SPECIAL REPORTS AND REVIEWS

Hemochromatosis Gene Modifies Course of Hepatitis C Viral Infection

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HFE is major histocompatibility class I–like (MHC) molecule that, unlike other known classical and nonclassical MHC proteins, has a function in cell and body iron metabolism. HFE is unable to bind iron but indirectly modulates the rate of iron accumulation in cells that are key in body iron trafficking and absorption. The disruption of the *HFE* gene in rodents leads to iron overload, whereas a homozygote mutation in the HFE protein in humans that changes cysteine at position 282 to tyrosine (C282Y) is responsible for iron overload and organ damage resulting in the most common hereditary disorder of iron metabolism, hemochromatosis (HC).[1,2] A second mutation, which changes histidine at position 63 to aspartic acid (H63D), is present in a minority of patients, but its role in the pathogenesis of the disease is uncertain.[3]

In hemochromatosis, individuals with otherwise intrinsically normal iron absorption machinery and erythroid iron needs have a positive iron balance of 1 to 2 mg of iron daily from birth.[4] During childhood and adolescence in male and in menstruated females, this abnormality does not cause marked iron accumulation caused by high growth demands and iron losses, respectively. Afterwards, iron overload in the liver, pancreas, heart, and joints develops, leading to parenchymal cell damage and organ disease. The underlying pathogenic defect resides in macrophagic and intestinal cells that are unable to retain transferrin iron: This determines on one hand a cell-specific iron-deficient state and uncontrolled iron absorption and, on the other hand, increased saturation of serum transferrin with iron, followed by iron overload of parenchymal cells in the liver, pancreas, and heart.[5] In its fully developed stage, organ structure and function are impaired. A distinction should be made between classical hemochromatosis and hereditary iron overload diseases because of mutations in other iron proteins (e.g., ferroportin, transferrin receptor 2, or hepcidin)[6–9] or conditions owing to a secondary cause (e.g., iron-loading anemias, transfusions, chronic liver disease).

After the hemochromatosis (*HFE*) gene discovery, it has become apparent that the mutated *HFE* genotype may contribute to risk of developing many chronic diseases (e.g., liver diseases, diabetes, heart diseases, arthritis and arthralgias, impotence, and infertility), perhaps producing cases of these conditions that are, for example, more serious and/or of earlier onset.[10] In this context, much interest has been focused on the possible synergism of HFE and the hepatitis C virus (HCV) hepatitis.

HCV is the major causative pathogen associated with non-A, non-B hepatitis. Following acute infection, a chronic state is established in as many as 85% of infected individuals, and virus replication may continue for decades.[11] Although many individuals carrying the virus remain asymptomatic, chronicity is often accompanied by altered liver function and progressive liver disease and culminates in cirrhosis or hepatocellular carcinoma in as many as 20% of infected individuals.[12] Indeed, end-stage liver disease, as a consequence of chronic HCV infection, accounts for 30%–40% of all patients undergoing orthotopic liver transplantation in many Western countries. Global estimates suggest that there could be up to 300 million chronically infected individuals. This represents a significant proportion of the human population who are disposed to increased risk of liver complications and who are a reservoir for further transmission of the virus.

Mild to moderate iron overload is common among patients with chronic hepatitis C (CHC): Up to 30%–40% may show increased serum transferrin-iron saturation and serum ferritin or increased hepatic iron concentration, the latter finding being particularly common in

Abbreviations used in this paper: β_2M, β_2-microglobulin; CHC, chronic hepatitic C; FeTf, ferric-iron-loaed transferrin; HCV, hepatitis C virus; MHC, major histocompatibility complex; Tf, transferrin; TfRI, transferrin receptor 1.

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subjects with end-stage liver disease.[13–15] Several factors may play a role in increasing hepatic iron deposits during CHC as will be discussed below. In general, necroinflammatory events because of an ongoing viral infection may contribute to biochemical or tissue iron excess. On the other hand, it is also very possible that the viral infection per se may modify iron trafficking and metabolism in liver cells directly or following immunologic and host defensive responses. In this context, the genetic predisposition of each individual to properly modify iron trafficking may be crucial in affecting iron distribution within the liver and, possibly, in determining the outcome of the infection. For instance, rerouting iron to macrophagic cells, a typical response to viral and microbic invasion in humans, may subtract iron for viral replication and allow macrophages to mount an effective immunologic response and cytokine production.[16–18] What is the role played by HFE in these complex pathways and how may a mutated HFE influence iron homeostasis and host response in the course of HCV infection? These questions are not trivial in both biologic and clinical terms. The level of iron in each cell, including liver cells, is tightly controlled in humans and depends on the coordinated activity of a discrete set of genes, which orchestrate cell iron uptake, storage, or egress.[19] At the body level, the only regulated step in iron trafficking in humans is intestinal iron absorption,[20] and the most important hereditary disease of metabolism in humans caused by deranged regulation of intestinal iron absorption and body iron trafficking is hemochromatosis. It has been estimated that several million individuals throughout the world may carry a mutated hemochromatosis allele. Therefore, because of the worldwide dissemination of both mutated *HFE* alleles and HCV infection, the disease burden arising from a reciprocal pathogenic interaction can be remarkable.

