

Outcome of therapy is influenced by various factors. Some host factors, such as age, sex, body weight, insulin resistance, and liver fibrosis have been reported as pretreatment factors affecting virological response to this combination therapy [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004; Romero-Gomez et al., 2005]. Recently, several genome-wide association studies identified single nucleotide polymorphisms (SNPs) near the interleukin (IL)-28B gene, which encodes interferon (IFN) lambda-3, as associated with response to Peg-IFN plus ribavirin treatment among patients infected with HCV of European [Suppiah et al., 2009], African [Ge et al., 2009], and Asian ancestry [Tanaka et al., 2009]. These studies suggest that host genetic variants may be associated strongly with response to IFN-alpha-based therapy. However, the ethical problem to perform host genetic search for all patients remains, and the sustained virological response rate is only 48–69% in patients having favorable IL-28B genotype to this combination therapy [Thompson et al., 2010].

Response-guided therapy is a dynamic approach to management of chronic hepatitis C patients based on the virological response at weeks 4 and 12 of treatment. At present, it is regarded as an excellent strategy for optimizing the treatment duration for individual patients. Earlier HCV RNA disappearance has been shown to lead to a higher sustained virological response rate [Ferenci et al., 2005; Berg et al., 2006; McHutchison et al., 2009], while patients without an early virological response, defined as showing an at least 2 log decrease from the baseline of HCV RNA levels at week 12 is recommended for discontinuing the treatment under the current guidelines [Anonymous, 2002; Strader et al., 2004; Dienstag and McHutchison, 2006].

In addition to viral kinetics during treatment, other viral factors have also been reported to be associated with this combination therapy outcome [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004; Shirakawa et al., 2008]. Previous studies indicated that amino acid (aa) 70 and/or 91 substitutions in the HCV core protein were independent pretreatment predictors of null or weak response to this combination therapy in genotype 1 patients [Akuta et al., 2007b,c]. The HCV core protein has been reported to inhibit signal transducer and activator of transcription (STAT)-1 phosphorylation, and disrupt the normal IFN-stimulated transcriptional response to viral infection [Lin et al., 2006]. It is supposed that the HCV core region might be associated with resistance to IFN therapy involving the Janus activated kinase (Jak)-STAT signaling cascade [Blindenbacher et al., 2003; Bode et al., 2003; Melen et al., 2004; de Lucas et al., 2005]. Recently, Okanoue et al. [2009] have demonstrated that wild type of core aa 70 and 91 are important for positive prediction of the virological response. However, the impact of core aa substitutions on the extent of HCV RNA decline during the treatment or virological relapse after completion of treatment has not yet been investigated in detail. Approximately 30% of genotype 1 patients who become

HCV RNA negative at the end of the treatment will experience relapse [Hadziyannis et al., 2004]. Being able to distinguish between end-of-treatment responders with a high probability of relapse and those with a low probability of relapse will be useful in reducing relapse rates and improving treatment outcome.

The aim of this study was to evaluate the impact of aa substitutions in the HCV core protein on viral kinetics and virological relapse in patients with HCV genotype 1 treated by Peg-IFN alpha-2b and ribavirin combination therapy.

PATIENTS AND METHODS

Patient Selection and Study Design

Patients considered to be eligible for this study were those who were infected with HCV genotype 1, had a viral load more than 10^5 IU/ml, had started Peg-IFN alpha-2b (Schering-Plough K.K. Tokyo, Japan) and ribavirin (Schering-Plough K.K.) combination therapy from December 2005 to June 2008 at Osaka University Hospital and three other medical institutions taking part in the Osaka Liver Forum, and had been examined with respect to the aa sequences at positions 70 and 91 in the HCV core protein with pretreatment serum samples. Patients with the following criteria were excluded: hepatitis B virus or human immunodeficiency virus co-infection; decompensated liver disease; severe cardiac, renal, hematological, or chronic pulmonary disease; poorly controlled psychiatric disease; poorly controlled diabetes; and immunologically mediated disease. As a result of screening at the institutions concerned, 187 patients with HCV genotype 1 were enrolled in this study. Liver biopsy had been performed within 12 months prior to the treatment, and histological results were classified according to the METAVIR scoring system [Bedossa and Poynard, 1996].

Written informed consent was obtained from each patient, and the study protocol was reviewed and approved according to the ethical guidelines of the 1975 Declaration of Helsinki by Institutional Review Boards at the respective sites.

Peg-IFN alpha-2b and ribavirin dosages were based on body weight according to the manufacturer's instructions: Peg-IFN alpha-2b was given subcutaneously weekly (45 kg or less, 60 µg/dose; 46–60 kg, 80 µg/dose; 61–75 kg, 100 µg/dose; 76–90 kg, 120 µg/dose; and 91 kg or more, 150 µg/dose), and ribavirin was given orally daily (60 kg or less, 600 mg/day; 61–80 kg, 800 mg/day; and 81 kg or more, 1,000 mg/day). The drug doses were also modified based on the manufacturer's instructions according to the severity of the adverse hematologic effects.

Detection of Amino Acid Substitutions in Core Region

The nucleotide sequence encoding aa 1–191 (the core protein of HCV) was analyzed by direct sequencing as described by Akuta et al. [2005, 2007b]. In brief, HCV

RNA was extracted from the serum samples and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows: the first PCR was performed using cc11 (sense, 5'-GCC ATA GTG GTC TGC GGA AC-3') and e14 (antisense, 5'-GGA GCA GTC CTT CGT GAC ATG-3') primers. The second PCR was performed using cc9 (sense, 5'-GCT AGC CGA GTA GTG TT-3') and e14 (antisense) primers. All samples were denatured initially at 95°C for 15 min. The 35 cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 µl of the first PCR product was transferred to the second PCR reaction. The conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. The amplified PCR products were purified by the QIA quick PCR Purification Kit (Qiagen, Tokyo, Japan) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the Big Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Tokyo, Japan). The obtained nucleotide and amino acid sequences were compared with the prototype sequence of genotype 1b HCV-J (GenBank Accession No. D90208) [Kato et al., 1990]. Wild types virus encoded arginine (Arg) and leucine (Leu) at aa 70 and 91, respectively, and the aa substitutions were glutamine (Gln) or histidine (His) at aa 70 and methionine (Met) at aa 91. If the intensities of the band were similar, the case was regarded as competitive. Two patterns of mutant and competitive were labeled as mutant. In this study, patients were categorized into four groups according to aa substitution patterns: double-wild group, Arg70/Leu91; 70-mutant group, Gln or His70/Leu91; 91-mutant group, Arg70/Met91; and double-mutant group, Gln or His70/Met91.

Virological Tests

Serum HCV RNA level was quantified by PCR assay (COBAS Amplicor HCV Monitor Test v2.0, Chugai-Roche Diagnostics, Tokyo, Japan), with a sensitivity limit of 5,000 IU/ml and a dynamic range from 5,000 to 5,000,000 IU/ml.

Serum HCV RNA was assessed by qualitative PCR assay (COBAS Amplicor HCV Test v2.0, Chugai-Roche Diagnostics), with a detection limit of 50 IU/ml.

Efficacy Assessments

Patients who achieved negative HCV RNA at week 12 were defined as having a complete early virological response. Patients who became HCV RNA negative between weeks 13 and 24 were defined as having a late virological response. According to the established guidelines, the treatment was considered to have failed if the patients showed an insufficient virological response at week 12 (a detectable HCV RNA and a decrease of <2 log from the baseline level) or at week 24 (a detectable

HCV RNA), and therapy was discontinued. The end-of-treatment response was defined as undetectable HCV RNA at week 48. Patients with end-of-treatment response and undetectable HCV RNA 24 weeks after completion of therapy were defined as having sustained virological response. Relapse was defined as a case in which HCV RNA had been undetectable at the end-of-treatment, but detectable during the 24-week follow-up after the treatment.

Drug Exposure

The amounts of Peg-IFN alpha-2b and ribavirin actually taken by each patient during the treatment period were evaluated by reviewing the medical records. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline; Peg-IFN alpha-2b expressed as µg/kg/week and ribavirin as mg/kg/day.

Data Collection

The medical records were retrospectively reviewed and the factors necessary for this examination were extracted: age, sex, body weight, body mass index (BMI), basic laboratory assessments, liver histology, quantitative and qualitative HCV RNA, dose of Peg-IFN alpha-2b and ribavirin received at each administration, and the response to treatment.

Statistical Analysis

Continuous variables are reported as the mean with standard deviation (SD) or median level, while categorical variables are shown as the count and proportion. In univariate analysis, the Mann-Whitney *U*-test (between two groups) or Kruskal-Wallis test (among more than three groups) was used to analyze continuous variables, while chi-squared and Fisher's exact tests were used for analysis of categorical data. For all tests, two-sided *P* values were calculated, and the results were considered statistically significant if *P* < 0.05. Variables that achieved statistical significance (*P* < 0.05) or marginal significance (*P* < 0.10) on univariate analysis were subjected to multivariate logistic regression analysis. Stepwise and multivariate logistic regression models were used to explore the independent factors that could be used to predict a virological response. Statistical analysis was performed using the SPSS program for Windows, version 15.0J (SPSS, Chicago, IL).

