Contributions of β 2-microglobulin–dependent molecules and lymphocytes to iron regulation: insights from *HfeRag1*^{−/−} and *β 2mRag1*^{−/−} double knock-out mice

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Abstract

Genetic causes of hereditary hemochromatosis (HH) include mutations in the *HFE* gene, coding for a β 2-microglobulin (β 2m)–associated major histocompatibility complex class I-like protein. However, iron accumulation in patients with HH can be highly variable. Previously, analysis of *β 2mRag1*^{−/−} double-deficient mice, lacking all β 2m-dependent molecules and lymphocytes, demonstrated increased iron accumulation in the pancreas and heart compared with *β 2m* single knock-out mice. To evaluate whether the observed phenotype in *β 2mRag1*^{−/−} mice was due solely to the absence of Hfe or to other β 2m-dependent molecules, we generated *HfeRag1*^{−/−} double-deficient mice. Our studies revealed that introduction of *Rag1* deficiency in *Hfe* knock-out mice leads to heightened iron overload, mainly in the liver, whereas the heart and pancreas are relatively spared compared with *β 2mRag1*^{−/−} mice. These results suggest that other β 2m-interacting protein(s) may be involved in iron regulation and that in the absence of functional Hfe molecules lymphocyte numbers may influence iron overload severity.

Introduction

The protein involved in hereditary hemochromatosis (HH), HFE, has been identified as a β 2-microglobulin (β 2m)–dependent, major histocompatibility complex class I (MHC-I)–like molecule,¹ complementing earlier findings that β 2m deficiency in mice leads to iron overload.² Significantly, knock-out of the *Hfe* gene clearly confers the HH phenotype to mice.^{3,4}

Although *HFE* mutations are clearly associated with iron overload, the marked variability of the phenotype among homozygous individuals for the same *HFE* mutations indicates that other environmental and genetic factors modify disease severity.⁵ Earlier reports show that defective numbers of peripheral lymphocytes and liver lymphocyte populations are associated with a more severe clinical expression of iron overload and damage in HH.^{6,7}

Previously, we investigated the effect of total lymphocyte absence in iron overload by crossing $\beta 2m^{-/-}$ mice, lacking all $\beta 2m$ -dependent MHC-I molecules,² with mice deficient in recombinase activator gene 1 (*Rag1*) required for normal B- and T-lymphocyte development.⁸ Immunodeficient $\beta 2mRag1^{-/-}$ mice develop iron overload and, unlike their single knock-out counterparts, when challenged with dietary iron loading, iron accumulation occurs not only in the liver but also massively in the pancreas and heart myocytes.⁹

In this report, we further investigate the possible involvement of $\beta 2m$ -dependent molecules and lymphocytes in iron regulation. We examine changes in iron metabolism in compound mutant *HfeRag1*^{-/-} and $\beta 2mRag1$ ^{-/-} mice placed on a standard diet and after dietary iron challenge.

Study design

All procedures were performed in accordance with guidelines of Canadian Council on Animal Care and approved by the Animal Care Committee of the Centre Hospitalier de l'Université de Montréal. *Hfe*^{-/-} mice have been described previously.³ $\beta 2m^{-/-}$ and *Rag1*^{-/-} mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mutant mice have been back-crossed a minimum of 5 times onto the C57BL/6 (B6) background. Compound mutants $\beta 2mRag1$ ^{-/-} and *HfeRag1*^{-/-} were obtained as described.⁹ Mouse peripheral blood cells were screened by flow cytometry in a Coulter Epics Elite counter (Coulter, Hialeah, FL) for the absence of T and B lymphocytes, using anti- $\alpha\beta$ TCR (T-cell marker) and anti-CD45R/B220 (B-cell marker) antibodies (PharMingen, San Diego, CA), and were genotyped by polymerase chain reaction (PCR) for $\beta 2m$ and *Hfe* mutations. All animals were 8 weeks old at the beginning of the experiments. They were given a commercial diet (Teklad Global 18% protein rodent diet; Harlan Teklad, Madison, WI), or, when indicated, the same standard diet supplemented with 2.5% (wt/wt) carbonyl iron (Sigma Immunochemicals, St Louis, MO). Iron levels were measured by acid digestion of tissue samples followed by iron quantification by atomic absorption spectroscopy.⁹ For histology, tissue sections were stained with Prussian blue for ferric iron detection (iron stain kit; Sigma Immunochemicals).