HFE and Chronic Hepatitis C: Basic Aspects

HFE as Regulator of Body Iron Homeostasis

Most HC patients carry the same mutation (C282Y) in the HFE protein. The C282Y mutation disrupts a critical disulfide bond in the α_3 domain of the HFE protein and abrogates binding of the mutant HFE protein to β_2-microglobulin (β_2M).[21] This results in reduced transport to and expression of HFE on the cell surface. The H63D mutation does not impair β_2M binding or cell surface presentation of HFE. The first indication that HFE can influence iron homeostasis came with the discovery that it associates with the receptor for transferrin, transferrin receptor 1 (TfR1), in a pH-dependent interaction, such that a nanomolar-binding affinity is observed at pH 7.5, with no detectable binding at pH 6 and below.[22,23] TfR1 is a homodimeric type II transmembrane glycoprotein that binds ferric-iron-loaded transferrin (Fe-Tf) at the slightly basic pH of the cell surface (pH 7.4). Fe-Tf-TfR1 complexes are endocytosed into acidic vesicles, where iron is released from transferrin (Tf). Iron-free Tf (apo-Tf) remains bound to TfR1 at acidic pH and is recycled to the cell surface where the basic pH of the bloodstream triggers its dissociation.[24] In solution, HFE binds TfR1 tightly at the pH of the cell surface, but not at pH 6, suggesting that HFE dissociates from TfR1 in acidified endosomes. The binding of HFE to TfR1, a key receptor for iron uptake, is undoubtedly central to the function of HFE in regulation of iron homeostasis. HFE binds to TfR1 in duodenal crypt enterocytes,[25] which regulate the absorption of dietary iron, in placenta[26] and in cell lines over-expressing HFE in which HFE associates with TfR1 throughout the biosynthetic pathway and colocalizes with Tf in intracellular acidic vesicles.[27] Although in vitro studies indicate that wild-type HFE inhibits Tf-TfR1-dependent iron uptake, suggesting that a mutated HFE leads to intracellular iron overload, it is likely that, in vivo, a faulty HFE/TfR1 interaction will result in intracellular iron deprivation.[5] The HFE-TfR1 cocrystal structure provides a detailed view of the protein complex that would exist at the pH of the cell surface.[28] The HFE molecule contacts each polypeptide chain of the TfR1 homodimer. The relative orientations of both molecules indicate that HFE and TfR1 associate on the same membrane, rather than between opposing membranes. It is likely that changes in the TfR1, rather than HFE, mediate the pH-dependent HFE-TfR1 interaction. Whereas HFE changes relatively little upon complex formation, the TfR1 structure undergoes rearrangements.[29] The changes, many of which are at the TfR1 dimer interface, might be extensive enough to propagate across the membrane to the TfR1 cytoplasmic tail, thereby communicating to cytoplasmic proteins that HFE is bound to TfR1. Although HFE is predicted to dissociate from TfR1 in acidic vesicles, the imprint of HFE-induced structural changes might infuence TfR1-facilitated release of iron from Fe-Tf at acidic pH. Alternatively, HFE may bind TfR1 only as a means to gain entry into endosomes, where it would then interact with other molecule(s) to regulate iron metabolism. Because neither His 63, the site of a common amino acid change found in humans, nor a cluster of histidines on HFE are at the interface with TfR1, one or both of these regions of HFE could mediate an interaction with a different molecule at low pH.

In view of these findings and considering the actual phenotypic manifestation of the disease (e.g., iron-deficient intestinal and macrophagic cells), the role of HFE in vivo might be that of facilitating iron entry/retention in the cell. In a recent study by Montosi et al.,[30] it was demonstrated that a lower Fe^{2+}-Tf accumulation is a primary defect of hemochromatosis macrophages, persisting in vitro, corrected by transduction and expression of a wild-type HFE. In agreement with this conclusion, in the *HFE* knock-out mice, spleens are relatively resistant to dietary iron loading, reflecting decreased accumulation of transferrin-bound iron by the $HFE^{-/-}$ splenic macrophages,[31] whereas crypt-cells show a defective accumulation of transferrin iron.[32]

Phenotypic Penetrance of a Mutated *HFE*

The assumption that a mutated *HFE* genotype invariably results in an iron-overload phenotype, and "hemochromatosis" is incorrect. The clinical penetrance of a mutated HFE is not 100% and may vary depending on ethnic, genetic, and environmental factors. The "biochemical" penetrance (increased transferrin saturation and/or serum ferritin) of the C282Y homozygote state may reach 60%–70% in certain populations, whereas the full clinical penetrance (i.e., overt organ disease) may be significantly lower.[33,34] When discussing the potential disease-modifying effect of a mutated allele in the general population and in specific disease populations (e.g., patients with CHC), we should also consider the phenotypic effect of the C282Y or H63D heterozygote states, which are unable per se to cause a hemochromatosis disease. In fact, in the absence of other inciting insults (e.g., alcohol), there is no "clinical" penetrance of the HFE heterozygote state.[35] However, a trend toward higher transferrin saturation and ferritin in HFE heterozygotes as compared with control populations has been reported in different countries.[35–40] The H63D change in the HFE protein has been considered a polymorphic variety with no clinical impact.[3] However, the mutation lies in the HFE loop which interacts with TfR1.[41] According to crystallographic studies on HFE/TfR1 complexation, the His 63 residue makes no direct contacts with the TfR1,[28] consistent with biochemical studies showing that alteration of His 63 and nearby residues does not affect affinity for TfR1.[42] Nonetheless, it cannot be ruled out that, in the presence of other molecular partners or pathogenic events (viruses? hepatotoxins?), this amino acid change may also modify the function of HFE protein. Interestingly, in other disease states, a positive correlation has been reported between the H63D HFE mutation and disease severity.[43,44] Therefore, also in hemochromatosis carriers, we may expect subtle changes in body iron turnover. Along this line, important questions may arise: What is the clinical significance and pathogenic role of "biochemical iron overload?" How does this translate into liver toxicity?

The Effect of a Mutated HFE on Liver Metabolism: The Central Role of Kupffer Cells

If we hypothesize that a mutated HFE is a modifier factor for CHC, we must first consider a scenario in which HFE acts through a specific effect on body or liver iron trafficking and causes increased iron turnover in the bloodstream and, eventually, enhanced hepatic iron deposition and, finally, increased liver toxicity.

