The findings of the present study suggest higher sensitivity to adefovir dipivoxil therapy of the V1753 and C2189 mutant viruses compared to the wild-type virus in vivo. However, in vitro transfection analysis showed no differences in susceptibility to adefovir, as well as to lamivudine, among the wild-type virus and the C1753 and C2189 mutant viruses. This indicates that the V1753 and C2189 mutant viruses may be eradicated more efficiently by adefovir dipivoxil therapy than the wild-type virus regardless of a direct antiviral effect of adefovir dipivoxil. The V1753 and C2189 mutant viruses may induce stronger immune responses against the viral pathogens than the wild-type virus, which might result in more frequent viral eradication under adefovir dipivoxil therapy in patients having the V1753 or C2189 mutant virus compared to those with the wild-type virus.

Of the 1421 HBV strains, whose nucleotide sequences of the BCP, precore and core regions had been identified and registered in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp), there were 259 (18%) strains with the V1753 mutation and 127 (9%) strains with the C2189 mutation. The V1753 mutation was found in strains of all HBV genotypes, whereas the C2189 mutation was found in strains of genotypes A, B, C, and E. Thus, the V1753 and C2189 mutations were not specific for genotype C but common in other HBV genotypes.

The V1753 mutation occurring in the BCP not only influences the core promoter activity but also causes the I127T/N/S amino acid change of the overlapping X gene. This mutation has been detected in a considerable proportion of chronic HBV carriers, especially coupled with the adjacent T1762/A1764 mutation [Kidd-Ljunggren et al., 1997; Takahashi et al., 1999]. Indeed, all 11 patients with the V1753 mutation possessed the T1762/A1764 mutation in the current study. It has also been shown that, among patients with type B chronic hepatitis of genotype C, the V1753 mutation was found more frequently in patients with HCC than in those without it [Tanaka et al., 2006]. In acute HBV infection, the frequency of mutation has been reported to be higher in patients with fulminant hepatitis than in those with non-fulminant hepatitis [Imamura et al., 2003; Ozasa et al., 2006]. In vitro transfection assay revealed that the C1753 mutant virus possessed similar replicative competence to the wild-type virus, though viruses having the G1753 and A1753 mutation were not examined. Also, the in vitro replicative competence did not differ between the wild-type and C1753 mutant viruses when the T1762/A1764 mutation was introduced into the backbone HBV structure (data not shown). According to these observations, the serious disease course and better response to adefovir dipivoxil therapy caused by the V1753 mutation, as suggested by the present study and other previous investigations [Imamura et al., 2003; Ozasa et al., 2006; Tanaka et al., 2006], may not be due to the modification of the viral replicative competence. Further studies should be done to clarify why the V1753 mutation is involved in the active liver disease and the better outcome of adefovir dipivoxil therapy in patients with HBV infection.

The C2189 mutation, which leads to the I97L amino acid change in the core gene, has also been shown to be detected frequently in patients with type B chronic hepatitis [Ehata et al., 1991; Bozkaya et al., 1996], although the relevance of the mutation to a particular disease course has not been elucidated fully. Previous in vitro transfection studies have suggested that the virus with the C2189 mutation resulted in excessive secretion of the immature virion and enhanced viral replication [Yuan et al., 1999; Suk et al., 2002]. This does not agree with the present result showing lower replicative competence of the C2189 mutant virus than the wild-type virus. This discrepancy may be due to the usage of HBV-expressing plasmids of different viral strains. The virological and clinical significance of the C2189 mutant virus should be assessed by further detailed investigation.

In summary, the results of the present study indicate that the presence of the two viral mutations, V1753 and C2189, may be associated with a better therapeutic effect of adefovir dipivoxil added to lamivudine based on the results of screening of the full-length HBV genome obtained from lamivudine-resistant patients with type B chronic hepatitis. As the present study examined a limited number of patients with HBV of genotype C, further studies with a larger number of patients with different genotypes should lead to a better understanding of how identifying these mutations can be useful in a clinical setting.

