Table 3. Demographic and clinical features of chronic hepatitis C genotype 1b patients who received telaprevir, PegIFN-α-2b, and ribavirin triple therapy, classified by age and sustained virological response or non-sustained response.

Characteristics	Group A; patients aged >60 (n = 64)			Group B; patients aged ≤60 (n = 56)		
	SVR (n = 49)	Non-SVR (n = 15)	*p* value*	SVR (n = 47)	Non-SVR (n = 9)	*p* value*
Men, n (%)	17 (34.7)	2 (13.3)	0.113	29 (61.7)	6 (66.7)	0.778
Average age (yr)	66 [7.0]	69 [7.0]	0.094	54 [12]	52 [5.5]	0.814
Body mass index (kg/m^2)	22.6 [3.1]	23.9 [5.2]	0.130	23.5 [3.9]	24.8 [4.0]	0.058
Baseline HCV RNA (log_{10} IU/ml)	6.4 [0.6]	6.3 [0.8]	0.691	6.6 [0.9]	6.4 [0.5]	0.433
HCV core amino acid substitution at position 70, wild/mutation, n	29/20	4/11	0.027	21/26	4/5	0.989
White blood cell count (×10^6/L)	4210 [1355]	4090 [580]	0.409	5100 [2600]	3700 [2715]	0.224
Hemoglobin level (g/L)	134 [17]	132 [22]	0.261	144 [22]	141 [33]	0.911
Platelet count (×10^9/L)	152 [53]	115 [63]	0.002	171 [81]	142 [68]	0.064
Serum albumin (g/L)	39 [5.0]	39 [8.0]	0.430	41 [5.0]	39 [8.0]	0.072
Aspartate aminotransferase (IU/L)	48 [43.5]	70 [70]	0.007	46 [34]	70 [31.5]	0.031
Alanine aminotransferase (IU/L)	53 [65.5]	72 [74]	0.026	53 [68]	64 [41]	0.255
γ-glutamyl-transpeptidase (IU/L)	26 [24]	53 [62]	0.0004	45 [60]	103 [63.2]	0.023
Estimated glomerular filtration rate (ml/min)	75.3 [17.7]	74.4 [15.9]	0.686	83.7 [20.2]	83.1 [20.5]	0.561
Liver histology						
Stage, F0-2/F3-4, n	20/14	1/7	0.018	22/6	2/4	0.027
Stage F0, n	5	0	0.007	2	0	0.067
Stage F1, n	10	0		16	1	
Stage F2, n	5	1		4	1	
Stage F3, n	7	0		4	3	
Stage F4, n	7	7		2	1	
Grade, A0-1/A2-3, n	15/19	0/8	0.019	18/10	1/5	0.033
Not determined, n	15	7		19	3	
Previous treatment outcome						
Treatment naïve, n	12	0	0.084	13	2	0.081
Prior relapse, n	29	6		26	3	
Prior non-response, n	5	9		8	3	
Prior unknown response, n	3	0		0	1	
IL28B SNP (rs8099917)						
TT, n	42	5	<0.0001	34	3	0.023
TG/GG, n	7	10		13	6	
ITPA SNP (rs1127354)						
CC, n	39	12	0.972	35	6	0.628
CA/AA, n	10	3		12	3	
Completed assigned total cumulative PegIFN-α-2b dosage ≥80% and RBV dosage ≥80%, n (%)	14 (28.6)	1 (6.7)	0.079	19 (40.4)	5 (55.6)	0.400
Completed assigned total cumulative PegIFN-α-2b dosage ≥80%, n (%)	44 (89.8)	7 (46.7)	0.0002	40 (85.1)	6 (66.7)	0.185
Completed assigned total cumulative RBV dosage ≥80%, n (%)	15 (30.6)	1 (6.7)	0.061	20 (42.6)	6 (66.7)	0.184
Discontinuation of PegIFN-α-2b and RBV, n (%)	1 (2.0)	7 (46.7)	<0.0001	1 (2.1)	6 (66.7)	<0.0001
Discontinuation of TVR, n (%)	1 (2.0)	6 (40.0)	<0.0001	1 (2.1)	4 (44.4)	<0.0001
Discontinuation of treatment, n (%)	1 (2.0)	7 (46.7)	<0.0001	0 -	7 (77.8)	<0.0001
RVR, n (%)	42 (85.7)	5 (33.3)	<0.0001	40 (85.1)	1 (11.1)	<0.0001

SVR, sustained virological response; HCV, hepatitis C virus; *IL28B*, interleukin 28B; SNP, single-nucleotide polymorphism; *ITPA*, inosine triphosphate pyrophosphatase; PegIFN-α-2b, pegylated interferon α2b; RBV, ribavirin; TVR, telaprevir; RVR, rapid virological response.
All patients were infected with HCV genotype 1b.
Continuous variables are expressed as median [interquartile range].
**p* Value draws a comparison between the SVR and non-SVR patients of each group.

We found that the median serum HCV RNA level at day 3 was significantly lower for RVR patients than for non-RVR patients in both older and younger patients. RVR patients have a high potential for achieving an SVR. After a single dose of TVR, the mean half-life is approximately 4 hours. At steady state, the effective half-life is approximately 9–11 hours. When TVR is administered at 750 mg every 8 hours, a steady state is reached from 3 to 7 days after the start of administration [11–14]. For these reasons, we investigated the correlation between the HCV RNA level at day 3 and virological response. We also showed that lower exposure (accumulated dosage <60%) to TVR within the first 4 weeks of treatment leads to a lower SVR rate; however, other accumulated dosages were not related to SVR. The quick steady state of TVR is presumed to be related to a rapid decline of the HCV RNA level, which leads to RVR and SVR, irrespective of age.

Table 4. Predictive factors associated with sustained virological response by chronic hepatitis C genotype 1b patients who received telaprevir, PegIFN-α-2b and ribavirin triple therapy, classified by age.

	Group A: Patients aged >60 (n = 64)					Group B: Patients aged ≤60 (n = 56)				
	Simple		Multiple			Simple		Multiple		
	Odds ratio	*p* value	Odds ratio	95% CI	*p* value	Odds ratio	*p* value	Odds ratio	95% CI	*p* value
Age (per 1 yr)	0.876	0.101				0.975	0.609			
Sex (male to female)	3.448	0.129				0.805	0.778			
Body mass index (per 1 kg/m²)	0.822	0.062				0.823	0.093			
γ-glutamyl-transpeptidase (per 1 IU/L)	0.971	0.003				0.998	0.709			
White blood cell count (per 1×10⁶/L)	1.348	0.093				1.000	0.242			
Hemoglobin level (per 1 g/L)	1.364	0.187				1.043	0.876			
Platelet count (per 1 × 10⁹/L)	1.316	0.004				1.173	0.076			
Histological stage (F3-4 to F0-2)	0.700	0.049				0.136	0.027			
Previous treatment response (treatment naïve and prior relapse to prior non-response)	12.34	0.0004	8.403	1.025-66.67	0.047	2.437	0.259			
IL28B SNP (rs8099917) (TT to TG/GG)	107.5	0.0006	14.93	1.600-142.9	0.017	4.231	0.005	25.34	1.848-1409	0.042
RVR	27.78	0.0003	7.498	1.014-65.42	0.009	45.71	<0.0001	78.35	6.618-4418	0.005

IL28B, interleukin 28B; SNP, single-nucleotide polymorphism; RVR, rapid virological response; PegIFN-α-2b, pegylated interferon α2b.

The *IL28B* gene-related SNP on chromosome 19 is the most important baseline predictor of SVR in the treatment of chronic hepatitis C patients with PegIFN-α plus RBV [6,17,18]. This study found the *IL28B* genotype to be a significant, independent pre-treatment factor for achievement of SVR in the TVR-based regimen. Several classes of DAA are under development, and it is expected that, when used in combination with PegIFN-α plus RBV, these new drugs will improve SVR and decrease the required duration of treatment [25]. The *IL28B* genotype has an influence on early viral kinetics, even during treatment with interferon-free DAA regimens. Interferon-free regimens produce no difference within 6 days after the start of treatment, but a significant difference in HCV RNA reduction after day 7 between patients with *IL28B* SNP (rs12979860) CC *vs.* the non-CC genotypes [26]. The host *IL28B* genotype has also been shown to affect the spontaneous clearance of HCV infection [27]. Thus, *IL28B* genotyping will continue to play an important role in determining the likelihood of anti-HCV treatment response.

Older patients have been reported to have a greater frequency of treatment discontinuation or to require a reduction in drug dosage during anti-HCV treatment due to laboratory abnormalities and adverse effects [9,10]. The efficacy and safety of PegIFN-α plus RBV treatment have been documented in a large-scale KULDS research of 1,251 Japanese patients [9]. The findings for HCV genotype 1 have shown that the discontinuation rates due to complications and adverse effects of older patients were almost twice those of younger patients (42.9% *vs.* 24.4% for the 48-week treatment course, respectively). The present study showed no difference in the SVR and discontinuation rates of older and younger patients. This may be attributed to the fact that the TVR-based regimen is completed in a short period, 24 weeks, and that it results in a higher RVR rate. Moreover, this study did not include the principle of response-guided therapy, but our physicians explained the patients' virological response during the treatment, thus most of the patients knew they were responding and were motivated to continue to receive the treatment.

Surprisingly, our older patient group included a higher female percentage than the younger group, however, no significant differences in virological responses were found. Post-menopause is one of the most predictive factors for failure to achieve an SVR, which links SVR to estrogen secretion [6]. Despite the good virological response in the older patients to TVR-based treatment, a higher relapse rate was found in the older patients than the younger, but the difference was not significant. Moreover, lower cumulative dosages of PegIFN-α and RBV resulted in lower SVR rates only in the older patients. Our study supports the better drug adherence to improve the chance of SVR for older patients with non-RVR and EOT.