In patients with hemochromatosis, a new form of redox-active iron appears in the bloodstream, the so-called nontransferrin bound iron NTBI.[45] Interestingly, hemochromatosis heterozygotes also appear to have measurable levels of NTBI in the serum.[46] Hepatocytes may take up this form of iron through different TfR1-independent mechanisms that are perfectly functional also during iron overload.[47–50] In addition, the recently described transferrin receptor-2, which is highly expressed in the hepatocytes,[51] does not interact with HFE but is capable of delivering transferrin iron, and appears to be normally expressed during liver iron overload.[52] Therefore, the cell, when facing high iron levels in the bloodstream, even if the TfR/HFE system is nonfunctional because of HFE mutations, may still be exposed to a continuous influx of iron. The iron-storage capacity of the liver is highly expandable and mainly relies on the induction of ferritin synthesis: Within the ferritin shells, iron is kept in a safe state. Nevertheless, this is not an inert state, and redox changes in the cytoplasm, xenobiotics, or other conditions may rapidly mobilize this iron and make it catalytically active.[53] We know that excessive redox-active iron in the hepatocytes may enhance liver damage via its catalytic role for hydroxyl-radical formation[54] and lead per se to hepatic fibrosis through activation of hepatic stellate cells or exacerbate an underlying liver disease caused by other toxins.[53]

In hemochromatosis, in addition to the pathogenic importance of hepatocellular iron overload, a leading role in the derangement of iron metabolism and possibly in disease progression is likely played by Kupffer cells. Immunostaining experiments have found a predominant signal for HFE in Kupffer cells, with negligible expression in hepatocytes.[55] Macrophages in hemochromatosis do not respond to an inflammatory challenge by increasing iron retention, as normal macrophages do.[56] In fact, HFE, through modification of iron handling and retention in Kupffer cells, could modify the immunologic

activities of macrophages during host response to bacterial and viral infection. The presence of a mutated HFE may lead to inability to set the proper level of intracellular iron critical for iron-mediated regulation of nitric oxide or tumor necrosis factor α (TNF-α) production, or for macrophage-effector functions (e.g., pathogen killing) via iron-mediated catalysis of radical formation. In fact, hemochromatosis patients have a higher susceptibility to specific pathogens.[57]

Another possibility is that HFE in macrophages synergizes with other iron proteins, either indirectly, through perturbation of iron homeostasis, or directly, through a physical interaction, as suggested by crystallographic studies.[28] There are at least 3 proteins in Kupffer cells that are potential targets for HFE: ferroportin (the product of the *SLC11A3* gene), natural resistance-associated macrophage protein(s) (Nramp1 and Nramp2), and hepcidin. The first 2 proteins are normally produced in macrophages; the third one is a circulating peptide secreted by the hepatocytes that has its physiologic cell target in macrophages.

Ferroportin is a transmembrane iron carrier responsible for iron export from the basolateral site of the enterocytes and macrophages to the circulation.[58] Its mutation leads to a hereditary iron-overload disease similar to hemochromatosis, in which the main pathologic feature is iron trapping in Kupffer cells.[6,7] Ferroportin is over-expressed in the duodenum of patients with homozygote hemochromatosis.[59] A similar up-regulation in macrophagic cells could contribute to the macrophagic iron-deficient state in hemochromatosis.[60] It is unclear whether, in macrophages, there might be also a physical interaction of HFE with ferroportin. This latter possibility has been proposed by a recent study: A mutated HFE in hemochromatosis, unable to bind TfR1, would preferentially complex with ferroportin and enhance iron egress from macrophagic cells.[61]

The natural resistance-associated macrophage protein (Nramp) gene family is composed of 2 members in mammals, Nramp-1 and Nramp-2. Nramp1 is an integral phagolysosomal protein composed of 12 highly hydrophobic transmembrane domains[62] expressed primarily in macrophages and may be centrally involved in innate immune defense against intracellular pathogens. Nramp-1 confers protection against infections with intracellular pathogens such as *Leishmania*, *Salmonella,* or *Mycobacteria* (for review see Bellamy[63]). Interestingly, Nramp-1 expression appears to be regulated by iron perturbations, with increased mRNA and protein levels in macrophages loaded with iron.[64] There is controversy on the role of Nramp-1 as an iron carrier: Some studies suggest that Nramp-1 may indeed transport iron in macrophages across the phagolysosomal membrane by proton-coupled transport,[65] whereas others suggest that the primary function of Nramp-1 is proton transport into the phagolysosome.[66,67] Nramp-2 (also named divalent-metal transporter 1, DMT1) is the main iron carrier in the apical membrane of enterocytes and in the endosome. A glycine to arginine substitution at position 185 in Nramp-2 leads to severe iron deficiency anemia in the microcytic anemia (mk) mice and in the Belgrade (b) rats because of impaired intestinal iron absorption and defective endosomal iron handling in peripheral tissues, respectively.[68–70] A recent study in macrophages has shown that Nramp-1 is expressed in the lysosomal compartment, whereas Nramp-2 is expressed primarily in recycling endosomes and also, to a lesser extent, at the plasma membrane, colocalizing with transferrin.[71] This interesting observation suggests that Nramp-2 plays a key role in the metabolism of transferrin-bound iron by transporting free Fe^{2+} across the endosomal membrane into the cytoplasm. So far, no data have been presented on a direct contact of HFE and Nramp proteins in macrophages. Hepcidin was independently discovered by 2 groups searching for novel antimicrobial peptides.[72,73] Expression of hepcidin is nearly confined to the liver. The knock-out mice for the transcription factors upstream stimulatory factor 2 (*USF2*) or *C-EBPα*, both required for hepcidin transcriptional control, have a hemochromatotic phenotype.[74,75] Transgenic animals over-expressing hepcidin die perinatally because of severe iron deficiency.[76] A rare form of severe juvenile iron overload is associated with a mutation in hepcidin.[9] In addition, a clear role of hepcidin in the cause of hypoferremia of chronic diseases (e.g., anemia of chronic disease), characterized by iron trapping in macrophages and decreased intestinal iron absorption, has been recently proposed.[77] These findings highlight the role of hepcidin as a key regulator of iron metabolism: Hepcidin, produced in the liver in response to iron or inflammatory stimuli, would act as a circulating peptide, which, upon interaction with TfR1/HFE or ferroportin in intestinal cells and macrophages, dictates the extent of iron release/transfer to the bloodstream. In this model, the protein would modulate both intestinal iron absorption and macrophage iron recycling. Whether hepcidin, per se, has a direct effect on HFE expression or function, or vice versa, is presently unknown.

