Table 3. Competing Risk Analysis for Onset of HCC and Death

		Cox analysis			Fine and Gray analysis		
Event	Factors	Relative risk	95% confidence interval	*P* value	Relative risk	95% confidence interval	*P* value
HCC	BCAA supplementation	0.43[a]	0.21–0.90	.026	0.45[a]	0.24–0.88	.019
	AFP	1.01	1.00–1.02	.003	1.01	1.01–1.02	<.001
	TIBC	1.01	1.00–1.01	.006	1.01	1.00–1.01	.037
	BCAA-to-tyrosine ratio	0.74	0.56–0.99	.040	0.74	0.57–0.97	.029
Death	BCAA supplementation	0.002[a]	3.09E^{-05}–0.11	.007	0.009[a]	0.0002–0.365	.015
	Child–Pugh score	3.81	1.72–8.44	.003	2.78	1.45–5.32	.003
	BUN	1.04	1.02–1.06	<.001	1.03	1.02–1.04	<.001
	Platelet count	0.64	0.46–0.90	.014	0.69	0.53–0.89	.011
	Male sex	0.08	0.01–0.56	.014	0.22	0.05–0.90	.042
	Iron	0.96	0.93–0.98	.002	0.98	0.95–1.01	.246
	HOMA-IR	1.15	1.02–1.30	.029	1.05	0.90–1.23	.569

[a]Relative risk is adjusted by other covariates.

Discussion

There has been an increasing body of evidence to support the potential benefits of BCAA supplementation in cirrhotic patients in terms of reducing the risk of specific complications of liver cirrhosis. However, a BCAA supplementation–derived reduction in risk of overall mortality and/or development of HCC has not been demonstrated. Our study with competing risk analysis yielded several important findings in this regard. Most notably, BCAA supplementation was a significant negative risk factor for mortality. In addition, the present study found that BCAA supplementation significantly reduced the risk of hepatocarcinogenesis. We also made the novel observation that the serum BCAA-to-tyrosine ratio is independently associated with the onset of HCC in cirrhotic patients.

We showed that BCAA supplementation significantly reduced the risk of hepatocarcinogenesis in cirrhotic patients. Our findings are in contrast to those of 2 previous studies in which a preventive effect of BCAA supplementation on hepatocarcinogenesis was not observed.[10,11] Although the reason for this discrepancy is unclear, the following are possible explanations. In an Italian study performed in 1996, the study period was 1–15.5 months,

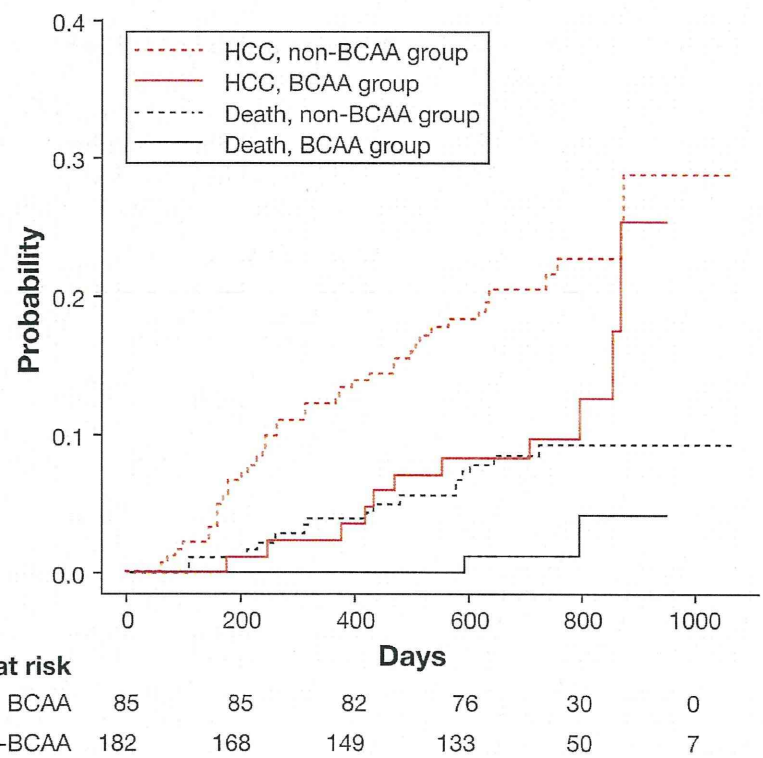

Figure 1. Cumulative incidences of HCC (*red lines*) and death (*black lines*) between BCAA and non-BCAA groups. *Solid lines* indicate the BCAA group, and *dashed lines* indicate the non-BCAA group.

and HCC developed in 5 patients.[10] The low rate of HCC onset would make it nearly impossible to observe a benefit in the treatment arm without a much larger number of events. In a Japanese study conducted in 1997, the obesity rate (BMI >25 kg/m^2) was 28.3%,[11] whereas the obesity rate in our study was 35.0%. BCAA supplementation is known to improve hepatic steatosis and reduce hepatic expression of insulin-like growth factors and the insulin-like growth factor-1 receptor, leading to suppression of obesity-related hepatocarcinogenesis in mice.[21] A previous study showed the preventive effect of BCAA supplementation on hepatocarcinogenesis in cirrhotic patients with obesity.[22] Although the event number is too small to perform a multivariate analysis stratified by BMI in the present study, the higher obesity rate could be a reason for the observed preventive effect of BCAA supplementation on hepatocarcinogenesis.

