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Inflammatory regulation of iron metabolism during thioacetamide-induced acute liver injury in rats

Takeshi Izawa *,[1], Hiroshi Murakami [1], Kavindra Kumara Wijesundera, Hossain M. Golbar, Mitsuru Kuwamura, Jyoji Yamate

Laboratory of Veterinary Pathology, Osaka Prefecture University, Japan

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ABSTRACT

Systemic iron homeostasis is tightly regulated by the interaction between iron regulatory molecules, mainly produced by the liver. However, the molecular mechanisms of iron regulation in liver diseases remain to be elucidated. Here we analyzed the expression profiles of iron regulatory molecules during transient iron overload in a rat model of thioacetamide (TAA)-induced acute liver injury. After TAA treatment, mild hepatocellular degeneration and extensive necrosis were observed in the centrilobular region at hour 10 and on day 1, respectively. Serum iron increased transiently at hour 10 and on day 1, in contrast to hypoferremia in other rodent models of acute inflammation reported previously. Thereafter, up-regulation of hepcidin, a central regulator of systemic iron homeostasis, was observed in hepatocytes on day 2. Expression of transferrin receptor 1 and ferritin subunits increased to a peak on day 3, followed by increases in liver iron content and stainable iron on day 5, in parallel with regeneration of hepatocytes. Histopathological lesions and hepatocellular iron accumulation disappeared until day 10. The hepcidin induction was preceded by activation of IL6/STAT3 pathway, whereas other pathways known to induce hepcidin were down-regulated. IL6 was expressed by MHC class II-positive macrophages in the portal area, suggestive of dendritic cells. Our results suggest that IL6 released by portal macrophages may regulate hepatocyte hepcidin expression via STAT3 activation during transient iron overload in TAA-induced acute liver injury.

1. Introduction

The liver plays a central role in the regulation of iron metabolism. It is the major storage site for iron and produces a variety of regulatory molecules. Hepcidin (HAMP) is a central regulator of systemic iron homeostasis, mainly synthesized by hepatocytes (Ganz, 2011; Hentze et al., 2010). HAMP regulates duodenal iron absorption, macrophage iron release and tissue iron storage by binding, internalization and degradation of ferroportin (FPN), the iron exporter expressed in iron-releasing cells. Circulating iron is bound to transferrin (TF), a major vehicle for iron delivery into cells via transferrin receptor 1 (TFR1) (Hentze et al., 2010). Cytoplasmic iron is sequestered by ferritin, consisting of 24 subunits of heavy (FTH) and light (FTL) chains (Hentze et al., 2010). Cellular iron is exported from cells into blood by FPN and then bound to TF. Since iron is continuously recycled by the body, coordination between these molecules is essential for systemic iron homeostasis.

Disruption of iron homeostasis is involved in the progression of chronic liver diseases. In chronic hepatitis C, patients with poor outcomes have higher scores of stainable iron in the liver than those without outcomes (Lambrecht et al., 2011). In patients with non-alcoholic fatty liver disease (NAFLD), increased iron deposition is associated with the severity of the disease (Nelson et al., 2011, 2012; Valenti et al., 2010). Oxidative stress is one of the pathogenetic mechanisms of iron-mediated tissue injury, since excess iron can generate reactive oxygen species via the Fenton reaction. A recent study suggested that iron deposition in reticuloendothelial (RES) cells is associated with advanced steatohepatitis, increased apoptosis, and increased oxidative stress in NAFLD (Maliken et al., 2013).

Molecular mechanisms of systemic iron regulation are being elucidated by using murine models of hemochromatosis, especially genetically-modified mice that lack iron regulatory molecules (Chua et al., 2011; Corradini et al., 2011a,b; Hentze et al., 2010; Pietrangelo, 2010; Ramos et al., 2011). However, these models have no apparent liver disease (Pietrangelo, 2010). Repeated hepatocellular injury and associated inflammation are most likely a

* Corresponding author at: Laboratory of Veterinary Pathology, Osaka Prefecture University, Rinku-Orai Kita, Izumisano, Osaka 598-8531, Japan.
Tel.: +81 72 463 5346; fax: +81 72 463 5346.
E-mail address: izawa@vet.osakafu-u.ac.jp (T. Izawa).
[1] These authors contributed equally to this work.

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cause of iron dysregulation in chronic liver diseases, although the underlying mechanisms remain obscure. However, the relationship between iron regulatory molecules and inflammation after hepatocellular injury is still unclear. In this study, we analyzed the expression profiles of iron regulatory molecules during transient iron overload in a rat model of acute liver injury. We also investigated early inflammatory responses to clarify the role of inflammation in iron regulation with acute hepatocellular damage.

2. Materials and methods

2.1. Animals

Seven week-old male F344 rats weighing 138 to 176 g (Charles River Laboratories Japan, Yokohama, Japan) were injected intraperitoneally with thioacetamide (TAA) (300 mg/kg body weight; Wako Pure Chemicals, Osaka, Japan). Control rats were administered an equal volume of saline. Rats were euthanized by isoflurane anesthesia, and blood and liver were collected at hour 10 and on days 1, 2, 3, 5, 7 and 10 after injection ($n = 4$ in each group). Rats were maintained in a room with controlled temperature and 12-h light–dark cycle. Food and water were provided ad libitum. All experiments were performed according to the Guidelines for Animal Experimentation of Osaka Prefecture University.