HFE as "Immunological" Factor in Chronic Hepatitis C

A further explanation for a pathogenic role of a mutated HFE may stem from a still unraveled immuno-

logic role of HFE. HFE is a nonclassical MHC class I molecule and does not seem to play a direct role in the classical pathway of antigen presentation, as MHC class I molecules normally do. HFE has not been found to bind antigenic peptides: Its crystal structure suggests that the ancestral peptide-binding groove is too narrow for such a function.[28,41] MHC class I molecules are capable of functions outside of antigen presentation. Some have been shown to interact with receptors such as the insulin receptor and the epidermal growth factor receptor and are likely involved in the formation of high-affinity, ligand-binding sites.[78–80]

On the other hand, MHC class I-like molecules are endowed with immunologic functions[81]: Q7 and Q9 fix and present a diverse array of peptides to $CD8^+$ α-β-lymphocytes; human HLA-E and its mouse ortholog Qa1 preferentially present peptides and control the activation of a subset of CD94/$NKG2^+$ natural killer cells; CD1d preferentially associate with lipids and interact with a restricted subset of T lymphocytes known as NK/T cells; the neonatal Fc receptor, without any bound material in its closed $\alpha 1$ $\alpha 2$ groove, interacts laterally with its ligand and promotes maternal IgG gut absorption, transplacental IgG transport, and IgG reabsorption from the initial urine filtrate.[82] It is, therefore, tempting to speculate that HFE also, in addition to its regulatory role in iron metabolism, could have preserved some immunologic function. However, C282Y homozygous patients and HFE knock-out mice do not present any detectable gross alteration of their immune system.[83] This, however, does not preclude that HFE molecules have a more specific immune function.

As a nonclassical MHC-I protein, HFE may be able to interact with immune cells. HFE could be the ligand for specific γ-lymphocytes in the intestine of mammalian species and communicate the body's iron status to T cells, which would then use cytokines as feedback modulators to achieve iron homeostasis. In fact, the ligand for specific T lymphocyte subsets that accumulate in the gut of mammalian species is still unknown.[84] HFE is one of the possible candidates. Because the γ-δ receptor knock-out mice develop iron overload, it has been suggested that γ-δ T cells may communicate with intestinal cells via γ-δ receptor HFE antigen interaction.[85] Such an intercellular communication between T lymphocytes and intestinal cells, possibly in the crypt where HFE is highly abundant, may induce immune effector functions (e.g., TNF-α formation) and modulate intestinal cell differentiation/apoptosis, and may thus be also of importance for the coordination of both intestinal immune response and iron absorption. Interestingly, it has been found that monocytes from HC patients produce significantly reduced amounts of TNF-α in vitro.[86]

In addition to a direct interaction of HFE with immune cells, there might be a more general and profound effect of HFE on the activity of immune cells, for instance, through its interaction with TfR and/or through modulation of iron homeostasis. In T lymphocytes, TfR is associated with the T-cell receptor ζ chain, 1 of the 2 subunits responsible for the transduction of an activation signal following antigen recognition.[87] The TfR, through this interaction, coparticipates in T-cell activation. It is unknown whether HFE also participates in this pathway or whether a mutated HFE might negatively affect this interaction. On the other hand, iron has turned out to be of pivotal importance for immunosurveillance because of its growth-promoting role for immune cells and its interference with cell-mediated immune effector pathways and cytokine activities. There is a critical iron level for immune cells for mounting proper defense mechanisms and immunologic response because both iron deprivation and iron excess may have detrimental effects.[88] An iron-deficient state may limit the availability of the essential nutrition factor iron to the pathogen, including viruses, as recently suggested for HCV and HBV.[89,90] In fact, experimental iron overload seems to enhance HCV pathogenicity.[91] The induction of iron overload also results in a shift of the ratio between T-helper ($CD4^+$) and T-suppressor/cytotoxic T cells ($CD8^+$), with a relative decrease of the latter.[92] In fact, the $CD4^+/CD8^+$ peripheral blood lymphocyte ratio is often high, because of a reduction in the number of circulating $CD8^+$ T lymphocytes, in most hemochromatosis patients with liver damage.[93] A similar reduction in the number of $CD8^+$ T lymphocytes was also documented in liver biopsy specimens from HH patients.[93] Alterations in CD4/CD8 ratios and in the CD8 receptor repertoire significantly correlate with the degree of liver damage and fibrosis in hemochromatosis patients.[94] These findings suggest that HFE has a direct role in the shaping of T-cell populations, and this effect may reflect on the outcome of the infection as well as the severity of liver disease.

Of great importance is also the potential effect of iron on subsets of lymphocyte populations, which may have a role in viral clearance and in the progression of liver disease. There exist 2 subsets of T-helper (Th) cells in humans, Th-1 and Th-2.[95,96] Th-1-derived cytokines such as interferon (IFN)-γ and interleukin (IL)-2 in addition to growth-promoting effects toward other immune cells are crucial for effective host defense in the acute phase of certain infections: These cytokines stim-

ulate the effector pathways of circulating monocytic cells or resident macrophages by inducing the formation of proinflammatory cytokines, such as TNF-α, IL-1, or IL-6, or by activating cytotoxic effector armies directed against invading pathogens, such as oxygen radical or nitric oxide (NO) formation. By contrast, increased activity of Th-2-derived cytokines such as IL-4, IL-5, IL-10, and IL-13 heightens susceptibility to many infections and causes exacerbations. Cellular iron availability also modulates the differentiation and proliferation of Th-1 and Th-2 cell subsets, with Th-1 clones being less resistant to iron deprivation. This suggests that Th-1-mediated immune effector function is more sensitive to changes in iron homeostasis.[97,98] Interestingly, iron challenge in macrophages causes an impaired response to stimulation by the Th-1 cytokine IFN-γ,[99] thus reducing the cytotoxic effector potential of such macrophages toward various intracellular bacteria or viruses.

