recently reported to be important for HCV infection by Vanwolleghem *et al.*,[47] who showed that 100% HCV infectivity was reached with high quality inocula in mice with human albumin levels of more than 1 mg/mL.

Spontaneous eradication of HCV has been reported to be higher in individuals with the rs12979860CC genotype.[20] In such individuals, components of both the innate and adaptive immune systems likely play a role in eliminating the invading virus in the absence of exogenous IFN. As no human or mouse adaptive immune effector cells are present in human hepatocyte chimeric mice, it is possible to isolate the effects of innate immunity in liver cells using this model. When liver cells are selected from donors with different *IL-28B* genotypes, the model provides an opportunity to study differences in innate immune responses associated with the *IL-28B* genotype.

Clonal infection with HCV genotypes 1a, 1b, 2a and 2b is now possible,[48–50] and reverse genetic approaches combining infectious HCV clones with the human hepatocyte chimeric mouse facilitate studying the effects of viral mutations and host factors, such as the *IL-28B* genotype, on infectivity of the virus. Several factors may influence infectivity of the virus, including serum lipid profile,[51] presence of neutralizing antibodies and presence of antibody-resistant viral quasispecies.[52,53] However, we found no difference in incidence of establishment of infection with genotype 1b clones among mice transplanted with different *IL-28B* genotype liver cells.[54] This result may derive from the specific infection route (intrahepatic injection of RNA to mouse liver) and the large RNA titers used in the study.

EXPRESSION OF INTERFERON-STIMULATED GENE (ISG) AND VIRAL LOAD IN HUMANS AND HUMAN HEPATOCYTE CHIMERIC MICE

ALTHOUGH THE MECHANISM underlying the *IL-28B* polymorphism remains unclear, a number of studies have reported that intrahepatic ISG expression levels are lower[55–57] and viral titers are higher[8,12] in patients with the eradication-favorable *IL-28B* genotype. Honda *et al.* showed that expression of a number of ISGs was higher in patients with the unfavorable rs8099917 TG or GG genotypes compared to those with the TT genotype and that non-responders were significantly over-represented among patients with high ISG expression levels.[57] Using paired liver biopsy samples, Sarasin-Filipowicz demonstrated that ISG expression levels in non-responders were initially higher prior to IFN treatment, but administration of IFN failed to induce ISG expression levels above this baseline level, whereas IFN induced a strong upregulation of ISG expression in patients who achieved a rapid virological response.[58] Shebl *et al.* measured ISG expression levels in uninfected cells and found no evidence of an association with the *IL-28B* genotype, suggesting that the association observed in HCV-infected cells does not reflect normal expression levels in healthy cells and reflects the response to HCV infection.[59]

The chimeric mouse provides a suitable model to test hypotheses concerning the role of the *IL-28B* genotype on innate immune responses and ISG expression. Using this model, we found that ISG expression levels were also lower and viral titers were higher in human hepatocyte chimeric mice with the favorable *IL-28B* genotype.[54] As illustrated in Figure 1, the virus appears to replicate more efficiently in mice with the favorable genotype, perhaps because whenever the viral load is low hepatocytes with the favorable genotype can efficiently clear the virus so that chronic infection only results under higher viral loads.[20,60] At the same time, ISG expression is lower in cells with the favorable allele, which may be advantageous by preventing saturation of the IFN signaling pathway through continual stimulation.[58] However, earlier reports demonstrated that induction of a strong response to IFN-α is dependent on a weak constitutive IFN signal to maintain IFN-dependent expression of insulin-related factor-7, an essential transcription factor involved in IFN signal transduction that has a short half-life.[61] The unfavorable *IL-28B* genotype might alter the balance of this feedback loop in the presence of HCV. Although we observed that ISG expression levels were lower in mice with the favorable genotype prior to IFN treatment (unpubl. data, K. Chayama, Hiroshima University, Japan), ISG expression increased sharply following IFN administration in mice with the favorable genotype and were significantly higher than in mice with an unfavorable genotype.[54]

ISG EXPRESSION LEVELS AND EFFECT OF IFN THERAPY IN HUMANS AND CHIMERIC MICE

PRETREATMENT ISG EXPRESSION levels may be a stronger predictor of outcome of combination therapy than the *IL-28B* genotype,[62] suggesting that the mechanism of the SNP is related to its role in ISG regulation. Lower ISG expression levels are associated with the eradication-favorable *IL-28B* genotype in chronic hepatitis C patients,[55–57] and high baseline expression levels of ISG such as *RIG-I*, *ISG-15* and *USP-18* correlate with poor response to therapy.[63] Using the human

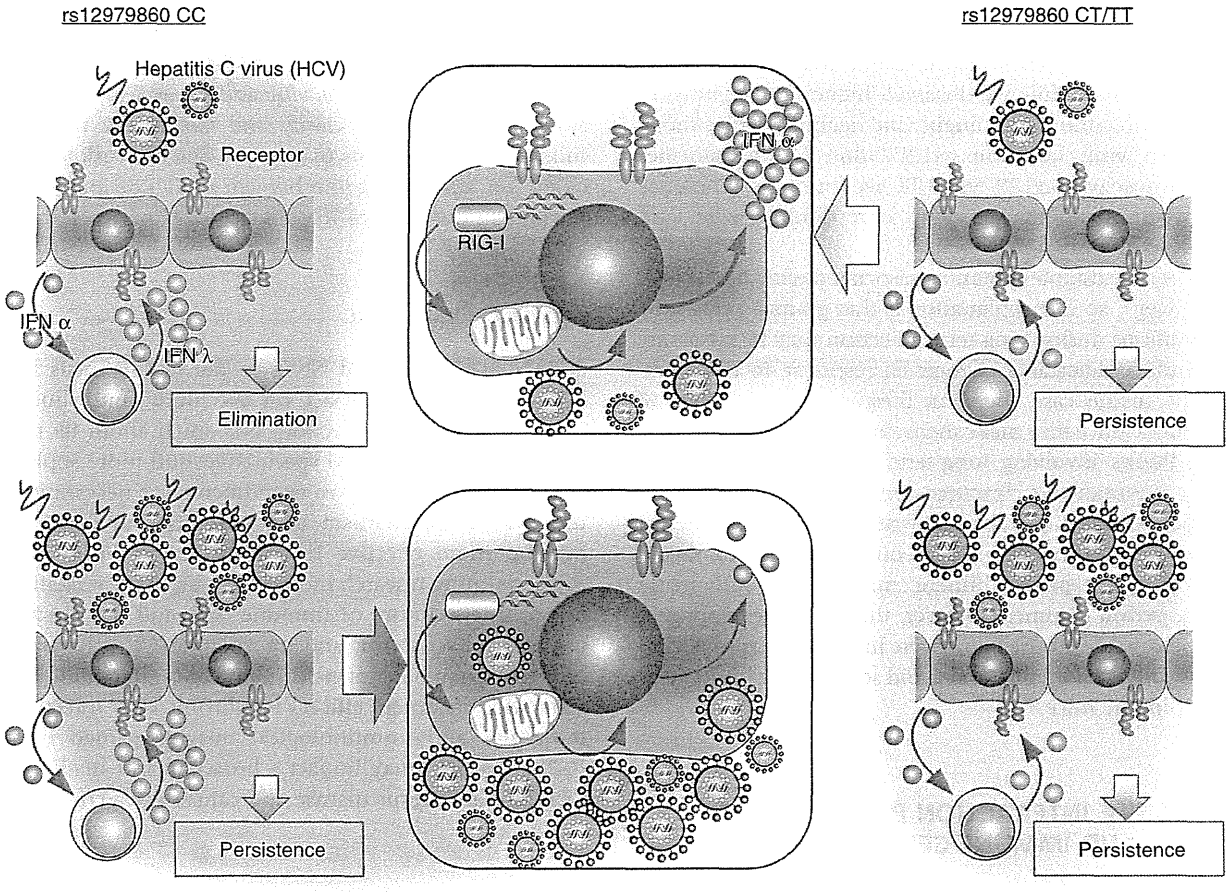

Figure 1 Hypothetical model of virus replication in hepatocytes with eradication-favorable (interleukin [IL]-28B rs12979860 CC or rs8099917 TT) or unfavorable (rs12979860 CT/TT or rs8099917 GT/GG) genotypes. HCV may be eliminated from individuals with the favorable genotype at low titer but may persistently infect those with high titers or the unfavorable genotype (upper right and left panels). Viral sensors continuously detect viral RNA during chronic infection (central panel), resulting in continuous activation of interferon-stimulated genes (ISG), including interferon inhibitory molecules such as *PIAS* and SOCS3. In hepatocytes with the unfavorable genotype, ISG expression may be refractory to further interferon stimulation, resulting in a poor response to therapy, but in hepatocytes with the favorable genotype, low baseline ISG expression may prevent overexpression of interferon signal inhibitors, resulting in stronger ISG induction and a better response to therapy.

hepatocyte chimeric mouse model, we showed that intrahepatic expression levels were significantly higher and HCV RNA reduction was significantly greater in mice with rs12979860CC hepatocytes following IFN administration.[54] Several genes that suppress IFN-dependent antiviral activity, such as *PIAS* and genes in the SOCS family, are themselves ISGs, and may serve as a negative feedback loop to regulate IFN signaling. Consequently, continuous low-level ISG expression in hepatocytes with the unfavorable genotype may dampen IFN sensitivity and prevent effective ISG induction when IFN is administrated during treatment.

