Hepcidin plays a negative role in liver regeneration

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Hepcidin is a small peptide produced by the liver, is a key regulator of iron absorption and homeostasis [4,5]. Hepcidin regulates extracellular iron by controlling its intestinal absorption and recycling by macrophages [6]. Consistent with its role as a negative regulator of iron absorption, hepcidin expression is up-regulated in response to iron overload [5]. Hepcidin is also induced by inflammation and the infection of pathogenic bacteria [7–9]. Our previous study demonstrated that hepcidin expression is regulated in livers of mice infected with Plasmodium berghei [10]. Up-regulation of hepcidin by inflammation is mainly mediated by pro-inflammatory cytokines IL-6 and IL-1 produced from macrophage [7,8]. Conversely, the expression of hepcidin is inhibited during erythropoiesis and hypoxia [11].

The alteration of hepcidin expression has been reported in response to hepatic damage, such as PH and CCl4 administration [12,13]. An increase in hepcidin expression is observed with a maximum expression between 8 and 16 h in 70% PH rat models. IL-6 probably is one of the principle mediators of hepcidin expression during liver regeneration [12]. Furthermore, a recent study has demonstrated that HGF suppresses hepatic hepcidin synthesis, in part through the phosphoinosidade 3-kinase (PI3K) and the mitogen-activated protein kinase (MEK)/extracellular signal regulated kinase (ERK) pathways [14]. Since both IL-6 and HGF are the two key modulators of liver regeneration, we examined the effect of hepcidin on hepatocyte proliferation.

Materials and Methods

Experimental animals and hepatectomy

Male BALB/c strain mice, 6 – 8 weeks old, were purchased from the Animal Center, Kunming Medical University (Kunming, China). The animals were fed one of the two diets: (i) control diet (LM-485 chow; Harlan Teklad,
Madison, USA) or (ii) high-iron diet (LM-485 chow supplemented with carbonyl iron; 8.3 g/kg) [15]. After being fed with the diets for 3 weeks, the mice underwent PH. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The protocol of the experiments was approved by the Animal Care and Use Committee of Yunnan University.

The mice underwent PH to remove 70% of total liver mass (left lateral, left median, and right median lobes) under sodium pentobarbital anesthesia (75 μg/g body weight). After the surgical operation, the animals were allowed to recover on a heating pad and later were returned to their cages and fed ad libitum. At the indicated time points, the mice were sacrificed by exsanguination from abdominal aorta. The serum and a part of the liver were harvested and frozen at −80°C. The remaining liver was fixed in formalin, dehydrated in graded ethylic alcohol, and embedded in paraffin.

Treatment with anti-HGF neutralizing antibodies
Rabbit anti-mouse HGF neutralizing antibodies (Santa Cruz Biotech, Santa Cruz, USA) or isotype control antibodies (rabbit IgG; Amersham Biosciences, Piscataway, USA) were injected (1 mg/kg) intravenously into the tail vein of mice 2 h before PH. Injection of the antibodies was repeated once a day for the next 3 days.

Animal infection with lentiviruses
Lentivirus vector expressing mouse hepcidin-1 and lentivirus particles were obtained from Shanghai R&S Biotechnology (Shanghai, China) [10]. Viral supernatants were injected intravenously into the tail vein of the mice. Each mice received ~1 × 10^8 transducing units of the respective infectious viral particles. The mice underwent PH 7 days after injection.

Quantitative real-time reverse transcription-polymerase chain reaction analysis
Mouse liver tissues (50 mg) were homogenized in liquid nitrogen. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA). Random-primed cDNAs were generated by reverse transcription of total RNA samples with SuperScript II (Invitrogen). Real-time polymerase chain reaction analysis was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) using SYBR Premix-Ex Tag™ (TaKaRa, Dalian, China). The primers used were: hepcidin, 5’-AGAGCTGCAGCCTTTGCAC-3’ (forward); 5’-GAAGATGCAGATGGGGAAGT-3’ (reverse); and β-actin, 5’-AGTTGAGCGTTGACATCCGTA-3’ (forward); 5’-GCCAGAGCAATCTCCTCCTC-3’ (reverse).

Detection of serum cytokines, transaminases, and iron levels
Serum levels of IL-6 and HGF in mice were determined by ELISA kits (R&D Systems, Shanghai, China). The assays were performed according to the manufacturer’s protocols. Serum levels of aminotransferase (AST), alanine aminotransferase (ALT), and iron levels were measured using a standard clinical automatic analyzer (Hitachi 7060; Hitachi, Tokyo, Japan).

