Brief report

Targeted disruption of the hepcidin 1 gene results in severe hemochromatosis

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We previously reported that mice made deficient for the transcriptional factor USF2 fail to express hepcidin 1 and hepcidin 2 genes as a consequence of targeted disruption of the Usf2 gene lying just upstream in the locus. These mice developed an iron overload phenotype with excess iron deposition in parenchymal cells and decreased reticuloendothelial iron. At that time, although the role of USF2 was still confounding, we proposed for the first time the role of hepcidin as a negative regulator of iron absorption and iron release from macrophages. Accordingly, we subsequently demonstrated that hyperexpression of hepcidin 1, but not hepcidin 2, resulted in a profound hyposideremic anemia. To analyze the consequences of hepcidin 1 deletion on iron metabolism without any disturbance due to USF2 deficiency, we disrupted the hepcidin 1 gene by targeting almost all the coding region. Confirming our prior results, Hepc1−/− mice developed early and severe multiscleral iron overload, with sparing of the spleen macrophages, and demonstrated increased serum iron and ferritin levels as compared with their controls. (Blood. 2006;108:1402-1405)

Introduction

Hepcidin is a small circulating 25-amino-acid cysteine-rich peptide first identified in human blood1 and urine.2 Hepcidin is the product of the HAMP gene, which consists of 3 exons and encodes a precursor protein of 84 aa (for a review see Nicolas et al3 and Ganz4). The hepcidin gene is expressed in the hepatocytes, secreted in the circulation, and cleared by the kidney. Whereas humans, rats, dogs, and pigs have a single gene in mice, Hepc1 and Hepc2.5,6 In mammals, convincing evidence indicates that hepcidin constitutes the master regulator of iron homeostasis; the circulating peptide acts to limit gastrointestinal iron absorption and serum iron by inhibiting dietary intestinal iron absorption and iron recycling by the macrophages.7 As befits an iron-regulatory hormone, hepcidin synthesis is induced by iron stores and inflammation and inhibited by anemia and hypoxia.5,8-10 Most of the iron overload syndromes known to date (primary hemochromatosis and secondary iron overload) imply a reduction of hepcidin secretion. In contrast, hypersecretion of hepcidin seems to play a determining role in anemia of inflammation (for a review see Ganz11). Four years after the discovery of the peptide, its mechanism of action was elucidated.12 To limit iron egress, hepcidin binds to ferroportin, the transmembrane iron transporter necessary for iron transfer out of intestinal epithelial cells and macrophages,13 thereby inducing its internalization and subsequent degradation, leading to decreased export of cellular iron.12,14,15

Much of the data concerning the involvement of hepcidin in iron metabolism were initially generated in mouse models. We previously reported that mice made deficient for the transcriptional factor USF2 fail to express either the Hepc1 or Hepc2 gene, as a consequence of targeted disruption of the Usf2 gene lying just upstream of the hepcidin genes.7 These mice presented with increased liver iron levels and developed an iron overload phenotype similar to that observed in hereditary hemochromatosis, with increased circulating iron, increased transferrin saturation, and increased reticuloendothelial iron. We assume that the phenotype was not due to USF2 deficiency since an independent Usf2 knockout (KO) line expressed a normal amount of hepcidin mRNA and had normal iron metabolism.16 We thus proposed the role of hepcidin as a negative regulator of iron absorption and iron release from macrophages. This hypothesis was further supported by our demonstration that transgenic mice overexpressing Hepc1 were born with severe iron deficiency.16 Interestingly, we recently showed that transgenic mice overexpressing Hepc2 presented with normal iron metabolism, suggesting that only Hepc1 is able to regulate iron homeostasis in mice.8

To analyze the consequences of Hepc1 deletion on iron metabolism without any disturbance due to USF2 deficiency (only 10% of Usf2−/− mice survive), we decided to disrupt the Hepc1 gene by targeting deletion of exons 1 and 2 by classic homologous recombination. Homozygous mice presented with normal viability and developed multiscleral iron overload, with sparing of the spleen macrophages. The mutant mice also demonstrated increased serum iron and ferritin levels as compared with their controls. The
Hepc1 KO mouse model will facilitate investigation into the pathogenesis of iron overload in hemochromatosis and provide opportunities to evaluate therapeutic strategies for prevention or correction of iron overload.