Another novel aspect of our study is the demonstration of a survival benefit for BCAA supplementation in cirrhotic patients. Chronic hepatic failure is one of the major causes of death in cirrhotic patients, with previous studies observing that hepatic failure is significantly prevented by BCAA supplementation.[10,11] In accordance with these previous reports, our results also suggest that BCAA supplementation prevents chronic hepatic failure and subsequent death. BCAAs are reported to promote the production of hepatocyte growth factor in hepatic stellate cells, asialoscintigraphic removal, and liver regeneration.[12,13] Thus, there are several possible mechanisms by which BCAA supplementation may suppress the progression of liver failure.

Bacterial infection increases mortality 4-fold in cirrhotic patients[23] and is an important issue in the management of cirrhosis.[16] In this study, bacterial infection accounted for 25.0% (4 of 16) of all causes of death and was a major cause of death along with hepatic failure in the non-BCAA group. However, no subject died of bacterial infection in the BCAA group, suggesting that BCAA supplementation prevented bacterial infection and consequent death. The preventive effects of BCAA supplementation on bacterial infection have been reported in liver transplant recipients.[24] In cirrhotic patients, BCAA supplementation increases lymphocyte counts and their natural killer activity[25] and improves the phagocytic function of neutrophils.[1,14] In addition, BCAAs reverse functional impairment and improve the maturation of dendritic cells, leading to an increase in interleukin-12 production.[15] Thus, BCAAs may exert a survival benefit through prevention of bacterial infection by up-regulation of immune functioning.

The final potentially important observation in this study was the predictivity of the BCAA-to-tyrosine ratio for the development of HCC. Decreased serum albumin levels have been reported to be a significant risk factor associated with HCC.[26] Although the serum albumin level was not a risk factor for HCC in this study, the BCAA-to-tyrosine ratio is a significant predictor of a decrease in serum albumin levels in patients with chronic liver disease.[27] In addition, a low BCAA-to-tyrosine ratio is important in the pathogenesis of insulin resistance, which is a known risk factor for HCC.[1] Moreover, our study showed that BCAA supplementation, which increases the BCAA-to-tyrosine ratio, was a negative risk factor for HCC. Taken together, these findings suggest that the BCAA-to-tyrosine ratio may be a useful predictor of the occurrence of HCC in cirrhotic patients.

Because BCAA supplementation has a bitter taste, compliance with BCAA supplementation is generally poor. In this study, the compliance rate was 73.9%, which was about 12% lower than the previous Japanese study (86%).[11] Although the reason for the difference is unclear, a possible reason could be that BCAA granules (12 g/day) were used in the previous Japanese study, whereas the present study used either BCAA granules or BCAA-enriched nutrients. The volume of BCAA-enriched nutrients is 200–600 mL/day, which could impact compliance with BCAA supplementation.

A limitation of this study is that this study is not a randomized controlled trial. Furthermore, the effects of BCAA supplementation on each cause of death remain unclear because of the small number of events that occurred during the study period. Therefore, our results are preliminary and would need to be confirmed by a randomized controlled trial with a much larger number of events.

In summary, this multicenter study showed that the BCAA-to-tyrosine ratio was independently associated with the onset of HCC in cirrhotic patients. In addition, this study demonstrated that BCAA supplementation prevented the onset of HCC and death in cirrhotic patients.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at http://dx.doi.org/10.1016/j.cgh.2013.08.050.

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Conflicts of interest
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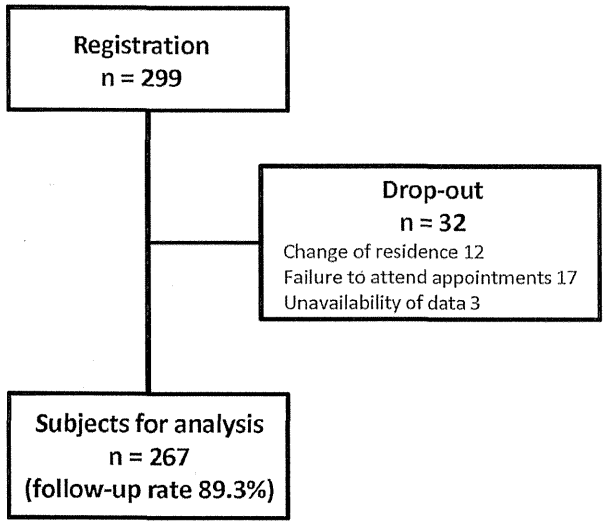

Supplementary Figure 1. Study design. A cohort of 299 cirrhotic patients without HCC were enrolled in 2009 and followed up until 2011. In the course of the study, 32 patients dropped out, and the remaining 267 patients were analyzed (follow-up rate, 89.3%).

Supplementary Table 1. Cause of Death and Incidence of Death in Each Group

Cause of death	All subjects (n = 18)	Non-BCAA group (n = 16)	BCAA group (n = 2)
Chronic hepatic failure (%)	27.8 (5/18)	25.0 (4/16)	50.0 (1/2)
Bacterial infection (%)	22.2 (4/18)	25.0 (4/16)	0 (0/2)
Rupture of varices (%)	11.1 (2/18)	12.5 (2/16)	0 (0/2)
Renal failure (%)	11.1 (2/18)	12.5 (2/16)	0 (0/2)
Pancreatic cancer (%)	5.6 (1/18)	6.3 (1/16)	0 (0/2)
Cardiovascular disease (%)	5.6 (1/18)	0 (0/16)	50.0 (1/2)
Trauma (%)	5.6 (1/18)	6.3 (1/16)	0 (0/2)
Unknown (%)	11.1 (2/18)	12.5 (2/16)	0 (0/2)

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Upregulation of iron regulatory hormone hepcidin by interferon α

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hepcidin, interferon, iron.

