Hepatocyte-targeted HFE and TFR2 control hepcidin expression in mice


Hereditary hemochromatosis is caused by mutations in the hereditary hemochromatosis protein (HFE), transferrin-receptor 2 (TfR2), hemojuvelin, hep-cidin, or ferroportin genes. Hepcidin is a key iron regulator, which is secreted by the liver, and decreases serum iron levels by causing the down-regulation of the iron transporter, ferroportin. Mutations in either HFE or TfR2 lower hepcidin levels, implying that both HFE and TfR2 are necessary for regulation of hepcidin expression. In this study, we used a recombinant adeno-associated virus, AAV2/8, for hepatocyte-specific expression of either Hfe or Tf2r in mice. Expression of Hfe in Hfe-null mice both increases hepcidin mRNA and lowered hepatic iron and Tf saturation. Expression of Tf2r in Tf2r-deficient mice had a similar effect, whereas expression of Hfe in Tf2r-deficient mice or of Tf2r in Hfe-null mice had no effect on liver or serum iron levels. Expression of Hfe in wild-type mice increased hepcidin mRNA and lowered iron levels. In contrast, expression of Tf2r had no effect on wild-type mice. These findings suggest that Hfe is limiting in formation of the Hfe/Tf2r complex that regulates hepcidin expression. In addition, these studies show that the use of recombinant AAV vector to deliver genes is a promising approach for studying physiologic consequences of protein complexes.

Introduction

Hereditary hemochromatosis (HH) is an autosomal recessive disease of iron metabolism characterized by increased intestinal iron absorption and hepatic iron overload. HH is caused by mutations in genes encoding proteins involved in iron homeostasis, including the HH protein, HFE; hemojuvelin; hepcidin; transferrin-receptor 2 (TfR2); and ferroportin. Accumulation of excessive iron results in hepatic cirrhosis, hepatocellular carcinoma, cardiomyopathy, arrhythmias, diabetes, arthritis, and hypogonadotropic hypogonadism.

HH type 1, the most common form of HH, is caused by a missense mutation in HFE resulting in a C282Y (numbering system includes the first 23 amino acid signal peptide) substitution and accounts for 85% of HH. HFE encodes an atypical major histocompatibility complex class I protein. Like the major histocompatibility complex class I proteins, HFE is a membrane protein that consists of a signal sequence, α1-α3 domains followed by a transmembrane domain, and a short cytoplasmic domain. HFE also forms a heterodimeric complex with β2-microglobulin. The C282Y mutation in HFE disrupts a disulfide bond in the α3 domain, leading to misfolding, lack of association with β2-microglobulin, and failure to traffic to the cell surface. The Hfe knockout mouse (Hfe−/−) also develops iron overload, confirming that the Hfe-C282Y mutation confers loss of rather than gain of function. Although the importance of HFE in iron regulation is apparent in patients with HH and murine models, the underlying mechanism by which HFE regulates iron metabolism is only beginning to be understood.

Type 3 HH is caused by a variety of missense and nonsense mutations in TfR2. The most common mutation is the nonsense mutation (Y250X), which results in a truncation of TfR2 at amino acid 250. The equivalent mutation in the mouse is Y2545X. TfR2, a homolog of TfR1, is expressed predominantly in the liver, in contrast to the ubiquitously expressed TfR1. Tf binds to TfR2 with a lower affinity than it binds to TfR1. Unlike TfR1, which is inversely regulated at the level of mRNA stability by intracellular iron, TfR2 is regulated at the level of protein stability by a novel mechanism, involving the stabilization of TfR2 on Tf binding. Although TfR2 has been investigated with respect to Tf-mediated iron uptake, binding affinity to Tf, tissue-specific expression, and posttranslation regulation by Tf, its role in regulation of iron homeostasis still remains to be clarified.

A characteristic finding in HH caused by mutations either in HFE or in TfR2 is a lower level of hepcidin than in either humans or mice with the same degree of iron loading from other causes. Hepcidin, a key iron regulator, is a peptide hormone synthesized by the liver and secreted into the circulation. Hepcidin modulates serum iron levels, by binding to and causing the down-regulation of the iron transporter, ferroportin. In murine studies, hepcidin expression (Hamp1) increases in response to iron loading, thus preventing further iron uptake. Conversely, during iron deficiency, hepcidin expression decreases.