The main adverse effects monitored in this study were rash, serious skin reactions, and anemia. Treatment discontinuation rates for these conditions were compared between older and younger patients. No significant differences in drug-induced skin disorders were found. There was a significant difference in the occurrence of severe anemia between the two groups. As expected, the older patients had higher rates of severe anemia than the younger patients, probably due to significantly lower blood counts at baseline of the older patients than the younger patients. Moreover, one man of the older group developed comorbid early gastric cancer and another had deteriorating diabetes. Care must be taken to protect against the occurrence of such disorders in older patients undergoing triple therapy.

The study has a number of limitations. First, the sample size might provide inadequate statistical power to detect definitive differences between the SVR and non-SVR data of both age groups. However, as far as we know, ours is the first study of a TVR-based regimen to show a significant clinical impact by age. Second, we studied only Japanese patients with HCV genotype 1b. Significant differences in virological response and drug mutations between HCV genotypes 1a and 1b have been reported in DAA-based treatment [28]. Among Japanese, the favorable *IL28B* genotype is found in the majority of the population (about 75%) [6,21,22]. Hence, our results may not be able to be extended to patients with other HCV genotypes or other racial cohorts. Third, our patients received a 24-week triple therapy. TVR has been approved since September 2011 for use in Japan only for the treatment of genotype 1 chronic hepatitis C with a high HCV RNA level ($\geqslant$5.0 log IU/ml). Also, the duration of triple therapy is 24 weeks, with all three agents for the first 12 weeks, then PegIFN-αb plus RBV dual therapy for the remaining 12 weeks [14]. However, in Europe, the USA, and Canada, TVR must be administered with PegIFN-α and RBV for all patients for 12 weeks, followed by a response-guided regimen of either 12 or 36

additional weeks of PegIFN-α and RBV, depending on early viral response of treatment naïve and prior relapse or prior partial and null response [11–13]. Our results show that patients with RVR can achieve high rates of SVR with 24 weeks of TVR-based triple therapy, irrespective of age. The fact that RVR is such a strong predictor for achieving an SVR may partly be explained by the fact that a 48-week course TVR-triple therapy is not necessary for patients with RVR [29]. Forth, our older patients had no severe baseline comorbidities such as heart, pulmonary, renal, and hematological diseases, namely under favorable baseline conditions, so that the drawn conclusion for the safety by TVR-based triple therapy may be limited. However, our findings that there were no serious newly occurred and deteriorated comorbidities are important. We believe the safety of this therapy is probably due to the shorter period.

In conclusion, in this prospective, multicenter study of HCV genotype 1b chronic hepatitis C patients, we found that older patients achieve a better virological outcome by TVR-based triple therapy than with the traditional dual therapy. *IL28B* genotyping and early virological response indicate the potential to achieve an SVR in these difficult-to-treat older patients.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Efficacy of pegylated interferon alpha-2b and ribavirin treatment on the risk of hepatocellular carcinoma in patients with chronic hepatitis C: A prospective, multicenter study☆

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Background & Aims: The effects of pegylated interferon (PegIFN) α and ribavirin (RBV) treatment of chronic hepatitis C on the incidence of hepatocellular carcinoma (HCC) have not been well established. This study investigated the impact of treatment outcome on the development of HCC by chronic hepatitis C patients treated with PegIFNα2b and RBV.

Methods: This large-scale, prospective, multicenter study consisted of 1013 Japanese chronic hepatitis C patients with no history of HCC (non-cirrhosis, n = 863 and cirrhosis, n = 150). All patients were treated with PegIFNα2b and RBV and the follow-up period started at the end of the antiviral treatment (median observation period of 3.6 years). The cumulative incidence rate of HCC was estimated using the Kaplan–Meier method, according to treatment outcome.

Results: Forty-seven patients (4.6%) developed HCC during the observation period. In the non-cirrhosis group, the 5-year cumulative incidence rates of HCC for the sustained virological response (SVR) (1.7%) and transient virological response (3.2%) (TVR: defined as relapse or breakthrough) groups were significantly lower than those of the non-virological response (NVR) group (7.6%) ($p = 0.003$ and $p = 0.03$, respectively). A significantly low rate of incidence of HCC by TVR patients in comparison with NVR patients was found for patients aged 60 years and over, but not for those under 60 years of age. In the cirrhosis group, the 5-year cumulative incidence rates of HCC for the SVR (18.9%) and TVR groups (20.8%) were also significantly lower than those of the NVR group (39.4%) ($p = 0.03$ and $p = 0.04$, respectively).

Conclusions: SVR and complete viral suppression during treatment with relapse (TVR) were associated with a lower risk of HCC development when compared with NVR.

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Keywords: Hepatitis C; Pegylated interferon; Ribavirin; Hepatocellular carcinoma.

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Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; IFN, interferon; PegIFN, pegylated interferon; RBV, ribavirin; NVR, non-virological response; TVR, transient virological response; KULDS, Kyushu University Liver Disease Study; AFP, α-fetoprotein; HIV, human immunodeficiency virus; EASL, European Association for the Study of the Liver; ALT, alanine aminotransferase; HbA1c, hemoglobin A1c; EPV, events per predictor variable; HR, hazard ratio; CI, confidence interval; DAAs, direct acting antivirals.

Introduction

Hepatitis C virus (HCV) is a major human pathogen responsible for chronic hepatitis, which often progresses to cirrhosis and hepatocellular carcinoma (HCC) [1–3]. While recent advances in HCV have led to a markedly improved treatment, HCC is at present the sixth most common cancer and the third cause of cancer death worldwide [4]; moreover, its incidence is increasing due to HCV infection [5].

Previous studies have reported that patients who achieved a sustained virological response (SVR) after interferon (IFN) monotherapy demonstrated improvement in liver fibrosis and a

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reduction in the incidence of decompensated liver disease and HCC compared with non-SVR patients [6–9]. In the past 10 years, a combination of pegylated IFN (PegIFN) α and ribavirin (RBV) has become the standard treatment and has resulted in an increased SVR rate [10–12]. Therefore, whether or not PegIFNα and RBV treatment is effective in preventing HCC is important, but its effect on the incidence of HCC has not been adequately studied, particularly in a large prospective study.

A recent prospective study from the United States reported that the cumulative incidence rate of HCC in an SVR group was significantly lower than in a non-virological response (NVR) group. It was also lower in a transient virological response (TVR) group than in an NVR group, although the difference did not reach statistical significance [13]. The number of aging chronic hepatitis C patients has been increasing in Japan, earlier than in other countries [14], thus investigation into the development of HCC by Japanese chronic hepatitis C patients treated with PegIFNα and RBV is highly important. Furthermore, the risk factors for the development of HCC by patients who achieve an SVR after treatment with PegIFNα and RBV have not been adequately clarified in a prospective study, although a recent report suggested that SVR reduced the risk of all-cause mortality in patients treated with PegIFNα and RBV [15]. Clarification of the demographic and clinical factors associated with HCC development, such as advanced age, lower albumin, lower platelet count and higher α-fetoprotein (AFP) level, is important.

The aim of this large-scale, multicenter, prospective study was to evaluate the relationships among pretreatment clinical factors, virological response, and development of HCC by chronic hepatitis C patients with no history of HCC, who were treated with PegIFNα2b and RBV.

Patients and methods

Patients

The Kyushu University Liver Disease Study (KULDS) Group consists of the Kyushu University Hospital and affiliated hospitals in the Northern Kyushu area of Japan. We conducted a prospective study to investigate the efficacy and safety of PegIFNα2b and RBV for chronic hepatitis C patients. The design of the KULDS project has been described previously [12,16,17]. This prospective study consisted of 1013 Japanese patients with chronic HCV infection aged 18 years or older, treated with PegIFNα2b and RBV between December 2004 and November 2009.

The exclusion criteria were: (1) history of HCC; (2) HCC development during antiviral treatment; (3) previous PegIFNα and RBV treatment; (4) positivity for antibody to human immunodeficiency virus (HIV) or positivity for hepatitis B surface antigen; (5) clinical or biochemical evidence of hepatic decompensation at entry; (6) excessive active alcohol consumption (a daily intake of more than 40 g of ethanol) or drug abuse; (7) other forms of liver disease (e.g., autoimmune hepatitis, alcoholic liver disease, hemochromatosis); or (8) treatment with antiviral or immunosuppressive agents prior to enrollment.

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Ethics Committee of each participating hospital. Informed consent was obtained from all patients before enrollment.

Antiviral treatment and patient follow-up

All HCV genotype 1 patients received a combination treatment of PegIFNα2b (PEG-Intron; MSD, Tokyo, Japan) and RBV (Rebetol; MSD) for 48 weeks: the same regimen was prescribed for 24 weeks for genotype 2 patients. In order to investigate the incidence of HCC after treatment, the length of the follow-up period was calculated from the end of antiviral treatment to the diagnosis of HCC or last follow-up visit. Serum AFP and abdominal imaging (ultrasonographic examination, or computed tomography) were performed every 3–6 months, for each patient. The HCC diagnosis was based on histology or non-invasive criteria according to the guidelines of the European Association for the Study of the Liver (EASL) [18].

Clinical and laboratory assessment

Clinical parameters included serum albumin, alanine aminotransferase (ALT), serum AFP, hemoglobin, platelet count, hemoglobin A1c (HbA1c), HCV genotype, and HCV RNA. All were measured by standard laboratory techniques in a commercial laboratory (SRL Laboratory, Tokyo, Japan). The HbA1c levels that we report are expressed as National Glycohemoglobin Standardization Program units (%). Body mass index was calculated as weight in kilograms/height in square meters.

Assessment of liver fibrosis

Liver biopsy for 613 (60.5%) of the 1013 patients was performed by experienced hepatologists. The antiviral treatment was initiated within 1 month after liver biopsy. The minimum length of liver biopsy was 15 mm and at least 10 complete portal tracts were necessary for inclusion. For each specimen, the stage of fibrosis was established according to the METAVIR score [19]. Liver cirrhosis in patients with no liver biopsy was diagnosed by ultrasonographic findings (nodules in the hepatic parenchyma, portal vein >16 mm) (mandatory inspection) at the time of antiviral treatment initiation. Moreover, the diagnosis of liver cirrhosis was made based on at least one of the following: (1) endoscopic findings (varices, portal gastropathy); (2) serological markers (aspartate aminotransferase to platelet ratio index >2.0; the cut-off value that indicates a negative predictive value for cirrhosis is 93%) [20]; or (3) transient elastography (FibroScan value ⩾14.9 kiloPascal; the cut-off value that indicates that the negative predictive value for cirrhosis is 100%) [21]. The EASL HCV guidelines of 2011 describe the accuracy of these non-invasive tests of liver fibrosis as sufficient for identifying patients with cirrhosis [22].