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Abstract

Background and Aim: Interferon (IFN) activates various immune systems *in vivo* and is administered to patients with diseases such as viral hepatitis B, C, and malignant tumors. Iron dysregulation has been reported during treatment with IFN; however, it remains unclear whether IFN itself affects iron metabolism. We therefore determined the effect of IFN on iron metabolism.

Methods: Mouse IFNα was administered to mice, and serum, spleen, bone marrow, liver, and duodenum tissue samples were subsequently collected. The messenger RNA (mRNA) and protein expression of genes involved in iron metabolism were then analyzed by real-time reverse transcription–polymerase chain reaction, Western blotting, and liquid chromatography-tandem mass spectrometry. Immunofluorescence for ferroportin was also performed.

Results: Among the gene expressions analyzed, we found that the expression of hepcidin, an iron regulatory hormone produced in the liver, was highly upregulated after IFNα treatment. Serum hepcidin levels and hepcidin mRNA expression in the liver were both found to be increased in the IFNα-treated mice. The expression of ferroportin (the target molecule of hepcidin) in the duodenum of the IFNα-treated mice was observed to be decreased, indicating that hepcidin upregulation could be physiologically functional. *In vitro* analysis of primary hepatocytes treated with IFNα and human hepatoma-derived cells showed an upregulation of hepcidin mRNA, including an activation of signal transducer and activator of transcription3, which was shown to be involved in the hepcidin upregulation.

Conclusions: Results indicate that iron absorption is decreased during IFN treatment; this favorable effect could inhibit iron overload during IFN treatment and may enhance the action of IFN.

Introduction

Iron is essential for almost all living organisms.[1,2] Excess iron however generate toxic free radicals, leading to organ damages, and even contribute to inflammatory disease and cancer. Iron in the body must therefore be tightly regulated.[3,4] Hepcidin is the main player in the regulation of iron metabolism.[5,6] Hepcidin is a peptide consisting of 20–25 amino acids and is produced mainly by the liver.[7,8] Hepcidin circulates and acts on enterocytes of the duodenum and macrophages in the spleen that trap and destroy senescent red blood cells. Hepcidin binds to the ferroportin (FPN), an iron exporter protein at the cell surface, leading to the internalization and degradation of FPN. This results in a decrease of iron efflux into blood.[9] Hepcidin therefore acts as a negative regulator of iron absorption from the gastrointestinal tract and iron excretion from the reticuloendothelial system.

The expression of hepcidin is important in maintaining iron homeostasis in the body, as dysregulation of hepcidin may result in diseases. Inappropriate low hepcidin expression caused by mutations in genes involved in the regulation of hepcidin expression leads to hereditary hemochromatosis.[10] On the other hand, high concentrations of serum inflammatory cytokines such as interleukin-6 (IL-6) and IL-1β in the inflammatory condition cause upregulation of hepcidin, resulting in the decrease of available iron in the serum and finally lead to anemia of chronic disease.[11–13]

In chronic liver disease caused by viral infection or alcohol, iron metabolism is also dysregulated.[14,15] In hepatitis C, iron overload is

an exacerbating factor,[16] while interferon α (IFNα) is a standard drug for treatment. Exogenously administered IFN activates the immune system *in vivo*. During treatment with IFN, iron dysregulation such as an increase in serum ferritin and hepcidin occurs especially when IFN therapy is effective.[17] Iron overload in chronic hepatitis C contributes to a poor response to IFN treatment, which lead to a poor prognosis.[18] It is therefore important to investigate iron metabolism during IFN treatment because it is unclear how exogenously administered IFN affects iron metabolism. Interesting findings in the relation between IFN and hepcidin have been reported; hepcidin expression was inhibited in patients with chronic hepatitis C,[19] and this inhibition was subsequently reduced by treatment with pegylated (PEG)-IFN with ribavirin, an antiviral agent.[20] However, it is still unclear whether IFN itself influences iron metabolism. We therefore studied the effect of IFN itself on iron metabolism using mouse models.

Materials and methods

Samples and tissues. Mouse IFNα (Miltenyi Biotec, Bergisch Gladbach, Germany) was subcutaneously administered to 6-week-old C57BL/6 mice (Clea Japan, Tokyo, Japan) at a dose of 10^4 IU/day or 10^5 IU/day for 3–7 days after which the mice were sacrificed, and blood, duodenum, spleen, bone marrow of thighbone, and liver were collected. Control mice were received subcutaneous injection of phosphate-buffered saline (PBS). *Ex vivo* culture of duodenums obtained from normal mice was performed, where the duodenums were washed with PBS and cultured with Dulbecco's modified Eagle's medium (DMEM) (Wako, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (Wako), 1000 IU/mL of mouse IFNα (Miltenyi Biotec), and 0.1 μg/mL of anti-mouse IFNα, β receptor antibody (eBioscience, San Diego, CA, USA) overnight. Protocols for animal experiments were approved by the Asahikawa Medical University Institutional Animal Care and Use Committee.

Measurements of markers involved in iron metabolism. Serum analysis for iron was performed using an automatic serum analyzer LAboSPECT008 (Hitachi, Tokyo, Japan). Quick auto neo Fe (Sino-test, Tokyo, Japan) was used in measuring serum iron. The iron contents of mouse liver, spleen, and bone marrow were measured by atomic absorption spectrometry (AAS), and the ratio of iron to dried tissues is calculated.