RELATIONSHIP BETWEEN *IL-28B* GENOTYPE AND HCV CORE AMINO ACID SUBSTITUTIONS

REVERSE GENETICS USING the chimeric mouse model could facilitate detailed studies of the effect of host and viral factors on IFN sensitivity and ISG expression. HCV core protein substitutions are predictive of outcome of peginterferon plus ribavirin combination therapy,[64,65] and patients with the unfavorable *IL-28B* genotype are more likely to host viruses having core substitutions.[66] To examine these interactions *in*

vivo, we created core amino acid 70 and 91 double wild and double mutant clones using the infectious genotype clone KT-9 and performed infection and IFN treatment experiments. While we observed higher ISG expression levels and a more prominent viral decline in mice transplanted with favorable rs12979860 CC hepatocytes than those with rs12979860 TT, we found no difference between mice infected with core double wild and double mutant HCV.[54]

The core double mutant is associated with steatosis of the liver,[67] so one explanation is that gradual metabolic alterations during long-term infection may be necessary to induce detectable changes in response to IFN treatment. In this case, the short lifespan and sensitivity of chimeric mice may make them unsuitable for examining conditions involving long-term hepatic change. Similarly, spontaneous clearance does not necessarily imply rapid clearance, and so it may not be possible to detect subtle differences in spontaneous clearance due to the *IL-28B* genotype in chimeric mice over a sufficiently long period of time. However, the recent development of permissive mouse hepatocyte lines may improve viral infection rates in mice and avoid some of the limitations of chimeric mice.[68]

POSSIBLE INTERACTION BETWEEN LIVER CELLS AND IMMUNE CELLS

TWO OF THE initial *IL-28B* reports showed that production of IL-28 was higher in individual leukocytes homozygous for the eradication-favorable allele (rs12979860 CC or rs8099917 TT),[8,10] suggesting that stronger IFN-λ expression was responsible for the better response to therapy, although Urban *et al.* found no association between *IL-28B* genotype and *IL-28B* or *IL-28A* expression.[56] Honda *et al.* also found no association between *IL-28B* genotype and *IL-28B* expression, although they point out that it may be difficult to detect differences in expression levels due to the high sequence similarity between *IL-28A* and *IL-28B* and the relatively low expression levels of these genes regardless of genotype.[57] Although IFN-λ inhibits replication of HCV genotypes 1 and 2[69] and induces essentially the same set of ISGs as IFN-α,[62] IFN-λ may act synergistically by enhancing the antiviral efficacy of sub-saturating levels of IFN-α.[69] IFN-α and IFN-λ also have different kinetics. Marcello *et al.* showed that IFN-α induces an early peak followed by a rapid decline, whereas IFN-λ induces slower but more sustained ISG expression.[69] Therefore, coordination of autocrine and paracrine IFN expression is likely complex and may involve interactions between hepatocytes and immune cells such as dendritic cells, cytotoxic T lymphocytes, natural killer cells and natural killer T cells. The use of *in vitro* and *in vivo* experimental systems may help to clarify and isolate the roles of individual components of the cytokine network to elucidate the underlying mechanism of *IL-28B* polymorphisms on outcome of IFN therapy.

FUTURE REMARKS

IDENTIFICATION OF SNPs in the *IL-28B* locus has had a dramatic impact on the study and clinical assessment of HCV infection, but much about its role remains paradoxical and speculative, and more sophisticated *in vitro* and *in vivo* experiments are necessary to uncover the mechanism and use this knowledge to develop more effective IFN therapies. Recommendations for future research include genetic characterization of cell lines, culture of immune cells and hepatocytes using a cell separator, and transplantation of immune cells to human hepatocyte chimeric mice. In addition to improving treatment efficacy for chronic HCV infection, molecular and immunological insights gained from *IL-28B* research may impact a broad area of medicine, including infectious disease and cancer research.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Reducing Peg-IFN doses causes later virologic response or no response in HCV genotype 1 patients treated with Peg-IFN alfa-2b plus ribavirin

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Abstract
Background The timing to the first undetectable hepatitis C virus (HCV) RNA level is strongly associated with sustained virologic response in pegylated interferon (Peg-IFN) plus ribavirin combination therapy for patients with chronic hepatitis C (CH-C) with genotype 1. This study was conducted to clarify the impact of drug exposure to Peg-IFN on the timing of HCV RNA negativity in Peg-IFN plus ribavirin combination therapy for CH-C patients with genotype 1.

Methods A total of 1409 patients treated with Peg-IFN alfa-2b plus ribavirin were enrolled and classified into four categories according to the Peg-IFN dosage. Furthermore, 100 patients were extracted from each Peg-IFN dosage category to adjust for characteristic factors, using the propensity score method.

Results Peg-IFN exposure was dose-dependently associated with the timing of HCV RNA negativity ($p \leq 0.001$). The HCV RNA negative rate at week 4 decreased from 12% with a Peg-IFN dose of >1.5 μg/kg/week to 1–3% with a dose of <1.5 μg/kg/week ($p \leq 0.001$), and at week 12 the rate had decreased from 44% with a dose of

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≥1.2 μg/kg/week to 18% with a dose of <1.2 μg/kg/week ($p = 0.001$). Treatment failure (patients without a 1-log decrease of HCV RNA at week 4 or a 2-log decrease of HCV RNA at week 12, or positive at week 24) was found in 54–66% of patients given <1.2 μg/kg/week ($p \leq 0.001$), and these patients accounted for 64% of the non-responders.

Conclusions The timing of HCV RNA negativity depends significantly on the Peg-IFN dose. Reducing the Peg-IFN dose can induce a later virologic response or non-response in HCV genotype 1 patients treated with Peg-IFN plus ribavirin.

Keywords Chronic hepatitis C · Pegylated interferon plus ribavirin · Drug adherence · HCV RNA negativity · Propensity score matched study

Introduction

The timing to the first undetectable hepatitis C virus (HCV) RNA level during pegylated interferon (Peg-IFN) plus ribavirin combination therapy for patients with chronic hepatitis C (CH-C) genotype 1 is strongly associated with a sustained virologic response (SVR), defined as undetectable HCV RNA at 24 weeks after the finishing of the treatment. The SVR rate was 87–100% in patients with undetectable HCV RNA at week 4, 73–81% at week 12, and 14–44% between weeks 12 and 24 in patients receiving the standard 48-week treatment [1–8]. These results suggest that HCV RNA negativity should be achieved as soon as possible during treatment in order to attain a higher SVR rate.

Previous studies have revealed that many factors affect the complete virologic response (c-EVR) and SVR, such as age, gender, degree of liver fibrosis, HCV genotype, HCV viral load, and the amount of drug exposure [1–3, 9–16]. Of these factors, only the amount of drug exposure can be controlled in order to try to improve the antiviral effect, as the other factors are fixed for individual patients. Also, recently, the single-nucleotide polymorphisms (SNPs) of the *IL28B* gene have been revealed to be associated with the antiviral effects of pegylated interferon-alpha and ribavirin therapy [17–19].

Peg-IFN has been reported to be dose-dependently correlated with c-EVR [13]. Patients' characteristic factors can be related to drug adherence, as aged patients, female patients, and patients with progression of liver fibrosis have a tendency to show low drug adherence. This suggests that patients with low drug adherence could be those who are difficult to treat. Therefore, patients with similar characteristic factors should be compared in order to precisely assess the actual impact of drug exposure on the timing to the first undetectable HCV RNA level.

Only a few randomized controlled trials (RCTs) have examined the relationship between drug dose reduction and antiviral effect with Peg-IFN plus ribavirin combination therapy [1–3, 20–23], and the findings are controversial. Manns et al. [1] reported that the SVR rate was significantly lower in patients given 0.5 μg/kg/week of Peg-IFN than in those given 1.5 μg/kg/week of Peg-IFN (34 vs. 42%, $p < 0.05$). McHutchison et al. [3] reported that the SVR rate did not differ between groups given 1.0 and 1.5 μg/kg/week of Peg-IFN (38 vs. 40%, $p = 0.20$). No detailed study of the relationship between dose reduction and delay of HCV RNA negativity or the relationship between dose reduction and an increase of non-responders to the treatment has been reported, and the real impact of drug exposure on the anti-viral effect remains unclear.

In this present work, we conducted a matched study in which characteristic factors other than drug exposure were adjusted using propensity scores. We investigated the impact of drug exposure to Peg-IFN on the timing to the first undetectable HCV RNA level.

Patients and methods

Patients

The present study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 1409 Japanese patients with CH-C treated with a combination of Peg-IFN alfa-2b plus ribavirin were enrolled in this study between December 2004 and July 2008.

Patients eligible for this study were those who were infected with HCV genotype 1 and had a viral load of $\geq 10^5$ IU/ml, but were negative for hepatitis B surface antigen and anti-human immunodeficiency virus. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcoholic liver disease, autoimmune hepatitis). Informed consent was obtained from each patient included in this study, which was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki.