HCV as Modifier of HFE and Iron Homeostasis?

Nonclassical MHC proteins, as homologs of MHC molecules, have diverse roles that include presentation of lipid antigens (by CD1), transport of immunoglobulins (by the neonatal Fc receptor), regulation of iron metabolism (by the hemochromatosis gene product HFE), and, most notably, deception of the host immune system (by viral homologs). During the millions of years they have coexisted with their hosts, viruses have learned how to manipulate host immune control mechanisms. One of the most common is interference with class I MHC-mediated antigen presentation to evade cellular immune responses.[100] Initiation of an immune response requires that antigenic fragments of pathogen-derived proteins be presented by the products of MHC. MHC class I products sample the cytosolic compartment and its topological equivalents and present peptides to antigen-specific receptors on CD8 T cells. Phagocytes and dendritic cells can also ingest materials, including host cells that express viral antigens, and have them processed and presented by class I products.[101] Class I-restricted antigen presentation is linked to the biosynthesis and intracellular trafficking of MHC molecules. When properly assembled and loaded with peptide, class I molecules are released from the endoplasmic reticulum (ER), enter the secretory pathway and are displayed at the cell surface. When pathogen-derived proteins make their appearance in the cytosol, can there be a contribution of pathogen-derived peptides to the surface-displayed pool of MHC-peptide complexes.[100] If the eradication of virus-infected cells relies on the activity of class I-restricted $CD8^+$ cytotoxic T lymphocytes (CTLs), then pathogens that attenuate class I expression would have a selective advantage: Through elimination of class I molecules from the cell surface, the infected cell becomes temporarily invisible to CTLs and allows the pathogen the time to proliferate. Every step in the assembly and trafficking of the class I complex present a suitable target for this strategy.[102] In particular, pathogens can rearrange the intracellular trafficking machinery without causing overt cytopathic effects. They could modify the endocytic pathway either directly or through control of cytokine production. Because this interference often involves direct interaction with class I heavy chains and because HFE is closely related to these antigen-presenting molecules, it can be postulated that HFE complexes might be modulated by viral antigens and might thus manifest another target for virus manipulation of cellular proteins. Several viral proteins are well known to manipulate antigen presentation by classical class I MHC molecules: Adenovirus E3/19K retains class I molecules in the endoplasmic reticulum and binds to TAP (transporter associated with antigen presentation)[103]; human cytomegalovirus (HCMV) US2 and US11 proteins target class I heavy chains for degradation[104,105]; human immunodeficiency virus Nef causes rapid endocytosis of cell surface MHC class I molecules[106]; and herpes simplex virus ICP47 inhibits peptide transport through the TAP channel.[107,108] In support of the hypothesis that HFE may be the target of an immunoevasion strategy, a recent study has shown that HCMV US2 but not other viral proteins prevents the expression of TfR-free and TfR-associated HFE complexes, as well as of free HFE heavy chains, by targeting HFE for rapid proteasome-mediated degradation in both human and mouse cells.[109] Thus, this virus employs a protein for altering cellular functions that might interfere with its survival and efficient replication. In fact, targeting HFE to degradation in liver cells may modify cellular iron status and favor viral replication, cell toxicity, or modify antiviral immune response. To what extent this mechanism can be effective in cells containing a mutated HFE remains to be explored. Nevertheless, the fact that pathogenic viruses have evolved mechanisms to inactivate HFE to escape the immune response strongly indicates the importance of this molecule in the defensive response against invading pathogens.

There is a surprising homology between HCV and iron protein biology, which raises an intriguing possibility for potential synergism/competition between HFE and HCV. HCV uses an alternative mechanism to initiate translation of its genome: An RNA element located in the 5′-noncoding region, termed *internal ribosome entry site* (IRES), drives translation initiation through direct

recognition of the 40S subunit and eIF3 by the IRES RNA tertiary structure, eliminating the need for the cap structure and other cap-binding factors usually used by eukaryotic RNA.[110,111] Interestingly, most iron proteins are controlled at the posttranscriptional level by the interaction of abundant cytoplasmic proteins, the iron regulatory proteins (IRPs) with RNA motifs (so-called iron-responsive element; IREs) located either at the 5′ (i.e., ferritin, ferroportin) or at the 3′ (DMT1, TfR1) untranslated region (UTR) of their RNA.[112] Recently, the molecular mechanism of this process has been dissected by using reconstituted, IRE-IRP-regulated, cell-free translation systems.[113] While for mRNA containing an IRE at the 3′ UTR binding of IRP hinders nuclease attack,[114] in the case of mRNA bearing 5′ UTR IRE, IRE-IRP binding prevents the interaction between the cap-binding complex eIF4F and the small ribosomal subunit and hence the formation of an efficient translation complex. The binding of IRP to IRE is stimulated by iron deprivation, cytokines, nitric oxide, and inflammatory stimuli.[112] These data along with those on the function of HCV-IRES raise the fascinating questions of whether there might be an interaction/competition at the molecular level for the ribosomal machinery to initiate translation between HCV and iron proteins or whether, during viral infection, the interaction of these various molecular players might be modified by viral proteins or immunological/inflammatory signals.

HFE and Chronic Hepatitis C: Clinical Observations

Although there are clear differences among the published studies in terms of aim, design, ethnic background of studied populations, analyses of data, methods employed for measuring hepatic iron status, and evaluation of the phenotypic penetrance of the genetic defect, we can identify 3 main areas of investigation of the relationship between *HFE* and CHC: prevalence of *HFE* mutations in CHC; relationship between *HFE* genotype and hepatic iron overload in CHC; and relationship between *HFE* genotype, fibrosis, and disease progression in CHC.