Human and mouse hepcidin standards were synthesized at Peptide Institute (Osaka, Japan). Mice hepcidin concentrations were measured by liquid chromatography (LC)-tandem mass spectrometry (MS) as previously reported.[21,22]

The concentrations of IL-6 in mouse serum were evaluated using commercially available multiplex bead-based sandwich immunoassay kits, Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad Laboratories, Hercules, CA, USA). Assays were performed following the manufacturer's instructions. Different sandwich immunocomplexes formed on distinct bead sets were measured and quantified using the Bio-Plex Suspension Array System (Bio-Rad Laboratories). Instrument control and data processing were performed using Bio-Plex Manager software (Bio-Rad Laboratories).

Real time reverse transcription–polymerase chain reaction. Total RNA was isolated from cells and tissues with the RNA purification system PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed with a high-capacity complementary DNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). Real-time reverse transcription–polymerase chain reaction (RT-PCR) was then performed using ABI 7300 system (Applied Biosystems) with TaqMan probes. Probes (Applied Biosystems) used included human hepcidin, mouse hepcidin, mouse transferrin receptor 1, mouse transferrin receptor 2, ferritin heavy chain, divalent metal transporter 1, FPN, human IL-6, mouse IL-6. 18S ribosomal RNA was analyzed as an internal control (18 s rRNA, Applied Biosystems), and the ratio of hepcidin to 18S ribosomal RNA was calculated.

Immunofluorescent study for FPN expression by the duodenum. Tissue samples of duodenum were fixed with 3.7% formaldehyde for 24 h and processed for paraffin embedding. The paraffin sections were sequentially treated by deparaffinization, rehydration, and antigen retrieval before applying primary antibodies as follows: for antigen retrieval, sections were immersed in retrieval solution (pH 9.0) at 125°C for 15 min in a pressure boiler. The sections were then incubated with the anti-SLC40A1/FPN1 rabbit polyclonal antibody (Medical & Biological Laboratories, Nagoya, Japan) and then with Alexa Fluora 594 conjugated anti-rabbit immunoglobulin G (IgG) antibody (Invitrogen). This was followed by nuclear staining with 4′,6-diamidino-2-phenylindole.

Cell culture. Human hepatocellular carcinoma cell line HepG2 was obtained from ATCC (Rockville, MD, USA) and cultured in DMEM, supplemented with 10% FCS and penicillin-streptomycin. Mouse primary hepatocytes were isolated from male 6-week-old C57Bl/6 male mice by the collagenase perfusion method, and the hepatocytes with more than 95% viability were prepared by the Percoll centrifugation method. The cells were then plated on collagen-coated dishes, and cultured in Williams' E medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS, 0.1 mg/mL of epidermal growth factor, 10 mg/mL of insulin and penicillin-streptomycin. These cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 21% O_2. The HepG2 cells were treated with 0–500 IU/mL of human IFNα (Miltenyi Biotec), and primary cultured mouse hepatocytes were treated with 0–1000 IU/mL of mouse IFNα for 24 h. The concentrations of human and mouse IFN were previously reported.[23–26] In some experiments, 0.1 mg/mL of anti-human IFNα, β receptor antibody (Abcam, Cambridge, UK), or 0.1 μg/mL of anti-mouse IFNα, β receptor antibody (eBioscience) was added to inhibit IFN from binding to its receptor at the cell surface.

Western blot analysis. The cell samples were lysed in a Radioimmunoprecipitation assay buffer, separated in Mini-protean TGX precast polyacrylamide gel (Bio-Rad Laboratories) and electro-transferred to nitrocellulose membranes. After blocking the membranes with 5% non-fat dry milk in a PBST buffer (PBS containing 0.05% Tween-20), they were probed with

anti-SLC40A1/FPN1 rabbit polyclonal antibody (Medical & Biological Laboratories), anti-signal transducer and activator of transcription3 (STAT3) (Cell Signaling Technology, Danvers, MA, USA), anti-phosho-STAT3 (Cell signaling technology), and anti-actin antibody (BD Biosciences, Franklin Lakes, NJ, USA), followed by incubation with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibody (R&D Systems, Minneapolis, MN, USA). Antibody binding was then visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA).

Statistical analysis. Statistical analysis was done using the Student paired *t*-test, and P values < 0.05 were considered to be statistically significant.

Results

The changes of serum iron and liver iron concentrations by IFNα in mice. To determine how IFNα affects iron metabolism, serum markers for iron metabolism and iron contents of the liver were evaluated in IFNα-treated mice. Serum iron was significantly decreased to 202.2 ± 28.6 μg/dL in 10^4 IU/day IFNα-treated mice compared with the 249.8 ± 25.8 μg/dL found in the control mice. Serum iron levels in 10^5 IU/day IFNα-treated mice however found to be 250.6 ± 12.5 μg/dL, showing no significant decrease as compared with the control mice (Fig. 1a). The liver iron contents determined by AAS were 231.6 ± 27.5 μg/dry g in control mice, 211.4 ± 38.8 μg/dry g in 10^4 IU/day IFNα-treated mice, and 200.7 ± 9.6 μg/dry g in 10^5 IU/day IFNα-treated mice; no significant differences between the IFNα-treated groups and control group were observed (Fig. 1b).

The effect of IFNα on the expressions of the molecules involved in iron metabolism. The messenger RNA (mRNA) expression of *TfR1*, *TfR2*, *ferritin heavy chain*, and *DMT1* in the liver were determined by real-time RT-PCR using TaqMan probes, and from the results obtained, there was no significant change in any of the genes expressed. The expression of *Hamp1* (the gene codes hepcidin) in the liver of the IFNα-treated mice, however, was found to be significantly upregulated. This effect was observed in both 10^4 and 10^5 IU/day IFNα-treated groups (Fig. 2).