The observation that mutations in either HFE or TfR2 lower hepcidin levels implies that both HFE and TfR2 play a role in the regulation of hepcidin expression. HFE, TfR2, and hepcidin are expressed predominantly in hepatocytes, suggesting that HFE and
TfR2 regulate iron metabolism upstream of hepcidin. The findings that macrosphere/granulocyte-specific depletion of HFE in mice (Hfe) has no detectable effect on Hamp1 expression, whereas hepatic-specific depletion of Hfe is sufficient to decrease Hamp1 expression reinforces the importance of Hfe in regulating hepcidin. TfR2 and HFE are capable of forming a complex, suggesting that the complex is involved in hepcidin regulation.

In this study, we used a recombinant serotype 2 adenovirus vector pseudotyped with serotype 8 capsid (AAV2/8) and carrying a hepatic-specific promoter to express either Hfe or Tf2 in mice to test the role of the HFE/TfR2 complex in the regulation of hepcidin. Our results indicate that the virally encoded Hfe and Tf2 are expressed in mouse livers. Expression of Hfe in Hfe-null mice both lowered hepatic iron levels and serum Tf saturation and increased hepcidin levels and HFE levels. Tf2-deficient mice responded similarly when Tf2 was expressed but not when Hfe was expressed. Expression of Hfe but not Tf2 in wild-type mice also increased hepcidin levels and lowered iron levels in the liver. However, expression of Hfe in Tf2-deficient mice or of Tf2 in Hfe-null mice had no effect on liver or serum iron levels. These findings imply that the HFE/TfR2 complex regulates the expression of hepcidin and that HFE may be limiting in the formation of the HFE/TfR2 complex to regulate hepcidin expression. They also validate AAV-mediated gene delivery to the liver as a strategy for the reduction of iron overload.

Table 1. List of primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Mouse gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh (459F/563R)</td>
<td>5'-AAATATGCAACTCAGCTCAAGATTGCA-3'</td>
<td>5'-CCCTTCCACAAAGCCAAAGT-3'</td>
</tr>
<tr>
<td>Hfe (718F/859R)</td>
<td>5'-TCTGGGCAGGAGAAGTGCTTCT-3'</td>
<td>5'-GCGATCCAGTGTTTGTGTT-3'</td>
</tr>
<tr>
<td>TfR2 (1409F/1514R)</td>
<td>5'-GCTGGGACGGAGGTACCTTT-3'</td>
<td>5'-GAGTTGTACCGAGCTACAGTACA-3'</td>
</tr>
<tr>
<td>Hepcidin (2559F/2660R)</td>
<td>5'-CTGAGACGACACCACCTATCTC-3'</td>
<td>5'-TGGCTCTAGGCTATGTTTGC-3'</td>
</tr>
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qRT-PCR indicates quantitative reverse-transcription–polymerase chain reaction.

Methods

Preparation of AAV2/8 Hfe and Tf2 vectors

Full-length mouse Hfe open reading frame was amplified from a mouse liver cDNA library by polymerase chain reaction (PCR) with the use of the Expand High Fidelity PCR System (Roche Applied Science) with the following primers: forward, 5'-TCTGAGAAGCTCACATGGCTGGCTCCC-3'; reverse, 5'-TCTGGGAAGATTTGACCTCTGCTGTTTGTTGCTGGACCTACAGTGGATG-3'. The reverse primer contains the FLAG sequence before the stop codon. The gel-purified PCR product was inserted into the pGEM-T vector (Promega) and isolated by using the Complete Mini Protease Inhibitor Cocktail (Roche Diagnostic) and was reduced and denatured with 3.6 M laemmlipuffer for 5 minutes at 100°C. The chromogen 10000 g at 4°C. Liver tissue was harvested and stored in liquid nitrogen for assays.