Efficacy of treatment

Successful treatment was an SVR, defined as undetectable HCV RNA at 24 weeks after the end of treatment. A TVR was defined as relapse of serum HCV RNA after treatment of patients whose HCV RNA level was undetectable at the end of treatment and the reappearance of HCV RNA at any time during treatment after virological response (breakthrough). An NVR was defined as a decrease in the HCV RNA level of less than 2 $\log_{10}$ IU/ml at week 12 (null response) and a more than 2 $\log_{10}$ IU/ml decrease in the HCV RNA level from baseline at week 12, but detectable HCV RNA at weeks 12 and 24 (partial response).

HCV RNA level and HCV genotype

Clinical follow-up of HCV viremia was done by real-time reverse transcriptase PCR assay (COBAS TaqMan HCV assay) (Roche Diagnostics, Tokyo, Japan), with a lower limit of quantitation of 15 IU/ml and an outer limit of quantitation of 6.9×10^7 IU/ml (1.2 to 7.8 log IU/ml referred to $\log_{10}$ IU/ml). HCV genotype determination was by sequence determination in the 5′ non-structural region of the HCV genome, followed by phylogenetic analysis [23].

Statistical analysis

Statistical analyses were conducted using SPSS Statistics 19.0 (IBM SPSS Inc., Chicago, IL, USA). Baseline continuous data are expressed as median (first-third quartiles) and categorical variables are reported as frequencies and percentages. Univariate analyses were performed using the Chi-square, Fisher's Exact, Mann–Whitney U tests or analysis of variance (ANOVA) as appropriate. Variables with $p < 0.05$ in univariate analysis were evaluated using multivariate logistic regression to identify those significantly associated with the incidence of HCC. As a rule of thumb, 10 events per predictor variable (EPV) are needed when performing a logistic regression analysis. However, 5 to 9 EPV with a large sample size (over 1000) showed robust results of as much as 10 to 16 EPV [24]. Thus, our sample size and 5 to 9 EPV might be sufficient to insure the robustness of our model. Results are expressed as hazard ratios (HR) and their 95% confidence interval (CI).

The main outcome of this study was HCC incidence. Cumulative incidence curves of HCC according to response to antiviral treatment were plotted using the Kaplan–Meier method. Differences between groups were assessed using

log-rank tests. The time frame for HCC incidence was defined as the time from the end of antiviral treatment to the diagnosis of HCC. A p value less than 0.05 was regarded as statistically significant in all analyses.

Results

Patient characteristics

The baseline characteristics of the 1013 studied patients at the start of antiviral treatment, as classified by the existence of cirrhosis and treatment outcome, are shown in Table 1. HCV genotype 1 was detected in 710 patients and genotype 2 in 303. Of all patients, 151 (14.9%) discontinued antiviral treatment because of adverse effects or other reasons (e.g., poor virological response, economic reasons, or dropout). The discontinuation rate of patients with HCV genotype 1 (129 of 710, 18.2%) was significantly higher than that of those with HCV genotype 2 (22 of 303, 7.3%) (p <0.001). Of the studied patients, 557 achieved SVR (55.0%), 304, including 20 with breakthrough, were TVR (30.0%), and 152 (15.0%) were NVR. The SVR rate of patients infected with HCV genotype 1 was 43.9% (312 of 710), significantly lower than the 80.9% (245 of 303) found for patients with genotype 2 (p <0.001).

In the non-cirrhosis group (n = 863), the three treatment outcome groups differed significantly for age, sex, HCV genotype, and laboratory values associated with liver and metabolic disease (e.g., ALT, platelet count, AFP and HbA1c). The SVR group was more likely to be infected with HCV genotype 2 and to have mild liver fibrosis, but less likely to have laboratory values associated with advanced liver and metabolic disease (e.g., low platelet count, or high AFP and HbA1c level) than the TVR and NVR groups. Independent comparisons of SVR and TVR patients extracted age (p <0.001), sex distribution (p = 0.01), ALT level (p = 0.01), platelet count (p <0.001) and HCV genotype (p <0.001). Likewise, independent comparisons of TVR and NVR patients extracted only AFP level (p = 0.01).

Liver cirrhosis was diagnosed according to clinical (n = 77) and histological (n = 73) findings. In the cirrhosis group (n = 150), however, no significant differences, except for ALT and HCV genotype, were found among the clinical and biochemical parameters of the three treatment outcome groups.

SVR and TVR patients had fewer deaths from any cause (four [0.7%] and four [1.3%], respectively) in comparison to NVR patients (six [3.9%]). Similarly, the frequency of SVR and TVR patients who developed ascites and encephalopathy, symptoms of hepatic decompensation, was lower than that of NVR patients (ascites: two [0.4%], six [2.0%] and eight [5.3%], and encephalopathy: two [0.4%], two [0.7%] and five [3.3%] patients with SVR, TVR and NVR, respectively). None of the patients underwent liver transplantation during the observation period.

Risk of HCC classified by treatment outcome

Of 1013 patients who were followed for a median of 3.6 (range 0.3–7.0) years, 47 (4.6%) developed HCC during the observation period. The baseline characteristics of these patients classified by the development of HCC are shown in Table 2. By univariate analysis, the development of HCC was associated with older age, male sex, higher ALT level, lower serum albumin, lower platelet count, higher AFP level, cirrhosis, and NVR. No significant difference in the duration of HCV RNA negativity was found between the HCC (median [first-third quartiles]: 30.0 [24.0–48.5] weeks) and non-HCC group (41.0 [27.0–48.0] weeks) (p = 0.36) in patients with TVR.

Multivariable logistic regression analysis of possible predictors of HCC development is shown in Table 3. We examined eight factors (age [<60 *vs.* ⩾60 years], sex [men *vs.* women], ALT [<40 *vs.* ⩾40 IU/L], platelet count [<150 *vs.* ⩾150 × 10^9/L], AFP [<10 *vs.* ⩾10 ng/ml], serum albumin [<40 *vs.* ⩾40 g/L], liver pathophysiology [non-cirrhosis *vs.* cirrhosis] and treatment outcome [SVR *vs.* TVR *vs.* NVR]). Significant independent pretreatment predictors of HCC were age 60 years and over (HR 2.81; 95%CI 1.39–5.69; p = 0.004), male sex (HR 2.98; 95%CI 1.46–6.05; p = 0.003), low platelet count (<150 × 10^9/L) (HR 4.04; 95%CI 1.57–10.44; p = 0.004), higher AFP level (⩾10 ng/ml) (HR 2.50; 95%CI 1.09–5.78; p = 0.03), cirrhosis (HR 3.22; 95%CI 1.28–8.13; p = 0.01), and NVR (HR 3.72; 95%CI 1.69–8.18; p = 0.001). Baseline ALT level, serum albumin level, and TVR were not associated with the development of HCC.

Table 1. Pretreatment characteristics of 1013 patients with chronic hepatitis C classified by the existence of cirrhosis and treatment outcome.

Characteristic	Non-cirrhosis n = 863				Cirrhosis n = 150			
	SVR n = 504	TVR n = 255	NVR n = 104	p value*	SVR n = 53	TVR n = 49	NVR n = 48	p value*
Age (yr)	54 (46-63)	61 (55-67)	61 (53-67)	<0.001	61 (57-67)	63 (53-68)	60 (54-68)	0.94
Male, n (%)	263 (52.2)	109 (42.7)	52 (50.0)	0.05	30 (56.6)	19 (38.8)	25 (52.1)	0.18
Body mass index (kg/m²)	22.9 (20.8-25.2)	23.3 (21.3-25.7)	23.1 (21.2-25.1)	0.12	23.0 (20.4-25.6)	23.7 (21.9-26.7)	24.6 (22.8-26.9)	0.07
ALT (IU/L)	52 (34-91)	47 (33-78)	51 (31-80)	0.02	88 (69-127)	65 (53-107)	66 (48-102)	0.01
Albumin (g/L)	42 (40-44)	42 (39-44)	42 (39-44)	0.26	37 (35-39)	37 (35-40)	37 (33-39)	0.87
Platelet count (x10⁹/L)	177 (144-212)	158 (129-194)	159 (130-197)	<0.001	103 (89-116)	97 (84-111)	99 (84-118)	0.26
Hemoglobin (g/L)	137 (129-148)	136 (128-147)	138 (127-149)	0.49	130 (122-140)	133 (123-142)	137 (126-147)	0.37
Ferritin (ng/ml)	156 (75-280)	174 (92-316)	213 (116-361)	0.16	200 (127-317)	202 (134-327)	250 (170-452)	0.05
α-fetoprotein (ng/ml)	4.1 (2.9-6.0)	4.8 (2.9-7.8)	5.9 (3.4-8.9)	<0.001	14.0 (9.2-36.0)	14.1 (9.3-31.3)	30.2 (15.4-42.9)	0.24
Hemoglobin A1c (%)	5.8 (5.7-6.3)	5.9 (5.7-6.4)	6.0 (5.7-6.7)	0.005	5.8 (5.4-6.4)	5.6 (5.3-6.4)	6.0 (5.4-6.6)	0.73
HCV genotype (1/2), n (%)	288/216 (57.1/42.9)	220/35 (86.3/13.7)	92/12 (88.5/11.5)	<0.001	24/29 (45.3/54.7)	43/6 (87.8/12.2)	43/5 (89.6/10.4)	<0.001

Data are expressed as number (%) or median (first-third quartiles).
SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response; HCV, hepatitis C virus; ALT, alanine aminotransferase.
*Comparison among the three groups.

Table 2. Risk factors for the development of HCC by chronic hepatitis C patients treated with PegIFNα2b and RBV.

