Hepcidin, a candidate modifier of the hemochromatosis phenotype in mice

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ABSTRACT

Hereditary hemochromatosis (HH) type I is a disorder of iron metabolism caused by a mutation in the HFE gene. While the prevalence of the mutation is very high, its penetrance seems very low. The goal of our study was to determine whether hepcidin, a recently identified iron-regulatory peptide, could be a genetic modifier contributing to the HH phenotype. In mice, deficiency of either HFE (Hfe-/-) or hepcidin (Usf2-/-) is associated with the same pattern of iron overload observed in patients with HH. We intercrossed Hfe-/- and Usf2+/+ mice and asked whether hepcidin deficiency increased the iron burden in Hfe-/- mice. Our results showed that, indeed, liver iron accumulation was greater in the Hfe-/- Usf2+/+ mice than in mice lacking Hfe alone. This result, in agreement with recent findings in humans, provides a genetic explanation for some variability of the HH phenotype.
INTRODUCTION

Hereditary hemochromatosis (HH) is a genetic disorder of iron metabolism resulting in a chronic increase in intestinal iron absorption. As the disease progresses, patients develop toxic iron overload and complications of tissue damage including liver cirrhosis, hepatocarcinoma, and heart disease. Most patients with HH are homozygous for a missense mutation (C282Y) that disrupts the conformation of HFE, an atypical major histocompatibility class I molecule. Similar to human patients, mice lacking the Hfe protein or producing a mutated protein analogous to the human C282Y protein develop increased hepatic iron levels and elevated transferrin saturation. While HH is among the most prevalent genetic disorders (about five people in 1000 are homozygotes for the C282Y mutation), the clinical penetrance of the mutation is low, suggesting that the HFE C282Y mutation is a necessary but not sufficient cause of clinical HH. Variable penetrance may be due to epigenetic, environmental and genetic factors.

In mice, there is a marked difference in hepatic iron loading between the C57BL/6 and DBA/2 Hfe-/- strains, indicating that other genes modify the murine HH phenotype. Several candidate modifier genes have been investigated in mice and humans. Here, we evaluated the iron regulatory peptide, hepcidin, as a potential modifier of iron loading. Hepcidin is believed to act as a negative regulator of iron release from absorptive enterocytes and from macrophages that mediate iron recycling from senescent red cells. Its complete absence in hepcidin-deficient mice (the Usf2/- model) leads to iron accumulation in parenchymal cells. Accordingly, homozygous mutations in HAMP, the gene encoding human hepcidin, have been identified in two families with severe juvenile hemochromatosis. While hepcidin synthesis is physiologically increased by dietary iron to avoid excess iron accumulation, this response is defective in patients homozygous for the C282Y mutation in HFE and in Hfe-/- mice. Furthermore, forced expression of hepcidin prevented iron overload in Hfe-/- mice. These data support the hypothesis that inappropriate hepcidin regulation contributes to the iron loading phenotype. In the present study, we asked whether haploinsufficiency for hepcidin would exacerbate the phenotype of Hfe-/- mice.

MATERIALS AND METHODS

Animals

Hfe-/- mice and hepcidin-deficient Usf2/- mice were described previously. It is noteworthy that the Usf2/- mice (originally mixed 129/Sv-C57BL/6) were bred to uniformity...
(8 backcrosses) on a 129/Sv genetic background. *Hfe* genotype analysis was performed on tail DNA using primers specific for the wild-type *Hfe* allele (forward 5’-TTCTTTAGATAGCCTCTCAC-3’ and reverse 5’-GTGGCGAGTACTTTCCA-3’) and the targeted *Hfe* allele (forward 5’-AGTTGGGAGTGGGTCCGA-3’ and reverse 5’-CTAGCTTCGGCCGTACG-3’), resulting in 502bp and 190 bp products, respectively. *Usf2* genotype analysis was performed as previously described. *Hfe*-/- and *Usf2*+/- mice were interbred and resulting double heterozygous progeny were crossed with *Hfe*-/-*Usf2*+/- mice. Due to the poor viability of *Usf2* -/- mice, *Hfe*-/-*Usf2* -/- animals could not be investigated.

**Liver iron determination**
Liver non-heme iron content was determined as described previously.

**RNA isolation and PCR analysis**
Total RNA was isolated and double-stranded cDNA was synthesized as described previously. Real-time quantification of transcripts was performed on 25 µl samples in an ABI PRISM® 7700 Sequence Detector (Applied Biosystems) using SYBR® Green PCR master mix (Applied Biosystems). Sequences of the primers were: *hepc1*, forward 5’-CCTATCTCCATCAACAGATG-3’ and reverse 5’-AACAGATACCACACTGGGAA-3’ ; *gapdh*, forward 5’-TGCACCACCAACTGCTTAG-3’ and reverse 5’-GGATGCAGGGATGATGTTC-3’.

**Statistical analysis**
Results are expressed as mean ± SD for n animals, and statistical analysis was performed using Student’s t-test (unpaired, two tailed). All statistical analyses were performed using StatView version 5.0 (SAS Institute Inc.). To study the effects of *Hfe* and *Usf2* genotypes on hepatic iron content and *hepc1* gene expression, a Kruskal-Wallis test was performed.

**RESULTS AND DISCUSSION**

We intercrossed *Hfe*-/- mice and *Usf2*+/- mice to further investigate the role of hepcidin in the pathogenesis of liver iron overload in HH. *Usf2*+/- mice carry only one functional allele for both hepc1 and hepc2, the gene products resulting from a tandem duplication of the hepcidin gene in mice, due to insertional disruption of one allele of *Usf2*, a gene that lies in close proximity to the murine hepcidin locus. Although the roles of these proteins are incompletely
understood, hepc1, the peptide most closely resembling human hepcidin, is known to be important in iron metabolism. Compound mutant mice were sacrificed at 4 or 8 weeks of age to determine liver iron content. The abundance of hepc1 transcripts was assessed by real-time PCR quantification.