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Activated liver dendritic cells generate strong acquired immunity in α-galactosylceramide treatment☆

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***Background/Aims*: α-Galactosylceramide (α-GalCer) presented by dendritic cells (DCs) activates NKT cells that in turn drive DC maturation. However, the potential of generating acquired immunity of liver DCs in α-GalCer treatment remains unclear.**

***Methods*: We examined the activation of acquired immunity in the α-GalCer treatment against liver or spleen tumor and the ability of liver and spleen DCs in the generation of acquired immunity.**

***Results*: Administration of α-GalCer resulted in generation of p53 peptide-specific cytotoxic T lymphocytes (CTLs) in mice bearing liver CMS4 tumor, aberrantly expressing p53, but not in mice bearing spleen CMS4 tumor. The growth of rechallenged CMS4 subcutaneous tumor was inhibited in α-GalCer-treated mice against liver CMS4 tumor, but not in α-GalCer-treated mice against CMS4 spleen tumor. The antigen presenting related functions of liver DCs were significantly higher than those of spleen DCs in α-GalCer-treated mice. Vaccination of normal mice with p53 peptide pulsed liver DCs isolated from α-GalCer treated mice resulted in generation of p53 peptide-specific CTLs, but that with p53 peptide pulsed spleen DCs did not.**

***Conclusions*: These results demonstrated that α-GalCer treatment induced unique immunologic activation of liver DCs in comparison with spleen DCs, which might be favorable to generate liver acquired immunity.**

***Keywords*: α-Galactosylceramide; Liver dendritic cells; Acquired antitumor immunity**

1. Introduction

α-Galactosylceramide (α-GalCer) presented by CD1d molecules expressing on dendritic cells (DCs) efficiently stimulates NKT cells implicated in innate immunity [1,2]. Recently, *in vivo* animal studies have shown that systemic administration of α-GalCer can lead to anti-tumor effects against metastatic liver tumor [3,4], suggesting that α-GalCer treatment might be promising for clinical application against liver tumor. Metastatic liver tumors resist conventional chemotherapy and radiotherapy, and present with a poor prognosis. Thus novel and more effective immunotherapy is needed, especially for metastatic liver cancer. Several phase I clinical studies have been done in cancer immunotherapy using intravenous administration of α-GalCer, but with limited clinical responses [5,6]. For further development of α-GalCer treatment in liver cancer patients, the antitumor effect of α-GalCer should be more precisely examined in the liver.

DCs effectively elicit immune responses to self and foreign antigens [7,8]. These specialized antigen-presenting cells (APCs) can induce the generation of both

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Abbreviations: DC, dendritic cell; APC, antigen-presenting cells; CTLs, cytotoxic T lymphocytes; α-GalCer, α-galactosylceramide; MNC, mononuclear cells.

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antigen-specific cytotoxic T lymphocytes (CTLs) and T helper cells. α-GalCer administration resulted in maturation of spleen DCs and activation of the CD8+ T cell immune response via costimulatory molecules expressed on the spleen DCs [9,10]. However, in contrast to well-characterized spleen DCs, the details of activation of liver DCs by α-GalCer treatment remains to be clarified because of the difficulty of procuring adequate numbers of isolated liver DCs for functional analysis [11]. Although most previous studies reported that α-GalCer treatment induces early activation of liver NKT and NK cells [3,4,12], which were the main effector cells to eradicate metastatic tumor cells, little is known regarding the induction of liver acquired immunity after early rejection of liver tumor. Nakagawa et al. reported that CD122+CD8+ memory T cells play critical roles in metastatic liver tumor rejection by α-GalCer treatment [13]. However, the ability of α-GalCer to activate liver DCs and generate acquired immunity remains to be clarified.

In the current study, we evaluated the induction of acquired immunity by α-GalCer activated liver DCs in comparison with spleen DCs. We demonstrated that α-GalCer treatment resulted in generating strong acquired immunity after liver tumor treatment, but not after spleen tumor treatment. We also show that α-GalCer treatment activated liver DCs more strongly with respect to the antigen-presenting function and antigen-specific CTL induction than spleen DCs. Thus, α-GalCer treatment resulted in unique immunologic activation of liver DCs, which might contribute to induction of acquired immunity in the liver.

2. Materials and methods

2.1. Mice and cell lines

Six-to-ten-week-old female BALB/c mice and C57BL/6 mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan). The animals were handled under aseptic conditions. Procedures were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals. CMS4 sarcomas (H-2^d) express mutated p53 and present the wild-type $p53_{232\ 240}$ epitope recognized by H-$2K^d$-restricted CTLs [14,15], and MC38 colon cancer cell lines were maintained as previously described [16]. α-Galactosylceramide (α-GalCer) was kindly provided by Kirin Pharma (Gunma, Japan) and prepared as previously described [15].