Treatment

All patients received Peg-IFN alfa-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (REBETOL; Schering-Plough). Peg-IFN alfa-2b was given subcutaneously once weekly at a dosage of 60–150 μg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight

(body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg), according to the standard treatment protocol for Japanese patients.

Dose reduction

As a rule, dose modification, which was performed according to the intensity of the adverse hematologic effects, was done by following the manufacturer's drug information. The dose of Peg-IFN alfa-2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to $<1500/mm^3$, the neutrophil count declined to $<750/mm^3$, or the platelet (Plt) count declined to $<8 \times 10^4/mm^3$, and was discontinued if the WBC count declined to $<1000/mm^3$, the neutrophil count declined to $<500/mm^3$, or the Plt count declined to $<5 \times 10^4/mm^3$. Ribavirin was also reduced from 1000 to 600 mg, or from 800 to 600 mg, or from 600 to 400 mg if the hemoglobin (Hb) level decreased to <10 g/dl, and was discontinued if the Hb level decreased to <8.5 g/dl.

Virologic assessment and definition of virologic response

Serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analyzed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/mL). The HCV RNA level was evaluated every 4 weeks during treatment. A rapid virologic response (RVR) was defined as undetectable serum HCV RNA at week 4, a c-EVR as undetectable serum HCV RNA at week 12, and a late virologic response (LVR) as detectable HCV RNA at week 12 but undetectable at week 24. Patients with <a 1-log decrease in the HCV RNA level at week 4 or <a 2-log decrease at week 12 compared with the baseline or detectable HCV RNA at week 24 were considered to have experienced treatment failure (non-response, NR) and had to stop treatment. If patients discontinued the treatment due to adverse events, without HCV RNA negativity being attained, they were also regarded as having had treatment failure.

Assessment of drug exposure

The amounts of Peg-IFN alfa-2b and ribavirin actually taken were evaluated by reviewing the medical records and calculating the amount taken from the start until the timing of the first undetectable HCV RNA level for the patients achieving HCV RNA negativity, and calculating the amount taken throughout the treatment for the patients not attaining HCV RNA negativity. For patients who discontinued the treatment, if their HCV RNA had become negative before discontinuation, the drug amount data were calculated from the start of treatment until the timing of the first undetectable HCV RNA level, and if HCV RNA had not become negative before discontinuation, the data throughout the treatment before discontinuation were used. The amounts of both drugs were divided individually on the basis of body weight at baseline as the average: Peg-IFN alfa-2b was expressed as μg/kg/week and ribavirin as mg/kg/day.

Evaluation of impact of drug exposure on HCV RNA negativity

We evaluated the relationship between the exposure to both drugs and HCV RNA negativity at week 24 by univariate and multivariate analyses for the patients who completed 24 weeks of treatment, using the mean administration doses of both drugs during the first 24 weeks and the characteristic factors other than drug exposure at baseline.

The patients were divided into four categories according to the Peg-IFN dose: up to 0.9 μg/kg/week of Peg-IFN; from 0.9 to less than 1.2 μg/kg/week; from 1.2 to less than 1.5 μg/kg/week; and from 1.5 μg/kg/week. The propensity score matching method was used to adjust the patients' characteristic factors among these categories. This score was calculated for each patient by logistic regression analysis, with four patient characteristic factors as independent variables; age, gender, Plt values, and history of IFN treatment. We then performed 1:1 nearest neighbor matching within a caliper of 0.15 standard deviation of the propensity score: one patient in each group with 0.9–1.2 μg/kg/week, 1.2–1.5 μg/kg/week, and ≥1.5 μg/kg/week to one patient with <0.9 μg/kg/week, and extracted 100 patients from each category.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients were expressed as means ± SD or median values. Factors associated with HCV RNA negativity at week 24 were assessed by univariate analysis using the Mann–Whitney *U*-test or the χ^2 test, and by multivariate analysis using logistic regression analysis. To analyze the difference between baseline data among the four Peg-IFN groups, analysis of variance (ANOVA) or the χ^2 test was performed. The significance of trends in values for the timing to the first undetectable HCV RNA level was determined with the Mantel–Haenszel χ^2 test. A two-tailed p value of <0.05 was considered significant. Statistical analysis was conducted with SPSS version 15.0J (SPSS, Chicago, IL, USA).

Table 1 Baseline characteristics of patients before matching

Factor	All patients	<0.9 μg/kg/week of Peg-IFN	0.9–1.2 μg/kg/week of Peg-IFN	1.2–1.5 μg/kg/week of Peg-IFN	≥1.5 μg/kg/week of Peg-IFN	*p* value
Number	1409	153	159	670	427	
Age (years)	56.3 ± 10.4	58.0 ± 9.9	57.3 ± 10.2	55.9 ± 10.6	56.3 ± 10.4	0.069
Sex: male/female	722/687	70/83	69/90	376/294	207/220	0.004
History of IFN treatment: naïve/experienced	862/547	98/55	96/63	408/262	260/167	0.894
White blood cells (/mm^3)	5060 ± 1532	4325 ± 1419	4566 ± 1394	5246 ± 1562	5215 ± 1456	<0.001
Neutrophils (/mm^3)	2578 ± 1073	2129 ± 1049	2258 ± 949	2699 ± 1080	2667 ± 1052	<0.001
Red blood cells (×10^4/mm^3)	440 ± 46	424 ± 42	429 ± 45	445 ± 46	441 ± 45	<0.001
Hemoglobin (g/dl)	14.0 ± 1.4	13.6 ± 1.2	13.7 ± 1.5	14.1 ± 1.4	14.1 ± 1.4	<0.001
Platelets (×10^4/mm^3)	16.3 ± 5.6	11.9 ± 3.9	13.2 ± 4.8	17.4 ± 5.7	17.4 ± 5.2	<0.001
ALT (IU/l)	78 ± 61	93 ± 64	87 ± 68	75 ± 58	73 ± 60	0.001
Serum HCV RNA (KIU/ml)[a]	1450	1300	1900	1700	1900	0.176
Histology (METAVIR)[b]						
Fibrosis, 0–2/3–4 (%)[c]	810/186 (17%)	78/34 (30%)	70/30 (30%)	392/77 (16%)	270/45 (14%)	<0.001
Activity, 0–1/2–3	527/468	43/67	38/62	259/210	187/129	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

[a] Data shown are median values

[b] Data missing for 413 patients

[c] Percent of patients with 3–4

Results

Clinical characteristics of all patients according to Peg-IFN dosage before matching

A total of 1409 patients were enrolled in this study, and the baseline characteristics of the patients are shown in Table 1. Based on the Peg-IFN dosage, these patients were classified into four categories. With the decrease of Peg-IFN dosage, the ratio of female-to-male patients increased, the peripheral blood cell count decreased, and the number of patients with progression of liver fibrosis (METAVIR fibrosis score 3 or 4) increased significantly ($p < 0.001$). Patients with a lower Peg-IFN dosage tended to be older ($p = 0.07$).

Next, we analyzed the factors associated with HCV RNA negativity at week 24 for the 1226 patients who completed 24 weeks of treatment, using the baseline characteristic variables, excluding liver histology, shown in Table 1 and the mean doses of both drugs during the first 24 weeks. The HCV RNA negative rate at week 24 was 68% (829/1226). The results of univariate analysis are shown in Table 2. The factors evaluated by multivariate analysis were those for which the p value was <0.10 by univariate analysis for HCV RNA negativity at week 24: age, gender, history of IFN treatment, WBC, neutrophils, red blood cells (RBC), Hb, Plt, alanine aminotransferase, and the mean doses of Peg-IFN and ribavirin during the first 24 weeks. By the multivariate analysis, in addition to the RBC value ($p = 0.02$), Plt value ($p < 0.001$), and history of IFN treatment ($p = 0.04$), the factor of Peg-IFN exposure was an independent factor for HCV RNA negativity at week 24 ($p < 0.001$) (Table 3). The mean dose of ribavirin did not show a significant correlation with HCV RNA negativity at week 24 ($p = 0.07$).

Table 2 Univariate analysis of factors associated with HCV RNA negativity at week 24

Factor	Negative	Positive	*p* value
Number	829	397	
Age (years)	55.1 ± 10.5	57.6 ± 10.1	<0.001
Sex: male/female	437/392	189/208	0.094
History of IFN treatment: naïve/experienced	523/306	229/168	0.069
White blood cells (/mm^3)	5175 ± 1498	4566 ± 1394	<0.001
Neutrophils (/mm^3)	2665 ± 1087	2429 ± 1059	<0.001
Red blood cells (×10^4/mm^3)	445 ± 44	434 ± 47	<0.001
Hemoglobin (g/dl)	14.1 ± 1.4	13.9 ± 1.4	0.004
Platelets (×10^4/mm^3)	17.2 ± 5.6	14.8 ± 5.3	<0.001
ALT (IU/l)	73 ± 55	83 ± 63	0.001
Serum HCV RNA (KIU/ml)	1750	1800	0.673
Mean Peg-IFN dose (μg/kg/week)	1.39 ± 0.24	1.25 ± 0.32	<0.001
Mean ribavirin dose (mg/kg/day)	10.6 ± 1.8	9.7 ± 2.2	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

Clinical characteristics of patients extracted from each Peg-IFN dosage category after matching

Patients in the four Peg-IFN categories were matched by the propensity score method and 100 patients were extracted from each category. The c-statics for the propensity score model between the patients with <0.9 μg/kg/week of Peg-IFN and those given different levels of Peg-IFN were 0.62 for 0.9–1.2 μg/kg/week of Peg-IFN, 0.82 for 1.2–1.5 μg/kg/week of Peg-IFN, and 0.82 for ≥1.5 μg/kg/week of Peg-IFN.