Changes in serum hepcidin levels after IFNα treatment were determined using LC-tandem MS. Although there was no significant difference in serum hepcidin in the 10^4 IU/day IFNα-treated group, serum hepcidin was significantly upregulated in the 10^5 IU/day IFNα-treated mice (Fig. 3a).

The change of FPN expression in the duodenum by IFNα. Because the target molecule of hepcidin is FPN, the expression of FPN was determined to find out if IFNα-induced hepcidin upregulation can physiologically decrease FPN expression. Immunofluorescent study for FPN in the duodenal tissues showed a decrease in FPN expression in both the 10^4 and 10^5 IU/day IFNα-treated groups (Fig. 3b). Furthermore, neither the mRNA nor the protein levels of FPN were affected by the *ex vivo* duodenum culture with IFNα and/or anti-IFN receptor antibody (Fig. 3c,d). To elucidate the role of IFN-treatment in iron recycling

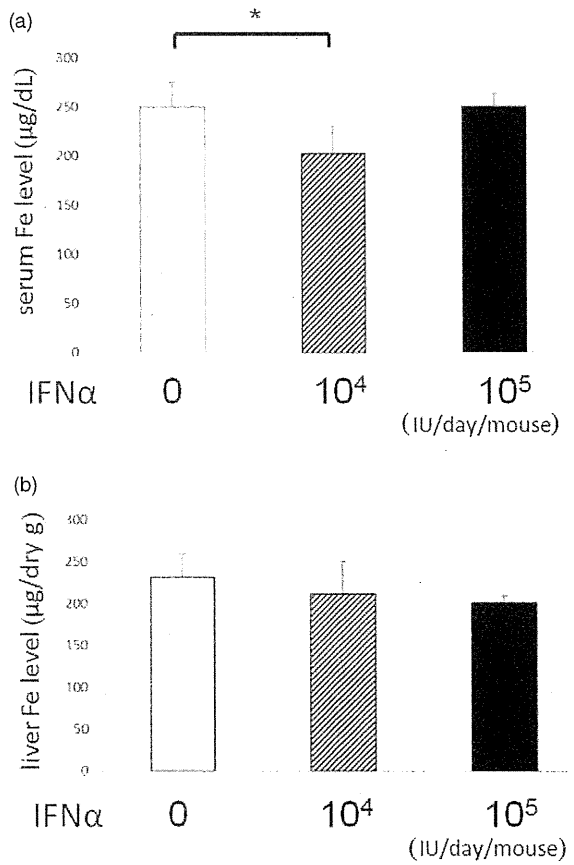

Figure 1 (a) Effect of interferon α (IFNα) on serum iron in mice. IFNα administration for 3 days at a dose of 10^4 IU/day significantly reduced serum iron level. However, no decrease in serum iron was observed in mice treated with 10^5 IU/day IFNα ($n = 5$ in each group). *$P < 0.05$. (b) Effect of IFNα on liver iron content in mice. Liver iron content evaluated by atomic absorption spectrometry showed no significant change after administration of IFNα ($n = 5$ in each group).

from macrophages, tissue iron contents in spleen and bone marrow were evaluated; the results showed no significant changes in both tissue iron contents between controls and IFN-treatments (Fig. 3e).

Hepcidin is upregulated via direct and indirect mechanism. Because IL-6 is a strong inducer of hepcidin expression, we measured IL-6 during IFNα administration. Serum IL-6 levels were 5.3 ± 2.6, 17.4 ± 5.9, and 23.6 ± 7.8 pg/mL in the control mice, 10^4 IU/day, and 10^5 IU/day IFN-treated mice, respectively; a significant increase in serum IL-6 was observed after IFNα administration (Fig. 4). This observation suggests that IFNα may indirectly upregulate hepcidin via the induction IL-6.

We then determined if IFNα has a direct effect on hepcidin upregulation in hepatocytes. The changes in hepcidin mRNA expression after IFNα treatment was determined using primary culture hepatocytes derived from mice and human hepatoma cell line HepG2 cells. Hepcidin mRNA expression was observed to be significantly upregulated in both the primary hepatocytes