2.2. IFN-γ ELISPOT assays for p53 peptide-reactive CD8+ T cells responses after α-GalCer treatment for CMS4 tumor and animal experiments

To examine the induction of the acquired antitumor immunity, BALB/c mice were injected intrahepatically or intrasplenically with 5×10^5 CMS4 cells on day 0 and treated intraperitoneally (i.p.) with α-GalCer (2 μg/100 μl) or 100 μl of vehicle on day 1. Fourteen days after α-GalCer treatment, CD8+ T cells were isolated from the spleen of immunized mice by using magnetic beads (MACS, Miltenyi Biotec, Gladbach, Germany). Next, CD8+ T cells (1×10^5 cells/well) and syngeneic bone marrow derived DCs (BMDCs) generated from normal BALB/c mice (2×10^4 cells/well) were cocultured with $p53_{232\ 240}$ peptide in ELISPOT culture plate. We used mouse IFN-γ ELISPOT kit (R & D Systems, Minneapolis, MN) to detect the $p53_{232\ 240}$ peptide-specific CD8+ T cell responses, as previously described [16]. To assess the systemic acquired immunity due to α-GalCer treatment, mice were injected in the liver or the spleen with 5×10^5 CMS4 cells or MC38 cells on day 0 and were injected i.p. with α-GalCer on day 1. On day 14 after α-GalCer treatment, 1×10^6 CMS4 cells or MC38 cells were injected as a rechallenge into the right flank of treated mice, respectively. Tumor size was assessed every 7 days.

2.3. Preparation of liver and spleen DCs and flow cytometry

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, hepatic mononuclear cells (MNC) and splenic MNC were prepared as previously described [15]. CD11c+ dendritic cells were isolated from liver MNC and spleen MNC by magnetic cell sorting using MACS (Miltenyi Biotec) according to the manufacturer's protocol. For phenotypic analysis of liver and spleen DCs, PE- or FITC- or APC-conjugated monoclonal antibodies against mouse cell surface molecules [CD11c (Miltenyi Biotec), CD40, CD80, CD86, MHC class II, CD8α and CD11b (all from BD-Pharmingen, San Diego, CA)] were used, and flow cytometric analysis was performed using a FACS Calibur (Becton Dickinson, San Jose, CA) flow cytometer. We defined DCs with CD11c+ MHC class II+ cells by flow cytometry and evaluated the expressions of these antigen presenting related molecules. Data were analyzed using FlowJo software (Tree Star, Ashland, OR) and reported as the mean fluorescence intensity (MFI).

2.4. Cytokine measurement

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, liver and spleen DCs were prepared as above. To assess cytokine production, we cultured 2×10^5 DCs in 1 ml of complete medium with LPS (R & D Systems Inc., 10 μg). After 48 h, cell culture supernatants were harvested and tested using a species-specific enzyme linked immunosorbent assay (ELISA) kit for IL-12, IFN-γ and TNF-α (BD-Pharmingen) according to the manufacturer's protocols.

2.5. T cell proliferation assay

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, liver and spleen DCs were prepared as above. The DCs were added in various numbers to 5×10^5 allogeneic T lymphocytes (purified using Thy-1.2 immunomagnetic microbeads from C57BL/6 mice) in 96-well U-bottom plates and then pulsed with [^{3}H] thymidine (1 μ Ci/well) on day 3 for an additional 20 h as previously described [17].

2.6. Immunization of p53 peptide-pulsed liver or spleen DCs from α-GalCer-treated mice

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, liver and spleen DCs were prepared as above. Isolated DCs were incubated with $p53_{232\ 240}$ peptide at concentration of 10 μg/mL per 10^6 DCs/mL for 2 h as previously described [14]. 1×10^6 $p53_{232\ 240}$ peptide pulsed liver or spleen DCs were injected i.p. into normal BALB/c mice. Five days after i.p. immunization, CD8+ T cells were isolated from the spleen of immunized mice by using magnetic beads (MACS) and were subjected to mouse IFN-γ ELISPOT assay as above described.