RESULTS

Baseline Characteristics of Study Groups

The total study population was predominately male (55.6%), with a mean age of 56.2 years. The baseline characteristics of all patients and the four study groups according to core aa substitution patterns are shown in Table I. Mean age of patients in the double-mutant group was higher than the other three groups (*P* = 0.003). More patients in the double-wild group had

TABLE I. Baseline Demographic and Viral Characteristics of Patients

Characteristic	Total (n = 187)	Double-wild (n = 92)	70-Mutant (n = 42)	91-Mutant (n = 31)	Double-mutant (n = 22)	P value ^a
Age (years)	56.2 ± 9.3	55.7 ± 9.2	57.0 ± 9.8	52.4 ± 9.9	61.8 ± 4.7	0.003
Sex (male/female)	104/83	51/41	26/16	18/13	9/13	0.444
Body weight (kg)	60.9 ± 11.6	60.9 ± 11.7	62.2 ± 11.7	62.5 ± 13.2	56.0 ± 7.5	0.193
Body mass index (kg/m ²)	22.8 ± 3.1	22.8 ± 3.0	22.8 ± 3.1	23.1 ± 3.6	22.1 ± 2.4	0.627
Past IFN therapy (naïve/experienced)	118/69	45/47	34/8	20/11	19/3	<0.001
HCV RNA (×10 ⁸ IU/ml) ^b	1,700	2,100	1,400	1,500	1,230	0.122
Fibrosis (0–2/3–4) ^c	105/29	56/11	22/6	14/7	13/5	0.366
Activity (0–1/2–3) ^d	83/50	42/24	18/10	11/10	12/6	0.771
White blood cell (×10 ⁶ /l)	4,980 ± 1,520	4,990 ± 1,420	5,180 ± 1,760	4,890 ± 1,430	4,660 ± 1,560	0.795
Red blood cell (×10 ¹² /l)	4.34 ± 0.46	4.33 ± 0.46	4.41 ± 0.52	4.39 ± 0.42	4.18 ± 0.32	0.145
Hemoglobin (g/dl)	13.9 ± 1.4	13.9 ± 1.4	14.0 ± 1.7	14.2 ± 1.4	13.5 ± 1.1	0.253
Platelet (×10 ⁹ /l)	161 ± 54	167 ± 49	165 ± 65	154 ± 60	138 ± 30	0.067
ALT (IU/l)	74 ± 61	73 ± 67	79 ± 56	81 ± 64	57 ± 37	0.263
γ-GTP (IU/l)	62 ± 74	47 ± 54	81 ± 89	70 ± 93	78 ± 78	0.032

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase.

^aP value for comparison among double-wild, 70-mutant, 91-mutant, and double-mutant.

^bValues expressed as median.

^cData for 53 patients are missing.

^dData for 54 patients are missing.

been treated previously for HCV infection ($P < 0.001$). Patients in the double-wild group had significantly lower gamma-glutamyl transpeptidase (γ-GTP) levels ($P = 0.032$).

Progress of Patients

The progress of patients in this study is shown in Figure 1. Of the 187 patients, 183 completed 4 weeks of treatment. Among them, 133 were assessed based on HCV RNA dynamics between baseline and week 4.

Those completing 12 weeks of treatment totaled 181, of which 154 were assessed for HCV RNA dynamics between baseline and week 12. Those completing 24 weeks of treatment totaled 153, and all were assessed for HCV RNA quantitatively or qualitatively at week 24. Those completing 48 weeks of treatment totaled 114. These 114 patients and the 55 patients who had discontinued treatment because of treatment failure entered a follow-up period. Among these 169 patients, 164 completed 24 weeks follow-up and the sustained virological response (SVR) rate

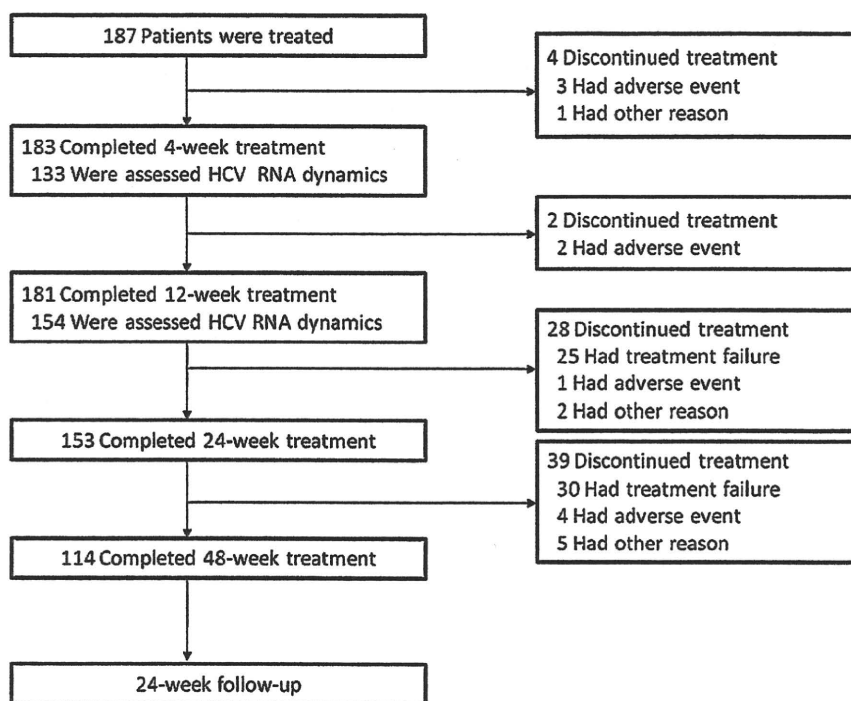


Fig. 1. Treatment and follow-up of the study patients. Treatment was discontinued for patients with <2 log decrease from the baseline HCV RNA level at week 12 or detectable HCV RNA at week 24.

TABLE II. Multivariate Analysis for Factors Associated With <1 log Decrease in HCV RNA Level at Week 4, <2 log Decrease at Week 12, Detectable HCV RNA at Week 24, and Relapse After Treatment

Factor	Category	Odds Ratio	95% CI	P value
HCV RNA <1 log decrease at week 4				
White blood cells ($\times 10^6/l$)	<5,000/5,000 \leq	—	—	NS
γ -GTP (IU/l)	<40/40 \leq	—	—	NS
Peg-IFN dose (μ g/kg/week)	By 0.1 μ g/kg/week	0.80	0.67–0.97	0.020
Core aa 70	Wild/mutant	1/2.80	1.16–6.75	0.022
HCV RNA <2 log decrease at week 12				
γ -GTP (IU/l)	<40/40 \leq	—	—	NS
Peg-IFN dose (μ g/kg/week)	By 0.1 μ g/kg/week	—	—	NS
Core aa 70	Wild/mutant	1/2.72	1.09–6.78	0.032
Detectable HCV RNA at week 24				
Platelet ($\times 10^9/l$)	<150/150 \leq	—	—	NS
γ -GTP (IU/l)	<40/40 \leq	1/2.46	1.02–5.95	0.045
Core aa 91	Wild/mutant	1/4.11	1.73–9.78	0.001
Relapse after treatment				
Ribavirin dose (mg/kg/day)	By 1 mg/kg/day	0.77	0.60–0.98	0.036
Virological response	Complete early virological response/late virological response	1/23.69	5.44–103.08	<0.001

CI, confidence interval; NS, not significant difference; γ -GTP, gamma-glutamyl transpeptidase; Peg-IFN, pegylated interferon; aa, amino acid.

was 48.2% (79/164), based on per-protocol set. Among the 106 patients who had an end-of-treatment response and completed follow-up, 27 showed relapse during the follow-up period; the relapse rate was 25.5% (27/106).

IMPACT OF CORE-RELAPSE AFTER TREATMENT (TABLE II)

Impact of core aa substitutions on <1 log viral decrease rate at week 4, <2 log at week 12, detectable HCV RNA at week 24, and virological relapse after treatment (Table II).

The impact of core aa substitutions on <1 log viral decrease rate at week 4, <2 log at week 12, detectable HCV RNA at week 24, and virological relapse after treatment (Table II).

The impact of the core aa substitutions on <1 log viral decrease at week 4, which is a predictor of non-sustained virological response; fewer than 5% of patients without 1 log decrease at week 4 had an sustained virological response [McHutchison et al., 2009] was examined. Among the 133 patients who completed 4 weeks of treatment, 31 failed to show a ≥ 1 log decrease of HCV RNA level at week 4. Univariate analysis for factors associated with <1 log decrease of HCV RNA level at week 4 was performed on the following variables: age, sex, body weight, BMI, history of past IFN therapy, baseline HCV RNA level, histological fibrosis and activity, white blood cell count, red blood cell count, hemoglobin level, platelet count, alanine aminotransferase (ALT) level, γ -GTP level, dose exposure of Peg-IFN and ribavirin, and aa substitutions in the HCV core protein. The results indicated that pretreatment white blood cell count, γ -GTP level, the mean dose of Peg-IFN during the first 4 weeks of treatment and single-spot substitution in the HCV RNA core position at aa 70 contributed to a <1 log decrease of HCV RNA level at week 4. Analysis of

these factors by multivariate logistic regression analysis showed that substitution of aa 70 (odds ratio (OR) 2.80, 95% confidence interval (CI) 1.16–6.75, $P = 0.022$) as well as the mean dose of Peg-IFN (OR 0.80, 95% CI 0.67–0.97, $P = 0.020$) was independently associated with viral decline (<1 log) at week 4.

Next, the impact of the core aa substitutions on <2 log viral decrease rate at week 12, which is presently considered to be the most reliable predictor of non-sustained virological response [Fried et al., 2002; Davis et al., 2003] was examined. Among the 154 patients who completed 12 weeks of treatment, 25 failed to show a ≥ 2 log decrease of HCV RNA level at week 12. Univariate analysis was performed on the same factors in the preceding examination. As a result, pretreatment γ -GTP level, the mean dose of Peg-IFN during the first 12 weeks of treatment and single-spot substitution in the HCV RNA core position at aa 70 contributed to a <2 log decrease of the HCV RNA level. These factors were then analyzed by multivariate logistic regression analysis; only substitution of aa 70 (OR 2.72, 95% CI 1.09–6.78, $P = 0.032$) was found to be independently associated with an insufficient virological response (<2 log HCV RNA decrease from baseline level) at week 12.

The impact of the core aa substitutions on detectable HCV RNA at week 24, which is another non-sustained virological response predictor [Davis et al., 2003] was also examined. Among 153 patients who completed 24 weeks of treatment, 30 still had detectable HCV RNA at week 24. Univariate analysis revealed that pretreatment platelet count, γ -GTP level, and single-spot substitution in the HCV RNA core position at aa 91 contributed to the HCV RNA remaining positive. Multivariate logistic regression analysis, using these factors, indicated that substitution of aa 91 (OR 4.11, 95% CI 1.73–9.78, $P = 0.001$) as well as γ -GTP level (>40 IU/l) (OR 2.46, 95% CI 1.02–5.95, $P = 0.045$) was

independently associated with detectable HCV RNA at week 24.

Next, the factors associated with virological relapse after the treatment was examined. Univariate analysis was performed on the virological response (complete early virological response or late virological response) in addition to the factors in the preceding examination, revealing the mean dose of ribavirin during the full treatment period and a late virological response, but not aa substitutions (single-spot substitution in the HCV RNA core position at aa 70, $P = 0.467$; aa 91, $P = 0.776$).