Immunohistochemistry
Liver DNA synthesis was measured by immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and bromodeoxyuridine (BrdU) incorporation. For PCNA staining, mouse anti-PCNA antibodies (Santa Cruz Biotech) were used. For BrdU staining, the mice were injected intraperitoneally with 100 mg/kg BrdU (Amersham Biosciences) 2 h before liver harvesting. BrdU incorporation was detected by mouse anti-BrdU antibodies (Ab3; Thermo Scientific, Fremont, USA). A hepatocyte labeling index (percentage of positive nuclei) was measured by counting 2000 nuclei in 10 high-power fields per animal at ×200 magnification.

Statistical analysis
Statistical difference between the groups was analyzed by using one-way analysis of variance followed by Student–Newman–Keuls test. Pearson correlation coefficient analysis was performed to determine the association between hepcidin and IL-6 or HGF. P values <0.05 were considered statistically significant. Statistical analyses were performed using SPSS11.1 software.

Results
Serum IL-6 and HGF are correlated with hepatic hepcidin expression after PH
The expression of hepatic hepcidin and iron status indicators were determined after 70% PH. As shown in Fig. 1(A), a significant increase in hepcidin expression was observed in the liver from 4 to 12 h after 70% PH. The expression of hepatic hepcidin declined to the baseline level at 24 h and attained the lowest level at 48 h after 70% PH, which was only 29% of the control value (sham operation).

Since IL-6 and HGF regulated the expression of hepcidin, we determined the serum levels of IL-6 and HGF. The IL-6 levels in serum were significantly increased from 2 to 12 h, with a maximal peak at 6 h after 70% PH [Fig. 1(B)], and declined to the baseline level at 24 h after 70% PH. In contrast to IL-6, the serum HGF levels were significantly increased at 6 h after 70% PH [Fig. 1(C)]. Then, the HGF levels reached the highest level at 12 h, and declined slowly but remained significantly higher at 72 h after PH.
To investigate the potential contribution of IL-6 and HGF to the expression of hepcidin, correlations between serum levels of IL-6 or HGF and mRNA levels of hepcidin were measured. We found that there was a positive correlation of hepcidin expression with concentrations of IL-6 ($P < 0.01$). In contrast, hepcidin mRNA levels were inversely associated with concentrations of HGF ($P < 0.01$).

Neutralization of HGF in mice restores hepcidin expression
A recent study has demonstrated that HGF suppresses hepatic hepcidin synthesis in mouse primary cultured hepatocytes, in part through the PI3 kinase and the MEK/ERK kinase pathways [14]. To further confirm the role of HGF in hepcidin expression, the mice received an intravenous injection of anti-HGF neutralizing antibodies or an equal dose of normal IgG antibodies. As shown in Fig. 1(C), mice receiving anti-HGF neutralizing antibodies exhibited a significant decrease in the levels of serum HGF. We found that the hepcidin expression in the livers of mice with treatment of anti-HGF neutralizing antibodies was significantly higher than that in control mice receiving normal IgG antibodies at 48 and 72 h after PH [Fig. 2]. These results suggest that HGF inhibits the expression of hepcidin during liver regeneration.

Gene delivery of hepcidin inhibits liver regeneration
Unlike human hepcidin, there are two isoforms of hepcidin in the mouse. However, only hepcidin-1 is involved in iron metabolism [16]. To study the role of hepcidin in liver regeneration, we overexpressed hepcidin-1 in mice via lentiviral vector-mediated gene transfer. Injection of lentiviral vector carrying hepcidin-1 (pLenti-Hep-EGFP) into mice via the tail vein led to a more than 3-fold increase in hepcidin expression in the livers at Day 7 [10]. After 70% PH, DNA synthesis was determined after lentiviral gene delivery. A quantitative time course of PCNA staining revealed that overexpression of hepcidin-1 caused a significant delay of hepatocyte proliferation [Fig. 3(A–F)]. The levels of PCNA staining in mice receiving pLenti-Hep-EGFP were significantly lower than those in control mice at 36 and 48 h after PH [Fig. 3(G,H)]. These results indicate that overexpression of hepcidin-1 delays liver regeneration.