Prevalence of HFE Mutations in Chronic Hepatitis C

A higher prevalence of *HFE* mutations in CHC may indicate a role for the mutated gene in the pathogenesis of the disease. In CHC patients, it might indicate that these individuals, in the presence of a mutated *HFE* allele, are more susceptible to contract acute HCV infection or, possibly, to develop CHC after acute infection. Most of the published studies in which HFE mutation analysis has been performed in HCV patients have shown no difference in the prevalence of C282Y or H63D mutations when compared with a control population.[115–121] However, CHC patients appear significantly more likely to have HFE mutations as compared with control population when C282Y homozygotes or C282Y/H63D compound heterozygotes are also included in the analysis.[118,122] This supports the concept of a synergistic deleterious effect of hemochromatosis (i.e., C282Y homozygosity or C282Y/H63D compound heterozygosity) and HCV on the underlying liver disease.[123] Similar conclusions have been reached by another recent study in HC patients with CHC.[124] In general, heterozygosity for HFE mutations does not seem to be overrepresented in CHC patients. More convincingly, a higher prevalence of HFE mutations has been reported in other liver diseases, such as porphyria cutanea tarda.[125–129]

HFE Mutations and Iron Overload in Chronic Hepatitis C

Another important issue addressed in the literature is the contribution of *HFE* genotype to iron overload in CHC. The majority of the published studies have found evidence for an association between *HFE* genotypes and biochemical iron overload (i.e., higher transferrin-iron saturation and/or serum ferritin levels),[115,116,118,120] even when considering only the H63D heterozygote state.[121] However, strikingly disparate results have been reported on the association between *HFE* gene status and hepatic iron overload: Some studies have found a positive correlation between *HFE* mutations and liver iron deposits in CHC[115,116,121,122]; other studies have not.[117,118,120,130–132] There is no straightforward explanation for this discrepancy, but some general comment can be put forth. First of all, it must be emphasized that different methodologies to measure hepatic iron are available, and each one of them may have advantages and limitations: standard histologic iron-staining procedure (Perl's reaction); semiquantitative scoring, including additional qualitative evaluation of lobular, hepatocytic, or sinusoidal iron distribution (histochemical hepatic iron index)[133]; and quantitative biochemical iron evaluation,[47] including atomic absorption spectrometry. The different sensitivity of the methods used may be particularly critical, especially when searching for low amounts of accumulated iron, as expected in hemochromatosis carriers. On the other hand, a qualitative evaluation of hepatic iron deposits may be very informative because, as discussed, iron can play different pathogenic roles depending on the hepatic cell in which it accumulates.

Another important aspect that must be considered in interpreting the available published data is the control for confounding variables such as demographic parameters, environmental factors, hepatic inflammatory activity, and, most importantly, duration of HCV infection. The date of infection with HCV is difficult to ascertain in most patients. In principle, if HFE had an effect in accelerating iron overload and liver damage in a shorter time frame in CHC, this effect could be blunted if the HFE group was compared with HFE wild-type patients with a much longer duration of disease. A longer disease duration may favor the occurrence of confounding factors that are independent from HFE status, such as development of spontaneous intrahepatic microshunting, spur cell anemia, or, in the opposite direction, chronic gastrointestinal blood loss. A recent report in 164 patients with antibodies to HCV demonstrated, after controlling for the duration of the disease, no relationship between *HFE* mutations and iron loading.[132] On the contrary, in a larger study in 316 patients with CHC that also included patients with end-stage liver disease, the presence of HFE mutations including C282Y heterozygosity was independently associated with biochemical and hepatic iron loading, after correction for the duration of the disease.[121] This correlation did not hold true in patients with end-stage liver disease, in which other nonspecific iron-loading factors may be present.

What is the source or cause of iron excess in CHC? Iron could be released by necrotic hepatocytes because of cytopathic and immune-mediated cell lysis. In addition, there might be a perturbation of iron trafficking because of the immunologic response to the invading pathogen, which may lead to intrahepatic iron decompartmentalization and redistribution. During infection, an underlying diversion of iron traffic leads to a withdrawal of the metal from the sites of erythropoiesis and the circulation to the storage compartment in the reticuloendothelial system, thus resulting, at the same time, in hypoferremia and hyperferritinemia.[134] Proinflammatory and antiinflammatory cytokines, acute-phase proteins, and radicals are prominently involved in causing these disturbances of iron homeostasis because they can affect transcriptionally or posttranscriptionally the expression of genes involved in iron uptake, storage, or iron sensing.[19] In all cases described, either following phagocytosis of necrotic hepatocytes or because of immunologic and inflammatory effects on iron trafficking, iron would preferentially accumulate in nonparenchymal liver (Kupffer cells). Indeed, in CHC, iron deposits have been preferentially found in sinusoidal/Kupffer cells.[50,135,136] However, this does not seem to be a general finding, because CHC patients with exclusive hepatocystic iron accumulation in the absence of HFE mutations or other secondary causes of iron overload can be also seen.[124,137] These observations open the possibility to other hypotheses, which may include, in addition to nonspecific causes of iron overload discussed above, a direct effect of the virus on cellular iron metabolism. An intriguing hypothesis, discussed above, is that HCV might affect the expression of proteins important in modifying iron trafficking during inflammation/host response, such as HFE, ferritin, ferroportin, or hepcidin.[77,138] This mechanism could explain, for instance, the occasional occurrence of increased serum ferritin and hepatic iron deposits in patients with CHC independently of ongoing necroinflammatory events or HFE status.