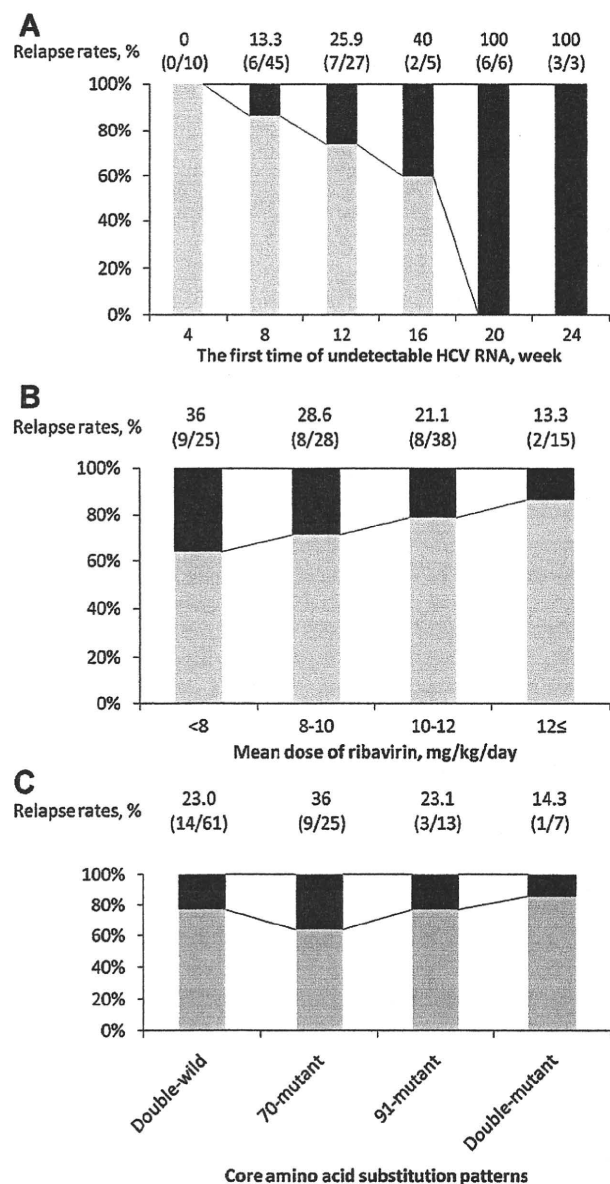


Fig. 2. Relapse rates according to the timing of HCV RNA disappearance (A), mean ribavirin dose (B), and core amino acid substitution patterns (C) in patients who had end-of-treatment response and completed 24-week follow-up. Relapse rates are shown as percentages and the number of patients with relapse in relation to the total number of patients examined is shown at the top of each column. Gray bar, sustained virological response; black bar, relapse.

J. Med. Virol. DOI 10.1002/jmv

These factors were analyzed by multivariate logistic regression analysis. This analysis revealed that the mean ribavirin dose (OR 0.77, 95% CI 0.60–0.98, $P = 0.036$) and a late virological response (OR 23.69, 95% CI 5.44–103.08, $P < 0.001$) were independently associated with relapse.

Relapse Rates According to the Timing of HCV RNA Disappearance, Ribavirin Dose, and Core aa Substitution Patterns

The relapse rates were indicated according to the time to the first non-detection of HCV RNA, mean ribavirin dose and core aa substitution patterns (Fig. 2). The relapse rate was 0% (0/10) in patients with undetectable HCV RNA during 1–4 weeks, and increased 13.3% (6/45) during 5–8 weeks, 25.9% (7/27) during 9–12 weeks, 40% (2/5) during 13–16 weeks, 100% (6/6) during 17–20 weeks, and 100% (3/3) during 21–24 weeks (Fig. 2A). Similarly, the relapse rates increased as the mean ribavirin dose decreased; 13.3% (2/15) in patients receiving ≥ 12 mg/kg/day of ribavirin, 21.1% (8/38) at 10–12 mg/kg/day, 28.6% (8/28) at 8–12 mg/kg/day, and 36% (9/25) at < 8 mg/kg/day (Fig. 2B). On the other hand, the relapse rates were similar among the four core aa substitution patterns; 23.0% (14/61) in patients in the double-wild group, 36% (9/25) in 70-mutant group, 23.1% (3/13) in 91-mutant group, and 14.3% (1/7) in double-mutant group (Fig. 2C). In the subgroup of patients receiving < 10 mg/kg/day of ribavirin, no significant difference of the relapse rates was observed between double-wild group and 70-mutant and/or 91-mutant group (31.3% (10/32) in double-wild group vs. 33.3% (7/21) in 70-mutant and/or 91-mutant group), and also in the patients receiving ≥ 10 mg/kg/day of ribavirin (13.8% (4/29) in double-wild group vs. 25% (6/24) in 70-mutant and/or 91-mutant group) (Fig. 3). Among patients with complete early virological response, the relapse rates were also similar between double-wild group and 70-mutant and/or 91-mutant group (13.7% (7/51) in double-wild vs. 18.4% (7/38) in 70-mutant and/or 91-mutant group). The impact of core aa substitutions on relapse rates in patients with late virological response could not be assessed because of the small number of patients.

DISCUSSION

Kobayashi et al. [2010] investigated the clinical and virological factors influencing these core aa substitutions in patients infected with HCV genotype 1 who had not received antiviral therapy, and found that HCV variants with wild type of core aa 70 and 91 significantly decreased with age, while those with the mutant type of core aa 70 and/or 91 significantly increased with age. Furthermore, they demonstrated that the proportion of patients with the mutant type of core aa 70 HCV variant significantly increased with an elevated γ -GTP level and a decrease in platelet counts. In this study, the significant differences of baseline demographics between patient groups according to core aa substitution pat-

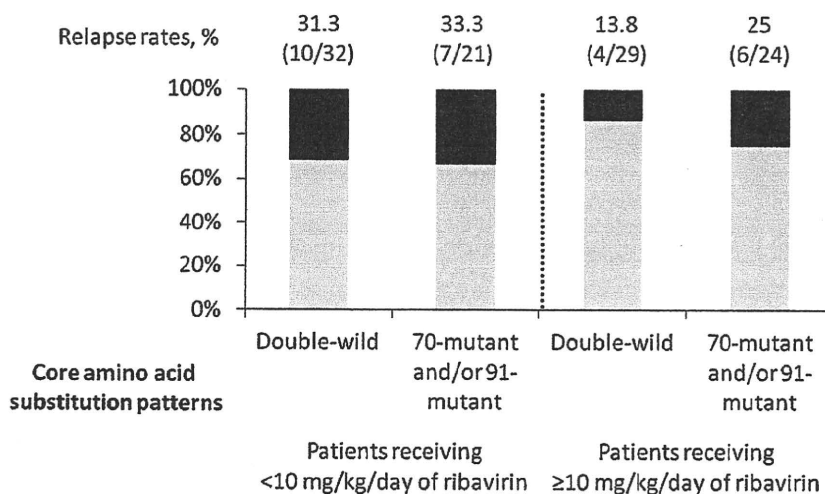


Fig. 3. Relapse rates according to core amino acid substitution patterns in patients receiving <10 mg/kg/day and receiving ≥ 10 mg/kg/day of ribavirin. Relapse rates are shown as percentages and the number of patients with relapse in relation to the total number of patients examined is shown at the top of each column. Gray bar, sustained virological response; black bar, relapse.

terns were similarly found in age, platelet count, and γ -GTP level. Accordingly, this study cohort had no specific bias and seems to reflect the natural background of the patients according to the HCV variance. In this study, the impact of HCV core aa substitutions on the virological response were evaluated by multivariate analysis, in order to resolve the bias of patient background factors among the groups classified according to the core aa substitution patterns. Recently, Abe et al. [2010] reported that the human genotype of the rs8099917 SNP at the IL28B locus was associated with lower γ -GTP level and viral wild type of core aa 70 and 91. Possibly these differences of IL28B genotype may influence the difference of patient background factors. Further studies are needed to clarify the relationship between human genetic variation and HCV core amino acid substitutions.

The HCV core protein has been reported to have an effect on a variety of cellular functions [Lai and Ware, 2000; Joo et al., 2005; Ariumi et al., 2007; Waris et al., 2007; Osna et al., 2008]. Currently, aa substitutions in the HCV core region has been thought to be related with outcome of antiviral therapy [Akuta et al., 2005; Donlin et al., 2007] and also the development of hepatocellular carcinoma [Akuta et al., 2007a; Hu et al., 2009]. Importance of core aa substitutions, especially at aa 70 and 91, comes to be recognized, and the new method to detect these substitutions easily has been proposed [Nakamoto et al., 2009]. As for the mechanism of antiviral activity on core aa substitutions, Ikeda et al. [2010] showed that core aa substitutions were not associated with intracellular antiviral response to IFN-alpha by in vitro analysis. The mechanism of antiviral activity and hepatocarcinogenesis on core aa substitutions has not been elucidated enough, so far. Further in vitro studies will be needed to clarify this.

Previous studies showed that patients with substitution of core aa 70 often had slow or no decrease in HCV RNA levels during the early phase of IFN-alpha treatment [Akuta et al., 2005, 2007b,c; Donlin et al., 2007]. Consistent with these reports, multivariate analysis in this study revealed that substitution of core aa 70 could be independently associated with insufficient viral decline during the first 12 weeks after the treatment (decline of <1 log from baseline at week 4, <2 log at week 12). This suggests that patients with substitution of core aa 70 are likely to fail to have a sustained virological response. On the other hand, dose exposure of Peg-IFN during the first 4 weeks of treatment was also independently linked to a minimal decline in HCV RNA (<1 log) at week 4 in this study. This suggests that maintaining the dose of Peg-IFN as high as possible until the disappearance of HCV RNA can help avoid treatment failure [McHutchison et al., 2002; Oze et al., 2009], especially in patients with substitution of core aa 70. On the other hand, substitution of core aa 91 was independently associated with detectable HCV RNA at week 24. This suggests that patients with substitution of core aa 91 are likely to achieve non-sustained virological response even if they had a ≥ 2 log decline in the HCV RNA level at week 12. The reason for the difference of the impact on virological response is not yet clear.