ImmunobLOTS

Murine liver pieces were solubilized on ice in 1% NET-Triton buffer (150mM NaCl, 5mM EDTA, 10mM Tris, 1% Triton X-100, pH 7.4) with Complete Mini Protease Inhibitor Cocktail (Roche Diagnostic) and was cleared by centrifugation at 16 000g for 30 minutes, and the supernatant was collected. Protein concentrations of the cell extracts were measured with the use of the BCA Protein Assay (Pierce Chemical). The liver tissue extracts (50 or 100 μg) were reduced and denatured with 3.6 M Laemmli buffer for 5 minutes at 100°C and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% or 12% gels. Proteins were transferred to nitrocellulose. Immunoblot analysis was carried out with the use of monoclonal anti–FLAG M2 and mouse antiactin antibodies from Sigma-Aldrich, and rabbit anti-mTfR2 from Alpha Diagnostic International. Bands were detected by horseradish peroxidase–coupled secondary antibody and enhanced chemiluminescence (SuperSignal WestPico; Pierce Chemical) or by fluorescently labeled secondary antibodies as described previously.

Nonheme iron assay

Nonheme iron in liver was determined as previously described with the use of the Nonheme iron kit (Qiagen) and treated with Dnase (Roche Applied Science) to remove any contaminating genomic DNA as previously described. Oligo (dT) primers and Superscript II reverse transcriptase were used to synthesize cDNA according to the manufacturer’s instructions. Hamp1, Hfe, Tf2, and Gapdh mRNA were measured with the use of the RNAeasy kit from Qiagen. Results were expressed as the level relative to the corresponding Gapdh. All primers were verified for linearity of amplification.

Quantitative reverse transcription PCR

Total RNA was isolated from mouse liver with the use of the RNAeasy kit from Qiagen and treated with Dnase (Roche Applied Science) to remove any contaminating genomic DNA as previously described. Total RNA was treated with DNase before reverse transcription with the use of Oligo (dT) primers and Superscript II reverse transcriptase. The Taqman probes and primers were used to synthesize cDNA according to the manufacturer’s instructions. Hamp1, Hfe, Tf2, and Gapdh mRNAs were measured with the use of the RNAeasy kit from Qiagen. Results were expressed as the level relative to the corresponding Gapdh. All primers were verified for linearity of amplification.
water) was added to the supernatants. Each sample was measured in triplicate.

**Blood iron analysis**

Total iron and Tf saturation were measured with the use of a serum iron/TIBC (total iron-binding capacity) Reagent Set (Teco Diagnostics) according to the manufacturer’s instructions. Serum hepcidin determinations were conducted as described previously.42

**Enzyme-linked immunoabsorbent assay of interleukin-6**

Serum samples were tested for interleukin-6 (IL-6) according to the manufacturer’s instructions (R&D Systems).

**Statistical analysis**

The standard deviation and the paired 2-tailed Student t test were used to compare 2 sets of data. The 1-way analysis of variance and Tukey test was used to compare 3 sets of matched data. The Kruskal-Wallis test was used to compare 3 sets of unmatched data.

**Results**

**Expression of Hfe in Hfe−/− mice results in lower iron in the liver, lower Tf saturation in the blood, and increased hepcidin expression**

To determine the feasibility of using the AAV2/8 virus to introduce genes into mice, we tested whether AAHV-Hfe could correct low levels of hepcidin and iron overload in Hfe−/− mice. AAV-Hfe was injected through the tail vein of 6-week-old male Hfe−/− mice on a 129/SvEvTac (129/S) background. The AAV2/8 vector used in our experiments is specifically targeted to liver and muscle,43 and the promoter/enhancing elements limit gene expression to hepatocytes.44 Mice injected with a virus (AAV-c) encoding glutaryl CoA-dehydrogenase, which is unrelated to iron metabolism, and animals not injected served as controls to test whether the vector injection increased hepcidin levels by causing inflammation. All animals not injected served as controls to test whether the vector injection increased hepcidin levels by causing inflammation. All animals not injected served as controls to test whether the vector injection increased hepcidin levels by causing inflammation. All animals not injected served as controls to test whether the vector injection increased hepcidin levels by causing inflammation. All animals not injected served as controls to test whether the vector injection increased hepcidin levels by causing inflammation. All animals not injected served as controls to test whether the vector injection increased hepcidin levels by causing inflammation.

