for the induction of apoptosis, and these residues are found on the hydrophobic face of the first α-helix of the D2 domain. Interestingly, the replacement of L119 with E did not affect LD association, while the replacement of L126 with E significantly reduced LD association (5). The contribution of V122 to LD association was not investigated. Consistently, the J6/JFH-1(V119L) virus, but not the J6/JFH-1(L126A) virus, replicated efficiently to produce infectious virus particles. Since L119 of the genotype 1b core protein, which occupies the crucial h2 position in the BH3 domain, is essential for its proapoptotic property but not for its association with LD, it is clear that the BH3 domain of the core protein is an independent motif that partially overlaps with the LD association domain.

Recently, Makes caterpillars floppy 1 (Mcf1), a bacterial toxin, was reported to contain a BH3-like domain (18). In addition, HBSP, a spliced hepatitis B viral protein, also contains a BH3-like domain (39). Here, we show that the HCV core protein is another BH3-like viral homologue, and it contributes directly to the induction of apoptosis during HCV infection. Our results also reveal that it is a bona fide BH3-only protein that appears to interfere with the prosurvival property of Mcl-1 in a manner similar to that of Noxa. Our observation that the enhanced apoptotic activity of the J6/JFH-1(V119L) virus is correlated with an increase in infectious progeny HCV release seems to be counterintuitive, as many viruses adopted strategies to prevent apoptosis in the infected cells so as to allow viral replication and the packaging of progeny genomes within the cells (14, 26, 51, 70). However, enhanced releases of virus from infected cells that are undergoing apoptosis also have been reported for other viruses, like the infectious bursal disease virus, adenovirus, and Aleutian mink disease parvovirus (4, 44, 73), indicating that apoptosis can be advantageous for viral spreading at the late stages of infection. Future studies to define the precise manner by which the BH3 domain of the core protein modulates apoptosis during infection will provide important insights into HCV replication as well as pathogenesis.

Besides the genotype 1b core protein, the properties of the genotype 1a core protein also have been examined in various studies. The apoptotic property of the genotype 1a core protein has yet to be studied using the JFH-1-based infectious clone system, although previous studies have attributed both prosurvival and proapoptotic properties to it (25, 30, 46, 57). Similar observations also have been described in overexpression studies using the genotype 1b core protein and appear to be dependent on the death stimuli and types of cells used (3, 9, 10, 36, 49, 53, 56, 60, 76). Several studies have identified domains or regions within the core protein that interfere with specific apoptosis pathways. For instance, the interaction of the N-terminal domain (residues 1 to 75) of the genotype 1a core protein with Hsp60 enhanced tumor necrosis factor alpha-mediated apoptosis, while its C-terminal region (residues 153 to 192) is required for Fas ligand-independent apoptosis (30, 46). The genotype 1b core protein (residues 1 to 153) binds to the death domain of FADD, resulting in enhanced apoptosis (76). However, an overlapping domain spanning the first 46 aa of the core protein is involved in ASPP2 interaction, which leads to the inhibition of p53-mediated apoptosis (9). These findings suggest that multiple domains present in the core protein contribute to the modulation of apoptosis via diverse pathways. Therefore, the net apoptotic effect of the core protein may be dependent on the relative strength of its prosurvival and proapoptotic properties. Unlike the genotype 2a core protein, the BH3 domains of the genotype 1b core protein and the genotype 1a core protein share an identical sequence (Fig. 1B) and are expected to function in a similar manner. However, we cannot rule out that there may be differences in the manner in which the core proteins of genotypes 1a and 1b modulate apoptosis during infection. For example, they may be involved in different virus-virus or virus-host interactions. Thus, more studies are needed to understand the contributions of genotype-dependent factors to the regulation of apoptosis during HCV infection.

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Double-Filtration Plasmapheresis plus IFN for HCV-1b Patients with Non-Sustained Virological Response to Previous Combination Therapy: Early Viral Dynamics

Soo Ryang Kim[a] Susumu Imoto[a] Masatoshi Kudo[d] Keiji Mita[a]
Miyuki Taniguchi[a] Ke Ih Kim[a] Noriko Sasase[a] Ikuo Shoji[b]
Motoko Nagano-Fujii[b] Ahmed El-Shamy[b] Hak Hotta[b] Tomoyuki Nagai[d]
Yoshiaki Nagata[d] Yoshitake Hayashi[c]

[a]Department of Gastroenterology, Kobe Asahi Hospital, [b]Department of Microbiology, and
[c]Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, and
[d]Department of Gastroenterology and Hepatology, Kinki University School of Medicine, Osaka-Sayama, Japan

Key Words

Chronic hepatitis C · Double-filtration plasmapheresis · Early viral dynamics · Genotype 1b · High viral load · Interferon β · Non-sustained virological responder · Peginterferon plus ribavirin combination therapy

Abstract

Double-filtration plasmapheresis (DFPP) was approved in Japan in April 2008 for the retreatment of chronic hepatitis C patients with genotype 1b and high viral loads, whose hepatitis C virus was not eradicated by earlier IFN therapy or by pegylated IFN plus ribavirin (PEG-IFN/RBV) combination therapy. In this study, we assessed the early viral dynamics of 9 patients with non-sustained virological response to the combination therapy. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks showed a reduction of ≥1 log in the viral load in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9) and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment. By contrast, DFPP plus consecutive intravenous IFN-β for 4 weeks reduced the viral load by ≥1 log in 33% (2/6), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks. The viral load declined by ≥2 log in 50% (3/6) at 4 weeks after the start of treatment. DFPP plus consecutive intravenous IFN-β for 4 weeks is a promising treatment for non-sustained virolgical response patients.

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Introduction

Hepatitis C virus (HCV) infection is the major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) in industrialized countries. HCV infection is manageable, however, and its complications can be prevented by antiviral therapy [1, 2]. Currently, the most effective treatment for chronic HCV infection is based on pegylated interferon plus ribavirin (PEG-IFN/RBV) combination therapy [3]. Nonetheless, sustained

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Soo Ryang Kim, MD
Department of Gastroenterology
Kobe Asahi Hospital
3-5-25 Bououji-cho, Nagata-ku, Kobe 653-0801 (Japan)
Tel. +81 78 612 5151, Fax +81 78 612 5152, E-Mail asahi-hp@arion.ocn.ne.jp

virological response (SVR) rates for those infected with the most resistant genotypes (HCV-1a and HCV-1b) still hover around 50% [3, 4].

To surmount this SVR rate with combination therapy, several trials have been undertaken, two of which are: (1) retreatment with combination therapy and (2) double-filtration plasmapheresis (DFPP). By the protocol-defined primary analysis of the former, the SVR rate has been 16% at most, even for a 72-week induction group [5].

The use of DFPP [approved in Japan in April 2008 for the retreatment of chronic hepatitis C (CHC) patients with genotype 1b and high viral loads] together with IFN administration has produced a substantial reduction in the viral load during the early stages of treatment and has effected a high SVR [6], suggesting that this treatment is a new modality for CHC patients in difficult-to-treat states. In this study, we used DFPP plus IFN to enhance the efficacy of the treatment of CHC patients whose HCV was not eradicated by earlier PEG-IFN/RBV combination therapy, and we assessed early viral dynamics associated with SVR.

Patients and Methods

Patients

Nine patients (aged 43–66 years) whose HCV had not been eradicated by earlier PEG-IFNα-2b plus RBV combination therapy carried out between 2008 and 2009 were enrolled in this study. The patients were divided into 2 groups: partial responders (PR; relapse after the end of therapy) and non-responders (NR; no disappearance of HCV RNA during therapy). All the patients were confirmed to be HCV RNA positive with high transaminase levels persisting for 6 months or longer, and with HCV RNA genotype 1b at levels exceeding 10^5 log IU/ml in blood (as determined before the start of therapy by real-time PCR). Also, the patients were negative for hepatitis B surface antigen. Patients with platelet counts of $\leq 10 \times 10^4/\mu l$, leukocyte counts of $\leq 3{,}000/\mu l$, or hemoglobin levels of ≤ 12 g/dl were excluded from the study.

Each patient gave written informed consent and agreed to receive concomitant DFPP, and the study was approved by the review board of the Kobe Asahi Hospital.

DFPP and Blood Collection

Blood collected from the peripheral vein for DFPP by a Plasmaflo™ OP-18W filter (Asahi Kasei Medical, Tokyo, Japan) was separated into plasma and cell components. The virus was then removed from the plasma by a second filter (Cascadeflo™ EC-50W; Asahi Kasei Medical) of an average pore size of 30 nm. For each session, the final volume of treated plasma was 50 ml/kg; the number of sessions was 5 over 2 weeks, and the time of DFPP, based on the reduced plasma fibrinogen levels during DFPP, was decided by the physicians and as required by the patients.