Multivariate logistic regression analysis also showed that the dose exposure of ribavirin during the full treatment period and having late virological response were independently associated with relapse. As for ribavirin exposure, it has been previously demonstrated that the relapse rate among patients responding to the treatment showed a decline in relation to the increase in the dose of ribavirin [Hiramatsu et al., 2009]. In this study, relapse rates were also decreased from 36% to 13.3% with increasing dose exposure of ribavirin among patients with end-of-treatment response. These results

confirm that maintaining a sufficient dose of ribavirin during the full treatment period could reduce the possibility of relapse, and that an extended duration of therapy for patients with late virological response could increase the chance of achieving sustained virological response, regardless of core aa substitution patterns [Berg et al., 2006; Pearlman et al., 2007; Ferenci et al., 2010].

In this study, the COBAS Amplicor HCV Test v2.0, with a lower limit of detection of 50 IU/ml, was used to assess the serum HCV RNA. Recently, real-time PCR-based HCV RNA assays with a higher sensitivity, COBAS TaqMan HCV assay (Chugai-Roche Diagnostics), with a lower limit of detection of 15 IU/ml, have been introduced. Sarrazin et al. [2010] compared virological response rates that were originally tested by COBAS Amplicor assay with those retested by COBAS TaqMan assay, using the same cohort. Among genotype 1 patients, complete early virological response and sustained virological response rates were similar when virological responses were defined as <50 IU/ml by Amplicor assay (77% and 87%) and <15 IU/ml by TaqMan assay (76% and 88%). Therefore, measuring HCV RNA by the Amplicor assay in this study would have little effects on the results.

In conclusion, the results have demonstrated that substitution of core aa 70 could be independently associated with an insufficient decline in HCV RNA level during first 12 weeks, and substitution of core aa 91 was independently associated with detectable HCV RNA at week 24, all of which were considered to be important negative predictors of attaining sustained virological response in patients with HCV genotype 1 treated with Peg-IFN plus ribavirin. On the other hand, only dose exposure of ribavirin and no complete early virological response was independent predictors of virological relapse among patients with end-of-treatment response, not substitution of core aa 70 or 91. The aa substitution patterns of the HCV core protein can be an important pretreatment predictor for non-response in patients with HCV genotype 1 treated with Peg-IFN plus ribavirin, but not for relapse after the completion of therapy.

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Original Article

α -Galactosylceramide activates antitumor immunity against liver tumor

Tomohide Tatsumi,¹ Tetsuo Takehara,¹ Takuya Miyagi,¹ Tsukasa Sugiyama,¹ Hiroshi Aketa,¹ Akira Sasakawa,¹ Tatsuya Kanto,¹ Naoki Hiramatsu¹ and Norio Hayashi^{1,2}

¹Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Osaka and ²Kansai-Rosai Hospital, Amagasaki, Hyogo, Japan

Aim: α -Galactosylceramide (α -GalCer) has been attracting attention as a novel approach to treat metastatic liver cancer. We investigated the detailed process of activating liver dendritic cells (DC) and immune cells after α -GalCer treatment in the mouse liver tumor model.

Methods: BALB/c mice bearing CMS4 liver tumor (p53 peptide-expressing tumor) were treated by α -GalCer. We evaluated the activation of liver DC and immune cells after α -GalCer treatment. Interferon (IFN)- γ enzyme-linked immunosorbent spot (ELISPOT) assay was performed to detect p53 peptide-specific cytotoxic T lymphocytes (CTL). To assess the impact of systemic acquired immunity by α -GalCer treatment, 28 days after liver tumor treatment, CMS4 cells or Colon26 cells were re-challenged s.c.

Results: The liver weights of α -GalCer-treated mice were significantly lighter than those of vehicle-treated mice. Depletion experiments revealed that natural killer (NK) cells were essential for the antitumor effect of α -GalCer. α -GalCer treatment

significantly increased the population of DC and NK cells in the liver. The expressions of co-stimulatory molecules on liver DC significantly increased with the peak at 1 day after α -GalCer administration. IFN- γ ELISPOT assay demonstrated that p53 peptide-specific CTL was generated efficiently in α -GalCer-treated mice. ⁵¹Cr-release assay revealed that CD8⁺, not CD4⁺, CTL against CMS4 cells were generated in α -GalCer-treated mice. The mice that had been protected from CMS4 liver tumor by α -GalCer injection became resistant against s.c. CMS4 re-challenge, but not against Colon26 re-challenge.

Conclusion: These results demonstrated the therapeutic potential of α -GalCer against liver cancer through activating liver DC and immune cells in the liver.

Key words: α -galactosylceramide, cytotoxic T lymphocytes, dendritic cells, liver, natural killer cells.

INTRODUCTION

THE GLYCOLIPID ANTIGEN α -galactosylceramide (α -GalCer) induced the activation of natural killer (NK) T cells in a CD1d-dependent manner.^{1,2} Recently, α -GalCer has been attracting attention as a novel anti-tumor therapy. In *in vivo* animal studies, systemic administration of α -GalCer showed antitumor effects against various tumors (including melanoma, sarcoma, colon carcinoma and lymphoma) in hepatic and lung

metastasis models.^{3,4} Based on the promising results of preclinical studies demonstrating the antitumor potential of α -GalCer, several phase 1 clinical studies on cancer immunotherapy by the i.v. administration of α -GalCer has been carried out, but clinical responses of α -GalCer has been limited.⁵ No clinical trial against liver cancer has been reported to date. In view of future α -GalCer treatment of liver cancer, the precise mechanism of activation of innate and acquired immunity in the liver by α -GalCer should be examined. However, these are still not fully understood.

The liver contains both a large compartment of innate immune cells (NK cells and NKT cells) and acquired immune cells (T cells).^{6,7} Dendritic cells (DC) can induce the generation of both antigen-specific cytotoxic T lymphocytes (CTL) and T-helper (Th) cells.^{8,9} Recent research of DC biology revealed that DC also contribute

Correspondence: Dr Tomohide Tatsumi, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Email: tatsumit@gh.med.osaka-u.ac.jp

Received 23 July 2010; revision 8 October 2010; accepted 18 October 2010.

to innate immune responses by activating NK cells^{10–14} and NKT cells^{1,15,16} through interleukin (IL)-12 secretion and direct cellular interaction. Thus, DC can be expected to play critical roles in activating abundant T cells, NK cells and NKT cells in the liver after α -GalCer administration. We previously reported that administration of α -GalCer stimulated hepatic NKT cells and led to activation of hepatic NK cells,⁴ and that α -GalCer activated liver DC have higher ability to generate acquired immunity.¹⁷ However, the detailed process of activating liver DC and immune cells after α -GalCer treatment should be elucidated.

In this study, we evaluated the liver DC activation and antitumor effect mediated by both innate and acquired immunity against mouse liver tumor after administration of α -GalCer. Administration of α -GalCer induced early activation of liver DC with upregulation of antigen presenting-related molecules and resulted in complete rejection of local liver tumor by NK cells. Followed by early rejection of liver tumor, tumor antigen-specific CTL were generated and complete rejection in s.c. re-challenge of tumor cells was observed. Sequential activation of liver DC, innate and acquired immune cells in the liver may be an attractive strategy for treatment of local and distant tumor of liver cancer.

METHODS

Mice

SIX-TO-EIGHT-WEEK-OLD female BALB/c mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan). All mice were maintained in micro-isolator cages. Procedures were performed according to approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

Cell lines and culture

CMS4 sarcomas (H-2^d) express mutated p53 and present the wild-type p53^{232–240} epitope recognized by H-2K^d-restricted CTL.¹⁸ Colon26, a mouse colon adenocarcinoma cell line, was kindly provided by Dr Takashi Tsuruo (Institute of Molecular and Cellular Bioscience, University of Tokyo, Tokyo, Japan). These cell lines were maintained as previously described.^{19,20}

α -GalCer

α -Galactosylceramide was purchased from Funakoshi (Tokyo, Japan) and prepared as previously described by Kawano *et al.*¹

Animal experiments

BALB/c mice were injected in the liver with 5×10^5 CMS4 cells on day 0. On day 1, BALB/c mice were injected i.p. with α -GalCer (2 μ g/100 μ L) or 100 μ L of vehicle. Two weeks after the tumor injection, the livers of treated mice were removed, and the weight was measured to examine intrahepatic tumor growth. To assess the impact of systemic immunity from i.p. injection of α -GalCer, mice were injected intrahepatically with 5×10^5 CMS4 cells on day 0 and i.p. treated with α -GalCer on day 1. On day 28 after α -GalCer treatment, 1×10^6 CMS4 cells or Colon26 cells were injected in the right flank of treated mice. To confirm the involvement of CD8⁺ T cells in this antitumor effect, we depleted CD8⁺ T cells before re-challenge of CMS4 cells in α -GalCer-treated mice. On day 1 and day 3 of re-challenge of CMS4 cells, anti-CD8 antibody (53–6.72 hybridoma, ATCC) was injected i.p. as previously described.¹⁹ Tumor size was assessed every 3 or 4 days and recorded in mm² by determining the product of the largest perpendicular diameters measured by vernier calipers. Data are reported as the average tumor area \pm standard deviation (SD).

NK cell depletion experiments

For NK cell depletion, mice were injected with anti-asialo GM-1 (ASGM1) antibody (Ab) (Wako, Osaka, Japan) on day 1, 5, 10, 15 and 20 after tumor inoculation. The efficiency of NK cell depletion was validated by flow cytometry analysis of splenocytes using phycoerythrin (PE)-conjugated anti-DX5 monoclonal antibody (BD-Pharmingen, San Diego, CA, USA). In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

Preparation of hepatic mononuclear cells and liver DC

Hepatic mononuclear cells (MNC) were prepared as previously described.⁴ CD11c⁺ dendritic cells were isolated from hepatic MNC by magnetic cell sorting using MACS (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's protocol.