Characteristic	All patients n = 1013	HCC n = 47	non-HCC n = 966	p value*
Age (yr)	58 (50-65)	67 (58-71)	58 (49-65)	<0.001
Male, n (%)	498 (49.2)	32 (68.1)	466 (48.2)	0.007
Body mass index (kg/m²)	23.0 (21.1-25.2)	23.6 (21.6-25.7)	23.0 (21.1-25.2)	0.15
ALT (IU/L)	54 (35-89)	74 (46-100)	54 (34-89)	0.008
Albumin (g/L)	41 (39-44)	40 (37-42)	44 (41-46)	0.002
Platelet count (x10⁹/L)	159 (120-199)	110 (88-132)	161 (123-201)	<0.001
Hemoglobin (g/L)	136 (127-147)	136 (128-149)	136 (127-147)	0.89
Ferritin (ng/ml)	165 (84-376)	187 (80-462)	167 (80-306)	0.68
α-fetoprotein (ng/ml)	4.9 (3.0-9.3)	11.7 (6.8-32.7)	4.8 (3.0-8.7)	<0.001
Hemoglobin A1c (%)	5.5 (5.3-5.9)	5.8 (5.4-6.3)	5.5 (5.3-5.9)	0.96
HCV genotype (1/2), n (%)	710/303 (70.1/29.9)	38/9 (80.9/19.1)	672/294 (69.6/30.4)	0.09
Non-cirrhosis/cirrhosis, n	863/150 (85.2/14.8)	19/28 (40.4/59.6)	844/122 (87.4/12.6)	<0.001
Treatment duration (wk)	47 (24-48)	43 (23-48)	47 (24-48)	0.58
Virological response (SVR/TVR/NVR), n (%)	557/304/152 (55.0/30.0/15.0)	13/13/21 (27.7/27.7/44.7)	544/291/131 (56.3/30.1/13.6)	<0.001

Data are expressed as number (%) or median (first-third quartiles).
All demographic and clinical data are those at the start of antiviral treatment.
HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response; ALT, alanine aminotransferase.
*Comparison between HCC and non-HCC.

Overall cumulative incidence of HCC classified by treatment outcome

The 5-year cumulative incidence rates of HCC of the SVR (3.1%) and TVR groups (5.8%) were significantly lower than those of the NVR group (18.8%) (both p <0.001), and the rate of the SVR group was lower, but not significantly, than that of the TVR group (p = 0.21).

Cumulative incidence of HCC classified by treatment outcome in the non-cirrhosis group

The Kaplan–Meier curves for the incidence of HCC classified by treatment outcome in the non-cirrhosis group are shown in Fig. 1A (p = 0.009 by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (1.7%) and TVR groups (3.2%) were significantly lower than those of the NVR group (7.6%) (p = 0.003 and p = 0.03, respectively), and the rate of the SVR group was lower, but not significantly, than that of the TVR group (p = 0.47).

Cumulative incidence of HCC classified by treatment outcome in the cirrhosis group

The Kaplan–Meier curves for the incidence of HCC classified by treatment outcome in the cirrhosis group are shown in Fig. 1B (p = 0.03 by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (18.9%) and TVR groups (20.8%) were significantly lower than those of the NVR group (39.4%) (p = 0.03 and p = 0.04, respectively), and the rate of the SVR group was lower, but not significantly, than that of the TVR group (p = 0.94).

Adjusted rates of HCC incidence classified by treatment outcome of non-cirrhotic patients under 60 years of age

The Kaplan–Meyer curves of the estimation of the incidence of HCC by non-cirrhosis patients under 60 years of age, classified by treatment outcome, are shown in Fig. 2A (p = 0.51 by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (0.9%) and TVR groups (1.7%) were lower, but not significantly, than those of the NVR group (2.6%) (p = 0.25 and p = 0.45, respectively).

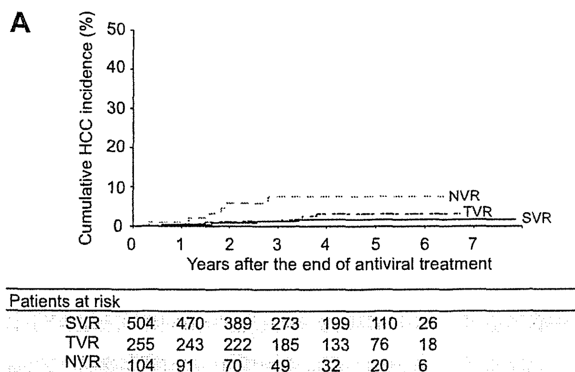
Table 3. Multivariate logistic regression analysis of possible predictors of HCC development.

Parameter	Hazard ratio	95% CI	p value
Age			
<60 yr	1		
≥60 yr	2.81	1.39-5.69	0.004
Sex			
Female	1		
Male	2.98	1.46-6.05	0.003
Platelet count			
≥150 x 10⁹/L	1		
<150 x 10⁹/L	4.04	1.57-10.44	0.004
α-fetoprotein			
<10 ng/ml	1		
≥10 ng/ml	2.50	1.09-5.78	0.03
Liver pathophysiology			
Non-cirrhosis	1		
Cirrhosis	3.22	1.28-8.13	0.01
Treatment outcome			
SVR	1		
TVR	1.50	0.65-3.44	0.34
NVR	3.72	1.69-8.18	0.001

HCC, hepatocellular carcinoma; SVR, sustained virological response; TVR, transient virological response; NVR, non-virological response.

Adjusted rates of HCC incidence classified by treatment outcome of non-cirrhotic patients aged 60 years and over

The Kaplan–Meyer curves of the estimation of the incidence of HCC in non-cirrhosis patients, aged 60 years and over classified by treatment outcome, are shown in Fig. 2B (p = 0.05 by log-rank test). The 5-year cumulative incidence rates of HCC in the SVR (3.5%) and TVR groups (4.2%) were significantly lower than those



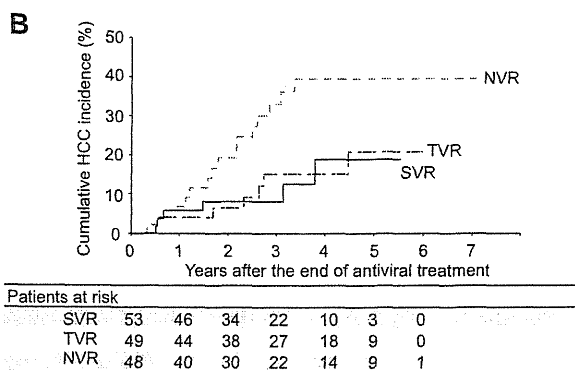
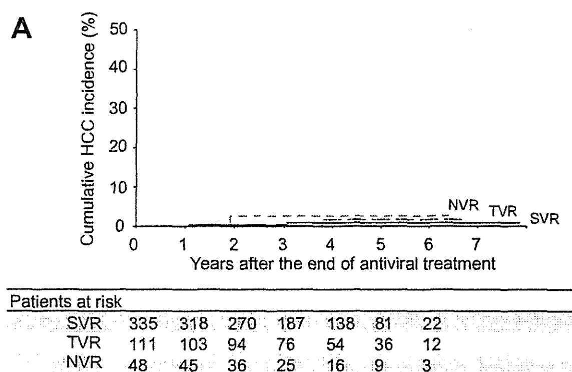


Fig. 1. Cumulative incidence of HCC after PegIFNα2b and RBV treatment stratified by treatment outcome (SVR: continuous line, TVR: long dashed-dotted line, NVR: dashed line). (A) Non-cirrhosis group (overall: $p = 0.009$; SVR *vs.* TVR: $p = 0.47$; SVR *vs.* NVR: $p = 0.003$; and TVR *vs.* NVR: $p = 0.03$ by log-rank test). (B) Cirrhosis group (overall: $p = 0.03$; SVR *vs.* TVR: $p = 0.94$; SVR *vs.* NVR: $p = 0.03$; and TVR *vs.* NVR: $p = 0.04$ by log-rank test).



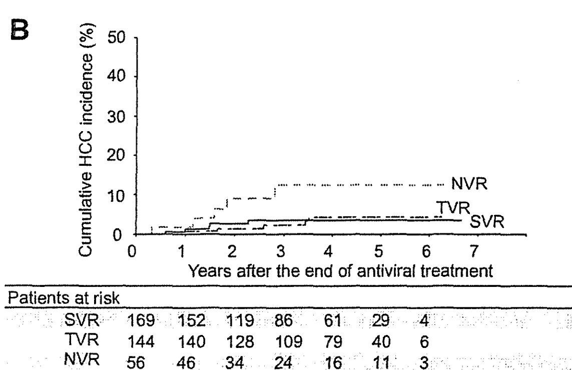


Fig. 2. Cumulative incidence of HCC after PegIFNα2b and RBV treatment stratified by treatment outcome of the non-cirrhosis group (SVR: continuous line, TVR: long dashed-dotted line, NVR: dashed line). (A) Under 60 years of age (overall: $p = 0.51$; SVR *vs.* TVR: $p = 0.94$; SVR *vs.* NVR: $p = 0.25$; and TVR *vs.* NVR: $p = 0.45$ by log-rank test). (B) Aged 60 years and over (overall: $p = 0.05$; SVR *vs.* TVR: $p = 0.96$; SVR *vs.* NVR: $p = 0.04$; and TVR *vs.* NVR: $p = 0.03$ by log-rank test).

of the NVR group (12.4%) ($p = 0.04$ and $p = 0.03$, respectively), and the rate of the SVR group was slightly lower, but not significantly, than that of the TVR group ($p = 0.96$).

The development of HCC by SVR patients

Thirteen patients who achieved SVR (2.3%) (6 non-cirrhotic and 7 cirrhotic patients) developed HCC during the follow-up period. Their individual pretreatment characteristics are shown in Table 4. Of these patients, 3 (patients 1–3) under 55 years of age had liver cirrhosis and the period from the end of antiviral treatment to the diagnosis of HCC was over 3 years. Of the remaining 10 patients (patients 4–13) aged 55 years and over, 6 did not have cirrhosis and the period from the end of antiviral treatment to the diagnosis of HCC was under 2.5 years.