Study design

Targeted disruption of the murine Hepc1 gene

The gene-targeting vector was constructed by replacement of the 2 first exons and part of exon 3 of the Hepc1 gene with a hygromycin cassette under the control of the PGK promoter. This cassette is flanked in the 5’ direction by a 4.7-kb homologous arm and in the 3’ direction by a 1.7-kb homologous arm. Isogenic homologous DNA arms were obtained by long-distance genomic polymerase chain reaction (PCR; Expand Long Template PCR System; Roche, Mannheim, Germany) using mouse 129/SvC57BL/C10 genomic DNA as template and subcloned. Primers for PCR-made fragments were as follows: for the 5’ homologous fragment (HF), forward 5’-CCGGGTACCTGATCAGAGCCAGGACAGGGC-3’ and reverse 5’-CGGGGTACCTGATCCTGCTAGAGGAGGAGCAGGCC-3’; and for the 3’ HF, forward 5’-ATTTGCGGCCGCTGATCAGCTAGAAATCAAG-3’ and reverse 5’-ATTTGCGGCCGCTGATCAGCTAGAAATCAAG-3’.

Iron measurements and immunohistochemistry

Serum and tissue iron concentrations were determined as previously described using the “IL test” (Instrumentation Laboratory, Lexington, MA). For histology, tissues were fixed in 4% formaldehyde and embedded in paraffin. Sections were immersed in Perl’s solution (1:1, 2% HCl and 2% potassium ferrocyanide) to visualize ferric (non-heme) iron and counterstained with nuclear fast red using standard procedures. Quantification of serum ferritin levels was performed on an Olympus AU4000 automat, using the Olympus human ferritin assay kit (Olympus, Hamburg, Germany).

Results and discussion

Targeted disruption of Hepc1 results in the same iron disorders as those observed in Usf2 KO mice. Targeted disruption of Hepc1 was obtained by replacing exons 1 and 2 and part of exon 3 with an hygromycin resistance cassette using homologous recombination in ES cells. Correctly targeted clones were identified by Southern blot analysis (using the indicated 5’ probe, Figure 1A) and PCR analysis (data not shown). Chimeric animals were bred with C57BL/6 females to produce inbred F1 offspring carrying the modified Hepc1 allele. Germline transmission of the targeted allele was confirmed by PCR analysis (not shown). We interbred Hepc1+/− animals to produce Hepc1−/− offspring, and obtained approximately 25% Hepc1−/− animals, indicating that there was no significant prenatal lethality. To assess the specificity of the Hepc1 targeting deletion, we checked by RT-PCR the absence of Hepc1 transcript in the liver of Hepc1−/− animals (Figure 1B). Furthermore, we showed that, in contrast to the inhibition of Hepc1 and Hepc2 gene transcription observed in the Usf2−/− mice, the expression of the neighboring genes of Hepc1, namely Hepc2 and Usf2, were still expressed in Hepc1−/− mice (Figure 1B).

Animals

All animals were cared for in accordance with the European convention for the protection of laboratory animals. Animals were maintained in a temperature- and light-controlled environment and were given free access to tap water and food (standard laboratory mouse chow, AO3, iron content 280 mg/kg; Usine d’Alimentation Rationelle [UAR], Epinay-sur-Orsay, France). Iron overload was induced by adding 2% carbonyl iron (reduced pentacarbonyl iron) to the diet (AO3; UAR) for 14 days.

Hematologic analysis of mice

Blood was obtained by retro-orbital phlebotomy before killing of mice and collected in heparinized tubes (capiject T-MLH; Laboratoires Terumo, Guyancourt, France). Blood-cell counts and erythrocyte parameters were determined using a MaxM coulter automatic analyzer (Coulter, Hialeah, FL).