2.7. Statistical analyses

The statistical significance of differences between the groups was determined by applying Student's *t*-test with Welch correction after each group had been tested with equal variance and Fisher exact probability test. The statistical significance of the differences in more than three groups was determined by applying one-way ANOVA. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Acquired antitumor immunity was induced by α-GalCer treatment of CMS4 liver tumor

We examined whether α-GalCer treatment for CMS4 liver or spleen tumor would induce acquired antitumor immunity. Mice bearing liver or spleen CMS4 tumor were treated i.p. with α-GalCer. Fourteen days after α-GalCer treatment, spleen CD8+ T cells from treated mice were prepared and subjected to IFN-γ ELISPOT. The high numbers of IFN-γ spots were detected in the CMS4 liver tumor model, but not in the CMS4 spleen tumor model (Fig. 1A).

We next analyzed whether the α-GalCer treatment of CMS4-treated liver or spleen would impact the progression of subcutaneous rechallenged CMS4 tumors. Fourteen days later after α-GalCer treatment, 1×10^6 CMS4 cells were rechallenged subcutaneously in the right flank. As shown in Fig. 1B, CMS4 subcutaneous tumors in α-GalCer treated mice bearing CMS4 liver tumor were significantly inhibited compared with those in non-treated mice, but those in mice bearing CMS4 spleen tumor were not. Colon26, BALB/c syngeneic colon cancer cell, subcutaneous tumors were not inhibited in mice receiving α-GalCer treatment for CMS4 liver or spleen tumor (data not shown). Strong acquired immunity could also be generated after α-GalCer treatment of MC38 liver tumors in C57BL/6 mice, but not of MC38 spleen tumors (Fig. 1C). These findings suggested that tumor-specific acquired immunity could be generated efficiently by α-GalCer treatment in the liver, but not in the spleen.

3.2. Administration of α-GalCer activated DCs and increased CD8- conventional DC fraction in the liver

Recent research revealed that NKT cells-DC interactions by α-GalCer are critically important in the sequential activation of effector cells in both innate and acquired immunity [12,18]. However, details of the DC activation by α-GalCer in the liver have not yet been evaluated.

First, we investigated the increase of liver and spleen DCs after α-GalCer or vehicle treatment. As shown in Fig. 2A, liver DCs increased significantly after α-GalCer administration whereas spleen DCs from α-GalCer treated mice did not. The proportion of liver DCs in liver MNCs also significantly increased by α-GalCer administration, but that of spleen DCs did not (data not shown). Next, we examined the change of DC subtypes after α-GalCer treatment by analyzing the relative surface expressions of the CD8α and the CD11b molecules [19]. The proportion and the number of CD8- conventional DCs (CD11b+CD8-) significantly increased in the liver by α-GalCer treatment, but not in the spleen. In marked contrast, those of CD8+ conventional DCs (CD11b-CD8α+) exhibited no significant change in both the liver and spleen by α-GalCer treatment (Fig. 2B and C).