Types of IFN for 4 Weeks with DFPP

During DFPP, the patients were treated with different kinds of IFN: patient 1 with PEG-IFNα-2b plus RBV for 4 weeks; patients 2 and 3 with IFN-β 3 MU twice daily for 2 weeks and PEG-IFNα-2a plus RBV for 2 weeks; patients 4 and 9 with IFN-β 3 MU twice daily for 2 weeks and IFN-β 6 MU daily for 2 weeks; patient 5 with IFN-β 3 MU twice daily for 10 days and IFN-β 6 MU daily for 18 days, and patients 6, 7 and 8 with IFN-β 3 MU twice daily for 4 weeks. The dose of PEG-IFNα-2b was 1.5 μg/kg and 180 μg of α-2a per week. The RBV dose was 800 mg/day with α-2b and 600–800 mg/day with α-2a. After DFPP plus IFN treatment for 4 weeks, all patients were scheduled to receive PEG-IFN/RBV combination therapy (patient 1: PEG-IFNα-2b 1.5 μg/kg per week plus RBV 800 mg/day; patients 2–9: PEG-IFNα-2a 180 μg per week plus RBV 600–800 mg/day).

Amino Acid Substitutions in the Core Region (aa 30 and aa 91) and Number of IFN Sensitivity-Determining Region Mutations

We measured pre-treatment factors such as prediction of clinical outcome of therapy, amino acid sequence variation in the NS5A region (referred to as IFN sensitivity-determining regions) and in the core protein regions (aa 70 and aa 91) of HCV with a given genotype, and the viral load.

HCV RNA Measurement

The quantity of HCV RNA was measured by real-time PCR (detection limit 1.2 log IU/ml), by HCV core antigen (detection limit 20 fmol/l), and by RT-PCR (Amplicor HCV monitor v 2.0; Roche; detection limit 50 IU/ml).

Virus Removal at Second Filter Inlet and Outlet

Plasma was collected twice from the inlet and outlet of the second filter during 1 session of DFPP: once when the treated plasma volume reached half of the target quantity, and once when DFPP was completed. The change in the quantity of HCV RNA was evaluated through the plasma samples collected.

Viral Reduction and Viral Response Rate

The quantity of HCV RNA was converted to a log value at the beginning of the treatment (A) and at each of the virus measurement points (B). Δlog was then calculated: $\Delta\log = \log A - \log B = \log (A/B)$.

Evaluation of DFPP Safety

The subjective and objective adverse events of DFPP were observed, and five clinical factors were measured (platelet and lymphocyte counts, and hemoglobin, albumin and fibrinogen levels) before the first session of DFPP, before successive sessions on the second, third, fourth, fifth and sixth days, and 2 weeks after the last session.

Statistical Analysis

Statistical analysis consisted of analysis of variance for patient background factors, and the paired t test for quantities of HCV RNA at the second filter inlet during DFPP. The t test was used for viral load reductions and Fisher's exact test for viral response rates among the groups. The t test was 2-tailed, and differences of $p < 0.05$ were considered significant.

Table 1. Early viral dynamics with DFPP plus IFN treatment

Case	Age/sex	Type of IFN for 4 weeks with DFPP	Viral dynamics after DFPP+IFN						Viral dynamics of previous treatment (PEG-IFN/RBV)				Viral mutation		
			before treatment	log drop				unit	before treatment	log drop	unit	outcome	aa 70	aa 91	ISDR
				24 h	1 wk	2 wks	4 wks			4 wks					
1	66/M	PEG-IFNα-2b/RBV 4 wks	6,510	0.5	0.6	0.6	1.1	fmol/l	452	0.7	KIU/ml	NR	wild	wild	0
2	65/F	IFN-β (3 MU 2/day) 2 wks → PEG-IFNα-2a/RBV 2 wks	7.5	0.4	1.3	2.6	1.0	log IU/ml	2,800	ND	KIU/ml	PR	wild	wild	0
3	52/F	IFN-β (3 MU 2/day) 2 wks → PEG-IFNα-2a/RBV 2 wks	5.8	0.4	1.0	1.6	+0.2	log IU/ml	6.3	0.2	log IU/ml	NR	wild	wild	1
4	47/F	IFN-β (3 MU 2/day) 2 wks → IFN-β (6 MU 1/day) 2 wks	6.8	0.6	0.3	0.4	0.4	log IU/ml	2,900	0.3	KIU/ml	NR	mutant	mutant	1
5	52/F	IFN-β (3 MU 2/day) 10 days → IFN-β (6 MU 1/day) 18 days	5.5	1.4	1.5	1.2	1.9	log IU/ml	782	0.6	fmol/l	NR	wild	mutant	1
6	61/F	IFN-β (3 MU 2/day) 4 wks	6.5	1.2	3.4	5.0	4.8	log IU/ml	8,450	2.6	fmol/l	NR	wild	wild	0
7	66/F	IFN-β (3 MU 2/day) 4 wks	5.3	0.0	0.8	1.2	1.3	log IU/ml	11,500	0.8	fmol/l	NR	mutant	wild	1
8	43/F	IFN-β (3 MU 2/day) 4 wks	3,460	0.5	0.2	1.3	2.2	fmol/l	745	0.1	fmol/l	NR	wild	mutant	1
9	43/M	IFN-β (3 MU 2/day) 2 wks → IFN-β (6 MU 1/day) 2 wks	7.2	0.6	1.4	2.5	2.9	log IU/ml	426	0.1	KIU/ml	NR	wild	wild	0

PEG-IFN/RBV: PEG-IFNα-2a (180 μg per week) plus RBV (600–800 mg/day) or PEG-IFNα-2b (1.5 μg/kg per week) plus RBV (800 mg/day). IFN-β: 3 MU twice daily or 6 MU daily. ND = Not done; aa = amino acid; ISDR = interferon sensitivity-determining region.

Results

Of the 9 patients, 1 was PR and 8 were NR. Virus mutation in the core region was as follows: wild type (7 patients) and mutant type (2 patients) at aa 70; wild type (6 patients) and mutant type (3 patients) at aa 91. IFN sensitivity-determining regions demonstrated mutation 1 (5 patients) and mutation 0 (4 patients), while mutation 2 was not seen in any patient. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks showed a reduction in the viral load of ≥1 log in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9) and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. The early viral dynamics after DFPP plus consecutive intravenous IFN-β treatment for 4 weeks showed a reduction in the viral load of ≥1 log in 33% (2 of 6 patients), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. The reduction of the viral load by ≥2 log was observed in 50% (3 of 6 patients) at 4 weeks after the start of treatment (table 1).

Discussion

New drugs to replace IFN as well as drugs that can be used in combination with IFN are being actively developed. Also, attempts are being made to find ways to physically remove HCV particles from the blood. Granulocyte apheresis, plasma exchange and hemofiltration have been applied to HCV-infected patients for the treatment of cryoglobulinemia and vasculitis, modalities which have been shown to reduce HCV RNA in the blood during treatment [6–11]. The mechanisms of the clinical results of plasmapheresis have been described, whereby HCV in the blood is related to the effects of IFN therapy that could be enhanced by removing the virus from blood [12–14]. Low-density lipoprotein-cholesterol apheresis and plasma exchange in hypercholesteremic patients with HCV infection reduces the quantity of HCV RNA in the blood of some patients [15]. Hemodialysis, hemofiltration and peritoneal dialysis in chronic dialysis patients infected with HCV significantly lower HCV RNA levels in the blood [16]. Combined granulocyte apheresis with IFN therapy for CHC [17–19] and the prerequisite for early reduction of the virus in the treatment of CHC [20, 21] are essential. Thus, the potential effectiveness of IFN therapy combined with early physical removal of the virus is of particular interest.

Asahina et al. [22] studied HCV dynamics in both serum and peripheral blood mononuclear cells in 44 patients, with HCV genotype 1b and high viral loads, randomly assigned to 4 treatment groups: (1) combination therapy with 6 MU daily of IFNα-2b plus 800 mg of RBV; (2) monotherapy with 6 MU daily of IFNα-2b; (3) monotherapy with twice-daily intravenous administration of 3 MU of IFN-β, and (4) monotherapy with daily intravenous administration of 6 MU of IFN-β. HCV RNA levels measured serially by highly sensitive real-time PCR and HCV dynamics in both serum and peripheral blood mononuclear cells have demonstrated a 'biphasic' pattern. The exponential decay slopes of the second phase have been significantly higher in the combination or the twice-daily dose regimen groups than in group 2 or 4 (0.10 ± 0.08 vs. 0.02 ± 0.09 or 0.16 ± 0.09 vs. 0.02 ± 0.04 day^{-1}; $p < 0.05$ and $p < 0.0005$, respectively) [22]. Kim et al. [23] observed that a daily dose of IFN-β 6 MU for 4 weeks effects a 2 log decrease in the HCV RNA load in 7 patients with genotype 1b and high viral loads.

