Brief report

Expression of *Rgmc*, the murine ortholog of hemojuvelin gene, is modulated by development and inflammation, but not by iron status or erythropoietin

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Mutations of hepcidin *(HAMP)* and hemojuvelin *(HJV)* genes have been recently demonstrated to result in juvenile hemochromatosis. Expression of *HAMP* is regulated by iron status or infection, whereas regulation of *HJV* is yet unknown. Using quantitative real-time polymerase chain reaction, we compared expression of *Hamp* and *Rgmc* (the murine ortholog of *HJV*) in livers of mice treated with iron, erythropoietin, or lipopolysaccharide (LPS), as well as during fetal and postnatal development. Iron overload increased *Hamp* expression without effect on *Rgmc* mRNA. Erythropoietin decreased *Hamp* mRNA, but *Rgmc* expression was unchanged. *Hamp* mRNA level decreased after birth by 4 orders of magnitude, without significant changes in *Rgmc* expression. Administration of LPS elevated *Hamp* mRNA levels, while markedly decreasing hepatic *Rgmc* mRNA levels (to ~5% after 6 hours). The responses of *Hamp* and *Rgmc* were quite different and suggested that human *HJV* expression could be modulated by inflammation. (Blood. 2004;104:4308-4310)

Introduction

During the past few years, a number of new genes participating in iron metabolism have been identified. Mutations in 2 genes, hepcidin (*HAMP*)[1] and hemojuvelin (*HJV*)[2,3] have been shown to result in juvenile hemochromatosis. Hepcidin, a small peptide synthesized predominantly in hepatocytes, is emerging as an important regulator of iron homeostasis, which inhibits iron absorption from the intestine and iron release from macrophages. Hepcidin expression is controlled by iron status and erythropoietic activity, as well as by inflammatory stimuli[4]; inappropriate expression of hepcidin probably plays a role in the pathophysiology of hereditary hemochromatosis and anemia of inflammation.[5] On the other hand, the function and regulation of hemojuvelin are at present unknown. Prior to identification of the *HJV* gene, it was speculated that its product could function in the hepcidin signaling pathway, possibly as a hepcidin receptor,[5] whereas a current concept proposes that hemojuvelin could modulate hepcidin expression.[2,6]

Orthologs of the *HJV* gene have been identified in zebrafish, mice, and rats[2]; the mouse *HJV* ortholog *Rgmc* is, like *HJV*, expressed mainly in skeletal muscle, heart, and liver.[7] The aim of the present study was to examine whether experimental conditions known to influence hepatic *Hamp* expression in mice will also change hepatic *Rgmc* mRNA levels and to compare possible similarities or discrepancies in the regulation of these 2 genes.

Study design

All animal experiments were approved by the Animal Care Committee of the First Faculty of Medicine. Male C57BL/6N mice (Charles River, Sulzfeld, Germany) were treated with lipopolysaccharide (LPS, serotype 0111:B4, 1 mg/kg intraperitoneally; Sigma Aldrich, Prague, Czech Republic) and humanely killed by cervical dislocation after 90 minutes or 6 hours. Iron overload (600 mg/kg) was induced by a single subcutaneous injection of iron polyisomaltosate (Ferrum Lek; Lek, Ljubljana, Slovenia); mice were humanely killed 1 week or 3 weeks after application. Erythropoietin (EPREX 10 000, Cilag AG, Schaffhausen, Switzerland) was administered at 50 U/mouse for 4 days, and mice were killed on day 5.

Liver RNA was extracted using RNABlue (Top-Bio, Prague, Czech Republic), treated with DNase I (Gibco, Life Technologies, Gaithersburg, MD), and 1 μg total RNA was reverse transcribed by the RevertAid First-Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania).

Levels of *Hamp* and *Rgmc* mRNA were determined by real-time polymerase chain reaction (PCR) on a Roche LightCycler instrument, using LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). Primer sequences were: β-actin forward 5′-GACATGGAGAAGATCTGGCA-3′, reverse 5′-GGTCTTTACGGATGTCAACG-3′; *Hamp* forward 5′-CTGAGCAGCACCACCTATCTC-3′, *Hamp* reverse 5′-TGGCTCTAGGCTATGTTTTGC-3′; *Rgmc* forward 5-CCCAGATCCCTGTGACTATGA -3, *Rgmc* reverse 5-CAGGAAGATTGTCCACCTCAG -3. *Rgmc* primers were designed to amplify a sequence from exons 3 and 4 of *Rgmc* DNA.[2] Because 5 possible splice variants have been reported for human *HJV*,[2] data from representative experiments were verified with 2 alternative primer pairs targeting sequences from exon 1 and exon 4, respectively.

Target mRNA amounts were normalized to β-actin mRNA, and calculated as described previously,[8] assuming exact doubling of amplified cDNA in each PCR cycle. Results are expressed as the relative amount of target mRNA in comparison to β-actin mRNA (Figure 1), or as percent of β-actin–normalized target mRNA in experimental groups versus control groups (Table 1). Statistical analyses were performed using the 2-tailed *t* test.