Fig. 1A shows the relationship between genotype and liver hepc1 expression. In agreement with previously reported data, we found a 40% reduction in hepc1 expression in Hfe-/-Usf2+/+ mice as compared to Hfe+/-Usf2+/+ mice. This reduction in the level of hepc1 was greater (75%) in Hfe-/-Usf2+-/- mice, indicating that haploinsufficiency for hepcidin expression results from inactivation of one allele. Fig. 1B shows, as described previously, that Hfe-/-Usf2+/- mice accumulate 3-times more iron than Hfe+-/-Usf2+/- mice. They also accumulate more iron than doubly heterozygous Hfe+/-Usf2+/- mice. However, there was no difference in iron loading observed when comparing Hfe-/-Usf2+/- and Hfe-/-Usf2+-/- mice at 4 weeks of age, despite decreased hepc1 expression in the Hfe-/-Usf2+-/- group.

We hypothesized that the physiological effects of decreased hepcidin synthesis might become more pronounced with increasing age, as a cumulative effect on intestinal iron absorption. To further investigate this possibility, we analyzed a second group of mice at 8 weeks of age. In contrast to 4 week old animals, we observed a sexual dimorphism in iron loading, as previously reported, with females accumulating more iron than males (data not shown). At 8 weeks we found that haploinsufficiency for hepcidin was associated with increased liver iron overload (Fig. 2B). The increase was approximately 40% for both sexes (females 721 ± 86 µg/g in Hfe-/-Usf2+/- [n=5] vs. 514 ± 72 µg/g in Hfe-/-Usf2+/- [n=6], P < 0.01; males 502 ± 32 µg/g in Hfe-/-Usf2+/- [n=5] vs. 354 ± 65 µg/g in Hfe-/-Usf2+/- [n=6], P < 0.01). These data strongly support the interpretation that hepcidin deficiency contributes to iron overload in Hfe-/- mice.

Our observations suggest that differences in basal hepcidin expression might contribute to phenotypic variability in clinical expression of HH in human patients. A similar conclusion was reached by Merryweather-Clarke et al., who identified heterozygous hepcidin mutations in two HH families. They noted a correlation between the severity of the iron overload and the presence of hepcidin (HAMP) mutations in individuals with HFE C282Y mutations. In the accompanying paper, Jacolot et al. extend those results, providing further evidence that co-
existence of mutations in HFE and HAMP genes may lead to new forms of hemochromatosis (manuscript submitted simultaneously).

It is worth noting that at 8 weeks, the amount of hepc1 remained low in the Hfe-l-Usf2+/− mice as compared to the other groups (Fig.2A). This difference however did not reach statistical significance most likely due to the wide range in hepc1 expression in the Hfe-l-Usf2+/+ group.

In conclusion, our result confirms the key role of hepcidin in the control of iron homeostasis and further highlight that decreased hepcidin contributes to the iron homeostasis abnormalities characteristic of HH. Although the mechanism of action of hepcidin has not yet been established, we speculate that hepcidin or its analogs may prove to be useful in the treatment of hemochromatosis.

**FIGURE LEGENDS**

**Fig.1**

A Hepc1 quantification (arbitrary units) in liver of 4-week old mice with different Hfe and Usf2 genotypes. Hepc1 amount was quantified by real-time PCR and normalized to the amount of gapdh as described in materials and methods. Results are expressed as mean ± SD for n animals.*p<0.01; **p<0.001; ***p<0.0001.

The Kruskal-Wallis test was : P < 0.0001 [mean rank : Hfe-l-Usf2+/− 10.75; Hfe-l-Usf2+/+ 21.35; Hfe+/−Usf2+/− 30.50; Hfe+/−Usf2+/+ 34.25]).

B Non-heme iron concentrations in liver samples from 4-week old mice with different Hfe and Usf2 genotypes. Iron content was measured in two pieces of liver for each mouse. Results are expressed as mean ± SD for n animals.

The Kruskal-Wallis test was: P < 0.0001 [mean rank : Hfe-/Usf2+/− 30.29; Hfe-/ Usf2+/+ 29.70; Hfe+/− Usf2+/− 11.50; Hfe+/− Usf2+/+ 5.50].

**Fig.2**

Hepc1 quantification (A) and non-heme iron concentration (B) as in Fig1 but in liver samples from 8-week-old mice. Results are expressed as mean ± SD for n animals (NS is for non significant). Results in B were normalized according to the sex. The sex ratio (female/male) for hepatic iron was 1.44 for Hfe-/Usf2+/− mice, 1.45 for Hfe-/Usf2+/+ mice, 1.28 for Hfe+/−Usf2+/− mice and 1.24 for Hfe+/−Usf2+/+ mice.
The Kruskal-Wallis test for hepc1 quantification was: $P < 0.01$ [mean rank : $Hfe-/ Usf2+/-$ 11.55; $Hfe-/ Usf2+/+$ 13.67; $Hfe+/ Usf2+/-$ 23.64; $Hfe+/ Usf2+/+$ 31.57] and for non-heme iron concentration $P < 0.0001$ [mean rank : $Hfe-/ Usf2+/-$ 29.80; $Hfe-/ Usf2+/+$ 21.92; $Hfe+/ Usf2+/-$ 10.71; $Hfe+/ Usf2+/+$ 4.29].

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REFERENCES


Fig. 1

A

hepc1/gapdh (arbitrary units)

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B

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Fig. 2

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hepc1/gapdh (arbitrary units)

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