In this study, early viral dynamics were assessed in the 9 patients non-SVR to the combination therapy. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks reduced the viral load by ≥1 log in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9), and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. DFPP plus consecutive intravenous IFN-β treatment for 4 weeks reduced the viral load by ≥1 log in 33% (2/6), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively.

The prerequisite for early virological response (EVR; indicating negative HCV RNA at 12 weeks) has been emphasized in predicting SVR and non-SVR in CHC patients undergoing IFN treatment; those who do not reach EVR fail to respond to further therapy. Treatment discontinued in patients not reaching EVR would reduce drug costs by more than 20%; consequently, early confirmation of viral reduction after initiating antiviral therapy for CHC is highly desirable [24].

To be able to predict SVR with PEG-IFN/RBV treatment, reduction of the HCV RNA viral load by week 4 is considered essential. A 2 log reduction in the HCV RNA viral load by week 4 is a prerequisite to achieving SVR with PEG-IFN/RBV treatment [25]. In our study of DFPP plus consecutive intravenous IFN-β treatment for 4 weeks, a reduction in the viral load of ≥2 log was achieved in 50% (3 of 6 patients) at 4 weeks after the start of treatment.

From the above considerations, DFPP plus consecutive intravenous IFN-β treatment for 4 weeks is a promising regimen for non-SVR patients with genotype 1b and high viral loads, previously treated with PEG-IFN/RBV therapy. Further study is needed to elucidate the SVR rate in a larger number of patients given DFPP plus IFN treatment, especially with consecutive intravenous IFN-β.

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Disclosure Statement

No conflict of interest exists.

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Outcome and Early Viral Dynamics with Viral Mutation in PEG-IFN/RBV Therapy for Chronic Hepatitis in Patients with High Viral Loads of Serum HCV RNA Genotype 1b

Noriko Sasase[a] Soo Ryang Kim[b] Masatoshi Kudo[e] Ke Ih Kim[a]
Miyuki Taniguchi[b] Susumu Imoto[b] Keiji Mita[b] Yoshitake Hayashi[c]
Ikuo Shoji[d] Ahmed El-Shamy[d] Hak Hotta[d]

Departments of [a]Pharmacy and [b]Gastroenterology, Kobe Asahi Hospital, [c]Center for Infectious Diseases and [d]Division of Microbiology, Kobe University Graduate School of Medicine, Kobe, and [e]Department of Gastroenterology and Hepatology, Kinki University School of Medicine, Osaka-Sayama, Japan

Key Words

Chronic hepatitis · Early viral dynamics · IFN/RBV resistance-determining region · HCV RNA genotype 1b · High viral load · PEG-IFN/RBV combination therapy · Virological response, prediction

Abstract

We investigated whether sustained virological response (SVR) and non-SVR by chronic hepatitis C patients to pegylated interferon plus ribavirin (PEG-IFN/RBV) combination therapy are distinguishable by viral factors such as the IFN/RBV resistance-determining region (IRRDR) and by on-treatment factors through new indices such as the rebound index (RI). The first RI (RI-1st; the viral load at week 1 divided by the viral load at 24 h) and the second RI (RI-2nd; the viral load at week 2 divided by the viral load at 24 h) were calculated. The subject patients were divided into 3 groups based on RI-1st and RI-2nd: an RI-A group (RI-1st ≤1.0), an RI-B group (RI-1st >1.0 and RI-2nd <0.7) and an RI-C group (RI-1st >1.0 and RI-2nd ≥0.7). The SVR rate was 71.4% (10/14) in the RI-A group, 46.2% (6/13) in the RI-B group and 20.0% (3/15) in the RI-C group (p = 0.005 between the RI-A group and the RI-C group). In IRRDR ≥6 and IRRDR ≤5 the SVR rate was 81.3% (13/16) and 23.1% (6/26) (p = 0.0002), respectively. By combining RI and IRRDR as a predicting factor, the SVR rate was 87.5% (7/8) in the RI-A group (≥6 mutations in the IRRDR) and 7.7% (1/13) in the RI-C group (≤5 IRRDR mutations) (p = 0.0003).

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Introduction

Recently, global consensus has obtained that a combination of IFN or pegylated IFN plus ribavirin (PEG-IFN/RBV) is the treatment of choice for chronic hepatitis C (CHC). Notwithstanding this treatment regimen, sustained virological response (SVR) rates of those infected with the most resistant genotypes [hepatitis C virus (HCV)-1a and -1b] still hover at ~50% [1, 2]. It is therefore worthwhile to identify the predictive factors that allow the selection of patients who would achieve eradication

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Soo Ryang Kim, MD
Department of Gastroenterology
Kobe Asahi Hospital
3-5-25 Bououji-cho, Nagata-ku, Kobe 653-0801 (Japan)
Tel. +81 78 612 5151, Fax +81 78 612 5152, E-Mail asahi-hp@arion.ocn.ne.jp

of HCV RNA either before or during therapy, especially since IFN/RBV combination therapy is costly and has several side effects [3].

Predictors of the effectiveness of IFN-based therapy can be classified into pretreatment and on-treatment factors. Pretreatment factors comprise: (1) host factors such as age, gender, obesity, alcohol consumption, hepatic iron overload, fibrosis, immune responses and co-infection with other viruses, and (2) viral factors that mainly include viral genotypes and loads, particular amino acid sequence variations in the NS5A region [4, 5] and in the core protein region of HCV [6] within a given genotype. Moreover, the mean number of mutations in variable region 3 (V3) plus its upstream flanking region of NS5A [amino acid 2334–2379, referred to as IFN/RBV resistance-determining region (IRRDR)] is significantly higher in HCV isolates obtained from patients who later achieve SVR by PEG-IFN/RBV than in those from non-SVR patients. On-treatment factors are mainly related to viral kinetics within the first few weeks of treatment [7].

In the current study, with the aim of investigating whether SVR and non-SVR can be distinguished by viral factors such as IRRDR and by on-treatment factors through new indices such as the rebound index (RI), we calculated the first RI (RI-1st; the viral load at week 1 divided by the viral load at 24 h) and the second RI (RI-2nd; the viral load at week 2 divided by the viral load at 24 h), as proposed by Nomura et al. [8].

Patients and Methods

The 42 patients included in this study, who all demonstrated high viral loads (>100 KIU/ml) of serum HCV RNA of genotype 1b, had been diagnosed with CHC on the basis of abnormal serum alanine aminotransferase persisting for at least 6 months, and of positive HCV RNA assessed by RT-PCR. None of the patients was positive for hepatitis B surface antigen or other liver diseases (autoimmune hepatitis, alcoholic liver disease). All the patients received a regimen of PEG-IFNα-2b (peginterferon alpha-2b; Peg-Intron; Schering-Plough, Kenilworth, N.J., USA) (1.5 μg/kg/week, subcutaneously) in combination with RBV (ribavirin; Rebetol; Schering-Plough) 600–1,000 mg/day for 48 weeks. RBV was administered at a dose of 600 mg/day (3 capsules) to patients weighing <60 kg, 800 mg/day (4 capsules) to those weighing <80 kg and 1,000 mg/day (5 capsules) to those weighing ≥80 kg.

The efficacy of the combination therapy was evaluated by HCV RNA negativity determined by qualitative RT-PCR analysis at the end of therapy (end of therapy response) and 6 months after the completion of therapy (SVR). The amount of HCV RNA was also measured quantitatively by RT-PCR (Amplicor HCV monitor v. 2.0; Roche) before therapy. The lower detection limit of the assay was 5 KIU/ml. Samples collected during and after therapy were also determined by qualitative RT-PCR (Amplicor; Roche), which has a higher sensitivity than quantitative analysis, and the results were labeled as positive or negative. The lower limit of the assay was 50 IU/ml.

SVR was defined as undetectable serum HCV RNA at 24 weeks after the cessation of treatment, and non-SVR as detectable HCV RNA at 24 weeks after the discontinuation of treatment. Informed consent was obtained from all patients enrolled in the study after thoroughly explaining the aims, risks and benefits of the therapy.

The amount of HCV core antigen was assessed by the IRM assay (Ortho Clinical Diagnostics, Tokyo, Japan), which provides a good correlation between the amount of HCV core antigen and the amount of HCV RNA, as shown in our previous study [9]. The HCV core antigen was measured on days 0, 1 (24 h), 7 (1 week) and 14 (2 weeks) according to the detection limit of 20 fmol/l established by the manufacturer.

RI-1st was defined as the coefficient derived by dividing the viral load of HCV core antigen at week 1 by that at 24 h, and RI-2nd was defined as the coefficient derived by dividing the viral load at week 2 by that at 24 h [8].

The patients were divided into 3 groups based on RI-1st and RI-2nd: group A (RI-1st ≤1.0), group B (RI-1st >1.0 and RI-2nd <0.7) and group C (RI-1st >1.0 and RI-2nd ≥0.7).