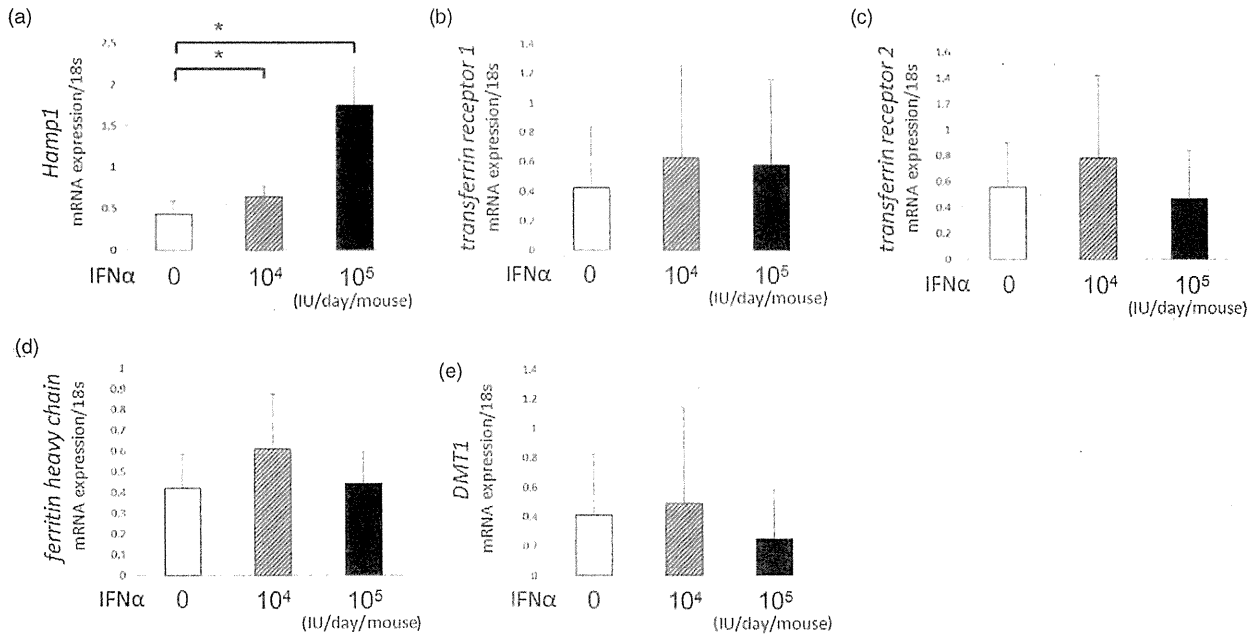

Figure 2 Changes in the expression of genes involved in iron metabolism in the liver of interferon α (IFNα)-treated mice. Quantitative polymerase chain reaction was performed to determine the expression levels of (a) *Hamp1*, (b) *transferrin receptor 1*, (c) *transferrin receptor 2*, (d) *ferritin heavy chain*, and (e) *divalent metal transporter 1 (DMT1)* using RNA extracted from the livers of mice treated with IFNα ($n = 5$ in each group). Relative expression levels were calculated by normalization using 18S expression. Only *Hamp1* expression was significantly increased by IFNα. $^*P < 0.05$.

and HepG2 cells, and this upregulation seemed to be IFNα concentration-dependent. The addition of a neutralizing antibody against IFN receptor prevented hepcidin upregulation (Fig. 5a,b).

Determination of the mechanism for direct upregulation of hepcidin by IFNα. We then focused on the direct mechanism by which IFNα may upregulate the expression of hepcidin. Commonly, binding of IFNα to IFN receptor on cell surfaces accelerates STAT1/STAT2 heterodimer formation via Janus kinase activation. However, there is no binding site for that heterodimer in the promoter region of the hepcidin gene. On the other hand, it has been reported that IFN can activate a STAT3 homodimer in human primary hepatocytes.[23] In addition, the promoter region of the hepcidin gene has a binding site for the STAT3 homodimer. Thus, it is possible that IFNα directly affects hepatocytes to enhance the transcription of the hepcidin gene via activated STAT3 homodimer. As hypothesized, phospho-STAT3 (Y705)/total STAT3 was observed to be significantly upregulated in both cultured primary mouse hepatocytes and HepG2 cells treated with IFNα (Fig. 6a,b). The addition of a neutralizing antibody against the IFN receptor cancelled STAT3 activation.

Although IL-6 is mainly produced by monocytes/macrophages and T-lymphocytes, hepatocytes are also known to produce IL-6; therefore, we determined if IFNα directly enhance IL-6 expression in hepatocytes. We determined if IFNα could induce *IL-6* mRNA expression in RNA extracted from mice liver (Fig. 7a) but found that no induction of *IL-6* mRNA expression after IFNα treatment. Because the liver contains not only hepatocytes but also other cells, such as Kupffer cells, we then examine the expression of IL-6 mRNA in primary cultured hepatocytes obtained from mice (Fig. 7b) and HepG2 cells (Fig. 7c) but did not observe any difference in *IL-6* mRNA expression with or without IFNα.

Discussion

Our results show that IFNα administration causes hepcidin upregulation in mice (Figs 2,3a). Serum IL-6 levels were increased in IFNα-treated mice (Fig. 4), so it is likely that IFNα-mediated IL-6 induction contributes to the upregulation of hepcidin as IL-6 is one of the most important inducible factors for hepcidin expression. We also confirmed that IFNα directly upregulates hepcidin expression in primary cultured mouse hepatocytes and HepG2 cells (Fig. 5a,b). IL-6 can be produced by hepatocytes; however, we excluded that possibility from the observation that IFNα treatment did not enhance IL-6 expression in hepatocytes (Fig. 7a–c). Together, IFNα directly affects hepatocytes to induce hepcidin expression and indirectly via IL-6 induction throughout the body.

There have been other reports on the effect of IFNα on hepcidin expression; for instance, Ryan *et al.* recently reported that IFNα treatment resulted in the expression of hepcidin.[27] However, they observed changes in hepcidin expression after IFNα treatment using monocytes, not hepatocytes. We used primary cultured hepatocytes from mice, thus providing more precise evidence regarding the direct effect of IFNα on hepcidin expression by hepatocytes. We then showed the cancellation effect of the anti-IFN receptor