To investigate whether overexpression of hepcidin-1 causes hepatic injury, we determined the serum levels of transaminases. We found that the levels of serum ALT and AST in mice receiving pLenti-Hep-EGFP were similar to those in control mice before and after PH (data not shown). These data suggested that overexpression of hepcidin-1 does not induce hepatic injury after PH.
Iron overload restores liver regeneration inhibited by hepcidin

To investigate whether the role of hepcidin in liver regeneration is related to iron status, we first compared the concentrations of iron in mice receiving pLenti-Hep-EGFP with those in the control mice. We found that the serum iron concentrations were significantly decreased from 6 to 12 h and returned to the basal level at 24 h after 70% PH [Fig. 4(A)]. The levels of serum iron maintained high levels from 48 to 72 h after 70% PH, compared with those in control mice. Next, we tested whether the overload of iron could rescue the inhibition of liver regeneration by overexpression of hepcidin-1. After being fed with a control diet supplemented with carbonyl iron (8.3 g/kg) for 3 weeks, the mice exhibited...
overexpression. The iron-regulatory hormone hepcidin plays a central role in iron homeostasis [4,5]. It has been well established that hepcidin expression is significantly induced by iron overload and inflammation [7–9], and is down-regulated in response to anemia and hypoxia [11]. Accumulating evidence indicates that hepcidin expression is also regulated in the liver in response to hepatic injury [12,13]. However, little is known about the role of hepcidin in liver regeneration. In this study, we demonstrate that hepcidin suppresses liver regeneration.

Our results demonstrate that hepcidin expression is up-regulated in the acute-response phase during liver regeneration and down-regulated during the late stage of liver regeneration (from 48 to 72 h after PH), which is consistent with the other observations [13]. Both in vitro and in vivo studies demonstrated that IL-6 induces hepcidin expression by the activation of STAT3 in hepatocytes and liver, respectively [12,17]. Since the serum levels of IL-6 are significantly elevated, Mollbrink et al. [13] suggested that IL-6 is responsible for the up-regulation of hepcidin expression, probably by inducing STAT3 protein levels during the acute-phase response after PH. However, the mechanism underlying down-regulation of hepcidin expression in the regenerating liver remains unknown. HGF suppresses hepatic hepcidin synthesis in hepatocytes [14], indicating a potential role of HGF in hepcidin expression during liver regeneration. In the current study, correlation analysis reveals an inverse relationship between the serum levels of HGF and hepatic hepcidin expression. Furthermore, the inhibition of hepcidin expression in the livers of mice after PH was significantly restored after the treatment of anti-HGF antibodies. Based on these results, we suggest that HGF is responsible for the suppression of hepcidin expression during liver regeneration.

A previous study has demonstrated that the treatment of HGF in the cirrhotic liver of mice effectively promotes liver regeneration [18]. As mentioned above, the expression of hepcidin is reduced which is mediated by HGF in the regenerating liver. These results imply that hepcidin plays a negative role in liver regeneration after the acute-phase response is completed. Indeed, a previous study has indicated that hepcidin inhibits proliferation and survival of erythroid progenitor cells [19]. In this study, our results demonstrate that overexpression of hepcidin-1 in mice inhibits hepatocyte proliferation in the regenerating liver. However, hepcidin-1 overexpression does not induce an increase in AST levels. Thus, enhanced vulnerability of the livers to surgical insult is unlike-ly to be a prima facie cause of hepatocyte proliferation inhibition by hepcidin. Recently, An et al. [20] demonstrated that chronic iron overload accelerates liver regeneration in rats after PH, probably due to the shortening of the G0–G1 transition. Since hepcidin reduces the levels of serum iron, the limitation of iron availability by overexpressing hepcidin is probably harmful to the cell cycle of the hepatocytes. Indeed, we demonstrate that iron overload restores hepatocyte proliferation after PH in mice overexpressing hepcidin-1. Thus, restoring iron levels by down-regulation of hepcidin may reflect an increased demand for iron during the late stage of liver regeneration.

In summary, the current study demonstrates that the inhibition of hepatic hepcidin expression is mediated by HGF at the late stage of liver regeneration. The down-regulation of hepcidin expression is beneficial for hepatocyte proliferation, which is probably associated with restoring iron levels after PH. These data may help us to further understand the role of hepcidin and iron in liver regeneration.

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References