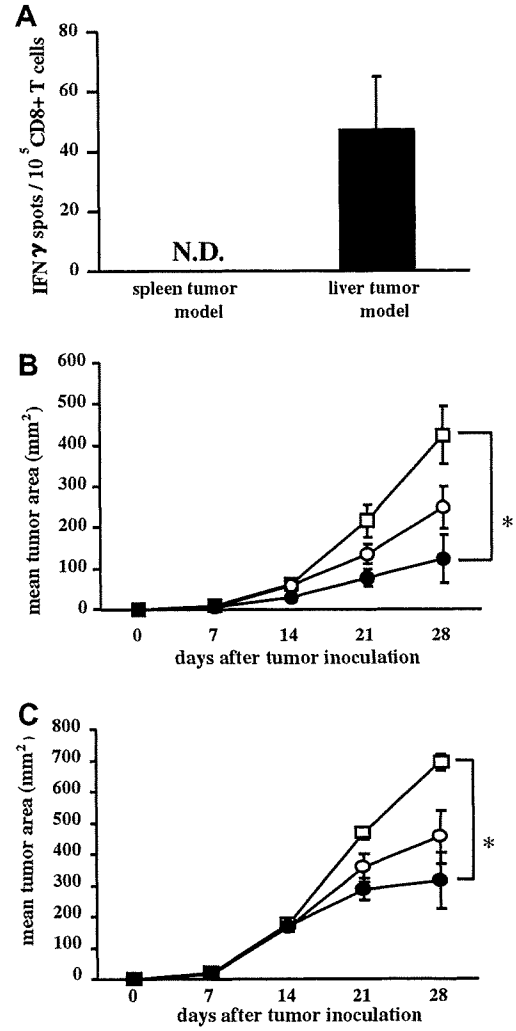

Fig. 1. Induction of local and systemic acquired antitumor immunity after α-GalCer treatment of CMS4 liver and spleen tumor. BALB/c mice were injected intrahepatically or intrasplenically with 5×10^5 CMS4 cells or MC38 cells. One day later, mice were injected i.p. with α-GarCer. (A) Fourteen days later, spleen CD8+ T cells were isolated from both the CMS4 liver and spleen tumor models and subjected to IFN-γ ELISPOT to analyze $p53_{232-240}$ peptide specific IFN-γ production. The results are shown as spots/100,0000 CD8+ T cells; mean ± SD of triplicate samples. CD8+ T cell reactivity against peptide-unpulsed BMDCs served as the negative control in all cases, and this value was subtracted from all experimental determination to determine p53-specific spot numbers. *$p < 0.05$. N.D., not detected. Similar results were obtained from two separate experiments. (B and C) Fourteen days later, mice were challenged subcutaneously with 1×10^6 CMS4 cells (B) or MC38 cells (C) in the right flank (all treatment groups $N = 8$). Tumor size was assessed every 7 days after subcutaneous injection of tumor cells (=on day 0). α-GalCer-treated CMS4 or MC38 liver tumor (•), α-GalCer-treated CMS4 or MC38 spleen tumor (○), non-treated mice (□). Each data point represents the mean tumor size ±SE. *$p < 0.05$.

We examined the CD80, CD86 and CD40 expressions of liver and spleen DCs after administration of α-GalCer. CD86 and CD40 molecules on both liver and spleen DCs from α-GalCer-treated mice were