NS5A sequence analysis (IRRDR) was performed as described [4]. Briefly, the sequences of the amplified fragments were determined by direct sequencing without subcloning with the use of a Big Dye Deoxy Terminator cycle sequencing kit and an ABI 337 DNA sequencer (Applied Biosystems, Japan). The aa sequences were deduced and aligned with Genetyx Win software v. 7.0 (Genetyx Corp., Tokyo, Japan). Numbering of aa throughout the manuscript is according to the polyprotein of HCV genotype 1b prototype HCV-J.

Statistical Analysis

Differences between the groups were assessed by the χ^2 test, Fisher's exact test or Student's t test, the Mann-Whitney test and the Kruskal-Wallis test. $p < 0.05$ was considered statistically significant.

Results

Of the 42 patients treated with combination therapy, 19 (45.2%) achieved SVR and 23 (54.8%) were still HCV RNA positive (non-SVR) 6 months after therapy. No significant differences were observed in patient characteristics between SVR and non-SVR, except in platelet counts and the degree of fibrosis (table 1), or among the RI-A, -B and -C groups (table 2).

The SVR rate was 71.4% (10/14), 46.2% (6/13) and 20.0% (3/15) in the RI-A, -B and -C groups, respectively, with a significant difference between the RI-A and -C groups (p = 0.005), but not significant between the RI-A and -B groups and the RI-B and -C groups (fig. 1). In the 14 patients of the RI-A group, HCV RNA turned negative

Table 1. Host-dependent, virus-related profile by response (SVR and non-SVR)

	SVR	Non-SVR	p value
Gender (M/F), n	11/8	13/10	NS
Age, years	56.7 ± 8.8	59.3 ± 10.5	NS
HCV RNA level, KIU/ml	1,685 ± 1,477	1,660 ± 1,363	NS
HCV core antigen, fmol/l	7,044 ± 6,763	9,343 ± 12,563	NS
Body weight, kg	59.9 ± 11.5	59.8 ± 13.6	NS
Treatment history (retreatment/naïve)	6/13	13/10	NS
Platelet count ($\times 10^4$/mm^3)	18.7 ± 4.4	14.8 ± 5.4	0.02
F0, 1/F2, 3	12/2	5/10	0.004

Table 2. Host-dependent, virus-related profile by response (RI-A, -B and -C groups)

	RI-A	RI-B	RI-C	p value
Gender (M/F), n	7/7	9/4	8/7	NS
Age, years	60.0 ± 5.9	58.5 ± 9.4	56.1 ± 12.8	NS
HCV RNA level, KIU/ml	1,401 ± 1,014	2,053 ± 1,286	1,593 ± 1,772	NS
HCV core antigen, fmol/l	6,084 ± 5,106	7,674 ± 5,038	11,000 ± 15,837	NS
Body weight, kg	62.1 ± 16.6	59.5 ± 10.4	58.2 ± 10.1	NS
Treatment history (retreatment/naïve)	3/11	7/6	9/6	NS
Platelet count ($\times 10^4$/mm^3)	15.3 ± 3.5	18.3 ± 5.9	16.3 ± 6.0	NS
F0, 1/F2, 3	7/3	5/4	5/5	NS

Table 3. SVR rate between IRRDR ≤5 and IRRDR ≤6 in RI-A, -B and -C groups

	RI-A		RI-B		RI-C	
	IRRDR ≤5	IRRDR ≥6	IRRDR ≤5	IRRDR ≥6	IRRDR ≤5	IRRDR ≥6
SVR	3	7	2	4	1	2
Non-SVR	3	1	5	2	12	0
SVR rate, %	50.0	87.5	28.6	66.7	7.7	100
p value	NS		NS		0.0024	
		0.0003 (RI-A IRRDR ≥6 vs. RI-C IRRDR ≤5)				

by week 4 in 3 patients, week 8 in 5 patients, week 12 in 5 patients and was positive in 1 patient throughout the treatment. In the 13 patients of the RI-B group, HCV RNA was negative by week 4 in 1 patient, week 8 in 2 patients, week 12 in 4 patients, at and after week 16 in 5 patients and remained positive throughout the treatment in 1 patient. In the 15 patients of the RI-C group, HCV RNA was negative by week 12 in 1 patient, on and after week 16 in 6 patients and remained positive throughout the treatment in 8 patients (fig. 2).

The SVR rate was 81.3% (13/16) in the group with ≥6 mutations in IRRDR, and 23.1% (6/26) in those with ≤5 (fig. 3), with a significant difference between the 2 groups ($p = 0.0002$).

By combining RI and IRRDR, the SVR rate was 87.5% (7/8) in the RI-A group (IRRDR ≥6) and 7.7% (1/13) in the RI-C group (IRRDR ≤5) (table 3), with a significant difference between the 2 groups ($p = 0.0003$).

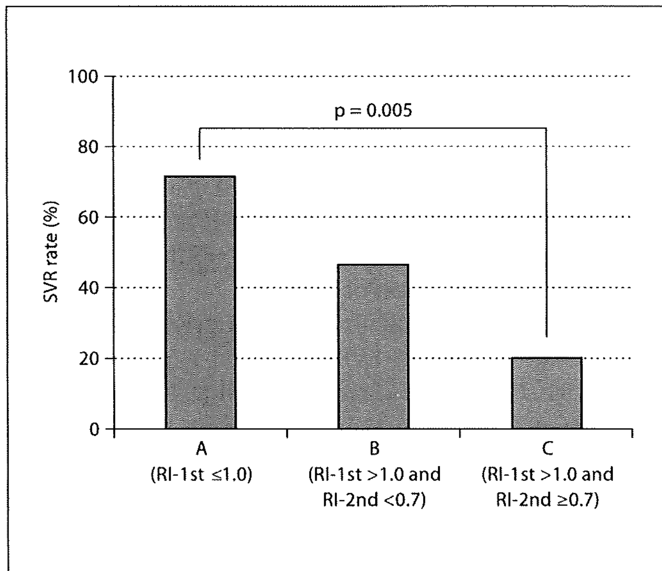

Fig. 1. SVR rate in RI-A, -B and -C groups. The overall SVR rate was 71.4, 46.2 and 20.0%, respectively. Significant difference in SVR rate is indicated.

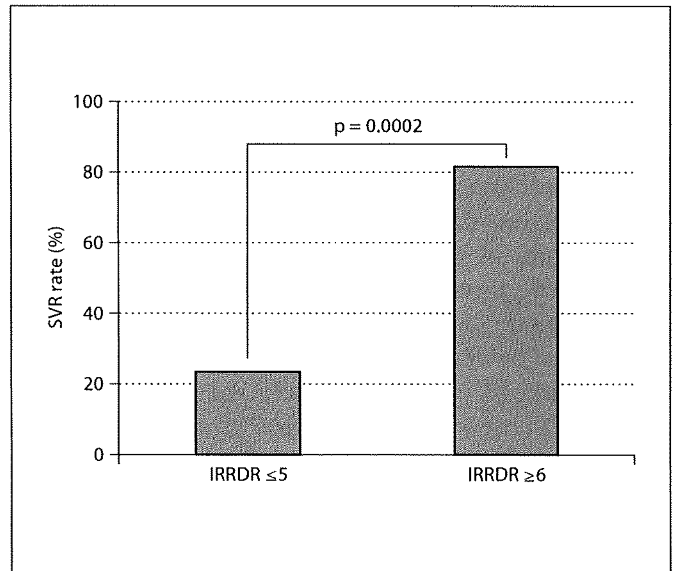

Fig. 3. SVR rate and IRRDR number. The SVR rate was 23.1% in IRRDR ≤5 and 81.3% in IRRDR ≥6, which was significantly different.

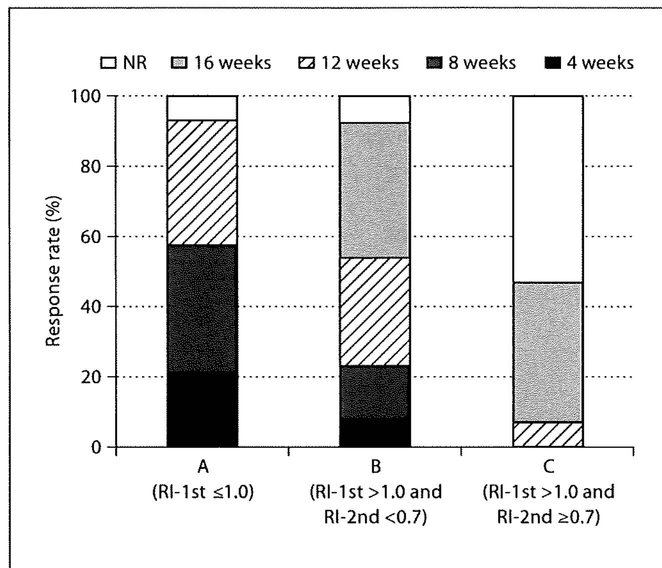

Fig. 2. Relation between response time and virus dynamics. In the 14 patients of the RI-A group, HCV RNA turned negative by week 4 in 3 patients, week 8 in 5 patients, week 12 in 5 patients and remained positive throughout the treatment in 1 patient. In the 13 patients of the RI-B group, HCV RNA was negative by week 4 in 1 patient, week 8 in 2 patients, week 12 in 4 patients, at and after week 16 in 5 patients and remained positive throughout the treatment in 1 patient. In the 15 patients of the RI-C group, HCV RNA was negative by week 12 in 1 patient, at and after week 16 in 6 patients and remained positive throughout the treatment in 8 patients.