Discussion

We here report the results of a prospective, long-term follow-up study done to evaluate the effect of treatment outcome on the development of HCC in a large cohort of Japanese patients with chronic hepatitis C, who were treated with PegIFNα2b and RBV. We found that those patients who achieved SVR or TVR had a lower risk of developing HCC within 5 years after the end of PegIFNα2b and RBV treatment when compared with NVR, in both cirrhosis and non-cirrhosis groups. Although SVR patients have been reported to have little risk of HCC incidence, a small number of our patients who achieved SVR did develop HCC, showing the necessity of a continued screening of patients with SVR.

Previously, the likelihood of HCC development by PegIFNα- and RBV-treated patients was difficult to determine because of the paucity of adequate long-term prospective studies. Based on the results of this prospective study, sex, age, platelet count, AFP level, and treatment outcome are significant, independent factors for the development of HCC. In addition to our present data, the incidence rate of HCC has been shown to be significantly lower for patients with TT genotype at rs8099917 and CC genotype at rs12979860 near the *IL28B* gene, which are associated with good response to antiviral treatment (data not shown). Of particular interest, the adjusted cumulative incidence of HCC was not significantly different between SVR and TVR for the 5 years after the end of treatment. Two randomized studies of maintenance therapy with low-dose PegIFNα to prevent hepatic decompensation and HCC have been recently reported [25,26]. However, maintenance therapy did not prevent HCC in presence of HCV viremia for at least 5 years, regardless of the degree of viral suppression. Our results showed that complete HCV sup-

Table 4. Individual characteristics of SVR patients who developed HCC.

Patient number	Age (yr)	Sex	Liver pathophysiology	Time to HCC* (yr)	HCV genotype	ALT (IU/L)	Albumin (g/L)	Platelet count ($x10^9/L$)	AFP (ng/ml)	HbA1c (%)
1	47	F	Cirrhosis	3.1	1	44	40	134	3.3	7.1
2	53	M	Cirrhosis	3.1	2	105	42	68	31.0	6.1
3	54	M	Cirrhosis	3.8	1	86	36	88	13.9	5.9
4	59	M	Non-cirrhosis	1.1	2	227	44	131	4.4	6.6
5	63	F	Cirrhosis	1.5	2	81	33	130	16.3	5.3
6	64	F	Non-cirrhosis	1.5	2	72	38	120	6.6	6.8
7	64	M	Non-cirrhosis	1.5	1	29	46	124	20.7	5.1
8	66	F	Cirrhosis	0.7	2	169	42	105	106.0	6.4
9	66	M	Non-cirrhosis	0.6	1	36	35	147	6.2	5.5
10	71	M	Cirrhosis	0.6	2	80	32	106	10.6	5.5
11	71	M	Non-cirrhosis	1.0	1	47	42	108	4.3	5.7
12	74	M	Non-cirrhosis	2.3	1	47	43	143	12.9	6.9
13	77	M	Cirrhosis	0.5	1	73	30	124	11.6	5.4

All data are those at the start of antiviral treatment.

SVR, sustained virological response; HCC, hepatocellular carcinoma; F, female; M, male; HCV, hepatitis C virus; ALT, alanine aminotransferase; AFP, α-fetoprotein; HbA1c, hemoglobin A1c.

*The time frame for HCC incidence starts from the end of antiviral treatment.

pression during antiviral treatment played an important role in preventing the development of HCC.

A recent prospective study that included Caucasian, Hispanic, and Black patients treated with PegIFNα2a and RBV reported that the adjusted mortality from any cause or liver transplantation, or of any liver-related outcome, was significantly lower in TVR patients than in NVR patients [13]. Similarly, the risk of decompensated liver disease, HCC and liver-related death was also lower in TVR patients than in NVR patients, although these differences did not reach statistical significance [13]. Therefore, the significantly low incidence rate of HCC, for the patients of this study with TVR in comparison with NVR, is an original finding, but the trend was true for cirrhotic patients of all ages and for non-cirrhotic patients aged 60 years and over. One possible explanation for this difference may be related to the rising incidence of HCC for NVR patients aged 60 years and over. Our results indicate that the duration of clinical benefit may outlast the period of actual viral suppression in the 5 years after treatment, however, it remains unclear how older age would explain why TVR resulted in a lower incidence of HCC that matched the incidence in SVR. Therefore, it will be necessary to investigate the development of HCC in SVR and TVR patients beyond five years.

Recently, a number of direct-acting antivirals (DAAs) have been designed and developed. Among them, telaprevir and boceprevir, non-structural 3/4A protease inhibitors, have shown promising results in various clinical trials and have led to an increased SVR rate when given in combination with PegIFNα and RBV, as compared with PegIFNα and RBV alone [27,28]. Furthermore, several IFN-free clinical trials, using regimens that combine several potent DAAs, are ongoing. As a result of advances in antiviral treatment, almost all patients can experience complete HCV suppression during treatment. We showed that TVR patients had a lower incidence rate of HCC than did NVR patients. It will be necessary to study the impact of virological response on the development of HCC by patients who undergo DAAs with and without IFN antiviral treatment.

Findings on the effect of SVR on liver-related preferable clinical outcomes have been reported in many previous reports [13,29–31], however, the analysis of the effect of SVR on the development of HCC is statistically difficult, because the number of events is too small to draw meaningful conclusions. In fact, there were only 13 patients with SVR who developed HCC during the observation period, reducing the validity of the analysis. Additional prospective studies that include a larger number of patients with SVR will be necessary to evaluate the relationship between SVR and the development of HCC.

Risk factors for HCV-related HCC have been reported previously, such as older age, male sex, obesity, diabetes mellitus, alcohol consumption, HCV genotype 1b, insulin resistance, complicated hepatic steatosis, and co-infection with hepatitis B virus or HIV [32,33]. Unfortunately, this study lacks data on insulin resistance and hepatic steatosis. Homeostasis Model Assessment of Insulin Resistance value is also related to a profound effect on PegIFNα2b and RBV treatment outcome [34], thus, there may be a significant difference in HbA1c level between the SVR, TVR and NVR non-cirrhotic groups, indicating differences in glucose metabolism. Moreover, it is known that hepatic steatosis occurs in about 40% of the chronic hepatitis C patients, when all common factors of fatty liver, such as alcohol abuse, obesity, and diabetes, have been excluded [35]. Therefore, it remains unclear whether or not there is a significant bias due to different rates of patients with insulin resistance or hepatic steatosis. Another limitation is the generalizability of the extremely high cumulative incidence rate of HCC, especially for cirrhotic NVR patients. The reasons for this exceedingly high rate are not well understood, although it may be explained by the increasing number of aging chronic hepatitis C patients in Japan, earlier than other countries [14]. Our results, therefore, may not be generalized to other ethnic groups that do not have such high rates of HCC.

In summary, this prospective study demonstrated that SVR and TVR patients had a significantly lower rate than NVR patients of HCC incidence within five years after the end of treatment, both for patients with and without cirrhosis. Because the risk of developing HCC remains present even after HCV eradication, long-term screening of patients with SVR is important.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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ORIGINAL ARTICLE

Hepcidin production in response to iron is controlled by monocyte-derived humoral factors

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Abstract Hepcidin, which is mainly produced by the liver, is the key regulator in iron homeostasis. Hepcidin expression is up-regulated by iron loading in vivo, but the mechanism underlying this process is not completely understood. In the present study, we investigated the mechanism, following the hypothesis that hepcidin production in response to iron loading is regulated by extra-hepatic iron sensors. We measured serum hepcidin concentrations and iron indices in Wistar rats treated with saccharated ferric oxide (SFO). Human hepatoma-derived HepG2 cells were stimulated using SFO-administered rat sera, and co-cultured with rat spleen cells, human monocyte-derived THP-1 cells, or human monocytes with diferric transferrin (holo-Tf), and hepcidin concentrations in the conditioned media were measured. SFO elevated rat serum hepcidin concentrations. SFO-treated rat sera increased hepcidin production from HepG2 cells, and this induction correlated with serum hepcidin levels, but not with iron indices. Holo-Tf up-regulated hepcidin concentrations in media from HepG2 cells co-cultured with rat spleen cells, THP-1 cells, or human monocytes with or without cell-to-cell contacts, while holo-Tf did not up-regulate hepcidin from HepG2 cells alone. Our results suggest the existence of humoral factors capable of inducing hepcidin production that are secreted by extra-hepatic cells, such as reticuloendothelial monocytes, in response to iron.

Keywords Hepcidin · Iron · Monocyte · Extra-hepatic iron sensor

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Introduction

Iron is an essential metal for hemoglobin synthesis, many oxidation–reduction reactions, cellular proliferation, and more, while excess iron accumulation may cause organ dysfunction through the production of reactive oxygen species (ROS) and redox reactions. Iron is strictly conserved by recovering and recycling about 20 mg/day of iron from hemoglobin of senescent red blood cells. Dietary iron is absorbed predominantly in the duodenum to replace small daily losses of about 1–2 mg/day. The absorption of iron is tightly regulated by several factors including hepcidin [1]. Hepcidin is a 25 amino acid peptide hormone mainly produced by the liver, and it is thought to be the key regulator in iron homeostasis. Hepcidin is produced as precursor protein which undergoes proteolytic processing resulting in the active 25 amino acid protein [2–4].

Hepcidin regulates intestinal iron absorption and iron release from reticuloendothelial cells by causing the internalization and degradation of the cellular iron exporter ferroportin [5]. Hepcidin is involved in various disorders, such as the anemia of chronic disease (ACD), in which inflammatory cytokines such as interleukin (IL)-6 and IL-1β up-regulate hepcidin expression and thus cause iron-deficiency anemia [6, 7].