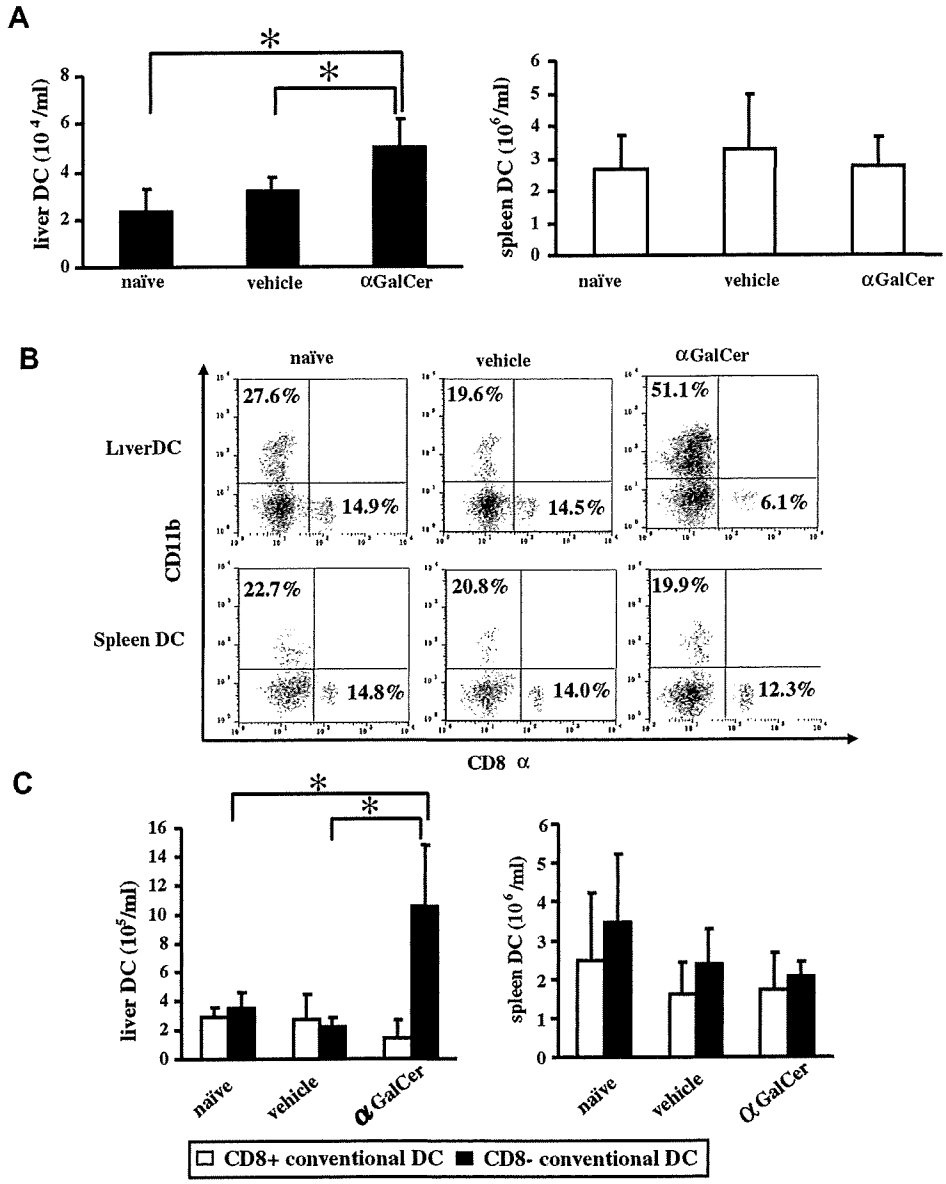

Fig. 2. α-GalCer treatment increased liver CD8- conventional DC. BALB/c mice were treated with α-GalCer or vehicle. Liver and spleen DCs were prepared on day 1 after α-GalCer treatment. (A) Liver DCs (left panel) increased significantly after α-GalCer treatment, but spleen DCs (right panel) did not. (B and C) The change of CD8+ or CD8- conventional DC subtypes after α-GalCer treatment was examined by flow cytometry. The data are represented as the average of numbers obtained from 5 separate experiments. $^*p < 0.05$.

expressed more strongly than those from vehicle-treated mice and non-treated mice. CD80 molecules on liver DC from α-GalCer-treated mice were expressed significantly more strongly than those from vehicle-treated or non-treated mice, but those on spleen DC showed no significant change by α-GalCer treatment (Fig. 3). The expressions of CD80, CD86 and CD40 molecules on liver DCs tended to be lower than those on spleen DCs in non-treated mice. However, after α-GalCer treatment, their expressions on liver DCs tended to increase to levels similar to those on spleen DCs.

3.3. Liver DCs from α-GalCer-treated mice could produce more Th1 cytokines and present higher T cell immunostimulatory ability than spleen DCs

Th1-cytokines, such as IL-12, INF-γ and TNF-α, play key roles in determining the strength and/or the phenotypes of the antitumor immune responses [20,21]. We next examined the production of Th1 cytokines from DCs after α-GalCer treatment. The production of these cytokines from DCs derived from vehicle-treated and non-treated mice were not detected in the

liver or the spleen. In marked contrast, all IL-12, INF-γ and TNF-α production from liver DCs derived from α-GalCer-treated mice were significantly higher than those from spleen DCs (Fig. 4A–C). To investigate the difference of the antigen-presenting function between liver DCs and spleen DCs, we examined the allostimulatory capacity of liver and spleen DCs using a mixed lymphocyte reaction (MLR). Liver DCs from α-GalCer-treated mice showed higher T cell proliferation ability than those from vehicle-treated or non-treated mice and spleen DCs from all treatment groups. Spleen DCs from all treatment groups and liver DCs from vehicle-treated or non-treated mice showed little T cell proliferation ability (Fig. 4D). These results suggested that α-GalCer treatment increased the function of DCs in the liver more strongly than those in the spleen.

