IREs). In the case of TfR1, the 3' untranslated portion of the TfR1 mRNA forms IRE stem loop structures in a region that contains a message-instability element. Under low iron conditions, IRP1 and IRP2 bind to the stem loop structures and protect the mRNA from degradation, resulting in increased TfR1 synthesis. Under high iron conditions, the IRPs do not bind to the IREs, exposing the nuclease-sensitive site and leading to mRNA degradation and decreased TfR1 expression. Ferritin, an intracellular iron storage protein is regulated by IRPs, but in contrast to TfR1, it is regulated at the level of translation. Under low intracellular conditions, the IRPs bind to an IRE stem loop structure upstream of the translation start site of ferritin mRNA and block translation. Under high intracellular iron concentrations, when it is desirable to synthesize ferritin in order to sequester iron and prevent iron-catalyzed oxidative damage to the cell, IRPs no longer bind to the IRE and ferritin translation is increased. The role of the IREs in mRNAs of two iron transporters, divalent metal transporter 1 (DMT1) and

ferroportin1 are only beginning to be understood (Hubert and Hentze, 2002; Lymboussaki et al., 2003). Other iron metabolism-related proteins that possess stem loop structures on the 3' untranslated portion of their mRNA include: g-amino levulinic acid synthetase, which is involved in heme biosynthesis; mitochondrial aconitase (Gray et al., 1996; Kim et al., 1996); and the iron-sulfur subunit of succinate dehydrogenase (Gray et al., 1996). These proteins are all regulated by intracellular iron concentrations through the IRPs.

Lower intracellular iron levels imparted by HFE expression

Transfection of HFE into in HeLa and HEK293 cells and the demonstration that HFE expression lowers iron levels within these cell lines provide important tools for determining the basis of HFE's effects. Evidence that HFE lowers intracellular iron levels include: decreased ferritin levels; increased TfR1 levels; increased levels of IRPs binding to IREs; and decreased Tf-mediated iron-uptake into cells (Corsi et al., 1999; Gross et al., 1998; Riedel et al., 1999; Roy et al., 2002; Roy et al., 2000; Roy et al., 1999). The effect of HFE expression in HeLa cells thus results in a lower iron phenotype, because HFE expression mimics the state of cells that are iron-deprived.

The molecular mechanism by which HFE lowers intracellular iron levels when expressed in these cells remains controversial. HFE binds to TfR1 (Feder et al., 1998; Parkkila et al., 1997a) and lowers the affinity of Tf to the TfR1 (Corsi et al., 1999; Feder et al., 1998; Gross et al., 1998; Lebron et al., 1998; Riedel et al., 1999). Careful analysis of the stoichiometry of the complex and binding studies using truncated soluble forms of HFE and TfR1 shows that HFE competes with Tf for binding to TfR1 (Lebron et al., 1998; Lebron and Bjorkman, 1999; Lebron et al., 1999; West et al., 2001). Competition of HFE with Tf for binding to TfR1 could result in less Tf-mediated uptake of iron and provides a superficial explanation for how expression of HFE would lead to lower intracellular iron. However, binding studies suggest otherwise. The affinity of HFE for TfR1 (Kd ~60 nM) is substantially less than that of Tf for TfR1 (Kd ~1 nM) (West et al., 2001). The concentration of diferric Tf in the blood is ~10 μM. Thus one would expect that HFE would never be associated with TfR1 in the presence of physiological concentrations of Tf. Recent studies on cells transfected with mutant HFE have confirmed these predictions (Zhang et al., 2003).