What about the pattern of iron accumulation in HFE mutated subjects with CHC? Although no data are available on large series of patients with both hemochromatosis and CHC, it is likely that a genetically determined hemochromatotic pattern of iron distribution (i.e., granular iron deposition in periportal zone I hepatocytes and only late involvement of Kupffer cells and periportal macrophage) would persist also during HCV-related liver disease. In the case of the HFE heterozygote state in CHC, the low penetrance of the mutated allele may not be able to modify the events leading to the nonhemochromatotic pattern of iron accumulation in the liver (e.g., preferential nonparenchymal cell iron accumulation).

HFE Mutation, Disease Severity, and Progression of Chronic Hepatitis C

On strict clinical ground, the most meaningful question is whether the mutated *HFE* affects hepatic disease activity and progression in CHC. Several studies have shown that *HFE* genotypes do affect histologic fibrosis score in CHC,[115,116,120–122,130] and, with the exception of 2 studies,[120,130] this effect is consistently related to hepatic iron accumulation. Yet, opposite findings have been reported in other studies in which prevalence of HFE mutations did not correlate with either iron accumulation or fibrosis scores.[117,132,137] The same reservations expressed above on the lack of control for confounding variables can apply to some of the published studies on the *HFE* status and disease progression in CHC. Indeed, Tung et al.,[121] by using multivariate regression analysis, and controlling for duration of HCV infection and histologic inflammation score, showed that patients with CHC who carried HFE mutations were significantly more likely to have bridging fibrosis or cirrhosis. Interestingly, this effect was more significant with shorter duration of disease and less pronounced with

longer duration, suggesting an effect of HFE in accelerating fibrosis progression. However, *HFE* mutations were not over represented in patients with HCV-related end-stage liver disease, again suggesting that other factors may play a role in iron deposition and liver failure during advanced liver disease.

The mechanisms described previously on iron toxicity and fibrogenesis may also apply to the synergism of a biochemically expressed HFE mutation and HCV: In conjunction with the toxic effect of excess iron in parenchymal cells, the alteration of macrophage and immune cell function may all cooperate in aggravating the underlying liver disease, as suggested by recent studies in patients with hemochromatosis and CHC.[124,139] Indeed, iron overload per se may aggravate the course and accelerate the progression of liver disease in CHC patients. In a recent study, two hundred eleven patients cured of thalassemia major by bone marrow transplantation, who did not receive any chelation or antiviral therapy, have been followed for a median follow-up of 5 years.[140] In a multivariate Cox proportional hazard model, the risk for fibrosis progression correlated with hepatic iron content and HCV infection.[140] Although none of the HCV-negative patients with hepatic iron content lower than 16 mg/g dry weight showed fibrosis progression, all the HCV-positive patients with hepatic iron concentration greater than 22 mg/g dry weight had fibrosis progression. Thus, iron overload and HCV infection are independent risk factors for liver fibrosis progression, and their concomitant presence results in a striking increase in risk. Nonetheless, because liver fibrosis per se is a highly dynamic process in which multiple genes interact with different external factors,[141] the pathogenic synergism of HCV and HFE has still to be considered within a more general and complex interaction of genetic susceptibility and environmental factors that will determine in the single patient the outcome of the chronic viral disease.

The impact of iron homeostasis on the immune response in HCV infection has also been emphasized by data showing that the clinical response to IFN-α is reduced in patients with increased hepatic iron stores.[119,142–152] This can be due to an inhibitory effect of iron on IFN-α action, in a fashion comparable with that shown for IFN-γ because type I and type II interferons share similar signal-transduction mechanisms in cells.[153] Patients with higher hepatic iron might also respond poorly to IFN-α because of a dominant Th-2 response that may inhibit some of the pathways induced by type I interferons toward target cells.[154] In this context, the inhibitory effect of iron overload on NO transcription may also have a key negative effect on an effective response to antiviral therapy.[155] In support of the above hypotheses, iron-removal therapy in CHC, although unable to achieve a sustained virologic response, has been able to improve in all published studies the serum transaminase activity and, in several studies, the histologic activity and fibrosis score.[119,151,152,156]

In the case of CHC in C282Y homozygotes without biochemical or clinical phenotype or heterozygotes with mild or absent iron abnormalities, we must consider other pathogenetic synergisms. In this case, a contribution of a mutated HFE to an accelerated or more severe liver disease in CHC, suggested by some studies,[120,130] may stem from a still unraveled role of HFE in immunologic or host defensive mechanisms. HFE could interact with another immunologic partner, possibly in specialized cells such as macrophages. If HFE has another partner beyond TfR1, which operates in pathways other than iron homeostasis, such as the immune response, the concept of "phenotypic penetrance" of the genetic defect should also be reconsidered. Conceptually, if HFE, for a role in iron homeostasis, has to reach the recycling endosomes,[5] possibly to operate in other pathways, other cellular compartments (cell surface?) may be targeted and other pathogenic mutations may be relevant. In this context, even the H63D mutated HFE, which has such a negligible effect on iron trafficking, being a H63D-mutated HFE still able to reach the recycling endosomes and cell membrane, could have other effects.

HCV infection takes a chronic course in the majority of the infected individuals, and the mechanisms underlying viral persistence are poorly understood.[157] Experimental and clinical data indicate that CD4 and $CD8^+$ T lymphocytes are involved in viral clearance and pathogenesis of liver disease in hepatitis C.[158–160] As mentioned in the previous section, perturbation of iron homeostasis may have profound effects on lymphocyte number and activity. In addition, it might be possible that HCV proteins interfere with HFE synthesis, processing, or presentation as a potential mechanism of immune evasion. A similar hypothesis has been recently tested for classical MHC class I in human osteosarcoma-derived tetracycline-regulated cell lines that allow the expression of HCV structural and nonstructural proteins.[161] In this study, however, the cells could efficiently process and present endogenously synthesized HCV proteins via MHC class I and serve as targets for HCV-specific HLAA2-restricted human CTL. Intracellular proteasome activity was not affected by the expression of HCV proteins. It is unclear whether these data can be extrapolated to other cells, such as hepatocytes and