The baseline characteristics of the patients extracted according to the Peg-IFN dosage category are shown in Table 4. There was no significant difference among the four Peg-IFN categories in any of the factors, indicating that the extracted cohort of 400 patients was well matched according to propensity score methods.

Table 3 Multivariate analysis of factors associated with HCV RNA negativity at week 24

Factor	Category	Odds ratio	95% CI	*p* value
Age	1 year	–	–	NS
Sex	Male/female	–	–	NS
History of IFN treatment	Naïve/experienced	0.756	0.581–0.984	0.037
White blood cells	$1 \times 10^3/mm^3$	–	–	NS
Neutrophils	$1 \times 10^3/mm^3$	–	–	NS
Red blood cells	$1 \times 10^4/mm^3$	1.004	1.001–1.007	0.02
Hemoglobin	1 g/dl	–	–	NS
Platelets	$1 \times 10^4/mm^3$	1.054	1.026–1.083	<0.001
ALT	1 IU/l	–	–	NS
Mean Peg-IFN dose	0.1 μg/kg/week	1.096	1.045–1.149	<0.001
Mean ribavirin dose	1 mg/kg/day	1.060	0.994–1.130	0.074

ALT alanine aminotransferase, *CI* confidence interval, *IFN* interferon, *NS* not significant, *Peg-IFN* pegylated interferon

Timing to the first undetectable HCV RNA level according to Peg-IFN dosage

We evaluated the relationship between the virologic response during the treatment and the drug exposure to Peg-IFN using our matched cohort of 400 patients (Fig. 1). Of the 400 patients, 23 had discontinued treatment due to adverse events by week 24 (<0.9 μg/kg/week, $n = 5$; 0.9–1.2 μg/kg/week, $n = 4$; 1.2–1.5 μg/kg/week, $n = 5$; ≥1.5 μg/kg/week, $n = 9$). The proportion of patients with treatment failure increased according to the decrease in the dose of Peg-IFN: 66% among patients with <0.9 μg/kg/week of Peg-IFN, 54% among those with 0.9–1.2 μg/kg/week of Peg-IFN, 35% among those with 1.2–1.5 μg/kg/week of Peg-IFN, and 32% among those with ≥1.5 μg/kg/week of Peg-IFN ($p < 0.001$). Additionally, the timing to the first undetectable HCV RNA level tended to shift to an earlier time

Table 4 Baseline characteristics of patients after matching

Factor	All patients	<0.9 μg/kg/week of Peg-IFN	0.9–1.2 μg/kg/week of Peg-IFN	1.2–1.5 μg/kg/week of Peg-IFN	>1.5 μg/kg/week of Peg-IFN	*p* value
Number	400	100	100	100	100	
Age (years)	56.9 ± 9.6	57.4 ± 10.0	56.6 ± 9.9	56.8 ± 9.5	56.7 ± 9.1	0.941
Sex: male/female	190/210	47/53	47/53	46/54	50/50	0.948
History of IFN treatment: naïve/experienced	286/114	70/30	71/29	73/27	72/28	0.970
White blood cells ($/mm^3$)	4557 ± 1344	4331 ± 1310	4532 ± 1372	4642 ± 1399	4725 ± 1280	0.186
Neutrophils ($/mm^3$)	2261 ± 955	2070 ± 855	2200 ± 883	2416 ± 1068	2357 ± 972	0.054
Red blood cells ($\times 10^4/mm^3$)	429 ± 43	423 ± 40	427 ± 42	432 ± 44	434 ± 46	0.300
Hemoglobin (g/dl)	13.8 ± 1.5	13.7 ± 1.3	13.7 ± 1.6	13.9 ± 1.4	14.0 ± 1.5	0.300
Platelets ($\times 10^4/mm^3$)	12.1 ± 3.7	11.8 ± 3.9	12.0 ± 3.8	12.1 ± 3.5	12.4 ± 3.6	0.625
ALT (IU/l)	89 ± 67	98 ± 65	92 ± 70	86 ± 61	80 ± 69	0.254
Serum HCV RNA (KIU/ml)[a]	1700	1400	1800	1700	1750	0.742
Histology (METAVIR)[b]						
Fibrosis, 0–2/3–4 (%)[c]	186/89 (32%)	47/22 (32%)	46/22 (32%)	48/21 (30%)	45/24 (35%)	0.958
Activity, 0–1/2–3	115/159	25/42	26/42	32/37	32/38	0.585

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

[a] Data shown are median values

[b] Data missing for 125 patients

[c] Percent of patients with 3–4

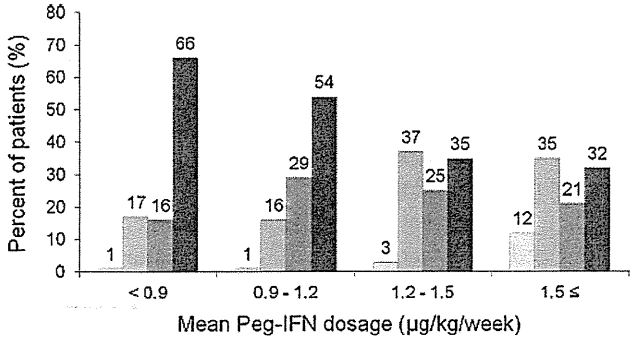
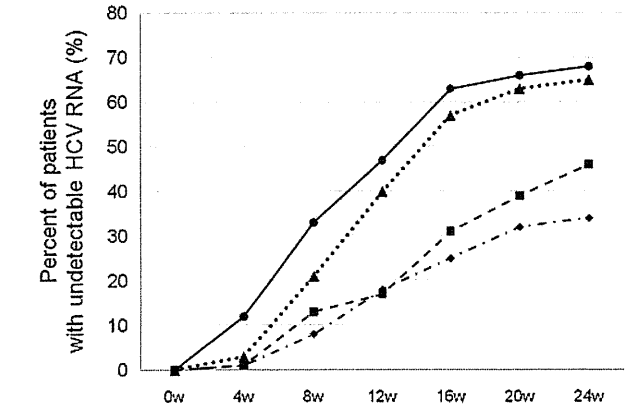


Fig. 1 Timing to the first undetectable hepatitis C virus (HCV) RNA level according to pegylated interferon (*Peg-IFN*) dosage. *Light gray bars* patients with undetectable HCV RNA at week 4. *Medium gray bars* patients with undetectable HCV RNA during 5 to 12 weeks. *Dark gray bars* patients with undetectable HCV RNA during 13–24 weeks. *Black bars* patients with treatment failure (patients with less than a 1-log decrease in HCV RNA level at week 4 or less than a 2-log decrease at week 12 compared with the baseline or detectable HCV RNA at week 24 and those with treatment discontinuance without HCV RNA negativity). Peg-IFN exposure was dose-dependently associated with the timing of HCV RNA negativity ($p \leq 0.001$)



Peg-IFN (μg/kg/week), %						
< 0.9	1	8	18	25	43	34
0.9 ≤ < 1.2	1	13	17	31	39	46
1.2 ≤ < 1.5	3	21	40	57	63	65
1.5 ≤	12	33	47	63	66	68

Fig. 2 Longitudinal negative HCV RNA rates from the start to 24 weeks of the treatment. *Filled circles* Peg-IFN ≥1.5 μg/kg/week, *filled triangles* Peg-IFN 1.2–1.5 μg/kg/week, *filled squares* Peg-IFN 0.9–1.2 μg/kg/week, *filled diamonds* Peg-IFN <0.9 μg/kg/week. The HCV RNA negative rate at week 4 was significantly higher among the patients with Peg-IFN ≥1.5 μg/kg/week than among those with Peg-IFN <1.5 μg/kg/week ($p \leq 0.001$). The HCV RNA negative rates at weeks 12 and 24 were significantly higher among the patients with Peg-IFN ≥1.2 μg/kg/week than among those with Peg-IFN <1.2 μg/kg/week ($p = 0.001$, $p = 0.002$, respectively). *w* week

during the treatment according to the increase in the Peg-IFN dose ($p \leq 0.001$).