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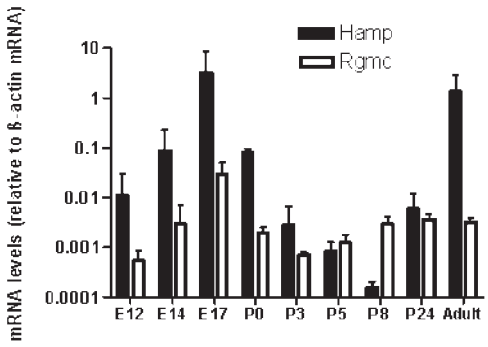

Figure 1. Levels of hepatic *Hamp* and *Rgmc* mRNA during prenatal and postnatal liver development. Liver samples were obtained at days 12.5, 14.5, and 17.5 of embryonic development (E12-E17), within 8 hours after birth (P0), during postnatal developmental days (P3-P24), or from adult mice aged 2 to 3 months. Values represent the amount of target mRNA compared to β-actin mRNA (means ± SD; n = 3).

Results and discussion

Real-time PCR allowed detection of *Hamp* and *Rgmc* mRNAs in adult as well as in fetal liver samples, with the amount of *Hamp* mRNA exceeding *Rgmc* mRNA in adult liver by more than 1 order of magnitude. Tissue-specific expression of *Rgmc* agreed with published data[2] for human *HJV* (results not shown).

Hepcidin expression increases during iron overload[9] and decreases following erythropoietin administration.[10] Subcutaneous injection of a single dose of iron (600 mg/kg) increased the amount of *Hamp* mRNA more than 4-fold when measured 1 week or 3 weeks after treatment; however, the amount of hepatic *Rgmc* mRNA was not significantly changed (Table 1). Administration of erythropoietin for 4 days decreased *Hamp* mRNA levels to less than 5% of control values, again without a statistically significant effect on hepatic *Rgmc* mRNA levels. These results indicate that, in contrast to *Hamp* mRNA, *Rgmc* mRNA content is not influenced by iron overload or increased erythropoiesis.

It has been previously shown that *HJV* is expressed in fetal liver.[2] Because *Hamp* expression displays significant changes during both prenatal and postnatal periods,[11] we examined whether the expression pattern of *Hamp* and *Rgmc* would be similar. Although both *Hamp* and *Rgmc* mRNAs increased during embryonic liver development, a striking difference was noted in the postnatal expression of the 2 genes (Figure 1). *Hamp* mRNA dropped by 4 orders of magnitude after birth and remained low until weaning, whereas *Rgmc* mRNA levels decreased only to about 30% at postnatal day 3 and reached adult levels at day 8. These results show that the 2 genes are regulated differently during the postnatal period.

In addition to iron homeostasis, expression of hepcidin is also regulated by inflammatory cytokines.[12,13] Hepcidin was originally described as an antimicrobial peptide,[14] and the link between hepcidin and the immune response has been further strengthened by the observations that urinary hepcidin levels rise by 2 orders of magnitude in patients with infections.[2,12] Human hepcidin has therefore been characterized as an acute-phase protein,[12] whose induction is probably responsible for the changes in iron homeostasis during anemia of inflammation. Accordingly, an increase of hepatic *Hamp* mRNA has been documented in experimental animals treated with LPS.[9,13,15] As shown in Table 1, a single injection of LPS slightly increased hepatic *Hamp* mRNA levels, measured 6 hours after LPS administration, while decreasing hepatic *Rgmc* mRNA levels by more than 1 order of magnitude. Thus, the response of *Hamp* and *Rgmc* to inflammatory stimuli appears to be fundamentally different.

Table 1. Relative amounts of liver *Hamp* and *Rgmc* mRNA after administration of iron, erythropoietin, or LPS

Treatment	*Hamp* mRNA, % of control	*Rgmc* mRNA, % of control
Iron, 1 wk	484 ± 26*	87 ± 16
Iron, 3 wks	408 ± 91*	79 ± 48
Erythropoietin	3 ± 2*	81 ± 19
LPS, 90 min	166 ± 66	53 ± 20
LPS, 6 h	128 ± 25	1 ± 1*

Iron was administered as a single 600-mg/kg dose 1 or 3 weeks prior to death, erythropoietin as a 50-U daily dose for 4 days preceding the day of death, and LPS as a single 1-mg/kg dose 90 minutes or 6 hours prior to death. Data are expressed as means ± SD (n = 4).

*Statistically significant compared to control group ($P < .05$).

The link between iron metabolism and inflammation has been well established, with expression of many of the proteins involved in iron metabolism responding to infection or LPS treatment.[16-18] LPS treatment decreases plasma iron concentrations and generally down-regulates iron export from the cells. In this respect, it is interesting to note that the response of *Rgmc* to LPS resembles the response of the *Slc40a1* gene,[18,19] which encodes the iron exporter ferroportin1. Both hepatic *Rgmc* and *Slc40a1* mRNAs show a similar decrease following administration of LPS to mice, with only slight changes at 90 minutes and substantial down-regulation 6 hours after LPS administration.

In conclusion, this study shows that, despite the postulated functional link between hepcidin and hemojuvelin,[2,20] murine *Hamp* and *Rgmc* genes respond differently to changes in iron status or inflammation. Although the results are based on mRNA quantification only, and as such do not reflect possible posttranscriptional regulation, they nevertheless indicate that whereas *Hamp* mRNA sensitively reacts to iron overload or increased erythropoiesis, hepatic *Rgmc* mRNA content is not significantly changed. In addition, hepatic *Hamp* and *Rgmc* mRNA levels respond in an opposite manner to bacterial LPS challenge. The decrease of hepatic *Rgmc* mRNA level following LPS treatment suggests that human *HJV* expression could be down-regulated during inflammation.

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