Results and discussion

To investigate whether the exacerbation of iron loading in $\beta 2mRag1$ ^{-/-} mice is due to the absence of only *Hfe* or to other $\beta 2m$ -dependent molecules, we generated mice lacking both *Hfe* and *Rag1* (*HfeRag1*^{-/-} mice). It was expected that if the only $\beta 2m$ -dependent molecule involved in iron regulation was *Hfe*, the $\beta 2mRag1$ ^{-/-} phenotype would be recapitulated in *HfeRag1*^{-/-} double mutants. Surprisingly, hepatic iron rose dramatically in *HfeRag1*^{-/-} double mutants, whereas iron levels in the heart and pancreas were not statistically different from *Hfe*^{-/-} single knockout mice (Table 1). The hepatic iron increment in *HfeRag1*^{-/-} was significant ($P < .01$) when compared with *Hfe*^{-/-} single knock-out mice. In contrast, liver iron stores remained similar in $\beta 2mRag1$ ^{-/-} compared with $\beta 2m^{-/-}$ mice.

In the heart, iron concentration in *HfeRag1*^{-/-} double mutants was similar to *Hfe* single knock-out mice (Table 1). In marked contrast, cardiac iron was significantly higher in

$\beta 2mRag1^{-/-}$ compared with $\beta 2m^{-/-}$ mice ($P < .01$) or to the remaining mouse strains ($P < .0005$). Similarly, in the pancreas, $HfeRag1^{-/-}$ did not differ from $Hfe^{-/-}$ mice, whereas $\beta 2mRag1^{-/-}$ mice showed a 3.7-fold increase in iron concentration over $\beta 2m^{-/-}$ single knockout mice ($P < .0001$) and all other mouse strains. Thus, on a standard diet, the phenotype of $HfeRag1^{-/-}$ mice is strikingly different from that of $\beta 2mRag1^{-/-}$, the main difference being the affected organs where introduction of the *Rag1* mutation leads to augmented iron loading.

To further investigate the responses to dietary iron loading in compound mutants, mice were challenged with a 2.5% wt/wt carbonyl iron-supplemented diet for 3 months. In the liver, the highest iron concentration was found in $HfeRag1^{-/-}$ mice, followed by $Hfe^{-/-}$, $\beta 2mRag1^{-/-}$, and $\beta 2m^{-/-}$ mice (Table 1). In the heart and pancreas, $\beta 2mRag1^{-/-}$ mice accumulated more iron than any other mouse strains. These differences were highly significant ($P < .0001$ and $P < .01$, respectively, in the heart and pancreas). Iron accumulation was visible in $\beta 2mRag1^{-/-}$ cardiac myocytes and pancreas sections stained with Prussian blue but remained undetectable in $HfeRag1^{-/-}$ mice (Figure 1).

Thus, the observed phenotype differences between mice lacking both *Hfe* and *Rag1* and mice lacking $\beta 2m$ and *Rag1* persist and are even enhanced after dietary iron challenge. The differences in phenotype are likely due to the lack of other molecules that interact with $\beta 2m$ in $\beta 2m$ -deficient mice. Involvement of other $\beta 2m$ -dependent molecules in iron regulation has previously been suggested by the demonstration of increased hepatic iron in $Hfe\beta 2m$ double-mutant mice compared with single knock outs¹⁰ and by the finding that *MHC-I*-deficient mice have elevated hepatic iron.¹¹ Intriguingly, the phenotype of $\beta 2mRag1$ double-deficient mice considerably resembles juvenile hemochromatosis (JH) in humans¹² and hepcidin-deficient mice¹³ in the severity of iron overload and the involvement of the heart and pancreas. JH is an autosomal recessive disorder, which leads to early-onset, severe iron overload. Heart failure and endocrine manifestations are the most prominent clinical features of JH, whereas liver involvement, although present, is clinically less relevant. Iron accumulation in the liver of hepcidin-deficient mice is not particularly different from $Hfe^{-/-}$ or $\beta 2m^{-/-}$ mice but rises dramatically in the pancreas and heart.¹³

In *Hfe*-deficient mice, hepatic hepcidin mRNA is inappropriately low, and the mice show a blunted response to iron loading.^{14–16} However, $\beta 2m$ -deficient mice have higher hepcidin levels than healthy mice, which is an appropriate, although insufficient response to iron loading.¹⁷ A possible explanation for this difference may reside in the fact that $\beta 2m^{-/-}$ mice are known to be functionally “leaky,” as residual functional *MHC-I* expression can still be detected.^{18,19} By analogy, residual *Hfe* expression may occur in $\beta 2m$ -deficient mice, giving them limited control over iron-induced hepcidin expression. This finding may explain why $Hfe^{-/-}$ and $HfeRag1^{-/-}$ mice accumulate more iron in the liver than do $\beta 2m^{-/-}$ and $\beta 2mRag1^{-/-}$ mice, respectively.