Figure 2 shows the longitudinal data of the HCV RNA negative rate. The data for patients with treatment failure were included until the end of each patient's treatment. The percentage of patients with undetectable HCV RNA at week 4 decreased from 12 to 1–3% if they were given <1.5 μg/kg/week of Peg-IFN ($p \leq 0.001$). As for the HCV RNA negative rates at week 12 and week 24, there was no significant difference between patients with 1.2–1.5 μg/kg/week and those with >1.5 μg/kg/week of Peg-IFN. The two groups with <1.2 μg/kg/week of Peg-IFN showed significantly lower HCV RNA negative rates than the other two groups given ≥1.2 μg/kg/week of Peg-IFN (week 12, 18 vs. 44%, $p = 0.0001$, week 24, 40 vs. 67%, $p = 0.0002$). The patients with <0.9 μg/kg/week tended to show a decreased HCV RNA negative rate at week 24 compared to the patients given 0.9–1.2 μg/kg/week (34 vs. 46%, $p = 0.08$).

Figure 3 shows the proportion of patients, according to Peg-IFN exposure, among those with undetectable HCV RNA at week 4 ($n = 17$) and at week 12 ($n = 122$), as well as the proportion of patients with detectable HCV RNA at week 24 ($n = 213$). The patients given ≥1.5 μg/kg/week of Peg-IFN accounted for 70% of the patients with undetectable HCV RNA at week 4, and those given ≥1.2 μg/kg/week of Peg-IFN accounted for 71% of the patients with undetectable HCV RNA at week 12. On the other hand, the patients given <1.2 μg/kg/week of Peg-IFN accounted for 64% of the patients with detectable HCV RNA at week 24.

Discussion

The association between drug exposure and HCV RNA negativity has been reported [9–13]. However, most studies have shown only the fixed-point relationship at week 12, and it remains unclear whether dose modification accelerates or delays the timing to the first undetectable HCV RNA level. The present study is the first to clarify this. Induction regimens in which a high dose (360 μg/week) of Peg-IFN alfa-2a was administered for the first 12 weeks failed to improve SVR rates compared to treatment with a standard dose of Peg-IFN in the CHARIOT [22] and PROGRESS [23] RCTs. In contrast, the adherence study of McHutchison et al. revealed that patients who received ≥80% of the planned dose of Peg-IFN and ribavirin for ≥80% of the full 48 weeks of treatment had a significantly higher SVR rate (51%) than those who received <80% of the planned dose of one or both drugs for ≥80% of the full 48 weeks of treatment (34%) ($p = 0.011$) [10]. These apparently paradoxical results for the relationship between drug dosage and antiviral effect imply that the dose-dependent increase of the antiviral effect was observed up to a certain dose and the antiviral effect then reached a plateau above the regular dose. This paradoxical effect could explain the impact of Peg-IFN reduction from a regular dose on the timing to the first undetectable HCV

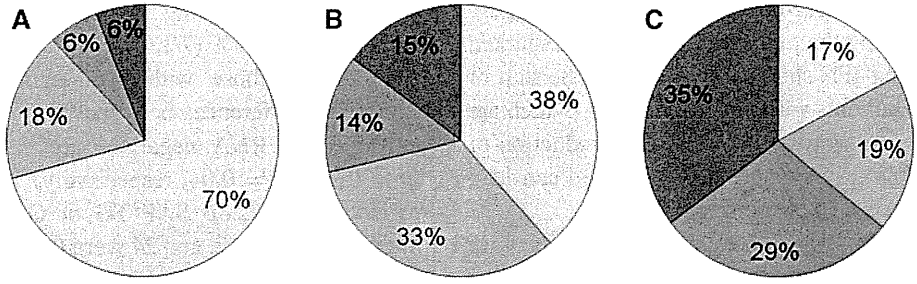


Fig. 3 Proportions of patients according to Peg-IFN exposure among patients with undetectable HCV RNA at weeks 4 and 12 and those with detectable HCV RNA at week 24. **a** Week 4 ($n = 17$), **b** week 12 ($n = 122$), **c** week 24 ($n = 213$). *Light gray segments* Peg-IFN <0.9 μg/kg/week. *Medium gray segments* Peg-IFN 0.9–1.2 μg/kg/week. *Dark gray segments* Peg-IFN 1.2–1.5 μg/kg/week. *Black segments* Peg-IFN ≥1.5 μg/kg/week

RNA level in the present study differing from that of the induction therapy with a high Peg-IFN dose in the above two studies.

In the present study, characteristic matched patients were extracted from a large retrospective cohort to examine the impact of Peg-IFN dosage on viral dynamics. The reason for using a matched cohort was that performing an RCT according to Peg-IFN doses poses an ethical problem, because a low dose of Peg-IFN is known to show little efficacy. The reason for our focusing on Peg-IFN dosage was based on the finding that ribavirin was indeed a significant factor for HCV RNA negativity at week 24 on univariate analysis, but not on multivariate analysis, and Peg-IFN was significantly correlated with HCV RNA negativity at week 24 in an independent manner in this study cohort. Our previous report that Peg-IFN, but not ribavirin, was correlated with c-EVR supports this [13].

To calculate the propensity score, we chose four covariates as candidates for adjustment: age, gender, Plt values, and history of IFN treatment, because there was a need to match universal features such as age, gender, and factors associated with HCV RNA negativity at week 24, such as Plt values and the history of IFN treatment. As shown in Table 4, the baseline characteristic factors in the different Peg-IFN patient categories were well matched after propensity score adjustment. That is, c-statics, the hallmark of application to logistic regression analysis, was regarded as adequate for random assignment. Only the c-statics for the patients given <0.9 μg/kg/week of Peg-IFN and the patients given 0.9–1.2 μg/kg/week of Peg-IFN showed a low value (0.62), because the number of patients in the Peg-IFN category of 0.9–1.2 μg/kg/week ($n = 153$) was not very large. However, the patient characteristic factors in two categories after extraction were well matched and were considered to be adequate for further analysis. In this study, the populations extracted after matching were composed of patients with relatively advanced liver fibrosis compared to the original population; the mean Plt value was lower and the proportion of patients with progression of liver fibrosis (METAVIR fibrosis score 3 or 4) was higher in the extracted population than in the original one (mean Plt value $12.1 \times 10^4/mm^3$ vs. $16.3 \times 10^4/mm^3$, proportion of patients with progression of liver fibrosis, 32 vs. 19%, respectively). The patients with <0.9 μg/kg/week of Peg-IFN, which was the smallest population among the four Peg-IFN categories and included more patients with advanced liver fibrosis, were used as the control for the propensity score matching.

Recently, the usefulness of extended therapy has been revealed for patients with LVR, defined as HCV RNA negativity between week 12 and week 24 (or week 36). In addition, we have reported that, even with extended treatment of 72 weeks, the timing of HCV RNA disappearance showed a strong correlation with relapse after treatment [24]. Accordingly, at present, it is necessary to verify how reducing drug doses affects the delay of the timing to the first undetectable HCV RNA level or treatment failure, and in the present study we demonstrated the appropriate dose of Peg-IFN required to attain HCV RNA negativity by 24 weeks. As shown in Fig. 1, Peg-IFN dose-dependently affected the timing to the first undetectable HCV RNA level during the treatment. These results indicate that dose reduction of Peg-IFN can cause a shift from c-EVR to LVR and a shift from LVR to HCV RNA-positivity at week 24. The proportion of patients with treatment failure among those given <0.9 μg/kg/week of Peg-IFN (66%) was decreased by half among the patients given ≥1.2 μg/kg/week of Peg-IFN (32–35%). Considering that the effectiveness of extended treatment for patients with LVR is obvious, if patients without a c-EVR were to attain HCV RNA negativity by 24 weeks, those patients would have the potential to attain an SVR with extended treatment. However, if patients do not attain HCV RNA negativity, those patients must discontinue the treatment. Therefore, causing patients to shift from HCV RNA negativity by week 24 to being HCV RNA-positive at week 24 would be missing the chance to obtain SVR even with extended treatment. As shown in Fig. 2, the longitudinal negative

rate of HCV RNA was dose-dependently affected by Peg-IFN at all points during the treatment. Therefore, a marked dose reduction of Peg-IFN should not be done at the start of treatment even for patients with lower Plt values (which are indicative of advanced fibrosis), because dose reduction of Peg-IFN before HCV RNA negativity is attained can lead to an increased possibility of treatment failure.

Next, as shown in Fig. 3, 70% of the patients with undetectable HCV RNA at week 4 were given ≥1.5 μg/kg/week of Peg-IFN, 71% of those with undetectable HCV RNA at week 12 were given ≥1.2 μg/kg/week, and 64% of those with detectable HCV RNA at week 24 were given ≤1.2 μg/kg/week. Therefore, in HCV genotype 1 patients treated with Peg-IFN plus ribavirin, the treatment goal for c-EVR or non-NR should be to maintain a Peg-IFN dose of ≥1.2 μg/kg/week, and that for RVR should be to maintain a Peg-IFN dose of ≥1.5 μg/kg/week. Using granulocyte–macrophage colony-stimulating factor for patients who develop a severe decrease of blood cells and are forced to decrease Peg-IFN can be beneficial, as long as HCV RNA is positive.