Many different mechanisms have been proposed to account for the lowered iron levels in cells expressing HFE. The expression of human HFE without β2-microglobulin in a Chinese hamster cell line produced lower ferritin levels, but HFE with human β2-microglobulin resulted in increased ferritin levels in the same cells (Waheed et al., 2002). This observation suggested that the lower intracellular iron levels originally observed in HeLa cells might be an artifact due to lack of β2-microglobulin expression in human cells transfected with HFE alone. More recent observations discounted this hypothesis (Wang et al., 2003). Transfection of a human lung carcinoma cell line with additional β2-microglobulin did not reverse the low ferritin levels and elevated TfR1 levels resulting from HFE expression. Furthermore, we demonstrated that in HeLa cells, which show a lower iron phenotype upon the induction of HFE, β2-microglobulin levels rise in accordance with HFE levels and are not limiting (Zhang et al., 2003). Other groups attribute the lower iron phenotype to a decrease in the rate of endocytosis of the HFE/TfR1 complex (Schwake et al., 2002) or a change in the ratio of Tf-mediated and non-Tf-mediated iron-uptake (Corsi et al., 1999).

To resolve some of these issues and to determine the effect of HFE on the trafficking of the TfR1 and on Tf-mediated iron-uptake into cells, HeLa cells in which the expression of HFE can be controlled by the tet-repressible promoter were used. The rates of endocytosis, exocytosis, and Tf-mediated ^{55}Fe uptake were measured. At saturating levels of Tf (100 nM) –

levels at which no differences in the binding of Tf are detected between cells expressing or not expressing HFE – the rate of endocytosis of TfR1 is the same. The rate of exocytosis of the TfR1 is similarly unaffected by HFE expression. These results are consistent with the finding that the distribution of the TfR1 between the cell surface and internal compartments are unchanged upon the induction of HFE (Gross et al., 1998; Roy et al., 1999). In contrast to the ^{125}I-Tf experiments, the amount of ^{55}Fe taken up from ^{55}Fe-Tf decreases 25-30%. We speculated that at physiological concentrations of Tf, HFE would not inhibit Tf uptake, but rather act to negatively regulate iron-uptake in the endosome of HeLa cells either by altering the release of iron from Tf, changing the pH of the endosome, interfering with the ferrireductase activity, or inhibiting the transporter responsible for iron transport from inside the endosome to the cytoplasm (Roy et al., 1999). Thus, with this cell line, HFE expression reduces the amount of Tf-mediated iron uptake without affecting the rates of Tf uptake or efflux. This data is consistent with the hypothesis put forth in this application that HFE directly or indirectly affects iron uptake via the iron transporter, DMT1, which is proposed to be the endosomal transporter.

The higher iron phenotype imparted by HFE expression

Three reports in the literature show that in contrast to HeLa and HEK 293 cells, HFE expression in some cell types results in a higher iron phenotype, decreased TfR1 levels, increased ferritin levels, and decreased levels of IRPs binding to IRE (Cairo et al., 1997; Davies and Enns, 2004; Drakesmith et al., 2002). In one case, HFE expression in monocytes isolated from people with HH type 1 produces a decrease in the IRP/IRE complex indicating a higher iron phenotype (Cairo et al., 1997). In another study, a macrophage cell line, THP1, infected with an adenovirus containing HFE showed increased intracellular ferritin levels (Drakesmith et al., 2002). The authors further demonstrated that iron efflux is decreased in the presence of HFE (Drakesmith et al., 2002). HT29 cells, a human colon carcinoma cell line, transfected with HFE show accumulation of intracellular iron and a decrease in iron-efflux (Davies and Enns, 2004). The data from the monocytic and intestinal cell lines implicates HFE in affecting the function of the iron transporter, ferroportin1. These studies combined with the studies on HeLa and HEK293 cells also raise the question of how HFE expression can produce different iron phenotypes in different cell lines.