Accumulating evidence suggests that iron does not directly regulate hepcidin expression in hepatocytes^{17,20} and that hepcidin levels may be indirectly regulated by other cells. In fact, hepcidin expression can be induced by interleukin 6 (IL-6),²⁰ a cytokine synthesized predominantly by macrophages and lymphocytes. Thus, lymphocytes may interfere with

hepcidin regulation either directly, by secreting IL-6, or indirectly, through effects on macrophage differentiation and function.²¹ Alternatively, the fact that phenotype differences between *Hfe*- and $\beta 2m$ -deficient mice are evident only after lymphocyte depletion may indicate that lymphocytes represent an important iron storage compartment.

Phenotype differences between *HfeRag1*^{-/-} and $\beta 2mRag1$ ^{-/-} mice may be attributable to the existence of other $\beta 2m$ -dependent, MHC-I-like molecules involved in iron regulation. *Hfe* is predominantly expressed in the liver, with the highest levels of *Hfe* mRNA found in hepatocytes.^{22,23} This is consistent with the finding that *Hfe* deletion primarily affects the liver. Presumably other $\beta 2m$ -dependent molecules might be more relevant in the heart and pancreas, which also express hepcidin.²⁴

In conclusion, the work presented here suggests that alterations in the expression of $\beta 2m$ -dependent, MHC-I-like molecules and lymphocyte numbers may relate to phenotype variation in HH patients.

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Figure 1. Storage of excess iron in the heart and pancreas of mice after 3 months on a diet supplemented with 2.5% wt/wt carbonyl iron (Prussian blue staining, n = 5)
 The animals were 5 months old at the end of the experiment. Heart section from representative $\beta 2mRag1^{-/-}$ (A) and $HfeRag1^{-/-}$ mice (B). Pancreas section from representative $\beta 2mRag1^{-/-}$ (C) and $HfeRag1^{-/-}$ mice (D). Original magnification, $\times 600$.

Table 1

Tissue iron concentration in mice fed a standard or iron-enriched diet (2.5% wt/wt carbonyl iron) for 3 months

Organ	µg iron/g dry wt					
	B6	Rag1 ^{-/-}	Hfe ^{-/-}	HfeRag1 ^{-/-}	β2m ^{-/-}	β2mRag1 ^{-/-}
Standard diet						
	n = 14	n = 9	n = 14	n = 12	n = 9	n = 11
Liver	287 ± 63	298 ± 43	1034 ± 253 [*]	1607 ± 730 ^{†‡}	788 ± 279 [*]	840 ± 292 [*]
Heart	358 ± 52	371 ± 59	409 ± 45	412 ± 90	439 ± 57	586 ± 134 [§]
Pancreas	160 ± 35	151 ± 30	173 ± 45	185 ± 42	178 ± 38	676 ± 276
Iron-enriched diet						
	n = 10	n = 10	n = 10	n = 10	n = 9	n = 11
Liver	4014 ± 1186	4279 ± 549	7276 ± 1247 [*]	12077 ± 4584 ^{†‡¶}	5742 ± 985 ^{§¶}	6118 ± 1462 [*]
Heart	467 ± 70	508 ± 72	500 ± 117	485 ± 84	501 ± 72	1181 ± 265
Pancreas	252 ± 72	287 ± 83	253 ± 23	279 ± 106	283 ± 46	3844 ± 2010 [§]

Mice (females) were 5 months old at the end of the experiment. Data are presented as mean ± SD.

^{*} $P < .005$ compared with B6 or Rag1^{-/-} mice by Student t test.
[†] $P < .01$ compared with Hfe^{-/-} mice by Student t test.
[‡] $P < .001$ compared with β2mRag1^{-/-} mice by Student t test.
[§] $P < .01$ by Student t test compared with all other mouse strains.
^{||} $P < .0001$ by Student t test compared with all other mouse strains.
[¶] $P < .05$ compared with Hfe^{-/-} mice by Student t test.