A limitation of the present study is that the actual SVR rate could not be compared among the four Peg-IFN categories because some patients with LVR were treated for 72 weeks and some were treated for 48 weeks; actual SVR rates were 20% in patients with Peg-IFN <0.9 μg/kg/week, 18% in those with 0.9–1.2 μg/kg/week, 36% in those with 1.2–1.5 μg/kg/week, and 48% in those ≥1.5 μg/kg/week. On the assumption that the SVR rate for patients with RVR is 90%, the SVR rate for those with c-EVR without RVR is 75% for 48-week treatment, and the SVR rate for those with LVR is 60% for 72-week treatment, the SVR rate of response-guided therapy was calculated to be 23% for patients given <0.9 μg/kg/week of Peg-IFN, 30% for those given 0.9–1.2 μg/kg/week of Peg-IFN, 45% for those given 1.2–1.5 μg/kg/week of Peg-IFN, and 50% for those given ≥1.5 μg/kg/week of Peg-IFN in the matched cohort in the present study. Thus, dose reduction of Peg-IFN can reduce the SVR rate even if response-guided therapy is done. Another limitation of this study is that the *IL28B* SNP, which is known to be a host factor affecting the antiviral effect, could not be examined in all cases, because the characteristic matched patients were extracted from a large retrospective cohort. However, we had the result of the *IL28B* SNP (rs8099917) for 290 patients; 214 patients had TT and 76 had TG or GG. The proportions of patients with the *IL28B* SNP TT were similar among the four Peg-IFN categories (≤0.9 μg/kg/week, 76%, 31/41; 0.9–1.2 μg/kg/week, 71%, 27/38; 1.2–1.5 μg/kg/week, 67%, 99/147; ≥1.5 μg/kg/week, 77%, 57/74, $p = 0.853$). Therefore, it would appear that there was no bias for any cases. Among the patients with the *IL28B* SNP TT, the HCV negative rates at weeks 4, 12, and 24 were 0% (0/58), 33% (19/58), and 69% (40/58) among the patients with <1.2 μg/kg/week of Peg-IFN and 4% (7/156), 62% (97/156), and 82% (128/156) among those with ≥1.2 μg/kg/week. There were significant differences between these two Peg-IFN groups in the HCV RNA negative rates at weeks 12 and 24 ($p = 0.002$, $p = 0.04$, respectively). Similarly, among the patients with *IL28B* SNP TG or GG, the HCV negative rates at weeks 4, 12, and 24 were 0% (0/21), 0% (0/21), and 10% (2/21) among the patients with <1.2 μg/kg/week of Peg-IFN and 2% (1/55), 9% (5/55), and 27% (15/55) among those with ≥1.2 μg/kg/week. The HCV RNA negative rates at weeks 12 and 24 tended to be higher in the patients with ≥1.2 μg/kg/week of Peg-IFN ($p = 0.06$, $p = 0.13$, respectively). From the above-mentioned results, it appears that the dose-dependent effect of Peg-IFN on the timing of HCV RNA negativity could be considered regardless of the *IL28B* SNP.

In conclusion, this matched study has demonstrated that, in patients with CH-C with genotype 1 receiving Peg-IFN plus ribavirin combination therapy, Peg-IFN dose-dependently affects the timing to the first undetectable HCV RNA level and the failure to attain HCV RNA negativity. Dose reduction of Peg-IFN to <1.2 μg/kg/week before HCV RNA negativity is attained delays HCV RNA clearance dose-dependently and increases the rate of treatment failure. Maintaining the Peg-IFN dose at ≥1.2 μg/kg/week, and preferably at ≥1.5 μg/kg/week, can accelerate the timing to the first undetectable HCV RNA level for CH-C genotype 1 patients treated with Peg-IFN plus ribavirin.

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MAJOR ARTICLE

Ribavirin Regulates Hepatitis C Virus Replication Through Enhancing Interferon-Stimulated Genes and Interleukin 8

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Background. The manner in which ribavirin (RBV) enhances the antiviral effects of interferon (IFN) against hepatitis C virus (HCV) remains unknown. We investigated whether RBV modifies IFN-stimulated genes (ISGs) in vivo and in vitro.

Methods. We measured the messenger RNA (mRNA) levels of ISGs in T lymphocytes from patients with HCV infection who were receiving IFN-α therapy with or without RBV. We added RBV and/or IFN-α to a plasmid-based HCV replication system containing a full-length HCV genotype 1a sequence in HepG2 and Huh7 cell lines and the JFH-1 HCV genotype 2a sequence in Huh7 cell lines and measured levels of ISGs and autocrine IFN-β.

Results. The expression of protein kinase R and myxovirus resistance A mRNA was enhanced more with IFN-α and RBV than by IFN-α alone in assays in vivo and in vitro. Such enhancement depended on autocrine IFN-β being enhanced by RBV. RBV upregulated interleukin 8 (IL-8) in the absence of IFN-α. The IL-8 upregulation induced by RBV was responsible for the activation of activator protein 1 (AP-1).

Conclusions. Ribavirin augments the anti-HCV effects of IFN-α induced by ISGs through enhancing autocrine IFN-β. Moreover, RBV can enhance IL-8 through activating AP-1. Improved understanding of ISG modulation by RBV would help to establish a means of eliminating HCV.

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, infecting about 170 million individuals worldwide [1]. Although ribavirin (RBV) plus pegylated interferon (IFN)–α has become a standard treatment for patients with chronic hepatitis C, this therapy eliminates HCV in only up to 60% of infected individuals [2, 3].

RBV (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a guanosine analogue with a broad range of antiviral activity against several RNA and DNA viruses [4]. This effect has transferred over into clinical applications against respiratory syncytial virus infection [5]. Of note, RBV together with IFN-α is used to treat HCV infection [3]. Since the discovery of RBV 30 years ago, various proposed mechanisms of action have included the inhibition of HCV RNA–dependent RNA polymerase [6], promotion of the Th1 immune response [7], inhibition of inosine-5-monophosphate dehydrogenase [8, 9], and mutagenesis leading to error catastrophe [10, 11]. However, the precise mechanism of how RBV acts against HCV remains undefined. Although some evidence supports these anti-HCV actions of RBV in vitro, RBV monotherapy only minimally impacts chronic hepatitis C infection in the clinical setting [12, 13]. That is, RBV can only eliminate HCV when combined with IFN.

IFN stimulates a large number of genes called IFN-stimulated genes (ISGs) that mediate its anti-HCV effects. Moreover, host liver cells can produce autocrine IFN-β through the pathways of Toll-like receptors induced by pathogens as a defense strategy [14]. The production of IFN is thought to be essential for protection against viral infections. Some ISGs, such

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as protein kinase R (PKR), myxovirus resistance protein A (MxA), and 2′-5′-oligoadenylate synthase (OAS) have powerful antiviral effects against HCV [15]. In particular, PKR activated (phosphorylated) by IFN phosphorylates eukaryotic initiation factor-2 subunit alpha (eIF-2α), which in turn stops protein synthesis and exerts antiviral effects [16].

Because the additive anti-HCV effects of RBV are clinically obvious [3], we postulated that RBV would enhance the antiviral effects of IFNs through ISGs. We thus investigated the mechanism of how RBV enhances ISGs.

MATERIALS AND METHODS

Patients and Blood Samples

Peripheral blood T lymphocytes were isolated from patients with chronic hepatitis C with use of the Pan T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's protocol. The patients were treated with either peg-IFN-α combined with RBV (n = 53) or peg-IFN-α alone (n = 12). Supplementary Table 1 shows the clinical features of the patients. All enrolled participants provided written informed consent to research testing under protocols approved by the institutional review boards of Ehime University (approval number 0710004). The study protocol conformed to the ethical guidelines of the Declaration of Helsinki.

Cell Lines, Infection, and Transfection

The human hepatoma cell lines Huh7, HepG2 (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen).

We used the plasmid-based binary HCV replication system [17, 18], in which the plasmid harbored an infectious full-length genotype 1a cDNA sequence corresponding to the H77 prototype strain [19], to yield pT7-flHCV-Rz (pH77; provided by Dr Raymond T. Chung). We transfected Huh7 and HepG2 using 3 μg of pH77 and Lipofectamine (Invitrogen) as described elsewhere [17, 18]. The cells were infected with a replication-defective adenovirus vector containing T7 polymerase (Ad-T7pol) at a multiplicity of infection of 10 at 24 hours after transfection.

We used the pJFH1-full (provided by Dr Takaji Wakita) HCV replication system encoding the HCV genotype 2a sequence in vitro [20]. Huh7 cells were resuspended in Opti-MEM I (Invitrogen) containing 10 μg of HCV RNA and electrically pulsed at 960 microfarads and 260 volts using a Gene Pulser II (Bio-Rad) for RNA transfection.

We added 100 IU/mL IFN-α (Schering-Plough) and/or 50 μM RBV (Sigma-Aldrich) as described elsewhere [10, 16, 17]. The expression of T7 polymerase was not remarkably altered by IFN-α and/or RBV (Supplementary Figure 1).

RNA Interference and Antibody Neutralization of IFN-β

Cells were transfected with 20 nM of a small interfering RNA (siRNA) targeting IFN-β or with a scrambled control siRNA (Invitrogen) using Lipofectamine RNAiMax (Invitrogen) at 1 day before transfection with pH77, as described elsewhere [18].

We neutralized IFN-β by adding 1.0 μg/mL of anti-human IFN-β antibody or isotype control antibody (R&D Systems) to the culture medium at 3 hours after infection and then incubated the cells for 48 hours as described elsewhere [18].