Hfe−/− mice injected with AAV-Hfe showed hepatic expression of Hfe approximately 3-fold lower than in wild-type 129/S mice. Yet this subphysiologic level of Hfe expression was sufficient to decrease liver nonheme iron levels and to increase hepcidin mRNA in the mice injected with AAV-Hfe did not simply show systemic effects on iron homeostasis in the injected mice compared with mice injected with a control virus and uninjected mice (Figure 1B). Thus, vector-mediated Hfe expression was sufficient to increase hepcidin levels and to decrease the iron overload in Hfe−/− mice.

Animals were examined for cell-specific expression of AAV2/8-derived Hfe by comparing Hfe in the livers, spleens, and intestine of Hfe−/− mice treated with AAV-Hfe (supplemental Figure 1A, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). All of these tissues contain macrophages. If the liver promoter was not specific for hepatocyte expression, then macrophages might express the AAV-derived Hfe. No Hfe could be detected in nonliver tissues.

**Expression of Tfr2 in Tfr2−/− mice lowers liver iron levels and serum Tf saturation and increases hepcidin expression**

To test the ability of viral vector to rescue iron overload further, we also generated an AAV2/8 virus that encodes mouse Tfr2 (AAV-Tfr2). Tfr2-deficient mice (Tfr2−/−) on a FVB/NJ (FVB)
background have a stop codon at amino acid 245 of Tfr2 and have iron overload.13 The equivalent mutation in humans produces HH.5 The mice have lower amounts of Tfr2 mRNA compared with wild-type mice and no detectable Tfr2 protein.12

The effects of Tfr2 expression on the iron overload in Tfr2245x/245x mice were examined. Tfr2245x/245x mice were injected with AAV-Tfr2 and killed 2 weeks later, similar to the procedures described for the injection of AAV-Hfe into the Hfe−/− mice. The average amount of liver Tfr2, measured by quantitative fluorescent immunoblot, was 53% of the levels in sex-, age-, and strain-matched wild-type controls (Figure 2A). This amount of Tfr2 was sufficient to lower nonheme iron in the liver by 23%, increase hepcidin mRNA by 1.9-fold, and lower serum Tf saturation by 13% (Figure 2B), indicating that the expression of Tfr2 by comparing Tfr2 in the livers and spleens of Tfr2245x/245x mice treated with AAV-Tfr2 (supplemental Figure 1B). No Tfr2 was detected in the spleens, indicating that other splenic cell types, including macrophages, do not express Tfr2. The difference in levels of Tfr2 mRNA and protein is consistent with the posttranslational control of Tfr2 observed previously.22,23 These results indicate that expression of Tfr2 in hepatocytes was able to increase hepcidin levels and to decrease Tf saturation and nonheme iron levels within 2 weeks.

We tried to estimate the percentage of the hepatocytes transduced by the AAV2/8 viruses. Using the AAV2/8 virus encoding Hfe or Tfr2, we were able to achieve 23% of the Hfe mRNA levels and 57% of Tfr2 protein levels compared with control mice. We were unable to determine the percentage of hepatocytes transduced by each virus with the antibodies currently available. Injection of an AAV-GFP virus encoding green fluorescent protein at a lower titer than we used for our other experiments resulted in GFP protein in 75% to 90% of the hepatocytes (supplemental Figure 1C). These results are consistent with the partial recovery of iron loading in the liver.