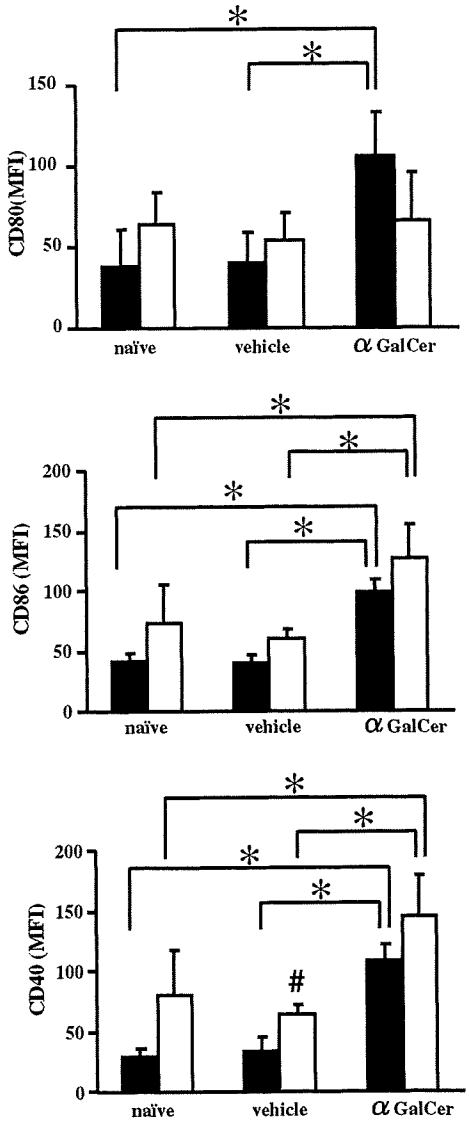

Fig. 3. α-GalCer treatment increased the expression of antigen presenting related molecules on both liver and spleen DCs. DCs were stained with PE- or FITC-conjugated monoclonal antibodies (CD11c, CD40, CD80, CD86 and MHC class II), and the expressions of these molecules were analyzed by flow cytometry. The data are represented as the average of MFI obtained from 5 separate experiments. $^{*}p < 0.05$ for each treatment group, $^{\#}p < 0.05$ between liver DCs (■ black bar) and spleen DCs (□ white bar). Naïve: DCs derived from non-treated mice; vehicle: DCs derived from vehicle-treated mice; α-GalCer: DCs derived from α-GalCer-treated mice.

3.4. Vaccination of $p53_{232-240}$ peptide-pulsed liver DCs isolated from α-GalCer-treated mice resulted in generating $p53_{232-240}$ peptide specific CTLs more efficiently than that of spleen DCs

Based on the above results, liver DCs had more antigen-presenting function than spleen DCs in α-GalCer-treated mice. We next evaluated the potential of tumor associated antigen specific CTL induction by vaccination of peptide-pulsed liver DCs or spleen DCs. We vaccinated normal mice i.p. with peptide-pulsed DC. Five days later, spleen CD8+ T cells were isolated and subjected to IFN-γ ELISPOT assay. As shown in Fig. 5, the numbers of IFN-γ spots observed for T cell responses against $p53_{232-240}$ peptide in mice vaccinated with α-GalCer-activated liver DCs were significantly higher than those in mice with vehicle- or non-treated-liver DCs. There were no detectable spots in mice vaccinated with spleen DCs from all treatment groups, suggesting that spleen DCs displayed no stimulatory activity for CTL induction regardless of the administration of α-GalCer *in vivo*. These results revealed that liver DCs in α-GalCer-treated mice have the highest potential for inducing tumor-associated antigen-specific CTLs, which might be associated with the *in vivo* generation of acquired immunity against liver tumor by α-GalCer treatment shown in Fig. 1.

4. Discussion

We and others previously reported that the early eradication of tumor cells in the liver mainly depended on NKT cells and NK cells [3,4]. In this study, we demonstrated that α-GalCer treatment resulted in generating stronger acquired immunity after eradication of primary CMS4 and MC38 liver tumor, but not after spleen tumor treatment. This suggests that liver, and not spleen, is an unique immunological organ that is favorable for generation of acquired immunity. We examined whether CTLs generated by immunization with peptide- and α-GalCer-pulsed BMDC could show equally antitumor effect in skin, liver and spleen in the normal mice. The generated CTLs in treated mice have equal access to all organs and are capable of killing tumor cells (Sasakawa, unpublished data). Thus, our data encour-