Iron regulation in animals

In spite of numerous animal studies on iron absorption, relatively little is known about the mechanisms by which HFE regulates iron homeostasis in the intestine and body. Iron replete individuals take up less iron than iron deficient ones, implying that the regulation of iron homeostasis in the intestine is controlled at the level of iron-uptake and that there is a mechanism for sensing iron levels in the body. Loss of iron from the body is not apparently regulated (reviewed in (Bothwell et al., 1995)). Iron is transported from the lumen of the duodenum into the body via apical (luminal facing) and basolateral (blood facing) transporters. DMT1, the apical transporter, is an integral membrane protein capable of divalent metal ion transport and has been identified in intestinal as well as other cells (Fleming et al., 1997; Gunshin et al., 1997). Single base pair mutations in the coding region of this transporter are associated with low iron content of enterocytes and severe defects in intestinal iron absorption in the *mk* mouse and Belgrade rat (Fleming et al., 1998; Fleming et al., 1997) indicating its important role in the initial stage of iron absorption. Ferroportin1, another divalent metal transporter, also is found predominantly in the duodenum and liver (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000) and participates in iron-efflux from cells.

Dcytb, a duodenal ferrireductase, and hephaestin, a ferroxidase (Li et al., 2003), also are implicated in the transport of iron

across intestinal epithelial cells. Both ferroportin1 and DMT1 transport the ferrous (Fe^{+2}) form of iron. Dcytb was cloned from intestinal tissue, and evidence suggests that it reduces iron from the Fe^{+3} form found in the lumen of the gut for transport into the villus cell via DMT1 (McKie et al., 2001). The *sla* mouse has a deletion mutation in hephaestin that results in anemia (Vulpe et al., 1999). The intestinal epithelia of these mice show increased levels of iron, which indicates that hephaestin plays a role in the iron-efflux from intestinal villus cells.

The liver plays a central role in iron homeostasis. It is the major site of the body's excess iron storage, which accounts for approximately 12.5% to 25% (0.5 to 1 g) of total body iron in a normal adult male (Andrews, 2000). Iron is mainly sequestered in hepatocytes in the core of ferritin. Under low iron conditions, the stored iron can be mobilized actively into circulation (Kim et al., 1985). Hepatocytes are also the source of Tf, the iron transport protein in blood; ceruloplasmin, a serum multicopper ferroxidase, which plays a role in the efflux of iron out of the liver; and hepcidin, a recently discovered peptide involved in the regulation of iron absorption from the intestine. Thus, hepatocytes play a major role in iron homeostasis in the body. In addition, Kupffer cells in the liver have an important role in recycling iron from senescent red blood cells.

The liver communicates with the intestine through the 'body iron stores regulator' to control intestinal iron-uptake. The regulator is most likely hepcidin. Mice lacking hepcidin suffer from severe iron overload (Nicolas et al., 2001), and mice overexpressing it suffer from severe iron deficiency anemia. Hepcidin expression increases several fold when mice are iron loaded with high-iron diets. These observations indicate that hepcidin negatively regulates intestinal iron uptake into the body (Nicolas et al., 2002). The effect of HFE on hepcidin expression in the liver is controversial. Increased hepcidin expression in β2-microglobulin knockout mice was reported (Pigeon et al., 2001). Because these mice do not express β2-microglobulin, they do not make functional HFE and suffer from iron overload similar to HFE knockout mice. These results are consistent with hepcidin secretion, stimulated by iron loading in the liver producing a negative regulation of iron uptake from the intestine. In contrast, two subsequent reports have measured either no difference in hepcidin levels between control and HFE knockout mice or lower amounts of hepcidin expression compared to control mice with comparable amounts of iron overload (Ahmad et al., 2002; Muckenthaler et al., 2003). In one report, hepcidin expression did not change when the HFE knockout mice were challenged with iron (Ahmad et al., 2002). The disparate results most likely reflect the different genetic backgrounds of the HFE knockout mice and the extent of iron loading due to other contributing factors.

In summary, a growing body of knowledge raises the question as to how HFE expression can produce different iron phenotypes in different cell lines. HFE appears to affect either iron import or export depending on the cell type. In addition, lack of functional HFE leads to a muted hepcidin response in mouse models of hereditary hemochromatosis.

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