Discussion

The importance of early virological response (EVR; signifying HCV RNA negative at 12 weeks) has been emphasized in predicting SVR and non-SVR in CHC patients undergoing IFN treatment; those not reaching EVR do not respond to further therapy. Discontinuation of treatment in patients not reaching EVR would reduce drug costs by more than 20%; consequently, early confirmation of viral reduction after initiating antiviral therapy for CHC is worth investigating [10].

Treatment with IFN induces a decline in HCV RNA levels that can be mathematically measured in 2 phases. The decline in the first phase, usually measured at 24 or 48 h, probably reflects direct inhibition of intracellular production and release of HCV [11], with IFN efficacy ranging from about 70% (approx. 0.7 log units) for standard IFN (given 3 times a week) to more than 90% (1 log unit) for high daily doses of standard IFN or PEG-IFN (given once a week) [12, 13]. The decline in the second phase, beginning after 24–48 h, is slower and more variable than that in the first phase, and is thought to reflect continued inhibition of replication and the gradual elimination of virus-infected cells [11]. The decay in the first phase has little correlation with the IFN dose, but is more rapid with PEG-IFN than with standard IFN preparations [10].

Lowering HCV RNA during the first phase is essential for efficient elimination of HCV during the second phase. Decreases in HCV RNA titers within the first 24–48 h after the start of IFN would, therefore, be a dependable estimate of antiviral efficacy [12, 13].

Early viral kinetics, determined up to week 2, are believed to express the therapeutic effect of PEG-IFN. The concentration of PEG-IFNα-2b in serum peaks after 24 h, then declines gradually [14, 15]. The viral load is thus reduced by 24 h but increases in week 1 [16, 17]; with a large dose of PEG-IFN at each administration, it decreases markedly at 24 h but then increases in week 1 regardless of the dose. In the responder group, however, the viral load continues to decline each week thereafter [17].

In this study, we used new indices proposed by Nomura et al. [8]: RI-1st and RI-2nd calculated from early viral kinetics. RI-1st was defined as the coefficient derived by dividing the viral load of HCV core antigen at week 1 by that at 24 h, and the RI-2nd was defined as the coefficient derived by dividing the viral load at week 2 by that at 24 h. In the SVR group, a number of patients demonstrated no increase in the viral load at week 1. Patients with a high RI-2nd were regarded as poor responders or non-responders to PEG-IFN. The RI-2nd of those other than non-responders was below 0.7; therefore, 0.7 was adopted as the reference value for RI-2nd, and the patients were divided into 3 groups based on RI-1st and RI-2nd: the RI-A group (RI-1st $\leq$1.0), the RI-B group (RI-1st >1.0 and RI-2nd <0.7) and the RI-C group (RI-1st >1.0 and RI-2nd $\geq$0.7). The SVR rate of the RI-A, RI-B and RI-C groups was 71.4% (10/14), 46.2% (6/13) and 20% (2/10), respectively ($p = 0.005$ between the RI-A group and the RI-C group). RIs are also associated with the early clearance of HCV RNA that is related to SVR.

In the RI-A group 21.4% (3/14), 35.7% (5/14) and 35.7% (5/14) became HCV RNA negative by weeks 4, 8 and 12, respectively. In the RI-B group 7.7% (1/13), 15.4% (2/13), 30.8% (4/13) and 38.5% (5/13) became HCV RNA negative by weeks 4, 8, 12, and at and after week 16, respectively. In the RI-C group 6.7% (1/15) and 40.0% (6/15) became HCV RNA negative by week 12, and at and after week 16, respectively. It is believed that the simplified RI-1st and RI-2nd are evidential indices for determining the therapeutic efficacy of PEG-IFN/RBV treatment.

We have previously reported that the high degree of sequence variation in IRRDR (IRRDR $\geq$6) significantly correlates with SVR, whereas the low degree of sequence variation in this region (IRRDR $\leq$5) correlates with non-SVR [4]. A significant correlation between the rapid reduction of HCV core antigen titers and the degree of sequence variation in IRRDR has been observed. This, in particular, suggests a possible influence of IRRDR $\geq$6 on HCV replication kinetics during IFN-based therapy, especially that the direct effect of IFN begins a few hours after the first dose.

In this study, the SVR rate was 81.2% (13/16) with IRRDR $\geq$6 and 23.1% (6/26) with IRRDR $\leq$5 ($p = 0.0002$), strongly suggesting that IRRDR $\geq$6 would be a useful marker for the prediction of SVR.

By combining RI and IRRDR as a predicting factor, the SVR rate was 87.5% (7/8) in the RI-A group (RI-1st $\leq$1.0) with IRRDR $\geq$6, signifying that about 90% of these patients turned SVR and were, thus, believed to be very good responders. An SVR rate of 7.7% (1/13) was obtained in the RI-C group with IRRDR $\leq$5 ($p = 0.0003$).

In conclusion, we propose that IRRDR combined with RIs is the most sensitive predictive factor for SVR and non-SVR. With the aid of RIs and IRRDR, a more effective PEG-IFN/RBV treatment could be within reach. A more detailed investigation with a larger number of subjects is needed to confirm the current results in patients given PEG-IFN/RBV combination therapy.

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Disclosure Statement

No conflict of interest exists.

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<速　報>

1b型高ウイルスC型慢性肝炎のPEG-IFN＋リバビリン併用療法（併用療法）無効例に対する二重濾過血漿交換療法（DFPP）＋IFN-β4週間連続投与の試み —早期ウイルスdynamicsを中心に—

金　守良[1)*]　井本　勉[1)]　三田　敬二[1)]　谷口　美幸[1)]
金　啓二[1)]　勝二　郁夫[2)]　長野　基子[2)]　堀田　博[2)]

緒言：1b型高ウイルス量C型慢性肝炎の併用療法の無効例に対して，併用療法による再治療のSVR率は約10〜15％であり[1)]，その対策は急務である．2008年4月から保険適用になったDFPP[2)]＋IFN治療の狙いはウイルス量の早期低下ひいては早期陰性化にある．preliminaryではあるが，併用療法無効例に対するDFPP施行症例の早期ウイルスdynamicsを検討した．

方法：対象は併用療法が無効であった8名(男1名，女7名)．DFPPは2週間で5回施行した．ウイルス量はリアルタイム法(log IU/m*l*)(ロシュ・ダイアグノスティックス(株))，アンプリコア法(KIU/m*l*)(ロシュ・ダイアグノスティックス(株))，又はコア蛋白抗原(fmol/*l*)(栄研化学(株))を用いた．尚，前回併用療法時のウイルスdynamicsが測定された症例は今回のそれと比較した．前回の治療の効果をPR（一過性有効）とNR(無効)に分けた．又，治療効果と関係する因子として，Coreアミノ酸の置換(R70QとL90M)とISDR変異数を調べた．

結果：結果は表の通りである(Table)．PRは1例，NRは7例であった．Coreアミノ酸の変異については，70番目のwildは6例，mutantが2例，91番目のwildは5例，mutantが3例で，ISDRの変異は1が5例，0が3例であった．8例全体の投与後24時間後の1 log低下は25％(2/8)，1週後の1 log低下は50％(4/8)，2週後の1 log低下は75％(6/8)，4週後の1 log低下は75％(6/8)であった．DFPP＋IFN-β300万単位1日2回2週間投与を行なった症例において1週後（症例2は1.3 log，症例3は1 log低下），2週後(症例2は2.6 log，症例3は1.6 log低下)とも開始時よりウイルス量は著明に低下したが，併用療法に切り替えたところ，4週後は2週後と比較してウイルス量は症例2は1.6 log，症例3は1.8 logの上昇を認めた．IFN-βの4週間連続投与の5症例のうち4症例では（症例5，6，7，8）では前回併用療法と比較しても早期にウイルス量の低下がみられ，24時間，1週後の1 log低下は40％(2/5)，2週，4週後の1 log低下は80％(4/5)，4週後の2 log低下は40％(2/5)にみられた．特に症例6においては，1週後で3.4 log，2週で5.0 log，4週で4.8 logの低下がみられた．DFPPにおいて血圧低下などの重篤な副作用はなく，又IFN-β投与の4週間連続投与の症例6においては投与4週間目に血小板減少（3万/mm^{3}以下）がみられたが，注意深い観察で治療を継続，問題は生じなかった．