2.2. Histopathology and biochemical analyses

Liver samples from the left lateral lobe were fixed in 10% neutral-buffered formalin, embedded in paraffin, cut at 5 μm and stained with hematoxylin and eosin (HE) for histopathological examination. Hepatic iron deposition was analyzed using diaminobenzidine (DAB)-enhanced Perls' stain as described previously (Izawa et al., 2010a; Meguro et al., 2003). Serum iron was measured by SRL Inc. (Tokyo, Japan). Liver iron content was analyzed using atomic absorption spectrophotometry (SRL Inc.).

2.3. Immunohistochemistry

Liver was fixed with periodate-lysine-paraformaldehyde solution and embedded in paraffin by acetone-methyl benzoate-xylene method (PLP-AMeX method) (Golbar et al., 2011; Suzuki et al., 2002). Primary antibodies used were as follows: rabbit anti-CD3 (1:200; Dako, Glostrup, Denmark) for T cells, mouse anti-CD79α (ready to use; clone HM57; Nichirei, Tokyo, Japan) for B cells, rabbit anti-granzyme B (1:500; Spring Bioscience, CA, USA) for natural killer (NK) cells, mouse anti-MHC class II (1:500; clone OX-6; AbD Serotec, Oxford, UK) for antigen-presenting macrophages, rabbit anti-ferritin (1:500; Sigma–Aldrich, MO, USA), mouse anti-transferrin receptor (1:250; clone OX-26; AbD Serotec), and mouse anti-heme oxygenase-1 (1:200; clone GTS-3; Takara, Shiga, Japan). After incubation with the primary antibodies, sections were treated with peroxidase-conjugated secondary antibody (Simplestain MAX-PO; Nichirei). Signals were visualized with DAB substrate kit (Nichirei).

2.4. In situ hybridization

For preparation of riboprobe, a 355-bp fragment of HAMP cDNA (NM_053469, nucleotide positions 13–367) and a 569-bp fragment of IL6 cDNA (NM_012589, nucleotide positions 179–747) were amplified by PCR, and subcloned into pGEM T-easy vector (Promega, WI, USA). DIG-labeled antisense and sense probe were synthesized with SP6 and T7 RNA polymerase (Roche, Penzberg, Germany). For preparation of tissue sections, liver was removed and immediately frozen at −80 °C. Ten-μm fresh frozen sections were cut on a cryostat. In situ hybridization was performed as previously described (Izawa et al., 2008, 2010b). DIG-labeled riboprobes were placed on each slide and the slides were incubated overnight at 65 °C. RNA hybrids were immunostained with alkaline phosphatase-conjugated anti-DIG antibody (1:100; Roche) overnight at 4 °C and were visualized with NBT/BCIP substrate (Roche). For identification of IL6-expressing cells, serial sections were subjected to IL6 in situ hybridization and MHC II immunohistochemistry.

2.5. Real-time reverse transcription (RT)-PCR

Liver samples from the right medial lobe were immersed in RNAlater reagent (Qiagen, Hilden, Germany) overnight at 4 °C and stored at −80 °C before use. Total RNA was isolated with the SV Total RNA isolation system (Promega, WI, USA) according to the manufacturer's instructions. Two μg of total RNA was transcribed with Superscript VILO reverse transcriptase (Life Technologies, CA, USA). Real-time PCR was performed using TaqMan gene expression assays (Life Technologies) in a Linegene system (Bioflux, Japan). The probe sets used are listed in Table 1. The data were analyzed using the comparative C_t method ($\Delta\Delta C_t$ method).

2.6. Western blot

Liver samples from the right medial lobe were homogenized in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 1 mM NaF, 100 μM Na_3VO_4, 1 mM PMSF and 1× proteinase inhibitor cocktail; Nakarai Tesque, Kyoto, Japan). After centrifugation at 13,000 × *g* for 10 min, the supernatant was mixed with an equal volume of 2×

Table 1
Probe sets used in this study.

Symbol	Name	Function	Assay ID
HAMP	Hepcidin	Central regulator of iron metabolism	Rn00584987_m1
TF	Transferrin	Iron transport	Rn01445482_m1
TFR1	Transferrin receptor	Iron uptake	Rn01474701_m1
DMT1	Divalent metal ion transporter 1	Iron uptake	Rn01533109_m1
FTL	Ferritin, light polypeptide	Intracellular iron storage	Rn00821071_g1
FTH	Ferritin, heavy polypeptide	Intracellular iron storage	Rn00820640_g1
FPN	Ferroportin	Iron export	Rn00591187_m1
HMOX1	Heme oxygenase 1	Degradation of heme, antioxidant	Rn01536933_m1
HFE	Hemochromatosis	Regulation of hepcidin expression	Rn00583982_m1
TFR2	Transferrin receptor 2	Regulation of hepcidin expression	Rn01481654_m1
BMP6	Bone morphogenetic protein 6	Regulation of hepcidin expression	Rn00432095_m1
HJV	Hemochromatosis type 2 (juvenile)	Regulation of hepcidin expression	Rn01265685_g1
IL6	Interleukin 6	Regulation of hepcidin expression	Rn01410330_m1
STAT3	Signal transducer and activator of transcription 3	Regulation of hepcidin expression	Rn00562562_m1
18s rRNA	18s ribosomal RNA	Internal control	Hs99999901_s1