Quantitative Real-time Reverse-Transcription Polymerase Chain Reaction

Cellular RNA was extracted using TRIzol (Invitrogen) and digested with DNase I using a DNA free-kit (Ambion) according to the manufacturer's protocol. Cellular messenger RNA (mRNA) was quantified using the reverse transcription of 1 μg of isolated RNA using rTth or an oligo $d(T)^{16}$ (Invitrogen) primer under standard conditions [10]. Real-time polymerase chain reaction (PCR) amplification proceeded using LightCycler technology (Roche Diagnostics) and SYBR green I dye, as described elsewhere [16]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IFN-β, OAS, MxA, and interleukin 8 (IL-8) (Search LC) were detected using primer sets under the recommended conditions. Real-time PCR amplification of HCV RNA and PKR proceeded as described elsewhere [16]. Data are expressed as copy numbers of HCV RNA or cellular mRNA per molecule of GAPDH mRNA.

Western Blotting

Protein extracted from cells was lysed with 100 μL of RIPA buffer [16]. Concentrations of extracted proteins in lysates were measured using the DC protein assay kit (Bio-Rad), and 30 μg of lysate was loaded in 4%–12% Bis-Tris gradient gels (Invitrogen). Proteins of interest were detected using antibodies to human PKR, eIF-2α (Santa Cruz Biotechnology), phosphorylated PKR (Invitrogen), phosphorylated eIF-2α (Cell Signaling Technology), T7 RNA polymerase (Novagen), and actin (Chemicon International), as well as species-specific conjugated secondary antibodies derived from kits (GE Healthcare).

Enzyme-Linked Immunosorbent Assay of IL-8 and HCV Core Protein

Concentrations of IL-8 were measured in cultured cell lysates (0.2 mg/mL) in RIPA buffer using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions. HCV core antigen in cell lysates (0.2 mg/mL) were quantified using HCV core antigen ELISA kits (Ortho-Clinical Diagnostics) [16]. The lower limits of detection for IL-8 and for HCV were 31.2 pg/mL and 44.4 fmol/L, respectively. All assays proceeded in duplicate.

PCR Array for Genes Related to IL-8 Transcription

We isolated and confirmed the quality of RNA and then performed PCR array analyses using Transcription PCR Array kits (Qiagen) according to the manufacturer's instructions. All

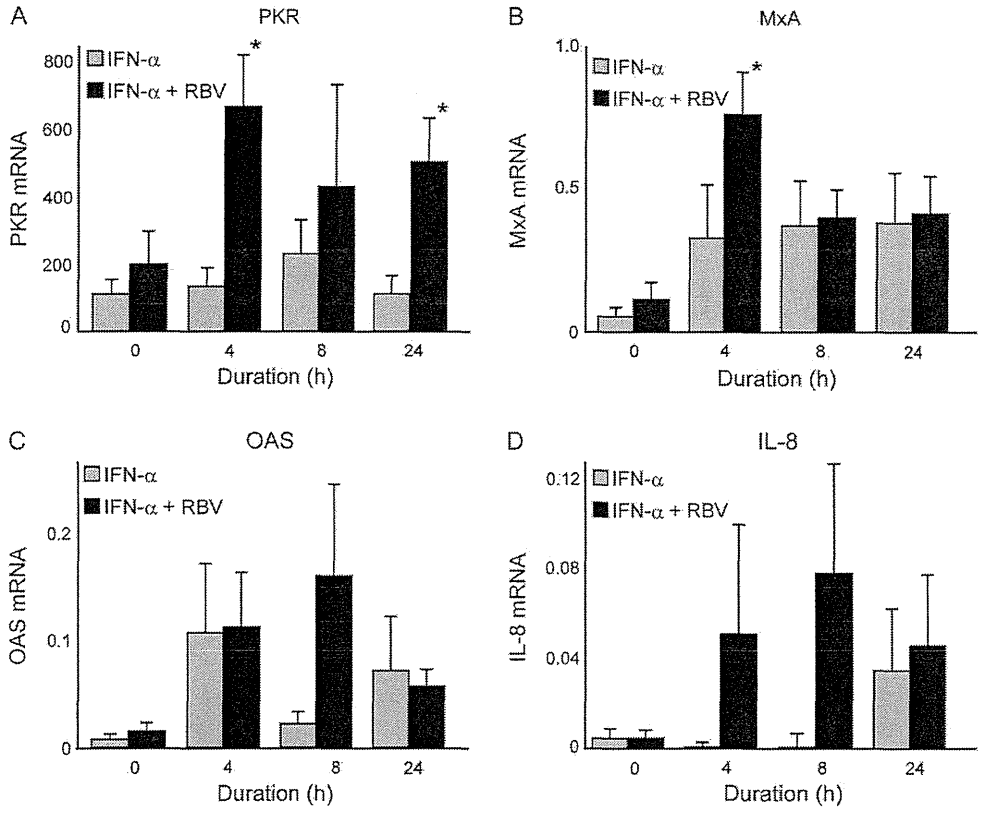

Figure 1. Combination of pegylated interferon (IFN)–α and ribavirin (RBV) enhances interferon-stimulated gene (ISG) messenger RNA (nRNA) levels in patients with chronic hepatitis C infection. Levels of ISG mRNAs in peripheral T lymphocytes from treated patients were measured. Levels of protein kinase R (PKR; *A*), myxovirus resistance protein A (MxA; *B*), 2′-5′-oligoadenylate synthase (OAS; *C*), and interleukin 8 (IL-8; *D*) mRNA were examined at several points. Data are shown as means ± standard error (SE). *$P < .05$ compared with IFN-α at each time point; Wilcoxon test.

individual experiments were performed 3 times, and data were analyzed using the recommended programs. Significant changes were identified in ΔCt (threshold cycle) data using Student *t* test.

Luciferase Assays

Cells were transfected with a wild type IL-8 promoter conjugated to firefly luciferase reporter constructs (pIL-8) or a mutant type (mutation of activator protein 1 [AP-1], nuclear factor κ–light-chain-enhancer of activated B cells [NF-κB], or the CCAAT/enhancer binding protein β [CEBPB] binding site), provided by Dr Charalabos Pothoulakis [21, 22], together with a control reporter plasmid conjugated with Renilla luciferase reporter constructs (phRL-TK^{Int-}; Promega). Luciferase activities in the samples were measured 48 hours later using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Micro-Tec). The level of transcription was evaluated as the ratio of firefly luciferase to renilla luciferase.

Statistical Analysis

All values are expressed as means and standard error (SE). Data were statistically analyzed using JMP, version 8.0 (SAS Institute). Differences in mean values were analyzed using the Wilcoxon test. *P* values of <.05 were considered statistically significant.

RESULTS

IFN-α Enhanced ISGs More With Than Without RBV in Patients Infected With HCV

We examined the effect of RBV combined with peg-IFN-α by measuring the time course of ISG mRNA levels in T lymphocytes isolated from peripheral blood mononuclear cells (PBMCs) collected from patients before and after therapy with IFN-α and/or RBV. Levels of PKR mRNA were significantly enhanced by the combination compared with IFN-α alone (mean ± SE at 4 hours, 668.5 ± 149.9 vs 132.5 ± 51.5; $P < .05$) (Figure 1). Moreover, MxA mRNA was upregulated at 4 hours after the combination was administered (mean ± SE, 0.76 ± 0.15 vs 0.33 ± 0.18; $P < .05$). However, OAS mRNA was not significantly changed. Mean levels of IL-8 mRNA were higher in patients treated with IFN-α and RBV than in those given IFN-α alone. However, the difference did not reach significance. The combination RBV and IFN-α rapidly