考察：朝比奈ら[3)]は1b型高ウイルス量患者のIFN治療において，IFN-β300万単位1日2回2週間連続投与が，IFN-α600万単位やIFN-β600万単位1日1回2週間連続投与よりも24時間後から2週後までの早期ウイルスdynamicsにおいて，ウイルス量が有意に低下したと報告している．又，Kimら[4)]によれば，1b高ウイルス量患者（初回治療例）に対するIFN-β600万単位4週間連続投与した7症例について検討し，4週後にいずれも2 log以下の低下を認めた．今回併用療法の無効例に対するDFPP＋IFN-β4週間連続投与の5例では，1例(症例4)を除いて持続的にウイルス量は低下しており，良好な治療効果(ウイルス陰性化)が期待される．4週以降のウイルス量の低下，SVR率については今後

1）神戸朝日病院消化器科
2）神戸大学大学院医学系研究科微生物学
*Corresponding author: asahi-hp@arion.ocn.ne.jp
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Table Early viral dynamics with double filtration plasmapheresis plus interferon treatment

Case	Age/Sex	Kind of IFN for 4 weeks with DFPP	Viral dynamics after DFPP + IFN						Viral dynamics of previous treatment (PEG-IFN/RBV)				Viral mutation		
			Before treatment	24h log drop	1w log drop	2w log drop	4w log drop	Unit	Before treatment	4w log drop	Unit	Outcome	aa70	aa91	ISDR
1	66M	PEG-IFN/RBV	6510	0.5	0.6	0.6	1.1	fmol/L	452	0.7	KIU/m*l*	NR	wild	wild	0
2	65F	IFN-β 2weeks (twice/day) → PEG-IFN/RBV	7.5	0.4	1.3	2.6	1.0	log IU/mL	2800	ND	KIU/m*l*	PR	wild	wild	0
3	52F	IFN-β 2weeks (twice/day) → PEG-IFN/RBV	5.8	0.4	1.0	1.6	+0.2	log IU/mL	6.3	0.2	log IU/mL	NR	wild	wild	1
4	47F	IFN-β 14days (twice/day) → IFN-β 14days (once/day) → PEG-IFN/RBV	6.8	0.6	0.3	0.4	0.4	log IU/mL	2900	0.3	KIU/m*l*	NR	mutant	mutant	1
5	52F	IFN-β 10days (twice/day) → IFN-β 18days (once/day) → PEG-IFN/RBV	5.5	1.4	1.5	1.2	1.9	log IU/mL	782	0.6	fmol/L	NR	wild	mutant	1
6	61F	IFN-β 4weeks (twice/day) → PEG-IFN/RBV	6.5	1.2	3.4	5.0	4.8	log IU/mL	8450	2.6	fmol/L	NR	wild	wild	0
7	66F	IFN-β 4weeks (twice/day) → PEG-IFN/RBV	5.3	0.0	0.8	1.2	1.3	log IU/mL	11500	0.8	fmol/L	NR	mutant	wild	1
8	43F	IFN-β 4weeks (twice/day) → PEG-IFN/RBV	3460	0.5	0.2	1.3	2.2	fmol/L	745	0.1	fmol/L	NR	wild	mutant	1

NOTE PEG-IFN/RBV: PEG-IFN α-2a + RBV or PEG-IFN α-2b + RBV
IFN-β: 6 MU/day (3 MU twice/day or 6 MU once/day)
DFPP: double filtration plasmapheresis
PR: partial responder
NR: non-responder
ND: not done
aa: amino acid
ISDR: interferon sensitivity determining region

の検討課題である.

索引用語：二重濾過血漿交換療法,
IFN-β 4 週間連続投与,
早期ウイルス dynamics

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英文要旨

Double filtration plasmapheresis (DFPP) plus consecutive intravenous interferon (IFN)-β treatment for 4 weeks for non-sustained virological responders with genotype 1b and high viral load previously treated with pegylated IFN plus ribavirin combination therapy
—focusing on early viral dynamics—

Soo Ryang Kim[1]*, Susumu Imoto[1], Keiji Mita[1], Miyuki Taniguchi[1], Ke Ih Kim[1], Ikuo Shouji[2], Motoko Nagano[2], Hak Hotta[2]

Double filtration plasmapheresis was approved in April 2008 in Japan for the retreatment of chronic hepatitis C patients with genotype 1b and high viral loads, whose hepatitis C virus was not eradicated by previous pegylated IFN plus ribavirin combination therapy. Eight patients with non-sustained virological response (SVR) to the combination therapy were enrolled in this study, and early viral dynamics were assessed. DFPP plus consecutive intravenous IFN-β treatment for 4 weeks reduced the viral load by 1 log in 2 of 5 (40%) patients 24 hours and 1 week after the start of treatment, and in 4 of 5 (80%), and by 2 log in 2 of 5 (40%) 4 weeks after the start of treatment. The present study suggests that DFPP plus consecutive intravenous IFN-β treatment for 4 weeks appears to be a promising treatment for non-SVR patients with genotype 1b and high viral load previously treated with PEG-IFN + RBV therapy.

Key words:
double filtration plasmapheresis,
consecutive intravenous interferon-β treatment,
early viral dynamics

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1) Department of Gastroenterology, Kobe Asahi Hospital, Kobe, Japan
2) Department of Microbiology, Kobe University Graduate School of Medicine, Kobe, Japan
*Corresponding author: asahi-hp@arion.ocn.ne.jp

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Involvement of Creatine Kinase B in Hepatitis C Virus Genome Replication through Interaction with the Viral NS4A Protein[▽]

Hiromichi Hara,[1,2] Hideki Aizaki,[1] Mami Matsuda,[1] Fumiko Shinkai-Ouchi,[3] Yasushi Inoue,[1,4] Kyoko Murakami,[1] Ikuo Shoji,[1,5] Hayato Kawakami,[6] Yoshiharu Matsuura,[7] Michael M. C. Lai,[8] Tatsuo Miyamura,[1] Takaji Wakita,[1] and Tetsuro Suzuki[1]*

Department of Virology II[1] and Department of Biochemistry and Cell Biology,[3] National Institute of Infectious Diseases, Tokyo 162-8640, Japan; Department of Internal medicine, Division of Pulmonary Diseases, The Jikei University School of Medicine, Tokyo 105-8461, Japan[2]; Mita Hospital, International University of Health and Welfare, Tokyo 108-8329, Japan[4]; Division of Microbiology, Kobe University Graduate School of Medicine, Hyogo 650-0017, Japan[5]; Department of Anatomy, Kyorin University School of Medicine, Tokyo 181-8611, Japan[6]; Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan[7]; and Department of Molecular Microbiology and Immunology, University of Southern California, Keck School of Medicine, Los Angeles, California 90033[8]

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Persistent infection with hepatitis C virus (HCV) is a major cause of chronic liver diseases. The aim of this study was to identify host cell factor(s) participating in the HCV replication complex (RC) and to clarify the regulatory mechanisms of viral genome replication dependent on the host-derived factor(s) identified. By comparative proteome analysis of RC-rich membrane fractions and subsequent gene silencing mediated by RNA interference, we identified several candidates for RC components involved in HCV replication. We found that one of these candidates, creatine kinase B (CKB), a key ATP-generating enzyme that regulates ATP in subcellular compartments of nonmuscle cells, is important for efficient replication of the HCV genome and propagation of infectious virus. CKB interacts with HCV NS4A protein and forms a complex with NS3-4A, which possesses multiple enzyme activities. CKB upregulates both NS3-4A-mediated unwinding of RNA and DNA in vitro and replicase activity in permeabilized HCV replicating cells. Our results support a model in which recruitment of CKB to the HCV RC compartment, which has high and fluctuating energy demands, through its interaction with NS4A is important for efficient replication of the viral genome. The CKB-NS4A association is a potential target for the development of a new type of antiviral therapeutic strategy.

Hepatitis C virus (HCV) infection represents a significant global healthcare burden, and current estimates suggest that a minimum of 3% of the world's population is chronically infected (4, 19). The virus is responsible for many cases of severe chronic liver diseases, including cirrhosis and hepatocellular carcinoma (4, 16, 19). HCV is a positive-stranded RNA virus belonging to the family *Flaviviridae*. Its ~9.6-kb genome is translated into a single polypeptide of about 3,000 amino acids (aa), in which the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B reside in the C-terminal half region (6, 34, 44). NS4A, a small 7-kDa protein, functions as a cofactor for NS3 to enhance NS3 enzyme activities such as serine protease and helicase activities. The hydrophobic N-terminal region of NS4A, which is predicted to form a transmembrane α-helix, is responsible for membrane anchorage of the NS3-4A complex (8, 44, 50), and the central region of NS4A is important for the interaction with NS3 (10, 44). A recent study demonstrated the involvement of the C terminus of NS4A in the regulation of NS5A hyperphosphorylation and viral replication (28).