Flow cytometry

For phenotypic analysis of liver DC, NK cells, NKT cells and CD4⁺ and CD8⁺ T cells, PE- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against mouse cell surface molecules (CD11c, CD40, CD80, CD86, T-cell receptor [TCR]- β , CD49b [DX5], CD4, CD8, CD69 [all from BD-Pharmingen], major his-

to compatibility complex [MHC] class II [Miltenyi Biotec]), and appropriate isotype controls were used, and flow cytometric analysis was performed using a FACScan (Becton Dickinson, San Jose, CA, USA) flow cytometer. The results of flow cytometric analysis are reported in positive cell rates (%) determined by using isotype controls. DC were identified as CD11c⁺/MHC class II⁺ cells. NK cells were identified as DX5⁺/TCR-β⁻ cells, NKT cells as DX5⁺/TCR-β⁺ cells, as previously described.²⁰

IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays for p53₂₃₂₋₂₄₀ peptide-reactive CD8⁺ T-cell responses

Bone marrow derived DC (BMDC) were generated from normal mice as previously described²¹ and were used as peptide-presenting cells. On day 14 after treatment of α-GalCer or vehicle, CD8⁺ T cells were isolated from the spleen cells of treated mice by using magnetic beads (Miltenyi Biotec). We used a mouse IFN-γ ELISPOT kit (R&D systems, Minneapolis, MN, USA) to detect the p53₂₃₂₋₂₄₀ peptide-specific CD8⁺ T-cell responses. To evaluate the p53₂₃₂₋₂₄₀ peptide, strongly expressing on CMS4 cells,¹⁸ specific CTL induction, isolated CD8⁺ T cells (1 × 10⁵ cells/well) were co-cultured with syngeneic BMDC (2 × 10⁴ cells/well) pulsed with p53₂₃₂₋₂₄₀ peptide in an ELISPOT culture plate. BMDC cells without p53₂₃₂₋₂₄₀ peptide served as the negative control, and this value was subtracted from all experimental determinations to establish p53₂₃₂₋₂₄₀ peptide-specific spot numbers. The data are represented as mean IFN-γ spots ± SD per 100 000 CD8⁺ T cells analyzed.

Cytolytic assays

Splenocytes from α-GalCer or vehicle-treated mice were harvested 14 days after tumor inoculation. After 5 days of *in vitro* stimulation with mitomycin-C (Kyowa Hakko, Tokyo, Japan)-treated CMS4 cells, lymphocytes were analyzed for their ability to kill CMS4 cells in 4-h ⁵¹Cr-release assays (effector cells/target cells ratio, 60:1), as previously described.²¹ CD4⁺ or CD8⁺ T cells were depleted by magnetic sorting using CD4 or CD8 microbeads (Miltenyi Biotec), respectively.

Statistical analyses

All experiments with three or more groups in which treatment was applied with a completely random design were first analyzed by a one-way factorial ANOVA. If the resulting *P*-value was less than 0.05, specific pairwise

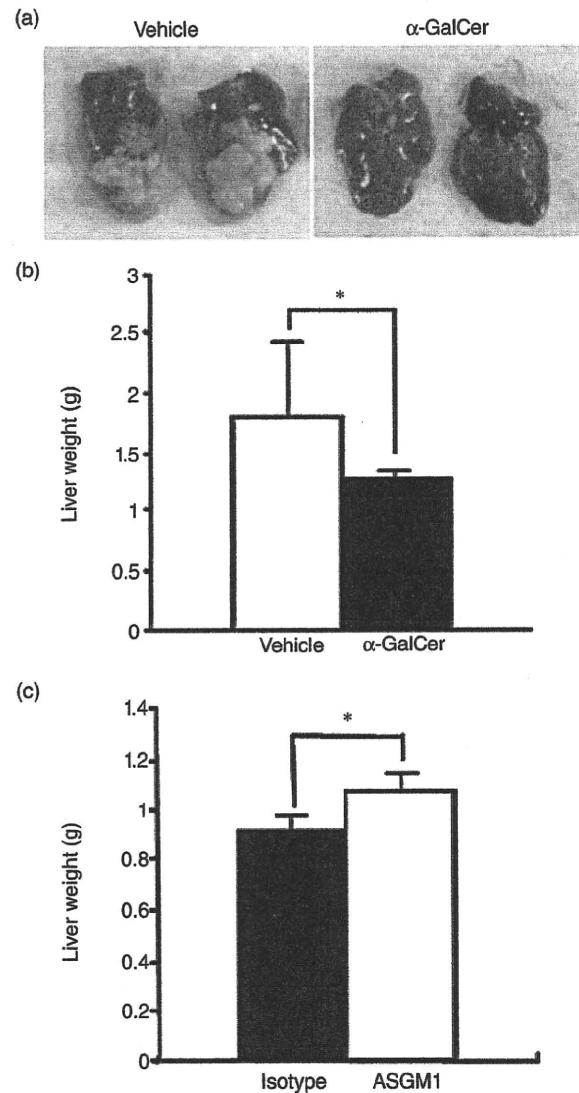


Figure 1 Therapeutic effectiveness of i.p. injection of α-galactosylceramide (α-GalCer) in CMS4 liver tumor model. BALB/c mice were injected intra-hepatically with 5 × 10⁵ CMS4 cells. One day later, BALB/c mice were treated with i.p. injection of α-GalCer or vehicle (all treatment groups, *n* = 7). Two weeks after the CMS4 tumor injection, the livers of treated mice were removed. (a) Representative liver macroscopic view of each group. (b) Comparison of liver weight of each group. **P* < 0.05. (c) To prove that the therapeutic benefit of α-GalCer treatment in the CMS4 liver tumor model is natural killer (NK)-cell dependent, *in vivo* depletion of NK cells was performed (as described in Methods, ASGM1). In control mice, isotype antibody (Ab) was injected i.p. (isotype). Both mice were treated by α-GalCer. Ab-mediated *in situ* depletion of NK cells markedly reduces the therapeutic efficacy of α-GalCer treatment (all treatment groups *n* = 5). **P* < 0.05.

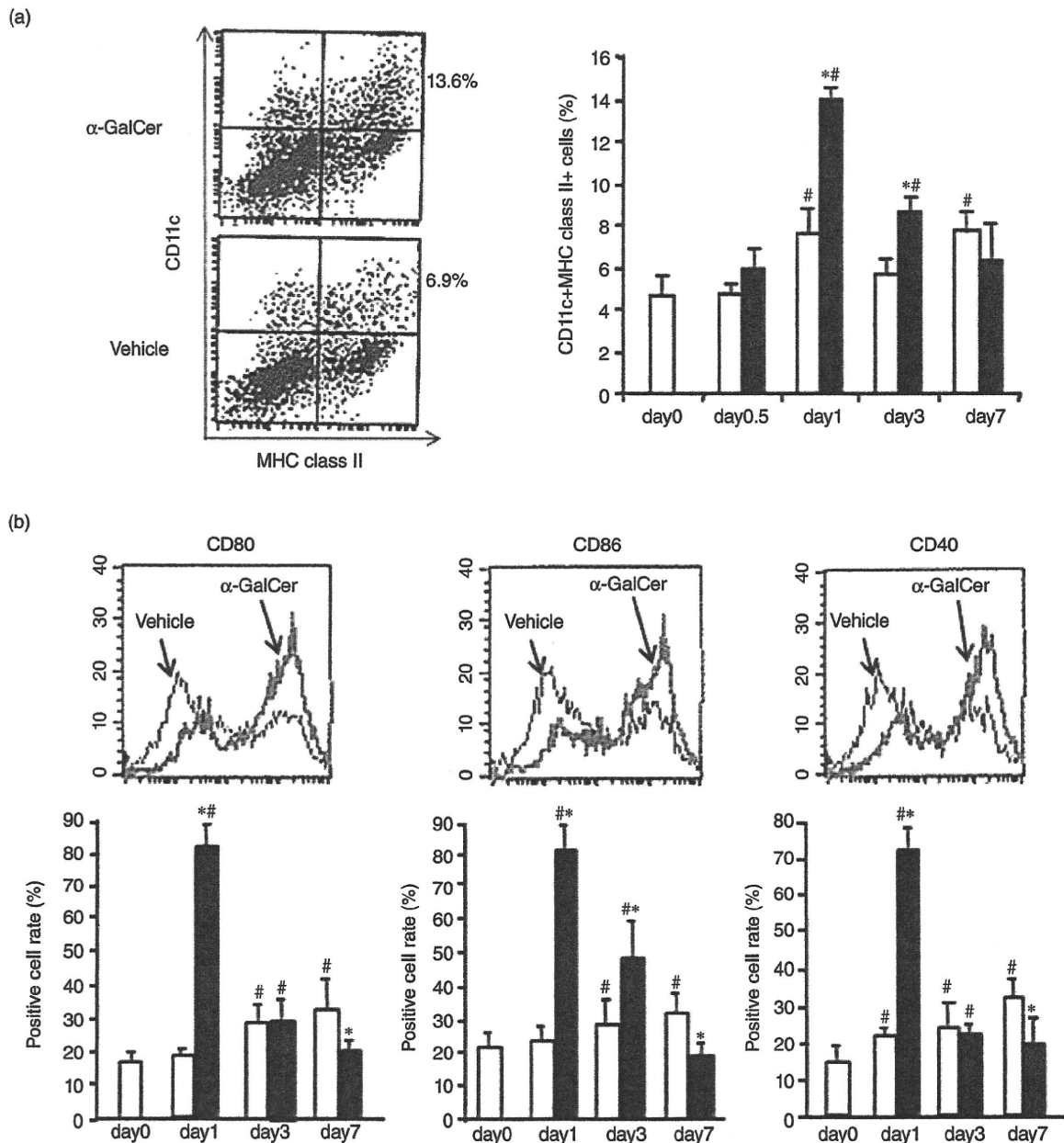


Figure 2 α -Galactosylceramide (α -GalCer) treatment increased dendritic cell (DC) population in the liver mononuclear cells (MNC) and activated DC functions. (a) BALB/c mice were treated with α -GalCer or vehicle. Hepatic MNC were prepared on days 0, 0.5, 1, 3 and 7. DC (CD11c⁺ major histocompatibility complex [MHC] class II⁺ cells) population was evaluated by flow cytometry. White bar, vehicle-treated mice; black bar, α -GalCer-treated mice. Representative dot plots of liver DC (CD11c⁺ MHC class II⁺ cells) at day 1 after α -GalCer or vehicle administration are shown in the left panels. The calculated percentages of liver DC are shown in the right. (b) BALB/c mice were treated with α -GalCer or vehicle. Hepatic MNC were prepared on day 0, 1, 3 and 7 and DC were isolated from liver MNC by a magnetic cell sorting system. For phenotypic analysis, liver DC were stained with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (CD11c, CD40, CD80, CD86), and the expressions of these molecules were analyzed by flow cytometry. White bar, vehicle-treated mice; black bar, α -GalCer-treated mice. * $P < 0.05$ vs vehicle-treated mice, # $P < 0.05$ vs non-treated mice. Representative histograms of the expressions of CD80, CD86 and CD40 on liver DC at day 1 after α -GalCer or vehicle administration are shown in the upper panels.

contrasts were tested with a Student's *t*-test with Welch's correction for unequal variance as needed.