**Lack of inflammatory response in AAV-infected mice**

The expression of hepcidin is sensitive to inflammation as well as to iron.45 Using the AAV-c virus, we showed that the AAV2/8 virus itself does not affect iron homeostasis (Figure 1). Despite these results, expression of genes in animals lacking the endogenous protein could still produce an inflammatory response. In particular, IL-6 can stimulate hepcidin expression through activation of signal transducer and activator of transcription 3, which is an acute-phase response protein. We used 2 approaches to look for evidence that expression of Hfe in Hfe−/− or Tfr2 in Tfr2245x/245x mice could elicit an inflammatory response. First, the histology of livers of 4 Tfr2245x/245x mice was compared with that of 4 Tfr2245x/245x mice injected with AAV-Tfr2. No evidence of lymphocytic infiltrates or other histologic evidence of inflammation was detected in the AAV-Tfr2–injected mice (supplemental Figure 2). Comparison of Hfe−/− mice and AAV-Hfe−/−injected mice yielded similar results (data not shown). Second, we used an enzyme-linked immunosorbent assay to test for changes in serum IL-6. IL-6 values in 4 Hfe−/− mice and in the 4 AAV-Hfe−/−injected mice were all below levels of detection. The average value of IL-6 in noninflammatory states in mouse serum is less than 7.8 pg/mL. The enzyme-linked immunosorbent assay is sensitive to approximately 2 pg/mL. The Tfr2245x/245x mice had a maximum of 2.8 pg/mL. IL-6 and an average value below the sensitivity of the assay. No increase in IL-6 could be detected in the mice injected with AAV-Tfr2. According to the manufacturer, a mouse injected with 15 μg of lipopolysaccharide has a level of 31 800 pg/mL 2 hours after injection. Thus, mice injected with AAV-Tfr2 or AAV-Hfe virus and killed 2 weeks later had no evidence of inflammatory processes that would cause the changes in hepcidin. These results are consistent with the lack of immune response detected when α-glucosidase was expressed in α-glucosidase knock-out mice using the same type of virus.38
Tfr2 and Hfe, individually, are insufficient to change the expression of hepcidin

The partial correction of iron overload in Hfe-null mice by injection with AAV-Hfe virus, and in Tfr2-deficient mice with AAV-Tfr2 virus, led us to test whether overexpression of Tfr2 in Hfe-null mice and overexpression of Hfe in Tfr2-deficient mice could compensate for the lack of Hfe and Tfr2, respectively. A normal level of hepcidin expression requires the presence of both Hfe and Tfr2.25,26,37,41 Overexpression of one in the absence of the other should be unable to correct for the lower hepcidin expression. To test this model, Tfr2245x/245x mice were injected with AAV-Hfe or AAV-c virus. Although increased expression of both Hfe protein and mRNA were observed (Figure 3A), no changes in iron parameters could be detected (Figure 3A-B).

In a complementary set of experiments, we observed that expression of Tfr2 in Hfe-null mice also had no effect on iron homeostasis. In AAV-Tfr2–injected animals, Tfr2 mRNA increased by 2.1-fold and Tfr2 increased 3-fold compared with the AAV-c–injected animals (Figure 4A), but the levels of iron and hepcidin in the livers and serum remained unchanged (Figure 4A-B). A similar level of viral-mediated Tfr2 expression in AAV-Tfr2–injected Tfr2245x/245x mice decreased nonheme iron and increased hepcidin mRNA in the liver, along with a decrease in Tf saturation (Figure 2). The combined results of these experiments indicate that neither overexpression of Tfr2 in Hfe-null mice nor overexpression of Hfe in mice that lack functional Tfr2 is sufficient to correct the iron overload of the mutant strain, supporting a model in which the 2 proteins are both required.
Figure 5. Increased expression of Hfe in wild-type 129/S and FVB mice increases hepcidin expression and lowers iron levels. (A-B) AAV-Hfe (●) or AAV-c (○) was injected into wild-type 129/S mice or wild-type FVB mice according to the protocol listed in Figure 1. Mice injected with AAV-Hfe (●) exhibit significantly reduced nonheme iron in liver compared with AAV-c (○) injected 129/S and FVB mice. No detectable changes in Tf saturation, total serum iron (TSI), or transferrin-binding capacity (TIBC) were detected. In each strain of mice, expression of HFE was detectable by immunoblot analysis, and increased Hfe mRNA could be detected in the AAV-Hfe- but not the AAV-c-injected animals. Three to 4 mice were used in each group. The results are shown for 1 group of mice. The experiment was repeated twice with similar results.