The development of HCV replicon technology several years ago accelerated research on viral RNA replication (7, 44). Furthermore, a robust cell culture system for propagation of infectious HCV particles was developed using a viral genome of HCV genotype 2a, JFH-1 strain, enabling us to study every process in the viral life cycle (27, 47, 54). RNA derived from genotype 1a, HCV H77, containing cell-culture adaptive mutations, also produces infectious viruses (52). Using these systems, it has been reported that the HCV genome replicates in a distinct, subcellular replication complex (RC) compartment, which includes NS3-5B and the viral RNA (2, 14, 33). The RC forms in a distinct compartment with high concentrations of viral and cellular components located on detergent-resistant membrane (DRM) structures, possibly a lipid-raft structure (2, 41), which may protect the RC from external proteases and nucleases. Almost all processes in viral replication are dependent on the host cell's machinery and involve intimate interaction between viral and host proteins. However, the functional roles of host factors interacting with the HCV RC in viral genome replication remain ambiguous.

To gain a better understanding of cellular factors that are components of the HCV RC and that function as regulators of viral replication, a comparative proteomic analysis of DRM fractions from HCV replicon and parental cells and subsequent RNA interference (RNAi) silencing of selected genes were performed. We identified creatine kinase B (CKB) as a key factor for the HCV genome replication. CKB catalyzes the reversible transfer of the phosphate group of phosphocreatine

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111. Fax: 81-3-5285-1161. E-mail: tesuzuki@nih.go.jp.
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(pCr) to ADP to yield ATP and creatine and is known to play important roles in local delivery and cellular compartmentalization of ATP (48, 51). The findings obtained here suggest that recruitment of CKB to the HCV RC, through CKB interaction with NS4A, is essential for maintenance or enhancement of viral replicase activity.

MATERIALS AND METHODS

Cell lines, antibodies, and reagents. Human hepatoma cell line Huh-7.5.1 (54) was kindly provided by Francis V. Chisari. Cell lines carrying subgenomic replicon RNAs, namely, SGR-N (41) and SGR-JFH1 (23), were derived from the HCV-N (17) and JFH-1 strains (24), respectively. Mouse monoclonal antibodies (MAbs) against HCV NS3 (Chemicon, Temecula, CA), NS4A (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), NS5A (Biodesign, Saco, ME), NS5B (2), FLAG (M2; Sigma-Aldrich, St. Louis, MO), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon), and Flotillin-1 (BD Biosciences, San Jose, CA) and polyclonal antibodies (PAbs) against CKB (mouse [Abnova, Taipei, Taiwan], goat [Santa Cruz]), hemagglutinin (HA; Sigma-Aldrich), and FLAG (Sigma-Aldrich) were used. Cyclocreatine (Ccr; also known as 2-imino-1-imidazolidineacetic acid), pCr, and phosphopyruvic acid (pPy) were purchased from Sigma-Aldrich. Recombinant CKB and pyruvate kinase (PK) were obtained from Acris (Herfold, Germany) and Calbiochem (San Diego, CA), respectively.

Proteome analysis. RC-rich membrane fractions of cells were isolated as described previously (2, 41). Briefly, cells were lysed in hypotonic buffer. After removing the nuclei, supernatants were treated with 1% NP-40 for 60 min, mixed with 70% sucrose, overlaid with 55 and 10% sucrose, and centrifuged at 38,000 rpm for 14 h. Proteins from membrane fractions were purified by using a 2D Clean-Up kit (GE Healthcare, Tokyo, Japan), followed by labeling with fluorescent dyes: Cy5 for replicon cells, Cy3 for parental cells, and Cy2 for the protein standard containing equal amounts of both cell samples. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) was performed using Immobiline DryStrip as the first-dimension gel and 12.5% polyacrylamide gel as the second-dimension gel. The 2D-DIGE images were analyzed quantitatively using the DeCyder software (GE Healthcare). Student *t* test was performed on differences between the tested samples using DeCyder biological variation analysis module. Samples were analyzed in triplicate. The protein spots of interest were excised from the gel, subjected to in-gel digestion using trypsin or lysyl endopeptidase and analyzed by liquid chromatography (MAGIC 2002 System; Michrom Bioresources, Auburn, CA) directly connected to electrospray ionization-ion trap mass spectrometry (LCQ-decaXP; Thermo Electron Corp., Iwakura, Japan). The results were subjected to database (NCBInr) search by Mascot server software (Matrix Science, Boston, MA) for peptide assignment.

Plasmids. A human CKB cDNA (43; kindly provided by Oriental Yeast Corp., Tokyo, Japan) was inserted into the EcoRI site of pCAGGS, yielding pCAGCKB. To generate expression plasmids for HA-tagged versions of wild-type and deletion mutated CKB, the corresponding DNA fragments were amplified by PCR, followed by introduction into the BglII site of pCAGGS. A fragment representing the inactive mutant CKB-C283S was synthesized by PCR mutagenesis. To generate FLAG-tagged NS protein expression plasmids, DNA fragments encoding either NS3, NS4A, NS4B, NS5A, or NS5B protein were amplified from HCV strains NIHJ1 (1) and JFH-1 (23) by PCR, followed by cloning into the EcoRI-EcoRV sites of pcDNA3-MEF (20). To generate an HA-tagged NS3 expression plasmid, a fragment encoding NS3 with the HA tag sequence at its N terminus was inserted into pCAGGS.

siRNA transfection. The small interfering RNAs (siRNAs) targeted to CKB (CKB-1 [5′-UAAGACCUUCCUGGUGUGGTT-3′] and CKB-2 [5′-CGUCAC CCUUGGUAGAGUUTT-3′]) and the scramble negative control siRNA to CKB-2 (5′-GGCGUACUAGCUUAUUCGCTT-3′) were purchased from Sigma. Cells in a 24-well plate were transfected with siRNA using HiPerFect transfection reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The siRNA sequences for the other genes used in the siRNA screening are available upon request.

HCV infection. Culture media from Huh-7 cells transfected with in vitro-transcribed RNA corresponding to the full-length JFH-1 (47) was collected, concentrated, and used for the infection assay (3).

Quantification of HCV core protein and RNA. To estimate the levels of HCV core protein, aliquots of culture supernatants or of cell lysates were assayed by using HCV Core enzyme-linked immunosorbent assay kits (5). Total RNA was isolated from harvested cells using TRIzol (Invitrogen, Carlsbad, CA). Copy numbers of the viral RNA were determined by reverse transcription-PCR (RT-PCR) (2, 36, 46).

Immunoprecipitation, immunoblot analysis, and immunofluorescence microscopy. The analyses, as well as DNA transfection, were performed essentially as previously described (42). Cells were lysed in immunoprecipitation lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol, 1 mM calcium acetate). For immunoprecipitation, supernatants of cell lysates were precipitated with anti-FLAG antibody and protein A-Sepharose Fast Flow beads (GE healthcare). For immunofluorescence microscopy, anti-CKB goat PAb and anti-NS4A MAb as primary antibodies and Alexa Fluor 555-conjugated donkey anti-goat immunoglobulin G (Invitrogen) and Alexa Fluor 488-conjugated rabbit anti-mouse immunoglobulin G (Invitrogen) as secondary antibodies were used and observed under an LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Immunoelectron microscopy. Postembedding immunostaining using the colloidal gold-labeling method was performed as described previously (38). Cells were fixed in 4% paraformaldehyde–1% glutaraldehyde at 4°C for 1 h. After dehydration through a graded series of ethanol, cells were embedded in LR White (London Resin Company, London, United Kingdom) and sectioned. After blocking, section grids were incubated with a mixture of anti-NS4A and anti-CKB antibodies at 4°C overnight, followed by treatment with a mixture of 18-nm colloidal gold-conjugated donkey anti-mouse immunoglobulin G and 12-nm colloidal gold-conjugated donkey anti-goat immunoglobulin G antibodies (Jackson Immunoresearch, West Grove, PA) at 4°C overnight. The sections were stained with uranyl acetate and observed under a transmission electron microscope.

Measurement of CK activity and cellular ATP level. Cells were lysed with passive lysis buffer (Promega, Madison, WI), and CK activities were measured based on Oliver methods (40), in which the activity of converting creatine phosphate and ADP to creatine and ATP was measured. ATP levels in cell lysates were measured by using a CellTiter-Glo luminescent cell viability assay (Promega).