RESULTS

α -GalCer administration inhibited CMS4 liver tumor mediated by NK cells

WE INITIALLY EXAMINED whether α -GalCer administration could induce antitumor effect against CMS4 liver tumor. As shown in Figure 1(a), no tumors were observed in the livers of α -GalCer-treated mice whereas large tumors were observed in the livers of vehicle-treated mice. The liver weight of the α -GalCer treatment group was significantly lighter than that of the vehicle treatment group (Fig. 1b). Depletion of NK cells significantly inhibited the antitumor efficacy of α -GalCer treatment (Fig. 1c), whereas depletion of neither CD4⁺ nor CD8⁺ T cells was inhibited (data not shown). These results suggested that administration of α -GalCer was therapeutic against CMS4 liver tumor and NK cells were the main effector cells in this antitumor immunity.

Administration of α -GalCer increased DC population in the liver MNC and activated DC functions

We investigated the population changes of DC in the liver MNC after α -GalCer or vehicle treatment. On day 1 after α -GalCer administration, liver DC proportion in α -GalCer-treated mice was higher than that in vehicle-treated mice (Fig. 2a). Liver DC proportion increased with the peak at 1 day after α -GalCer administration and the liver DC proportion at 7 days decreased to the same level with that from non-treated mice (Fig. 2a). In contrast, liver DC proportion in vehicle-treated mice exhibited weaker change than those in α -GalCer-treated mice (Fig. 2a). The liver DC number also exhibited increase at the peak of 1 day after α -GalCer administration whereas that from vehicle-treated mice exhibited no change (data not shown). We examined the CD40, CD80 and CD86 expressions of liver DC after administration of α -GalCer, which is an indicator of DC activation. On

day 1 after α -GalCer administration, CD40, CD80 and CD86 on liver DC from α -GalCer-treated mice expressed more strongly than those from vehicle-treated mice (Fig. 2b). The expressions of all these molecules on liver DC increased with the peak at 1 day after α -GalCer administration and the expression levels of these molecules at 7 days decreased to the same levels on liver DC from non-treated mice (Fig. 2b). In contrast, the expressions of these molecules on liver DC exhibited weaker change in vehicle-treated mice.

Activated NK cells composed the major subpopulation of hepatic MNC that increased after α -GalCer treatment

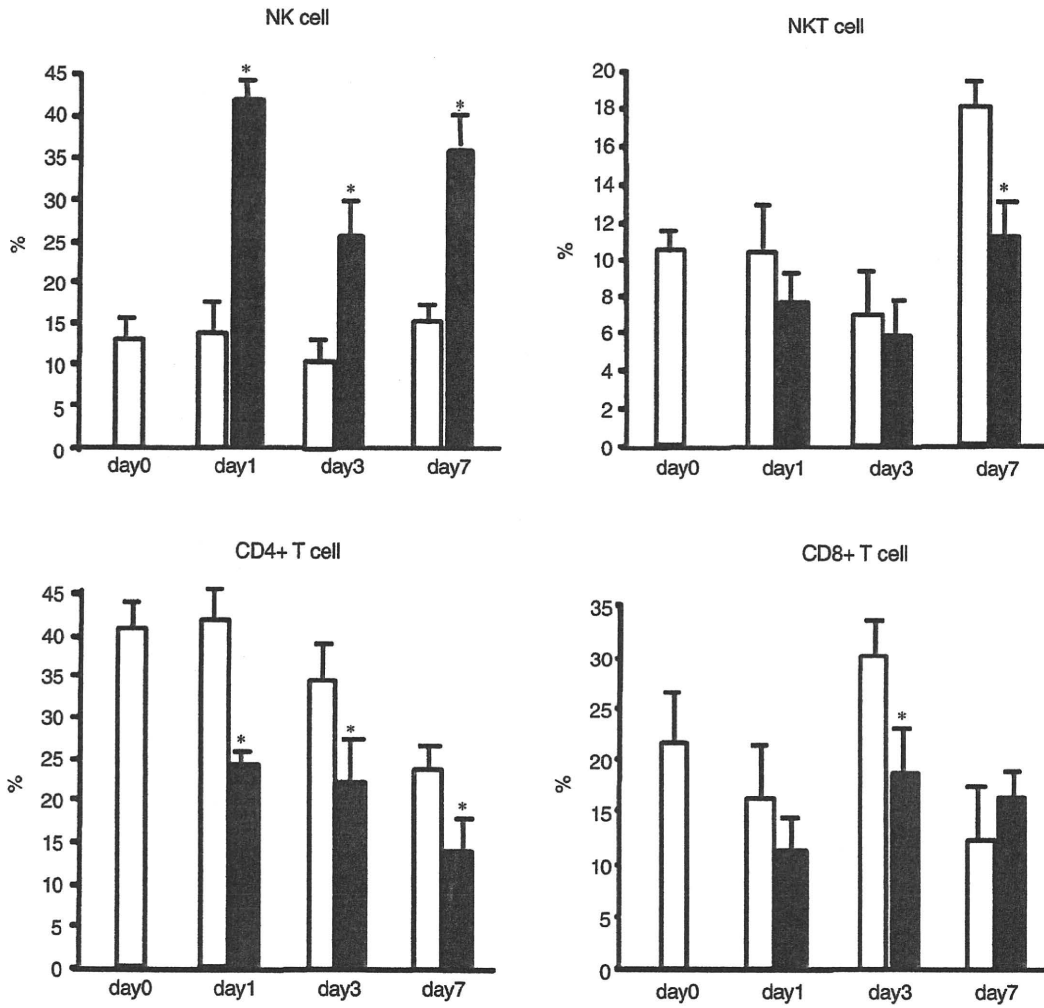
We examined the population change of MNC from the livers after α -GalCer or vehicle administration. It is notable that NK cells strikingly increased in proportion after α -GalCer administration, but not after vehicle administration (Fig. 3a). In contrast, the NKT cells decreased at 1 and 3 days after α -GalCer administration and recovered at day 7 after α -GalCer administration. Both CD4⁺ and CD8⁺ T cells decreased in proportion after α -GalCer administration, but not after vehicle administration. We also examined the CD69 expressions of NK cells, which is an indicator of lymphocyte activation. The CD69 expressions on liver NK cells increased with the peak at 1 day and gradually decreased at 7 days after α -GalCer administration (Fig. 3b). In contrast, those did not change after vehicle administration. These results demonstrated that the activated NK cells were the major subpopulation of MNC that increased in the liver after α -GalCer administration.

p53₂₃₂₋₂₄₀ peptide-specific CTL were generated after α -GalCer treatment of liver tumor

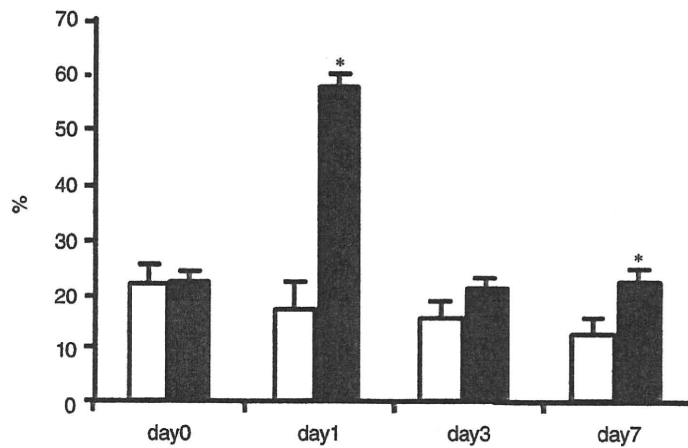
We evaluated whether p53₂₃₂₋₂₄₀ peptide-specific CTL were generated after α -GalCer treatment of liver tumor. CD8⁺ T cells were isolated from the spleen cells of treated mice and then co-cultured with syngeneic DC pulsed with p53₂₃₂₋₂₄₀ peptide strongly expressed on CMS4 cells. As shown in Figure 4(a), the number of

Figure 3 Activated natural killer (NK) cells composed the major subpopulation of hepatic mononuclear cells (MNC) that increased after α -galactosylceramide (α -GalCer) treatment. (a) BALB/c mice were treated with α -GalCer or vehicle. Hepatic MNC were prepared on days 0, 1, 3 and 7. NK cells, NKT cells, CD4⁺ T cells and CD8⁺ T cells in liver MNC were evaluated by flow cytometry. (b) The expressions of CD69 on liver NK cells were also evaluated by flow cytometry. White bar, vehicle-treated mice; black bar, α -GalCer-treated mice. **P* < 0.05 vs vehicle-treated mice.