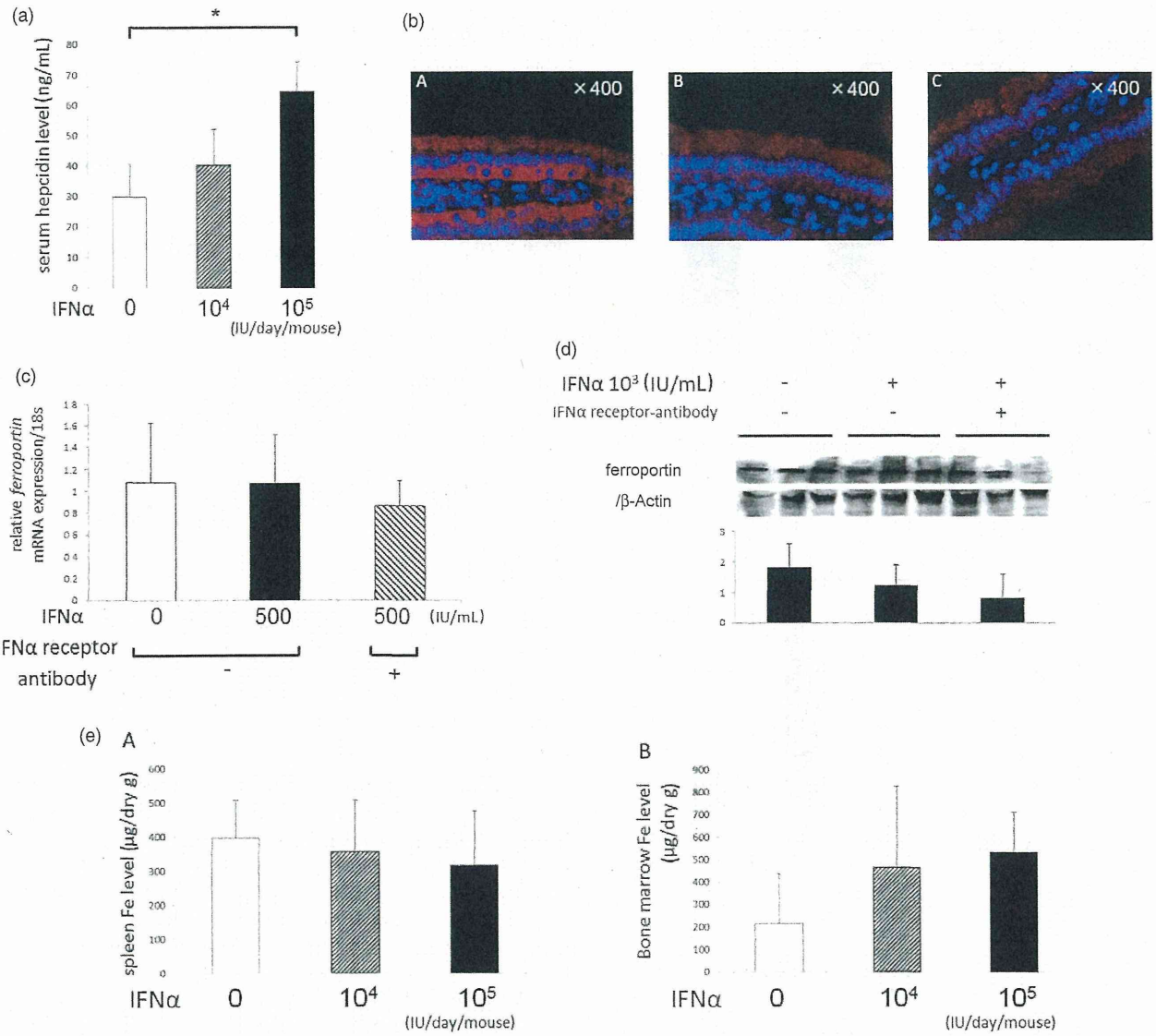

Figure 3 (a) Concentrations of hepcidin in the serum of mice treated with interferon α (IFNα). Hepcidin levels were measured using liquid chromatography-tandem mass spectrometry ($n = 5$ in each group). Significant increases in hepcidin concentration were observed in mice treated with 10^5 IU/day of IFNα. $^*P < 0.05$. (b) Immunofluorescent staining for ferroportin (FPN) in mouse duodenum tissue. The expression of FPN was observed on the basolateral side of the duodenum of (A) control mice, but disappeared after IFNα treatment (B) 10^4 IU/day and (C) 10^5 IU/day. (c) FPN mRNA expression and (d) FPN protein expression in mice duodenums treated with 10^3 IU/mL of IFNα and/or anti-mouse IFNα, β receptor antibody. (e) Tissue iron contents analyzed by atomic absorption spectrometry in spleens (A) and bone marrows (B).

antibody on IFNα-induced hepcidin upregulation, indicating that the binding of IFNα to its own receptor on the cell surface of hepatocytes is necessary for increasing hepcidin expression. Binding of IFNα to its receptor usually induces the activation of the STAT1/STAT2 heterodimer, however, our results showed that IFNα-induced upregulation of activated STAT3 may be involved in hepcidin upregulation (Fig. 6a,b).

In the present study, we also confirmed that IFNα-induced hepcidin upregulation is physiologically functional, as the expression of FPN in duodenum was significantly reduced in the IFNα-treated mice (Fig. 3b). The possibility that IFNα directly decrease FPN expression seemed to be unlikely because we did not observe any significant changes of FPN expression in the duodenum treated with IFNα in the present study (Fig. 3c,d). FPN is expressed on the basolateral cell membrane of enterocytes and functions in iron transport from enterocytes to blood. FPN has also been reported to be internalized and degraded when hepcidin binds to FPN on the cell surface, resulting in a decrease in iron absorption from the gastrointestinal tract. The IFNα treatment may therefore result in a decrease in iron absorption. Besides, we observed

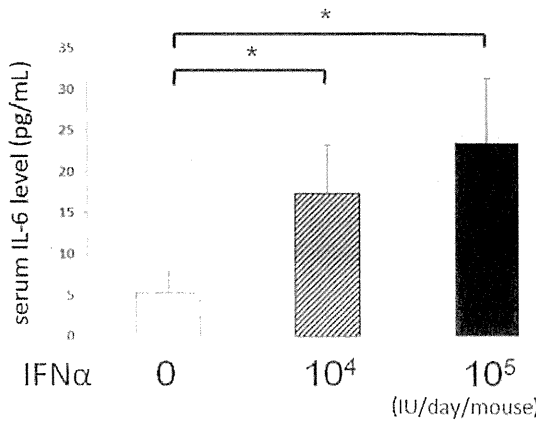

Figure 4 Change in interleukin (IL)-6 in the sera in interferon α (IFNα)-treated mice ($n = 5$ in each group). IFNα treatment significantly increased serum IL-6 levels with the administration of 10^5 IU/day of IFNα. $^*P < 0.05$.