Hepatic iron accumulation in Hepc1−/− mice

Hepatic iron accumulation was assessed by Perl’s staining. As shown in Figure 1C, massive iron accumulation was present in the livers and pancreata of 4-month-old Hepc1−/− mice and significant iron was also detected in the hearts. In contrast, splenic iron content was markedly decreased in Hepc1−/− mice as compared with wild-type mice. Excess non-heme iron was also present in kidney, lung, muscle, and brain (data not shown). Further examination of liver iron accumulation showed that iron accumulated in liver parenchymal cells (hepatocytes), with sparing of the resident macrophages (Kupffer cells). Surprisingly, iron deposition was found more prominent in the centrolobular areas (Figure 1D, left panel). To determine whether this iron deposition was due to an intrinsic incapacity of Hepc1−/− mice to load iron in the perportal region, control and mutant animals were given a 2% iron carbonyl-rich diet for 14 days. It is indeed well documented that dietary carbonyl iron overload is associated with specific iron deposition in the perportal hepatocytes. This perportal staining was evidenced in the experimentally iron-loaded control mice (Figure 1D) as well as follows: 5’-TCATGCAGACAGACAAAGACA-3’ (forward) and 5’-TCAGTCCGGGTACTCTCGC-3’ (reverse).
as in the Hepc1−/− mice, leading to a panlobular staining of the hepatic lobule of the mutant mice. We then examined the age-dependent variation of hematologic and iron parameters in control and Hepc1−/− mice from 2 to 8 months of age. Controls consist of both Hepc1+/+ and Hepc1−/− since no significant difference was observed between these animals. As shown in Figure 2, liver iron content was already 5-fold increased in 2-month-old mutant mice and continued to increase over the duration of the study. At 8 months, Hepc1−/− mice had almost 15-fold more liver iron than control mice. At that age, mutant mice presented with a 30-fold increase of iron in the pancreas (2056 ± 85 μg iron/g of wet tissue in Hepc1−/− mice, n = 3, versus 66 ± 6 in control mice, n = 4; P < 10−7), a 5.5-fold increase in the heart (513 ± 145 μg iron/g of wet tissue in Hepc1−/− mice, n = 3, versus 92 ± 30 in control mice, n = 4; P = .002) whereas splenic iron was decreased by almost 3-fold (273 ± 30 μg iron/g of wet tissue in Hepc1−/− mice, n = 3, versus 784 ± 23 in control mice, n = 4; P < .001). Serum ferritin levels increased in parallel to the liver iron increase. In contrast, serum iron increased at 2 months and remained constant during the following months.
Collectively, these features of iron disorders observed in Hepc1−/− mice appeared very similar to those described in the Usf2−/− mice. They confirm our first hypothesis that iron deregulation of the Usf2−/− mice was due to the suppression of Hepc1 gene expression, and highlight the nonredundant role of Hepc1 and Hepc2 in mice.

The phenotype reported here for the Hepc1−/− mice is very similar to the one recently reported for Hfe−/− mice that presented with a complete deficit in hepcidin production.19,20 These 2 mouse models are characteristic of the severe, early onset of human juvenile hemochromatosis caused by mutations in the hemojuvelin gene and, more rarely, in the hepcidin gene (for a review see Roetto and Camaschella21). The gradual accumulation of liver iron in the Hepc1−/− mice suggests that hepcidin is the final setpoint regulator of iron homeostasis and that, in its absence, there is no effective regulatory mechanism to decrease iron uptake. This result differs from that observed in the Hfe−/− mice, which do not completely lack hepcidin and still retain their ability to regulate intestinal iron absorption.22 The most intriguing result reported here, which seems in fact a unique feature of the Hepc1−/− mice, is the centrolobular accumulation of liver iron. Indeed, when documented, the iron accumulation of the other forms of hemochromatosis, primary or secondary, appeared to be perisplenic. The specific zonation seen in Hepc1−/− mice is interesting but difficult to explain. In this respect, it will be interesting to analyze the specific zonation of the iron-related proteins in the liver, a still poorly investigated domain.

References

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