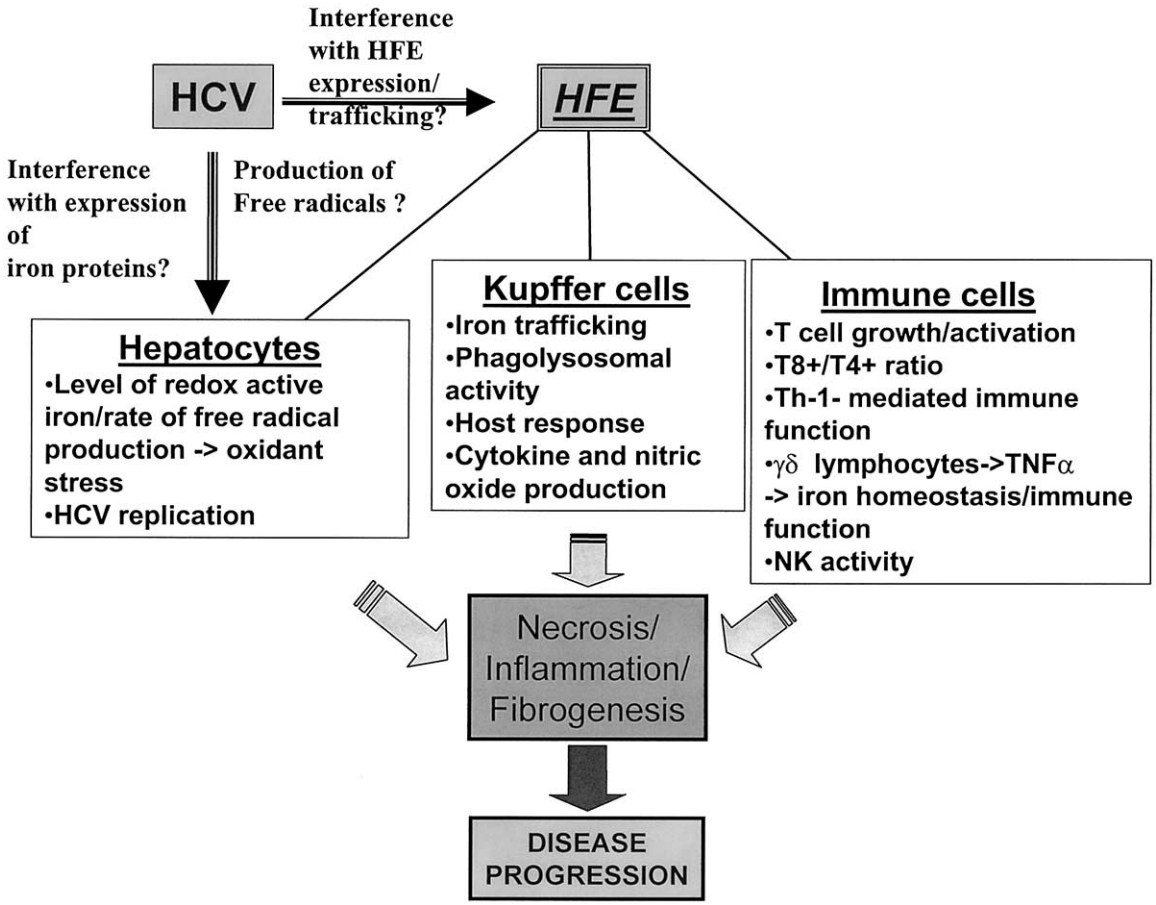

Figure 1. Possible pathways of HFE and HCV synergism during chronic hepatitis C.

Kupffer cells, which may be more relevant for both HCV infection and iron metabolism, or extended to the case of a nonclassical MHC molecules such as HFE.

Conclusions

HFE is a nonclassical MHC protein needed for setting a proper level of iron in cells, such as macrophagic and intestinal crypt cells, which control intestinal iron absorption and body iron trafficking but also immune response and host defensive mechanisms. The narrow ancestral groove of HFE impedes a peptide presentation function but allows an interaction with the main cellular receptor for iron-loaded transferrin and possibly with other iron proteins, which are involved in modification of iron trafficking during inflammation. HFE may be also able to interact with immune cells or affect their differentiation, proliferation, and activity either directly or through modulation of iron availability. The function of HFE may be key in macrophagic cells in which iron is essential for mounting proper defensive mechanisms and activates immunologic armies against pathogens, including viruses. A mutated HFE in humans may lead to the most common hereditary disease of metabolism, hemochromatosis, or, through an increased turnover of redox active iron in the bloodstream and within the liver, aggravate or accelerate the course of several chronic diseases, including chronic hepatitis C. During hepatitis C infection, HFE mutations may influence both cytopathic and immunopathic pathways involved in activity and progression of liver disease (Figure 1). The pathogenic role of iron in these settings is emphasized by the beneficial effect of iron-removal therapy on biochemical and histologic index of disease activity. On the other hand, HCV infection per se appears to modify iron homeostasis in the liver possibly because of mechanisms related to immunologic and host response or to still unraveled interaction with HFE and other iron proteins in immune cells. A proportion of individuals carrying the pathogenic amino acid substitution in the HFE protein will never develop a hemochromatosis disease; seemingly, when these subjects will experience an HCV infection, they may not present an accelerated or more severe liver disease as compared with CHC patients carrying a wild-type HFE. HFE may need other environmental and/or

genetic factors to produce life-threatening conditions. A still unidentified molecular (immunologic?) partner for HFE, for instance, in macrophagic or intestinal progenitor cells, would be an ideal candidate for such a cofactorial role. For millions of years, viruses have studied cell biology and immunology the hard way to acquire and defend their ecologic niche and to learn how to manipulate immune control mechanisms. To understand viral pathogenesis, our knowledge of viral gene functions must be integrated into virus-host interaction networks: HFE, an MHC I homolog, which happens to be a cofactor for an iron receptor, seems to be placed right at the center of these complex and largely unraveled pathways.

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