(a)



(b)



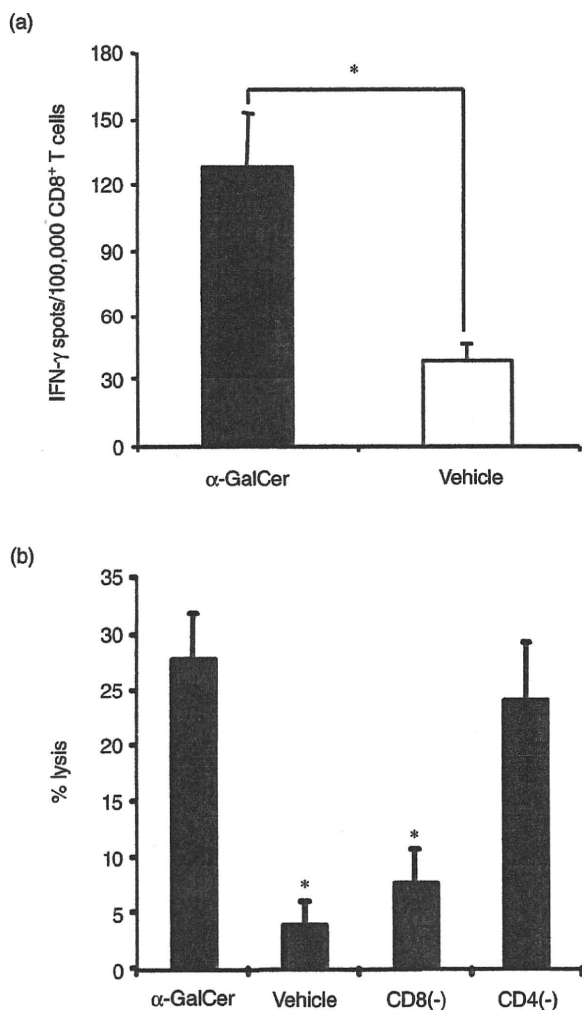


Figure 4 Evaluation of p53₂₃₂₋₂₄₀ peptide-specific CD8⁺ cytotoxic T lymphocytes (CTL) in α-galactosylceramide (α-GalCer)-treated mice. (a) CD8⁺ T cells were isolated from the spleen cells of treated mice 14 days after α-GalCer or vehicle treatment. The frequency of p53₂₃₂₋₂₄₀ peptide-specific CD8⁺ CTL was evaluated by interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assay. The results are shown in spots/100 000 CD8⁺ T cells; mean ± standard deviation of triplicate samples. **P* < 0.05. (b) Splenocytes from α-GalCer- or vehicle-treated mice were harvested 14 days after tumor inoculation and were analyzed for their ability to kill CMS4 cells in 4 h ⁵¹Cr-release assays (effector cells/target cells ratio, 60:1). CD4⁺ or CD8⁺ T cells were depleted by magnetic sorting using CD4 or CD8 microbeads (Miltenyi Biotec), respectively. CD8⁻, CD8⁺ T-cell-depleted splenocytes. CD4⁻, CD4⁺ T-cell-depleted splenocytes. **P* < 0.05 vs the cytolytic activity of splenocytes from α-GalCer-treated mice.

IFN-γ spots (per 100 000 CD8⁺ T cells) observed for T-cell responses against p53₂₃₂₋₂₄₀ peptide in α-GalCer-treated mice were significantly higher than that in vehicle-treated mice. These results suggested that strong p53₂₃₂₋₂₄₀ peptide-specific CTL were generated by α-GalCer treatment of liver tumor. Splenocytes from α-GalCer-treated mice displayed strong cytolytic activity against CMS4 cells, while those from vehicle-treated mice did not (Fig. 4b). CD8⁺ T-cell-depleted splenocytes from α-GalCer-treated mice displayed significant weak cytolytic activity against CMS4 cells, but CD4⁺ T-cell-depleted splenocytes did not. These results demonstrated that CD8⁺ T cells (i.e. CTL) played essential roles in the cytolytic activity against CMS4 cells in α-GalCer-treated mice.

Systemic therapeutic antitumor immunity was induced by α-GalCer treatment of CMS4 liver tumor

Because strong p53₂₃₂₋₂₄₀ peptide-specific CTL were generated in α-GalCer-treated animals, we next chose to analyze whether the treatment of a CMS4 lesion in the liver would impact the progression of subcutaneous untreated CMS4 tumors. BALB/c mice were intrahepatically injected with CMS4 tumors and treated by administration of α-GalCer. Twenty-eight days later, 1 × 10⁶ CMS4 cells or Colon26 cells were injected s.c. in the right flank. As shown in Figure 5(a), the non-treated CMS4 tumors in mice receiving α-GalCer treatment were completely rejected in all mice. The growth of non-treated Colon26 tumor in α-GalCer-treated mice was not inhibited (Fig. 5b). These results suggested that systemic CMS4-specific antitumor immunity could be induced by α-GalCer treatment. To confirm the involvement of CTL in this antitumor effect, we depleted CD8⁺ T cells before re-challenge of CMS4 cells (s.c. injection of 1 × 10⁶ CMS4 cells) in α-GalCer-treated mice bearing CMS4 liver tumor. On days 1 and 3 of re-challenge of CMS4 cells, anti-CD8 antibody (53-6.72 hybridoma, ATCC) was injected i.p. As shown in Figure 5(c), antitumor effect against re-challenged CMS4 subcutaneous tumor was diminished in CD8⁺ T-cell-depleted mice. These results supported that CD8⁺ T cells (i.e. CTL) play essential roles in the antitumor effect against re-challenge of CMS4 cells in α-GalCer-treated mice.

DISCUSSION

WE PREVIOUSLY DEMONSTRATED that administration of α-GalCer activated both NKT cells and NK cells in the liver, and that liver NK cells were the

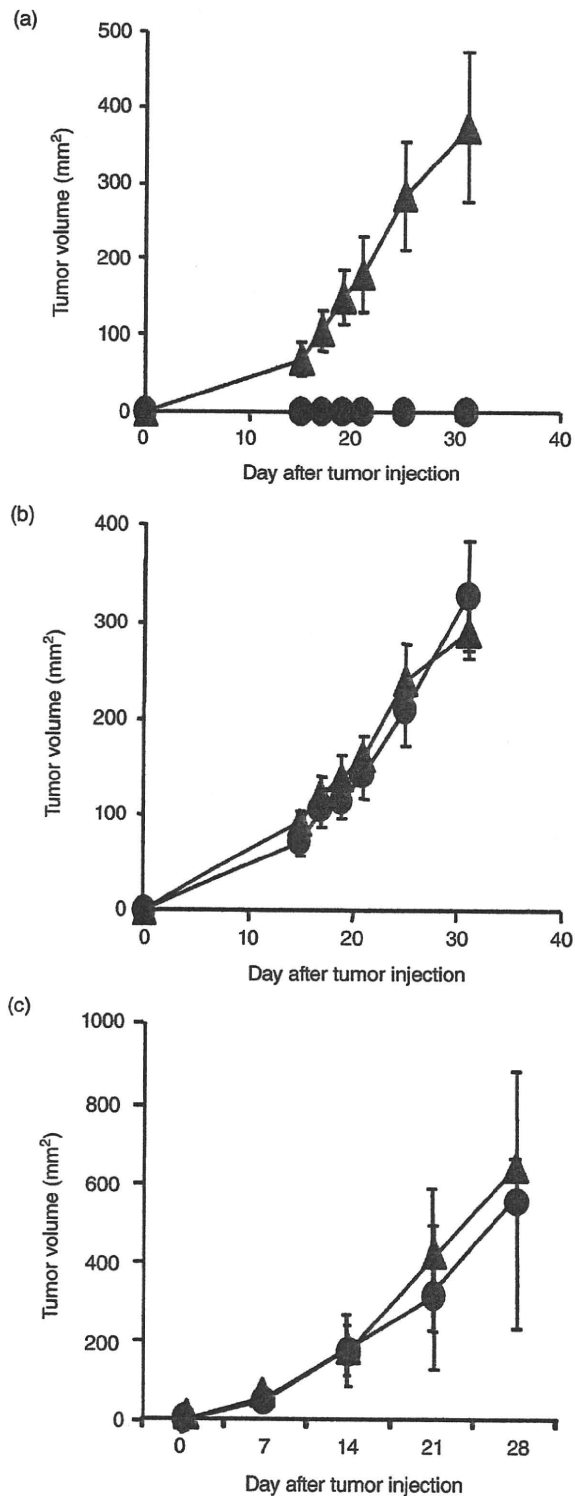


Figure 5 α -Galactosylceramide (α -GalCer) therapy results in the development of systemic antitumor immunity that protects distal tumor re-challenge. BALB/c mice were injected intrahepatically with CMS4 tumors. Twenty-four hours later, the mice were treated with α -GalCer. Twenty-eight days after treatment, α -GalCer-treated mice were re-challenged s.c. with 1×10^6 CMS4 cells (a) or Colon26 cells (b) in the right flank (all treatment groups, $n = 8$). To confirm the involvement of cytotoxic T lymphocytes (CTL) in this antitumor effect, we depleted CD8⁺ T cells before re-challenge of CMS4 cells in α -GalCer-treated mice bearing CMS4 liver tumor (c). On days 1 and 3 of re-challenge of CMS4 cells, anti-CD8 antibody was injected i.p. Tumor size was assessed every 3 or 4 days after s.c. injection of tumor cells (on day 0). As control mice, naïve mice were injected s.c. with 1×10^6 CMS4 cells (a, $n = 8$; c, $n = 6$) or Colon26 cells (b) ($n = 8$) on day 0. (●) α -GalCer-treated mice, (▲) control mice. Each data point represents the mean tumor size \pm standard deviations.

main effector cells to kill disseminated hepatoma cells injected from spleen in α -GalCer treatment.⁴ In this study, we evaluated α -GalCer treatment in local injected liver tumor, and the α -GalCer treatment resulted in complete rejection of local liver tumor, which had a similar antitumor effect as α -GalCer in a previous metastatic liver tumor model. These findings suggested the ability of α -GalCer treatment to activate the liver NK cells efficiently, which may mainly contribute to eradication of local liver tumor cells. A normal liver contains lymphocytes that are usually enriched with NK and NKT cells; namely, 25% NK cells and 30% NKT cells in contrast to peripheral blood that contains only 10% NK and 5% NKT cells.^{6,7} Thus, activation of innate immune cells, NK cells and NKT cells must be important to develop more effective immunotherapy against liver cancer. We believe that α -GalCer treatment must be a good candidate for human liver cancer treatment.

Recently, activated DC have been implicated in the activation of NKT and NK cells in both mice and humans,^{1,5,8-12,22} suggesting that DC play crucial roles in the activation of abundant immune cells in the liver. To establish more efficient α -GalCer treatment in liver cancer, the precise mechanism of liver DC activation is needed. Our results demonstrated that the proportion of liver DC in liver MNC increased immediately and reached the peak 1 day after α -GalCer treatment. The infiltration of tumors by mature DC has been reported to correlate with a better prognosis in cancer patients.^{23,24} Thus, the increase of liver DC by α -GalCer might contribute to generation of antitumor effect against liver cancer. The expressions of co-stimulatory

molecules on liver DC also increased early after administration of α -GalCer. IL-12 production from DC is key Th1-cytokine to enhance NK and CTL functionality,^{25,26} IL-12 production from liver DC after α -GalCer treatment was significantly higher than that after vehicle treatment.¹⁷ These results suggested that α -GalCer treatment resulted in rapid activation of liver DC, which might play important roles in activating liver NK cells and might contribute to the subsequent establishment of acquired immunity against liver cancer. Pillarisetty *et al.* identified new DC subsets, NK-DC, which presented in the liver of mice,²⁷ which may affect the interpretation of the activation of liver NK cells by α -GalCer. However, we previously demonstrated that α -GalCer had no direct effect on liver NK cells in mice.⁴ These results supported the idea that α -GalCer activated liver DC, which activated the liver NK cells secondary.