Overexpression of Hfe, but not Tfr2, in wild-type mice increases hepcidin expression and lowers iron levels

To determine whether Hfe or Tfr2 was limiting in the regulation of hepcidin expression and iron homeostasis, wild-type mice, which already express normal levels of both Tfr2 and Hfe, were injected with AAV-Hfe, AAV-Tfr2, or control (AAV-c) virus. When either of 129/S or FVB mouse strains were injected with AAV-Hfe, Hfe mRNA increased. Nonheme iron levels decreased and hepcidin mRNA levels increased (Figure 5A). Serum hepcidin levels similarly increased, but serum Tf saturation and iron levels in the blood remained unchanged (Figure 5B). In contrast, injection of AAV-Tfr2, produced a 2- to 3-fold increase in the levels of Tfr2 protein and mRNA in the liver, but it did not generate any significant changes in levels of hepcidin or iron in either the liver or serum (Figure 6). These results indicate that Hfe is limiting for hepcidin production in wild-type mice but Tfr2 is not.

Discussion

The liver plays a major role in the maintenance of iron homeostasis in both humans and mice by controlling the transcription of hepcidin, which is primarily expressed in this organ. Both direct and indirect evidence points to the importance of the HFE/TfR2/Tf complex in the regulation of hepcidin. Mice lacking functional Hfe or Tfr2 have a blunted hepcidin response in comparison to wild-type mice with a comparable iron load. In the present study, we used an AAV2/8 vector to express either Hfe or Tfr2 under the control of a hepatocyte-specific promoter. Expression of Hfe in Hfe-null mice alleviated iron overload in Hfe-null mice but not in mice deficient in Tfr2. Similarly, expression of Tfr2 corrected the iron overload of mice lacking functional Tfr2 but not in Hfe-null mice. These results are consistent with previous findings that hepatocyte-specific ablation of Hfe or Tfr2 in mice is sufficient to result in low hepcidin levels and increased iron accumulation. Importantly, we determined that, in wild-type mice, Hfe mRNA is a limiting factor in hepcidin signaling and that overexpression of Hfe increases hepcidin levels, which in turn decreases iron levels. This result is consistent with the observation that the molar amount of HFE protein is lower than that of TIR1 and TIR2 in solubilized human liver extracts. Superficially, these results appear to be in conflict with the idea that type 1 HH is a recessive disease. Both mouse studies and, to a lesser extent, human studies indicate that, although heterozygous persons do show detectably higher levels of iron loading, their iron loading is not close to what would be expected for a haplotype insufficiency. The protein levels of Hfe in Hfe+/− mice as well as in the AAV-Hfe–transduced mice remain to be determined.

HFE and TFR2 form a complex when expressed in tissue culture cells, leading to the hypothesis that they interact to form a sensor to detect the degree of Tf saturation. HFE also interacts with TIR1, but in contrast to TIR2, which forms a complex with Tf and HFE, Tf competes with HFE for binding to TIR1. Both crystallographic and extensive mutational analyses indicate that TIR1 interacts with the α1α2 domain of HFE. Recently, Schmidt et al demonstrated that the 2 different types of interaction are physiologically important, by showing that mice that express a mutant Tfr1, which fails to interact with Hfe, have higher hepcidin mRNA levels and lower iron levels. These results suggest that TIR1 serves to sequester HFE and prevents its interaction with TIR2. Our results show that HFE mRNA is indeed limiting and, combined with those of Schmidt et al, imply 2 functions for iron-bound Tf beyond just delivering iron to cells: the release of HFE from TfR1 and the formation of an HFE/Tfr2/Tf signaling complex.

In addition, we demonstrated that the AAV2/8 vector allows the controlled expression of proteins. Thus, this virus provides another tool to study the physiologic consequences of proteins that interact with each other or to study the disruption of protein interactions in vivo.

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**Authorship**

Contribution: J.G. and J.C. performed the experiments, analyzed data, and wrote the manuscript; I.D.D. performed experiments and analyzed data; D.M.K., C.O.H., and D.D.K. provided the viral vectors and assisted with experimental design and experiments; R.E.F. provided the Tfr2245x/245x mice and analyzed the data; and C.A.E. supervised the project, designed experiments, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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**References**


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