RNA replication assays in permeabilized replicon cells and in vitro. The RNA synthesis assay using permeabilized replicon cells was based on a previously described method (33). Briefly, SGR-JFH1 cells were treated with 5 μg of actinomycin D/ml for 2 h, followed by permeabilization with 50 μg of digitonin/ml for 5 min. The resulting mix was incubated with 500 μM concentrations of ATP, GTP, and CTP; 10 μCi of UTP ([α-^{32}P]UTP); 50 μg of actinomycin D/ml; and 5 mM pCr with or without 20 U of CKB/ml for 4 h at 27°C. RNA was extracted by using TRIzol and analyzed by 1% formaldehyde agarose gel electrophoresis. The cell-free RNA replication assay was performed as described previously (2).

In vitro helicase assays. Helicase activity on double-stranded RNA (dsRNA) was investigated as described previously (11) with some modifications. The 5′ end of the release strand was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Ambion). The dsRNA substrate was obtained by annealing the labeled RNA with a template strand RNA at a molar ratio of 1:1. The helicase assay mixture contained 5 nM dsRNA, helicase enzyme (80 nM NS3 or NS3-4A [kindly provided by R. De Francesco]), 6 mM ATP, in the presence or absence of 20 U of CKB/ml in an assay buffer (25 mM MOPS-NaOH [pH 7.0], 2.5 mM dithiothreitol, 100 μg of bovine serum albumin/ml, 3 mM $MgCl_2$, 5 mM pCr, 2.5 U of RNase inhibitor/ml). After the helicase reaction, samples were electrophoresed in a native 8% polyacrylamide gel and autoradiographed.

To determine the effect of PK/pPy system on the helicase activity, PK and pPy were used instead of CKB and pCr. Helicase activity on dsDNA was measured based on homogeneous time-resolved fluorescence quenching using a Trupoint helicase assay kit (Perkin-Elmer, Waltham, MA) according to the manufacturer's instructions.

In vitro protease assay. In vitro HCV protease activity of NS3-4A or NS3 was analyzed by using a SensoLyteHCV protease assay kit (AnaSpec, San Jose, CA) according to the manufacturer's instructions.

RESULTS

Identification of host factors involved in HCV RNA replication by comparative proteomic analysis of DRM fractions and RNAi silencing. To identify host proteins involved in the HCV RC, proteome profiles of the RC-rich membrane fraction in Huh-7 cells harboring subgenomic replicon RNA derived from genotype 1b, N isolate (SGR-N) were compared to those of parental cells by 2D-DIGE. We confirmed that the DRM fraction obtained from SGR-N cells is functionally active in a

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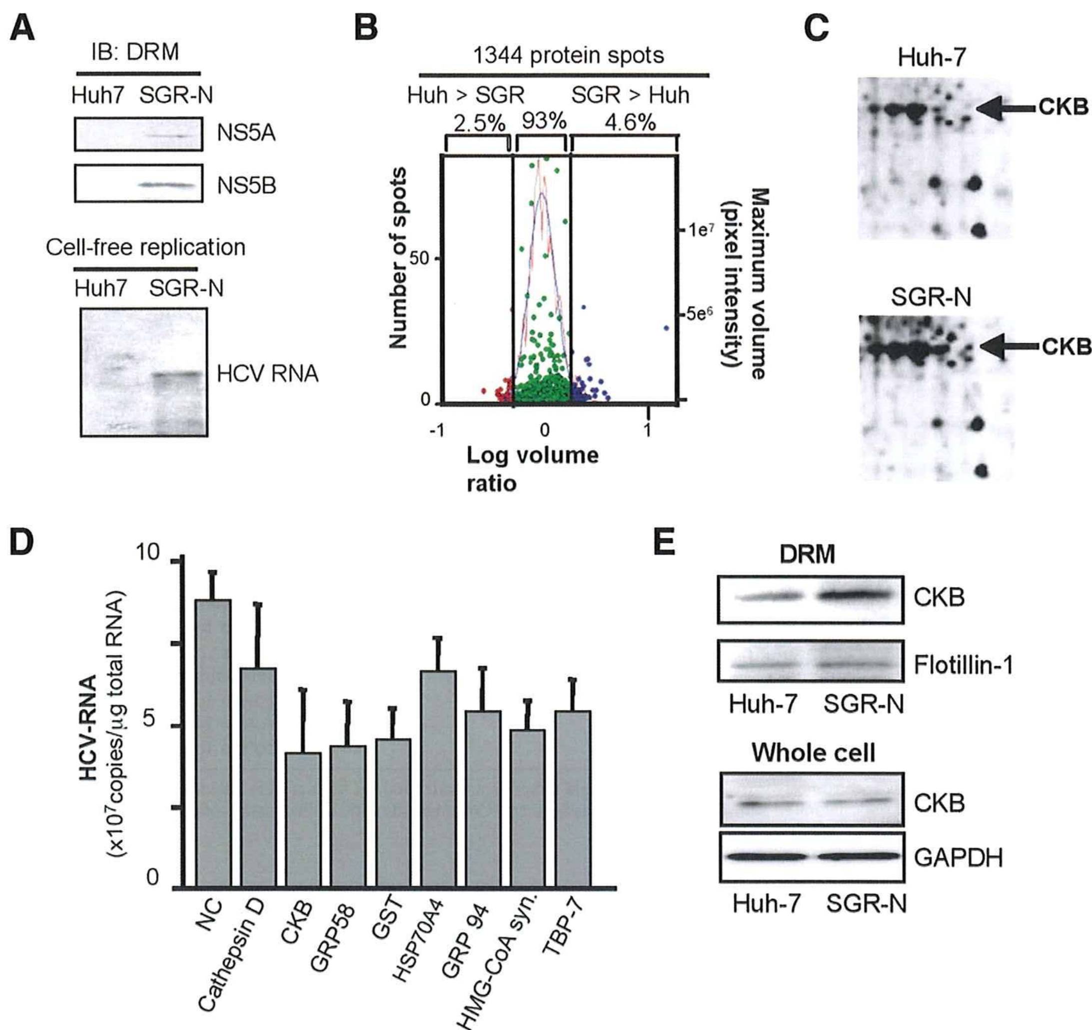

FIG. 1. Comparative proteomic analysis of DRM fractions and RNAi silencing. (A) Preparation of functionally active RC fraction for proteome analysis. DRM fractions obtained from SGR-N cells and parental Huh-7 cells were analyzed by immunoblotting with anti-NS5A and anti-NS5B antibodies (upper panel) and by the cell-free RNA replication assay (lower panel). (B) Histogram representation of proteins detected in 2D-DIGE. Images were analyzed quantitatively by the DeCyder software. The left and right *y* axis, respectively, indicate the spot frequency and the maximum volume of each spot, given against the log volume ratio (*x* axis). (C) Comparison of 2D-DIGE maps of proteins from DRM fractions of SGR-N cells and Huh-7 cells. Enlarged 2D-DIGE gel images of regions containing protein spots of CKB (arrows) are shown. (D) Effects of siRNAs of genes selected from comparative proteome analysis on HCV RNA replication. SGR-N cells were transfected with siRNA specific to cathepsin D, CKB (siCKB-1), GRP58, GST, Hsp70 protein 4, GRP94, HMG-coenzyme A synthase, or Tat binding protein 7 or with nontargeting (NC) siRNA. At 48 h posttransfection, total RNA was isolated and HCV RNA levels were assessed by real-time RT-PCR. (E) Enrichment of CKB in the DRM of HCV replicon cells. Equal amounts of DRM fractions from SGR-N and parental Huh-7 cells, or whole-cell lysates from both cells were analyzed by immunoblotting with antibodies against CKB, flotilin-1 or GAPDH.

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cell-free replication assay (Fig. 1A). Three independent proteome experiments were performed for a reliable analysis of protein expression. Approximately 1,300 spots were resolved in each gel, and 4 to 5% of the protein spots represented a >2-fold increase in the membrane fraction of replicon cells in each experiment (Fig. 1B). The protein spots that exhibited high reproducibility (an example shown in Fig. 1C) were excised, digested by trypsin or lysyl endopeptidase, and analyzed by mass spectrometry, which identified the corresponding proteins in 27 cases (Table 1). Among the proteins implicated in a variety of functional categories, 10 were involved in protein folding, mainly as chaperones, 7 were metabolic and biosynthesis enzymes including proteins for redox regulation or energy pathways, 3 were involved in cytoskeleton organization, and 3 proteins were related to cellular processes, mainly proteolysis pathways. The viral NS proteins identified as differentially expressed proteins in the analysis were not listed.

In order to identify host factors involved in HCV replication, we examined the effects on viral RNA replication of transfection of SGR-N cells with siRNAs against genes encoding nine proteins belonging to diverse classes of biological functions (Table 1). Each siRNA reduced the HCV RNA level to 47 to 76% of the level of the siRNA control (Fig. 1D). None of the siRNAs tested exhibited considerable cytotoxicity against the replicon cells, ruling out overt toxicity as a mechanism for inhibition of viral RNA replication. Among the candidate