The regulatory mechanism of hepcidin production is complicated and still under investigation. Hepcidin transcription is regulated by stimuli such as inflammation, erythropoietic activity, and iron loading. Inflammation has a potent effect on iron homeostasis, reducing intestinal iron absorption, sequestering iron in macrophages, and thereby decreasing serum iron levels. The stimulatory effect of IL-6 on hepcidin is transcriptional and depends on a signal transducer and activator of transcription (STAT) 3 interactions with a STAT3-binding element in the hepcidin promoter [8, 9]. The erythroid regulator pathway also has a strong effect on hepcidin expression [10]. Several groups reported that the administration of erythropoietin (EPO) decreased urinary hepcidin or circulating hepcidin levels in healthy volunteers, patients with chronic kidney diseases (CKD), and patients on hemodialysis (HD) [11–13]. There is evidence in a mouse study that bone marrow cells are involved in suppression of hepcidin after EPO treatment [14–16], but the molecular events are not yet clear. Patients with β-thalassemia have ineffective erythropoiesis, and this is involved with iron overload resulting from increased gastrointestinal iron absorption due to low hepcidin levels. The molecule responsible for hepcidin down-regulation in β-thalassemia was identified as growth differentiation factor (GDF)-15, a transforming growth factor (TGF)-β super family, but GDF-15 is not responsible for physiologic hepcidin regulation [11, 12, 17].

Hepcidin production is regulated by iron levels in the body. The mechanism of this regulation has been very difficult to determine and is under investigation currently. In humans, oral administration of iron increases urinary hepcidin excretion [18], and in a mouse model hepcidin-1 mRNA expression was induced by iron loading [19]. Although hepcidin induction after in vivo iron loading has been observed, inconsistent findings have been reported in experiments in vitro. Addition of diferric transferrin (holo-Tf) did not up-regulate hepcidin production or hepcidin mRNA expression in hepatoma-derived cell lines or primary hepatocytes [18, 20]. Limitations of these experiments weaken their significance. For example, transcriptional experiments would not be expected to cause active hepcidin production because of the complicated processing pathway. The mechanisms by which hepatocytes sense iron and control hepcidin expression are not completely understood, although a recent report suggests that epithelial cells of the small intestine may be one of the iron sensors [21]. Bone morphogenetic protein (BMP) 6, the endogenous regulator of hepcidin expression [22, 23], has been reported to be expressed by small intestinal cells in response to iron loading and induce hepcidin production in the liver, but other groups insisted that iron overload induces BMP6 expression in the liver but not in the duodenum [24]. It seems likely that small intestinal enterocytes sense iron and regulate hepatic hepcidin production because the small intestine is the only organ which absorbs iron, but this hypothesis is controversial. Another possibility is that extra-hepatic cells that store iron, such as reticuloendothelial cells, sense body iron status and regulate hepatic hepcidin production.

We, therefore, hypothesized that sensors to detect body iron status are located in extra-hepatic sites and these sensors mediate hepatic hepcidin production to maintain iron homeostasis.

To test the hypothesis, we developed a quantitative method for measuring levels of hepcidin concentrations in rat serum and culture media of human cell lines by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI–MS/MS). These methods were used to investigate the mechanism of hepcidin production in response to iron in iron-loaded rats and cultured human hepatoma-derived cells and monocyte-derived cells in vitro.

Materials and methods

Hepcidin standards

Human hepcidin-25 was obtained from the Peptide Institute, Inc. (Osaka, Japan). Rat hepcidin and [$^{13}C_{18}$, $^{15}N_3$]-human hepcidin were synthesized at the Peptide Institute, Inc.

Chemicals and antibodies

Holo-Tf was purchased from R&D Systems (Minneapolis, MN, USA). Human IL-6 was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Japan Bioserum Co., Ltd. (Hiroshima, Japan). Minimum essential medium eagle (E-MEM), L-glutamine, sodium bicarbonate, and albumin solution from bovine serum (BSA) were from Sigma-Aldrich Corp. (St. Louis, MO, USA). Penicillin streptomycin solution, sodium pyruvate, and non-essential amino acids (NEAA) were provided by Life Technologies Corporation (Carlsbad, CA, USA). Saccharated ferric oxide (SFO) was from Nichi-Iko Pharmaceutical Co., Ltd. (Toyama, Japan). Otsuka glucose injection 10 % was from

Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). Tocilizumab, humanized anti-human IL-6 receptor antibody [25], was produced by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals and solvents were of analytical reagent grade.

Animals

Nine-week-old male Wistar rats were purchased from Japan SLC., Inc. (Shizuoka, Japan). All animals were allowed to acclimatize and recover from shipping-related stress for 1 week prior to the study. Rats were fed rodent chow and water ad libitum. All studies were approved by our Institutional Animal Care and Use Committee and conducted according to guidelines for the care and use of laboratory animals from Chugai Pharmaceutical Co., Ltd.

Animal treatment

SFO was diluted in Otsuka glucose injection 10 % used as a vehicle in appropriate concentrations. Two or 5 mg/kg of SFO or equal volumes of vehicle was administered intravenously into rats which were sacrificed 1, 3, 6, 12, 18, 24 and 30 h after SFO injection. Three rats from each group were used.

Specimen collection

Rats were anesthetized with isoflurane and blood was collected into evacuated blood-collecting tubes (TERUMO Corporation, Tokyo, Japan), and serum was isolated according to the manufacturer's instructions. Spleen cells were isolated from Wistar rats.

Measurement of iron indices

Serum iron was measured using TBA-120FR biochemistry automatic analyzer (Toshiba Medical Systems, Tochigi, Japan). Non-transferrin bound iron (NTBI) was determined by metal-free high-performance liquid chromatography (HPLC) (Waters Corporation, Milford, MA, USA) [26].

Cell cultures

Human hepatocellular carcinoma cell line, HepG2 and human monocytic cell line, THP-1 were obtained from American Type Culture Collection (Manassas, VA, USA). Human peripheral blood monocytes were purchased from Biopredic international (Rennes, France).

HepG2 cells were cultured at 37 °C in a humidified incubator with 5 % CO_2 in E-MEM with 10 % (v/v) FBS supplemented with 0.1 mM NEAA, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. HepG2 cells were seeded at a density of 1.0×10^6 cells/well in 6-well plates (Corning Incorporated, Corning, NY, USA), and the cells were stimulated with 2.4 mg/mL holo-Tf, 20 ng/mL IL-6, or 10 % (v/v) rat serum in 2 mL of growth medium for 48 h. After incubation, cultured media were collected and human hepcidin concentrations in the media were analyzed.

Each treatment was performed in triplicate.

Cell co-culture system

All cells were cultured at 37 °C in a humidified incubator with 5 % CO_2 in E-MEM with 2 % (v/v) FBS and 1 % (v/v) BSA supplemented with 0.1 mM NEAA, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. HepG2 cells were seeded at a density of 1.0×10^6 cells/well in 6-well plates, and the medium in each well was replaced by 2 mL of growth medium containing 1.0×10^6 of normal rat spleen cells, THP-1 cells or human peripheral blood monocytes. To investigate the effect of IL-6 signaling on hepcidin production, cells were incubated with 100 μg/mL tocilizumab. After incubation, cultured media were collected and human hepcidin concentrations in the media were analyzed.

To inhibit cell–cell contact in cell co-culture system, we used cell culture inserts (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). HepG2 cells were seeded at a density of 1.0×10^6 cells/well in 6-well plates, and the medium in each well was replaced by 2 mL of growth medium. HepG2 cells were overlaid by cell culture inserts with additional 1 mL growth medium containing THP-1 cells or not. After incubation, total 3 mL cultured media were collected and human hepcidin concentrations in the media were analyzed.

Each treatment was performed in triplicate.

Sensitive LC/ESI–MS/MS analysis of human and rat hepcidin

In this study, concentration of human hepcidin-25 was specifically determined as previously reported, and that of rat hepcidin with the following modification [27]. Human hepcidin was used as an internal standard to measure rat hepcidin. LC/ESI–MS/MS was performed using an AB SCIEX Triple Quad™ 5500 System (AB SCIEX, Foster City, CA, USA) equipped with prominence $UFLC_{XR}$ systems (Shimadzu corporation, Kyoto, Japan). Analytical chromatography of human and rat hepcidin was performed on a PLRP-S (5 μm, 300 Å, 150 mm × 2.1 mm i.d.; Polymer Laboratories Ltd., Shropshire, UK). Instrument control and data processing were run by Analyst™ software version 1.5.1 (AB SCIEX). Selected reaction monitoring (SRM)

transitions were as follows: rat hepcidin, m/z 905.060 → 1118.300; human hepcidin, m/z 558.800 → 693.700. Mobile phase A was 0.1 % aqueous formic acid, and mobile phase B was 0.1 % formic acid in acetonitrile.

Statistical analysis

In in vivo study, statistical significances between iron-treated groups and the vehicle group were analyzed by the Tukey test. In in vitro studies, statistical significances were analyzed by Student's t test. A P value < 0.05 was used to estimate statistical significance. Data were represented as mean and SD.

Results

SFO administration increased serum hepcidin concentrations in rat

Wistar rats received 2 or 5 mg/kg of SFO as well as vehicle solution. Changes in serum hepcidin concentrations after SFO administration are shown in Fig. 1. Serum hepcidin concentrations in SFO-treated rats were gradually elevated 12 h after SFO injection with subsequent decrease. This effect was dose-dependent and serum hepcidin concentrations in the vehicle group were not increased.

SFO-administrated rat sera increased hepcidin production from HepG2 cells

>We screened hepatocyte-derived cell lines for hepcidin production with various stimuli as previously reported.

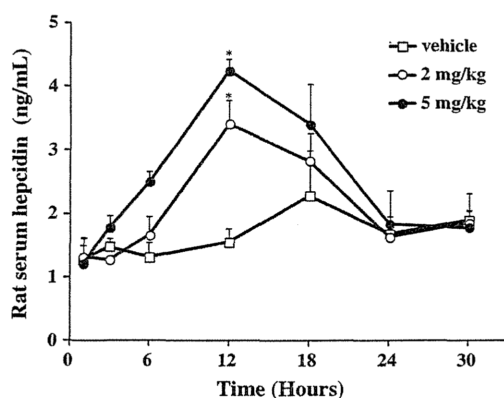

Fig. 1 SFO administration increased serum hepcidin concentrations in rats. Wistar rats received 2 mg/kg (*open circles*) or 5 mg/kg (*filled circles*) of SFO as well as vehicle solution (*open squares*). Serum was analyzed for hepcidin concentration by the LC/ESI-MS/MS method. Results are expressed as mean and SD. Three rats from each group were used. $*P < 0.05$ was found for SFO-treated groups compared with the vehicle group by the Tukey test

None of the tested cell lines produced hepcidin in response to holo-Tf stimulation, while some cell lines, including HepG2, secreted hepcidin after IL-6 stimulation [28]. Among these cell lines, we selected the HepG2 cell for subsequent in vitro experiments because of its higher hepcidin production. Hepcidin production from HepG2 cells was not increased by holo-Tf stimulation, while IL-6 significantly augmented hepcidin production from HepG2 cells as previously reported (Fig. 2a).