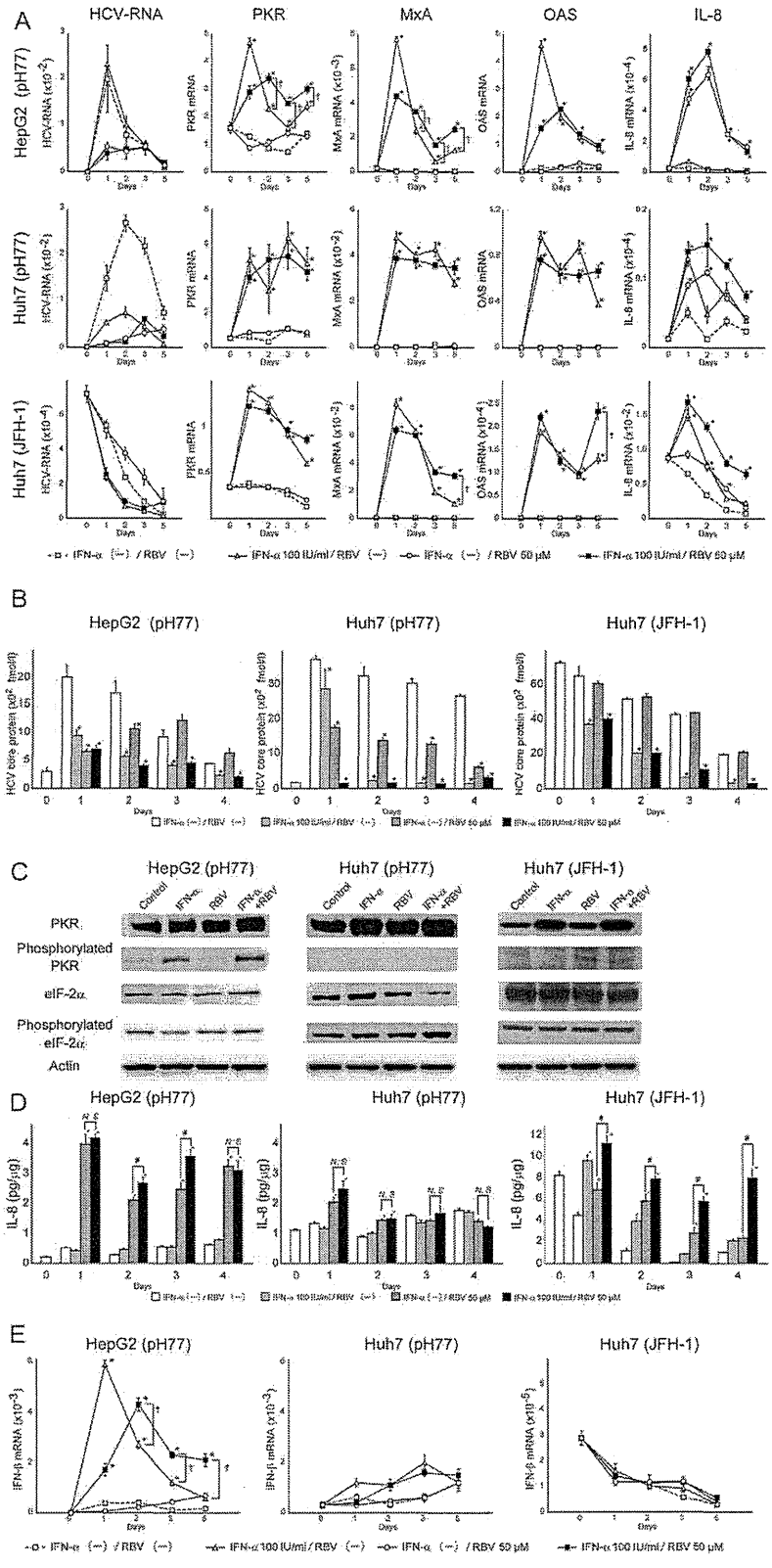

Figure 2. Interferon (IFN)–stimulated genes (ISGs) are modified by ribavirin (RBV) combined with IFN-α. *A*, Hepatitis C virus (HCV) RNA and messenger RNA of ISGs (protein kinase R [PKR], myxovirus resistance protein A [MxA], 2′-5′-oligoadenylate synthase [OAS], and interleukin 8 [IL-8]) were evaluated

upregulated both PKR and MxA mRNA, compared with IFN-α alone, in patients with HCV infection.

IFN-α and RBV Suppress HCV Replication

We assayed the effects of RBV combined with IFN-α in vitro. The response to autocrine IFN-β is somewhat incomplete in Huh7 cells that are used with major HCV replication systems [23]. Therefore, we investigated the anti-HCV mechanisms of RBV using a plasmid-based HCV replication system in the hepatocyte-derived cell lines HepG2 (pH77-HepG2) and Huh7 (pH77-Huh7), as well as in JFH-1 cells derived from Huh7 cells that are generally used to study HCV replication. We added IFN-α and/or RBV to these cells. IFN-α, RBV, and a combination of both each diminished HCV RNA levels (Figure 2*A*) and diminished the expression of HCV core proteins (Figure 2*B*).

RBV Additively Upregulates ISGs With IFN-α in HepG2 but Not in Huh7 Cells

IFN-α significantly induced PKR, MxA, and OAS mRNA during days 1–5 in all cell lines (pH77-HepG2, pH77-Huh7, and JFH-1) in vitro (Figure 2*A*), whereas RBV alone could not induce these ISG mRNAs without IFN-α. Of note, RBV plus IFN-α additively upregulated PKR and MxA mRNA more than did IFN-α alone during days 2–5 in pH77-HepG2 cells (PKR: 3.4 ± 0.1 vs 2.3 ± 0.1 on day 2 [$P < .05$]; MxA: $3.5 \times 10^{-3} \pm 0.1 \times 10^{-3}$ vs $2.4 \times 10^{-3} \pm 0.2 \times 10^{-3}$ on day 2 [$P < .05$]). However, neither PKR nor MxA, both of which were additively upregulated by RBV, was identified in either pH77-Huh7 or JFH-1 cells.

We also assayed PKR and its related proteins to determine how RBV and IFN-α modulate their activities (Figure 2*C*). RBV and IFN-α alone and in combination elicited PKR overexpression in the 3 cell lines. The expression of PKR and of phosphorylated PKR (activated form of PKR) was most upregulated in pH77-HepG2 cells by RBV plus IFN-α rather than by either alone. Moreover, phosphorylated eIF-2α, which indicates activated PKR function, was most upregulated by RBV plus IFN-α in pH77-HepG2 cells. These data indicate that PKR and its activation are additively upregulated by RBV when combined with IFN-α rather than by IFN-α alone in pH77-HepG2 cells.

On the other hand, RBV upregulated IL-8 mRNA (Figure 2*A*), and unlike other ISGs, RBV further upregulated IL-8 mRNA when combined with IFN-α. We measured the amount of IL-8 protein expressed in cell lysates using ELISA (Figure 2*D*). We found that RBV significantly upregulated IL-8 expression with or without IFN-α in all 3 cell lines and that IFN-α additively upregulated IL-8 in pH77-HepG2 and JFH-1 cells.

The RBV-induced upregulation of ISGs was more evident in pH77-HepG2 than in either pH77-Huh7 cells or in JFH-1 cells derived from Huh7 cells. We therefore investigated the effect of autocrine IFN-β in pH77-HepG2 cells, because the response of Huh7 cell lines to this cytokine is impaired.

IFN-α and RBV Enhanced Autocrine IFN-β in HepG2 Cells

Autocrine IFN-β was significantly upregulated by RBV plus IFN-α, compared with IFN-α alone in pH77-HepG2 cells ($4.3 \times 10^{-5} \pm 0.3 \times 10^{-5}$ vs $2.7 \times 10^{-5} \pm 0.2 \times 10^{-5}$ on day 2 [$P < .05$] and $2.1 \times 10^{-5} \pm 0.3 \times 10^{-5}$ vs $0.6 \times 10^{-5} \pm 1.0 \times 10^{-6}$ on day 5 [$P < .05$]) (Figure 2*E*), but not in pH77-Huh7 or JFH-1 cells. The effect of the additive upregulation of autocrine IFN-β by RBV in pH77-HepG2 cells was similar to that of PKR and MxA (Figure 2*A*). These results suggest that PKR and MxA are upregulated via the RBV-induced upregulation of autocrine IFN-β.

Knockdown of IFN-β Diminished the Additive Effects of RBV Plus IFN-α

We examined the effects of IFN-β inhibition to determine whether the upregulation of ISGs by RBV depends on an increase in autocrine IFN-β. We used pH77-HepG2 cells at day 2 after adding reagents, because ISGs were significantly upregulated with autocrine IFN-β enhancement under these conditions. Autocrine IFN-β was downregulated by >90% by IFN-β siRNA in the presence of IFN-α, RBV, or both (Figure 3*A*). We further assessed the modulation of ISG mRNAs via IFN-β mRNA downregulation.

IFN-α continued to upregulate PKR and MxA after IFN-β siRNA downregulation. The additive upregulation of PKR and MxA by RBV plus IFN-α was diminished (Figure 3*B*). These results indicated that the upregulation induced by RBV depends on its ability to increase autocrine IFN-β.

The additive upregulation of IL-8 induced by RBV plus IFN-α was diminished, compared with that induced by IFN-α alone. However, RBV continued to obviously upregulate IL-8, compared with the absence of RBV (Figure 3*B*). This behavior differed from that of other ISGs and suggested that RBV-induced IL-8 upregulation partly depends on autocrine IFN-β. However, the main mechanism of RBV-induced IL-8 upregulation does not depend on autocrine IFN-β.

Figure 2 continued. in plasmid-based HCV replication systems with IFN-α and/or RBV in HepG2 or Huh7 cells transfected with pH77 and infected with Ad-T7 and in JFH-1 cells derived from Huh7 cells. *B,* HCV core protein expression was evaluated in these 3 cell lines with IFN-α and/or RBV. *C,* Western blots of PKR-related proteins from all 3 cell lines. *D,* Levels of IL-8 protein evaluated by enzyme-linked immunosorbent assay (ELISA) from total cellular extracts treated with IFN-α and RBV. *E,* Ribavirin plus IFN-α upregulated IFN-β more than IFN-α alone only in Ad-T7-infected HepG2 cells transfected with pH77. Data are shown as means ± standard error (SE) of 6 replicates. $^{*}P < .05$, compared with absence of IFN-α and RBV; $^{\#}P < .05$ compared with RBV. $^{\dagger}P < .05$ compared with IFN-α at each time point; Wilcoxon test.