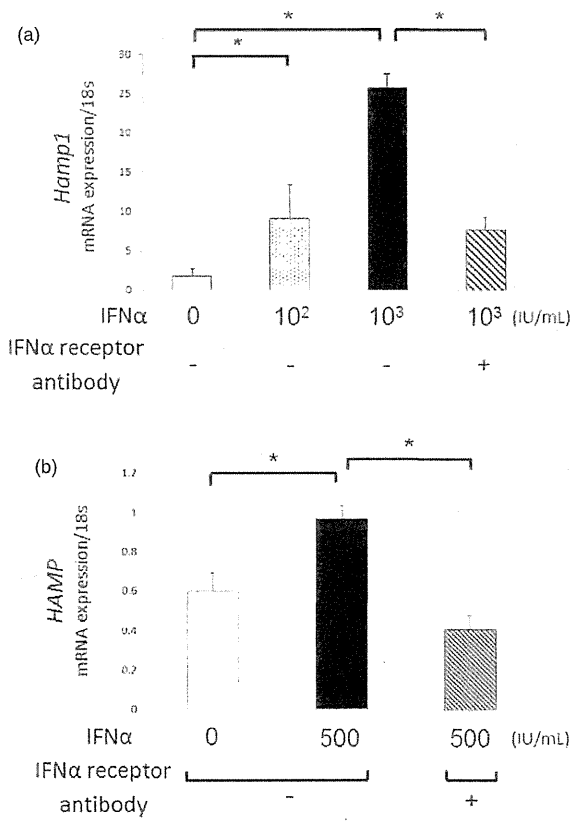

Figure 5 Expressions of hepcidin messenger RNA (mRNA) were evaluated in (a) mouse primary hepatocytes and (b) HepG2 cells. Quantitative polymerase chain reaction (PCR) was performed and the relative expression levels were calculated by normalization using 18S expression. In both cells, the expressions of hepcidin mRNA were significantly increased by the administration of interferon α (IFNα). These increases were cancelled by the neutralizing antibodies against IFNα receptors. $^*P < 0.05$.

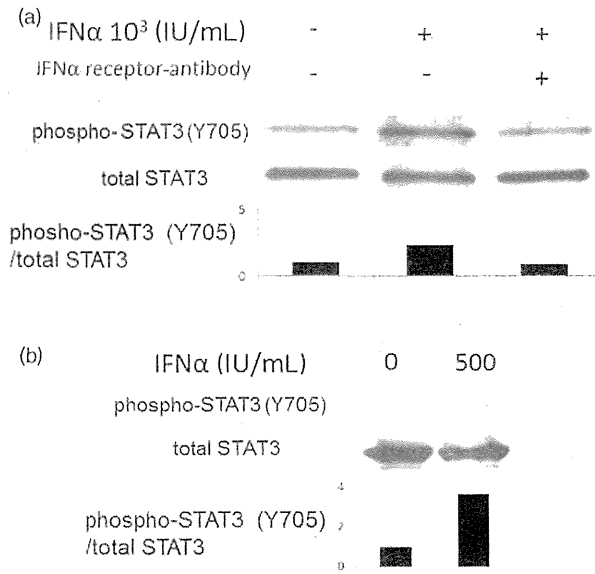

Figure 6 Phosphorylation of signal transducer and activator of transcription3 (STAT3) was determined in (a) mouse primary hepatocytes and (b) HepG2 cells treated with interferon α (IFNα). Densities of the bands corresponding to phosphorylated STAT3 levels were normalized by levels of total STAT3 shown. In both cell types, phosphorylated STAT3 was increased by IFNα treatment but cancelled using antibodies against IFNα receptors.

no significant accumulation of iron in the liver of the IFNα-treated mice (Fig. 1b), and this finding is supported by the absence of any increase in *TfR1* and *DMT1* mRNA in the liver (Fig. 2). Thus, IFN itself does not accelerate iron accumulation in the liver. However, further study will be necessary, as hepatocytes are thought to possess various pathways for iron uptake, including a transferrin receptor-independent route for the uptake of transferrin-bound iron.[28]

The decrease in FPN expression in the duodenum may explain the decrease in serum iron observed in the 10^4 IU/day IFNα-treated mice (Fig. 1a). There was a possibility that the inhibition of iron release from reticuloendothelial system contributed to the decrease of serum iron, but no significant changes of iron contents were observed in the spleen and the bone marrow (Fig. 3e). Ryan *et al.* reported that a single dose of PEG-IFNα/ribavirin resulted in a 50% reduction in serum iron and transferrin saturation over a 24-h period,[27] which is compatible with our findings in 10^4 IU/day IFNα-treated mice.

However, no significant reduction in serum iron was observed in the 10^5 IU/day IFNα-treated mice (Fig. 1a). It is however difficult to know the appropriate concentration of mouse IFNα that will have a substantially equal effect as the concentration of human IFN used in the treatment of diseases such as hepatitis. We therefore used two different concentrations of IFNα in the present study, but we have observed that the 10^5 IU/day IFNα may be too high. We also speculated that erythropoiesis may have been inhibited during the 10^5 IU/day IFNα treatment, resulting in reduced iron utilization in erythropoiesis decreased. A preliminary evaluation of cell numbers in 10^5 IU/day IFNα-treated mice thighbone