To confirm the involvement of humoral factors in hepcidin production induced after SFO administration, we examined the induction of hepcidin by serum from SFO-treated rats or vehicle-treated in HepG2 cells. HepG2 cells were cultured with media containing 10 % (v/v) rat serum for 48 h, and then media were collected and analyzed. As shown in Fig. 2b, SFO-loaded rat serum obviously induced hepcidin production from HepG2, while vehicle-treated rat serum did not induce hepcidin production. Hepcidin production from HepG2 cells stimulated with rat serum correlated with rat serum hepcidin concentrations (Fig. 2c).

Hepcidin concentrations were not correlated with iron indices and cytokines in SFO-administrated rat sera

Serum levels of iron indices and cytokines were examined in SFO-treated rats that have been reported to increase hepcidin production. Serum iron levels were higher in SFO-treated groups than in vehicle-treated group 1 h after SFO injection. Serum iron levels then gradually decreased, and returned to normal levels in both groups (Fig. 3a). NTBI levels were not significantly changed after SFO or vehicle injection (Fig. 3b). Serum IL-6 and IL-1β concentrations, determined by enzyme-linked immunoabsorbent assay (ELISA), were not detected in either serum (data not shown).

Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and rat spleen cells

We then hypothesized that the spleen is the putative extra-hepatic iron sensing organ and induces hepcidin production from liver in response to iron, because it is one of the major iron storage sites in the body. HepG2 cells were co-cultured with normal rat spleen cells with or without holo-Tf stimulation, and after 48 h incubation, cultured media were collected and human hepcidin concentrations in the media were analyzed. Although hepcidin production was not up-regulated when HepG2 cells alone were cultured with holo-Tf, holo-Tf stimulation significantly augmented hepcidin production from HepG2 cells co-cultured with rat spleen cells (Fig. 4).

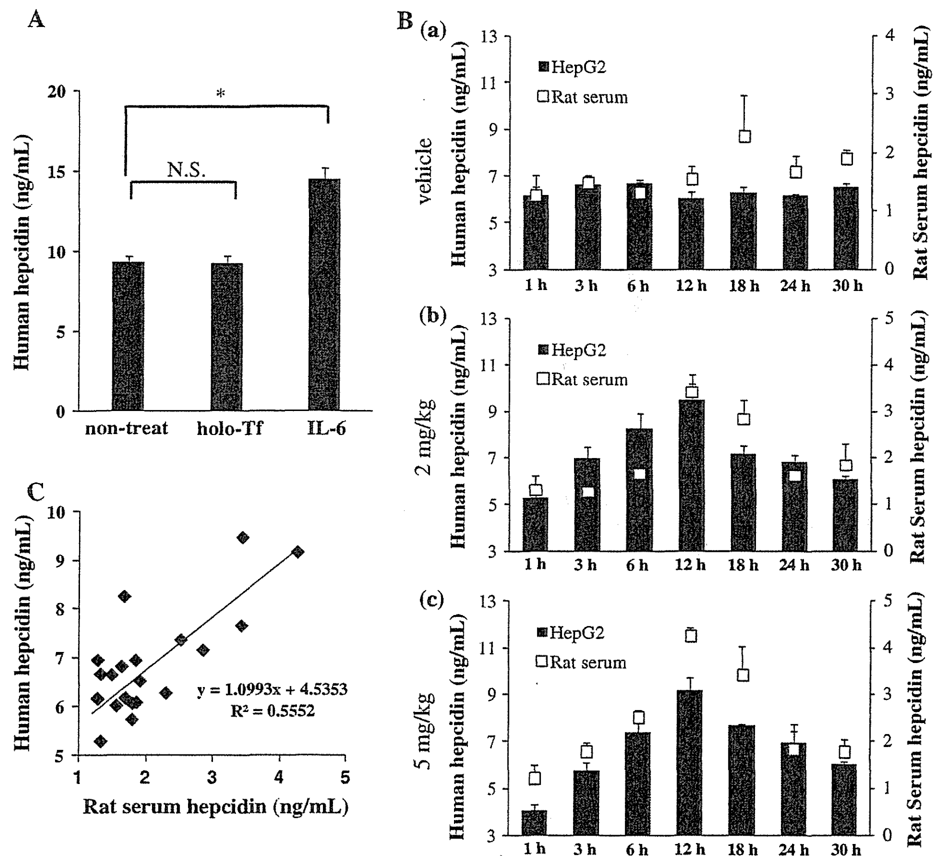

Fig. 2 SFO-administrated rat sera increased hepcidin production from HepG2 cells. **a** HepG2 cells were stimulated with 2.4 mg/mL holo-Tf or 20 ng/mL IL-6 for 48 h, and human hepcidin concentrations in cultured media were analyzed. **b** HepG2 cells were stimulated with 10 % (v/v) rat serum for 48 h, and human hepcidin concentrations in cultured media were analyzed. Results of vehicle-treated rats (*a*), 2 mg/kg SFO-treated rats (*b*), or 5 mg/kg SFO-treated rats (*c*) are shown. **c** The figure shows the correlation between rat serum hepcidin concentrations and hepcidin production from HepG2 cells incubated with rat serum. Results are expressed as mean and SD. Each treatment was performed in triplicate. Statistical significances were analyzed by Student's *t* test with a P value < 0.05

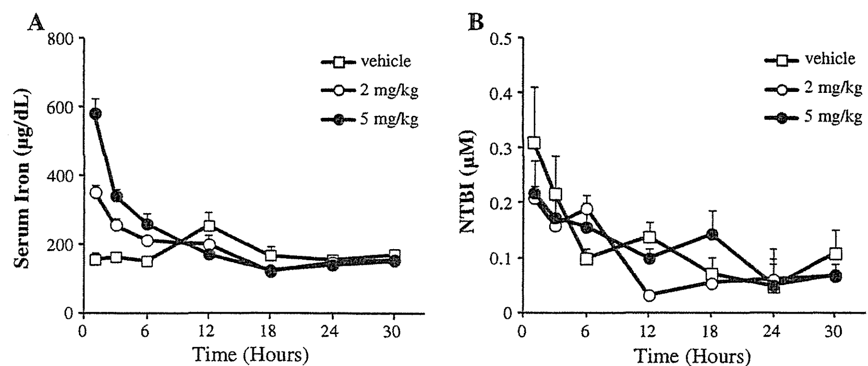

Fig. 3 Iron indices transitions in SFO-administrated rat sera were independent from hepcidin concentrations. Wistar rats received 2 mg/kg (*open circles*) or 5 mg/kg (*filled circles*) of SFO as well as vehicle solution (*open squares*). Serum iron levels (**a**) and serum NTBI levels (**b**) in each-treated rat sera were analyzed. Results are expressed as mean and SD. Three rats from each group were used

Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and THP-1 through humoral factors

To determine which cells in spleen affect hepcidin production in response to iron, we next tested whether reticuloendothelial monocytes are iron sensing cells, because spleen produces and stores monocytes [29] and monocytes are known to be involved in modulation of iron metabolism [30, 31]. We selected the human monocytic cell line, THP-1, for subsequent experiments. We observed the expression of CD71 which is needed for cellular import of iron by flow cytometry (FCM). An increase of iron uptake in THP-1 cells after addition of holo-Tf was detected by inductively coupled plasma atomic emission spectroscopy (data not shown). HepG2 cells were co-cultured with THP-1 cells with or without holo-Tf stimulation. After 48 h incubation, cultured media were collected and human hepcidin concentrations in the media were analyzed. As shown in Fig. 5a, hepcidin production from HepG2 cells was up-regulated by coexistence of THP-1 cells, while holo-Tf stimulation further increased hepcidin production from

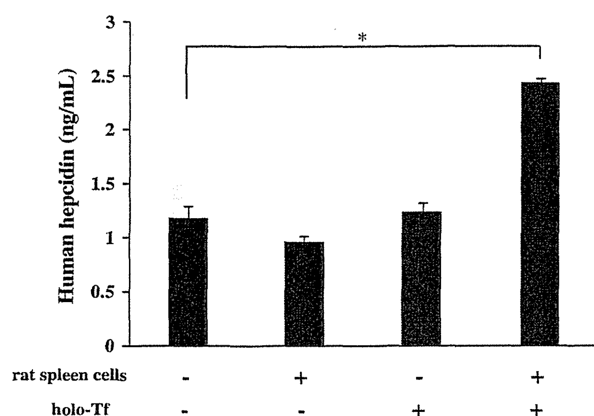

Fig. 4 Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and rat spleen cells. HepG2 cells were co-cultured with normal rat spleen cells with or without holo-Tf, and after 48 h incubation, cultured media were collected and human hepcidin concentrations in cultured media were analyzed. Results are expressed as mean and SD. Each treatment was performed in triplicate. Statistical significances were analyzed by Student's t test with a P value < 0.05

HepG2 cells significantly. Tocilizumab could not inhibit hepcidin production from HepG2 cells co-cultured with THP-1 cells with holo-Tf. We next investigated the requirement of cell–cell contact for cross-talk between HepG2 cells and THP-1 cells in mediating hepcidin regulation. As shown in Fig. 5b, we found up-regulation of hepcidin production from HepG2 cells with holo-Tf stimulation in noncontact system of HepG2 cells and THP-1 cells.

Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and human peripheral blood monocytes

HepG2 cells were co-cultured with human peripheral blood monocytes with or without holo-Tf stimulation. After 48 h incubation, cultured media were collected and human hepcidin concentrations in the media were analyzed. Holo-Tf stimulation augmented hepcidin production from HepG2 cells co-cultured with human peripheral blood monocytes significantly (Fig. 6).

Discussion

In this study, we investigated the mechanism of hepcidin production in response to iron to clarify the existence of putative extra-hepatic iron sensors.

Several groups reported that iron loading leads to elevated transcription of the *HAMP* gene and increased serum hepcidin levels in vivo [18, 19]. We detected elevated hepcidin levels in rat serum after SFO administration (Fig. 1). We have established a method for measuring hepcidin levels in media of cultured cell lines. In screened hepatocyte-derived cell lines, none of the tested cells up-regulated hepcidin production following holo-Tf addition, although IL-6 up-regulated hepcidin production and N-terminally truncated hepcidin isoforms were increased by holo-Tf in some cell lines [28]. These findings lead us to hypothesize that these cell lines lack iron sensory system so that the iron loading signal is mediated by humoral factors

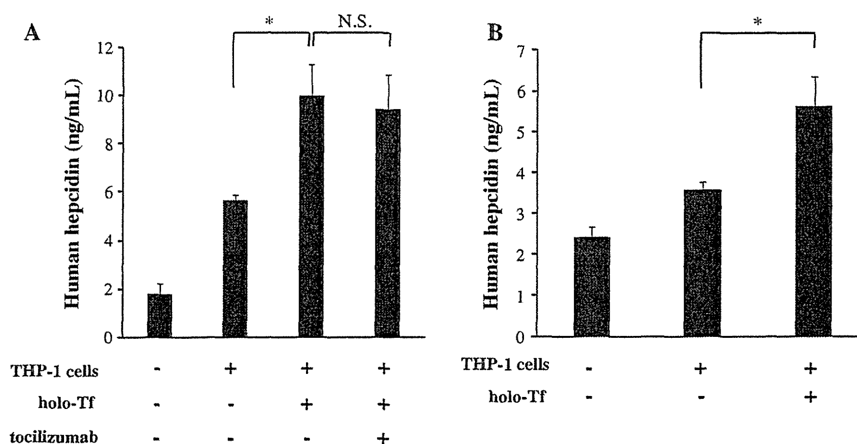

Fig. 5 Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and THP-1 through humoral factors. **a** HepG2 cells were co-cultured with THP-1 cells with holo-Tf alone or in combination with tocilizumab. After 48 h incubation, cultured media were collected and human hepcidin concentrations in cultured media were analyzed. **b** HepG2 cells were co-cultured with THP-1 cells with or without holo-Tf using cell culture inserts to inhibit cell–cell contact. After 48 h incubation, cultured media were collected and human hepcidin concentrations in cultured media were analyzed. Results are expressed as mean and SD. Each treatment was performed in triplicate. Statistical significances were analyzed by Student's t test with a P value < 0.05

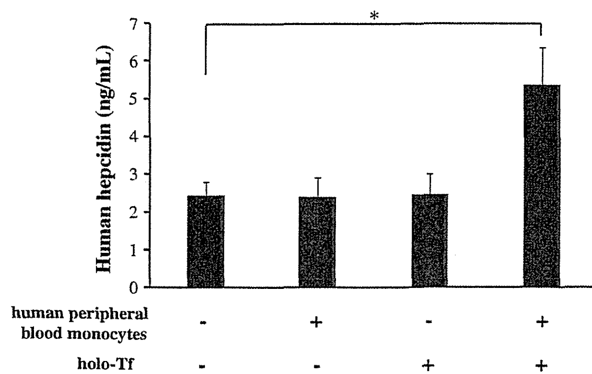

Fig. 6 Holo-transferrin stimulation increased hepcidin production in co-culture of HepG2 and human peripheral blood monocytes. HepG2 cells were co-cultured with human peripheral blood monocytes with or without holo-Tf, and after 48 h incubation, cultured media were collected and human hepcidin concentrations in cultured media were analyzed. Results are expressed as mean and SD. Each treatment was performed in triplicate. Statistical significances were analyzed by Student's *t* test with a P value < 0.05

such as cytokines like BMP participating in iron homeostasis.

To confirm the involvement of humoral factors induced by SFO administration in hepcidin production, we examined levels of hepcidin in HepG2 cells cultured in serum from SFO-treated rats. As shown in Fig. 2b, SFO-loaded rat serum significantly induced hepcidin production from HepG2 cells. The LC/ESI–MS/MS method for hepcidin detection, which we have established, allows discriminating human hepcidin from rat hepcidin from the difference between the molecular masses of precursor and product ions. Rat hepcidin in rat serum did not interfere with quantification of human hepcidin (data not shown). Hepcidin production from HepG2 cells stimulated with rat serum was correlated with rat serum hepcidin concentrations (Fig. 2c), but not correlated with iron indices and cytokines such as IL-6 and IL-1β that have been reported to induce hepcidin production (Fig. 3). These results suggest that humoral factors, whose relation with hepcidin regulation have previously been unsuspected, mediate iron loading signals for up-regulation of hepcidin; iron sensors may therefore be located on extra-hepatic cells.

It has been reported that an indirect mechanism may function for the up-regulation of hepatic hepcidin expression by iron [32]. THP-1 cells were differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA), and conditioned medium was collected after holo-Tf stimulation for 24 h. Hepcidin mRNA expression levels in HepG2 cells with activated THP-1 conditioned medium were up-regulated suggesting that iron stimulation induces hepcidin mRNA synthesis from hepatocyte via cross-talk with macrophages. Another report suggests that IL-1β is the predominant macrophage factor involved in inducing hepatic *HAMP* expression. Cross-talk between macrophages and hepatocytes may induce hepcidin expression, with these two cell types taking an active role in regulating hepcidin during inflammation [33]. Macrophages are produced by differentiation of monocytes in damaged tissues and have a low chemotactic activity. We therefore hypothesize that extra-hepatic iron sensors maintain the iron homeostasis. Reticuloendothelial monocytes circulate in the bloodstream and are known to be involved in modulation of iron metabolism.

The spleen is a site for storage and rapid development of monocytes [29]. HepG2 cells were co-cultured with normal rat spleen cells with or without holo-Tf stimulation. Holo-Tf increased hepcidin production in a co-culture system (Fig. 4). A human monocytic cell line, THP-1, was chosen as a model of reticuloendothelial monocytes model expressing transferrin receptor CD71 for subsequent experiments. These cells took up iron after addition of holo-Tf (data not shown). THP-1 did not produce hepcidin in transcriptional and protein levels (data not shown). HepG2 cells were co-cultured with non-activated THP-1 cells with or without holo-Tf. Holo-Tf increased hepcidin production in the co-culture system both with and without cell–cell contact (Fig. 5). These experiments showed that direct cell–cell contact between HepG2 cells and THP-1 cells was not required for hepcidin production in response to iron. Humoral factors increasing hepatic hepcidin production in response to iron might be secreted into blood. In this co-cultured system, tocilizumab could not inhibit hepcidin production from HepG2 cells co-cultured with THP-1 cells with holo-Tf, while induction of hepcidin production in response to iron was independent of IL-6 signaling. Finally, HepG2 cells co-cultured with human peripheral blood monocytes with or without holo-Tf showed that holo-Tf increased hepcidin production in the co-culture system (Fig. 6).

A molecular mechanism of hepcidin regulation by iron was recently reported [34]. Extracellular iron in the form of holo-Tf binds with transferrin receptor (TfR)1 and TfR2, and TfR2 is known to be one of the key iron sensors [35]. TfR2 is highly expressed in the liver, and faintly expressed in spleen and bone marrow [36]. TfR2 associates with HFE, which is essential for hepcidin expression, and BMP signaling via Sma- and Mad-related proteins (SMADs) for hepcidin induction is modulated by these components [37–39]. We observed the expression of TfR2 as well as HFE in HepG2 cells. Hemojuvelin (HJV) expression was also detected by quantitative PCR (data not shown). Hepcidin production from HepG2 cells was induced in response to BMP2 [28], indicating that HepG2 cells must express all components for iron sensing except for humoral factors secreted in response to iron. BMP6 is also thought to be a key endogenous regulator of hepcidin expression and iron metabolism [22, 23].

BMP6 interacts with HJV and BMP receptors, and induces hepcidin through translocation of SMADs to the nucleus. BMP6 is believed to be expressed mainly in the liver for sensing intracellular iron [24, 40]. We checked BMP6 protein levels in media from HepG2 cells co-cultured with THP-1 cells with or without holo-Tf, but BMP6 levels needed to increase hepcidin production from HepG2 cells were not detected (data not shown). Thus, other factors than BMP6 and IL-6 may be secreted from monocytes in response to iron to induce hepcidin production, and detailed mechanisms should be clarified in the future.

Various assays have been developed for quantification of hepcidin on mass spectrometry technique including surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF–MS) and LC/ESI–MS/MS [27, 41, 42]. Recently, immunochemical assays such as competitive radio-immunoassays (RIA) and ELISA to measure hepcidin have also been developed [13, 43, 44]. Hepcidin has the potential to be a novel clinical biomarker in iron metabolism disorders, because the involvement of hepcidin detected in blood and urine in the dysregulation of iron homeostasis in various disease, such as CKD, hereditary hemochromatosis and hepatitis C virus (HCV) infection has been reported [45–47]. On the other hand, hepcidin may reflect not only iron metabolism disorders but also other disease states, because hepcidin expression is controlled by several stimuli such as inflammation. Monocyte-derived humoral factors we suggested are thought to be iron-responsive, and then these factors are believed to allow for more elaborate diagnosis of iron metabolism disorders and develop into therapeutic target for them.

In conclusion, our results indicate the existence of humoral factors secreted in response to iron loading which could induce hepcidin production was clarified. Our data also suggested that they were secreted by extra-hepatic cells, such as reticuloendothelial monocytes to maintain physiological iron homeostasis. The nature of the factors awaits future identification.

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Conflict of interest The authors declare that they have no conflict of interest.

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HEPATOLOGY

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

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