Interferon- γ ELISPOT assay revealed that the frequency of CD8⁺ T cells isolated from α -GalCer-treated mice in liver tumors in response to p53_{232–240} peptide were much higher than that from vehicle-treated mice. Mayordomo *et al.* reported that immunization of p53_{232–240} peptide-pulsed DC induced peptide-specific CTL in immunized mice that showed cytolytic activity against CMS4, p53 overexpressing cells.¹⁸ In this study, ⁵¹Cr-release assay demonstrated that CD8⁺ T cell, not CD4⁺ T cells, played essential roles in the cytolytic activity against CMS4 cells in α -GalCer-treated mice, which is consistent with the IFN- γ ELISPOT results. The detection of p53_{232–240} peptide-specific CTL means the generation of CMS4 tumor-specific CTL after eradication of liver tumor by α -GalCer treatment. The activation of NKT cells was associated with an expansion of antigen-specific CTL, as might be expected if the DC that matured *in vivo* in response to NKT cells were capturing antigens.^{28–31} Our results suggested that the activation of hepatic DC might be associated with the efficiency of generation of tumor antigen-specific CTL.

Additional experiments using an s.c. re-challenge with tumor demonstrated that α -GalCer treatment of liver tumors not only blocked treated CMS4 liver tumor progression but completely protected against consequent "recurrence" of that same tumor at a distant site. In contrast, Colon26 re-challenge tumor was not inhibited in treated mice, suggesting that CMS4-specific immunity was generated after liver tumor treatment. These results were consistent with the activation of acquired immunity evaluated by IFN- γ ELISPOT assay with increase of the frequency of p53_{232–240} peptide-specific CTL. Taken together, we believe that α -GalCer treatment of liver

tumors resulted in rejection of both local liver tumor and distant metastatic tumor.

In summary, we have shown that α -GalCer treatment activated both innate and acquired immune cells in the liver. These findings suggested that the use of α -GalCer might represent a particularly promising approach to suppress tumor growth and to promote regression of metastatic lesions in liver cancer patients.

ACKNOWLEDGMENTS

THIS WORK WAS supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health, Labour and Welfare of Japan.

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Original Article

Hepatitis C virus-specific CD8⁺ T cell frequencies are associated with the responses of pegylated interferon- α and ribavirin combination therapy in patients with chronic hepatitis C virus infection

Tomohide Tatsumi,¹ Tetsuo Takehara,¹ Takuya Miyagi,¹ Shoichi Nakazuru,² Eiji Mita,² Tatsuya Kanto,¹ Naoki Hiramatsu¹ and Norio Hayashi^{1,3}

¹Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, and

²Department of Gastroenterology, National Hospital Organization Osaka National Hospital, Chuo-ku, Japan, and

³Kansai-Rosai Hospital, Amagasaki, Hyogo, Japan

Aim: Hepatitis C virus (HCV)-specific cytotoxic T lymphocytes (CTLs) play critical roles in elimination of the HCV-infected hepatocytes. However, the mechanism of HCV elimination by pegylated interferon- α (peg-IFN α) plus ribavirin is not fully understood. We examined HCV-specific CTL responses during this combination therapy.

Methods: CD8⁺ T cells were isolated from 16 HCV infected patients treated by this combination therapy and were subjected to IFN- γ enzyme-linked immunospot (ELISPOT) assay.

Results: The numbers of IFN- γ spots against HCV Core or NS3 protein-derived peptides in HCV patients before treatment were similar to those in healthy donors, and those in HCV patients significantly increased 4 weeks after the initiation of combination therapy. All HCV Core or NS3 proteins-derived peptides specific CD8⁺ T cells responses in pre-treated patients were not associated with ALT levels and HCV viral loads of HCV patients before treatment. And those

in pre-treated patients were similar between sustained virologic responder (SVR) patients and non-SVR patients. Significant increase of HCV Core or NS3 proteins-derived peptides specific CD8⁺ T cells responses between before and 4 weeks after this combination therapy were observed in SVR patients, but not in non-SVR patients.

Conclusions: These results demonstrated that significant increase of HCV-specific CD8⁺ T cells at 4 weeks after the initiation of IFN treatment might be associated with the elimination of HCV. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of the combination therapy of peg-IFN α and ribavirin.

Key words: chronic hepatitis C, HCV-specific CTL, IFN- γ ELISPOT, peg-IFN α , ribavirin

INTRODUCTION

CHRONIC INFECTION OF Hepatitis C virus (HCV) often leads to cirrhosis and hepatocellular carcinoma (HCC), which causes the poor prognosis of HCV-infected patients.^{1,2} Combination therapy of pegylated interferon- α (Peg-IFN α) plus ribavirin is standard treat-

ment for patients with chronic hepatitis C (CH-C), and sustained virologic response (SVR) in this combination therapy occurs in about 40–60% of genotype 1 patients,^{1,2} which can improve the prognosis of HCV-infected patients. HCV-specific cytotoxic T lymphocytes (CTLs) is believed to play essential roles in determining the course of chronic infection,³ and the insufficient activation, dysfunction, suppression of CTLs may cause persistent infection of HCV.^{4–6} The elimination of HCV by HCV-specific CTLs is believed to consist of second slope of decay after viral decay during the first 24–48 h of IFN therapy.⁷ However, the detail immune mechanism of HCV elimination by this combination therapy is not fully understood. In addition to direct antiviral

Correspondence: Dr Tomohide Tatsumi, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, JAPAN.
Email: tatsumit@gh.med.osaka-u.ac.jp
Received 28 June 2010; revised 20 August 2010; accepted 24 August 2010.

property of Peg-IFN α and ribavirin against HCV infection, this combination therapy might have immunomodulatory activity. IFN- α enhances the maturation of antigen-presenting cells and CD4+ T cell function, but with little effect on CTLs. In contrast, ribavirin could induce a switch from Th2 to Th1 profile.⁸ Although the base line immune responses of CTLs have been reported to be associated with the achievement of SVR in a few reports^{7,8}, even now there are relatively little reports examining the detail of HCV-specific CTL responses during this combination therapy.

IFN- γ enzyme-linked immunospot (ELISPOT) assay allows detection of finally differentiated effector CTLs, which means the ELISPOT data reflect the *in vivo* situation.^{9–11} In the current study, we evaluated the HCV Core and NS3 proteins-derived peptides specific CD8+ T cells responses of the HCV infected patients by IFN- γ ELISPOT assay and examined the relationship between CTL activity and the clinical outcome of the combination therapy of Peg-IFN α plus ribavirin. The frequencies of HCV-specific CD8+ T cells in pre-treated HCV patients were not associated with antiviral activity of this combination therapy in SVR. However, the significant increase of HCV-specific CD8+ T cells at 4 weeks after the starting of IFN treatment could be observed in SVR patients, but not in non-SVR patients. Our findings suggest that the reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

MATERIALS AND METHODS

Patients

SIXTEEN PATIENTS CHRONICALLY infected with HCV were examined for HCV specific CTL responses during the combination therapy of Peg-IFN α plus ribavirin. All patients enrolled in this study were infected with HCV genotype 1b with a high viral load and were HLA-A2 positive. The patients who were infected with other viruses (Hepatitis B virus, Human immunodeficiency virus) or had other forms of liver disease (alcohol liver disease, autoimmune hepatitis) were excluded from this study. Informed consent, under an Institutional Review Board-approved protocol, was obtained from each patient. All patients received Peg-IFN α -2b (PEGINTRON, Schering-Plough, Kenilworth, NJ) plus ribavirin (REBETOL, Schering-Plough) for the duration of the study of 48–72 weeks. In only one patient (Patients#11), treatment was stopped at 24 weeks because this patient remained HCV-RNA positive after

24 weeks and developed significant side effect. To evaluate the antiviral activity, serum HCV RNA levels were quantified during the combination treatment. Serum HCV RNA level was quantified using the COBAS AMPLI-CORE HCV MONITOR test (version 2.0; Roche Diagnostics, Branchburg, NJ). SVR was defined as the absence of detectable serum HCV RNA at 24 weeks after the end of the combination therapy. All treated patients were assessed the antiviral responses (SVR or non-SVR) as previously described.¹² The characteristics of patients with chronic HCV infection were summarized in Table 1.

CD8+ T cells isolation from peripheral blood mononuclear cells (PBMC)

PBMC was obtained from 16 treated HCV infected patients before IFN treatment (pre-IFN) and 4 weeks after starting of this combination therapy (IFN-4week) and six healthy donors. CD8+ T cells were isolated from PBMC by magnetic cell sorting using CD8 MicroBeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). More than 95% of the cells were CD8+ lymphocytes.

IFN- γ ELISPOT assays for HCV Core and NS3 protein-derived peptide-specific CD8+ T cells responses

To evaluate the frequencies of CD8+ T cells recognizing peptide epitopes, IFN- γ ELISPOT assay were performed as previously described.¹¹ Briefly, 96-well multiscreen hemagglutinin antigen plates (Millipore, Billerica, MA) were coated with 10 μ g/mL of anti-human IFN- γ Ab (1-D1K; Mabtech, Stockholm) in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI 1640/10% human serum (1 h, 37°C), 1×10^5 CD8+ T cells were co-cultured with 2×10^4 T2.DR4 cells (HLA-A2 positive peptide-presenting cells generously provided from Dr Walter J. Storkus, University of Pittsburgh, School of Medicine, Pittsburgh, PA) pulsed with HCV Core and NS3 derived peptides (a final concentration of 10 μ g/mL). HLA-A2-restricted HCV Core protein derived peptides (Core_{E35–44}, YLLPRPGPRL, Core_{E131–140}, ADLMGYIPLV) or NS3 protein derived peptides (NS3_{1073–1081}, CINGVCWTV, NS3_{1406–1415}, KLVALGINAV) were synthesized as previously described.¹³ Negative control wells contained CD8+ T cells with T2.DR4 cells pulsed with HIV-nef_{190–198} peptide (AFHHVAREL). After 24 h incubation of the plates, cells were removed from the ELISPOT well by washing and captured cytokine was detected at sites of their secretion