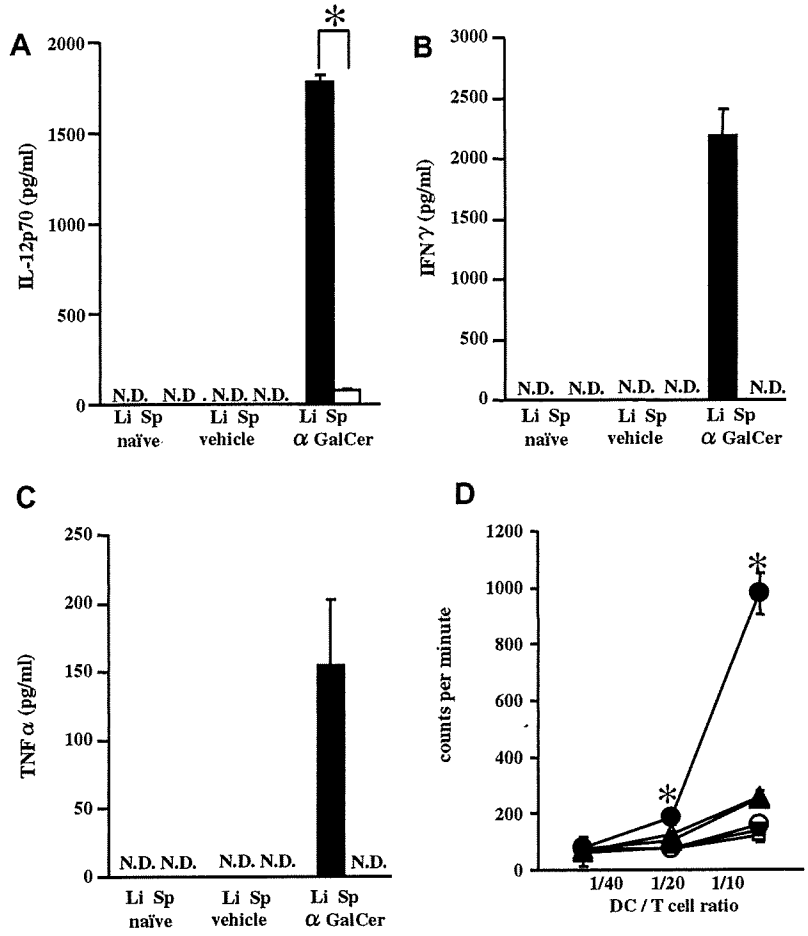

Fig. 4. Th1 type cytokine production of liver DCs from α-GalCer treated mice. Liver and spleen DCs were prepared 24 h after i.p. treatment of α-GalCer or vehicle. 2×10^5 DCs were stimulated with LPS (10 μg), and the supernatants of the DC cultures were subjected to specific ELISA. IL-12 (A), IFN-γ (B) and TNF-α (C). N.D., not detected. (D) We examined the allostimulatory capacity of liver and spleen DCs by MLR. Liver DC from non-treated mice (■), vehicle-treated mice (▲), and α-GalCer-treated mice (●). Spleen DC from non-treated mice (□), vehicle-treated mice (△), and α-GalCer-treated mice (○). Each data point represents the mean tumor size ± SD. *$p < 0.05$ counts per minute (CPM) of liver DCs vs CPM of spleen DCs from α-GalCer, vehicle or non-treated mice, respectively. Similar results were obtained from three separate experiments.

aged us to investigate the ability of liver DC to generate acquired antitumor immunity in comparison with spleen DCs.

In the current study, we investigated the activation of liver and spleen DC function after α-GalCer treatment. The expressions of antigen-presenting related molecules on liver DCs were weaker than those on spleen DCs in normal or vehicle treated mice. Pillarisetty et al. reported that liver DCs are generally weak activators of immunity in contrast to spleen DCs in normal mice and the expressions of MHC and costimulatory molecules on liver DCs were lower than those on spleen DCs in normal mice [22]. This is consistent with our results. In marked contrast, α-GalCer administration resulted in a significant increase of DCs in the liver and the expressions of antigen-presenting related molecules was more strongly upregulated in the liver than in the spleen. It has been reported that the expression of CD8α molecule is an activating marker of conventional DCs from progenitor cells [23]. We demonstrated that α-GalCer administration induced not only an increase of total DCs but also a significant increase of CD8- conventional DCs in the liver, which suggested that α-GalCer treatment resulted in developing progenitor DCs efficiently to matured conventional DCs. More strikingly, the production of Th1 type cytokine from α-GalCer-treated liver DCs were significantly more than from α-GalCer-treated spleen DCs. Previous reports demonstrated that the capacity of Th1 type cytokine to link between innate and adaptive immunity by interacting with DCs and T cells, is important for the induction of adaptive antitumor immune response and long-term therapeutic effect [24]. Furthermore, liver DCs showed higher T cell proliferation ability

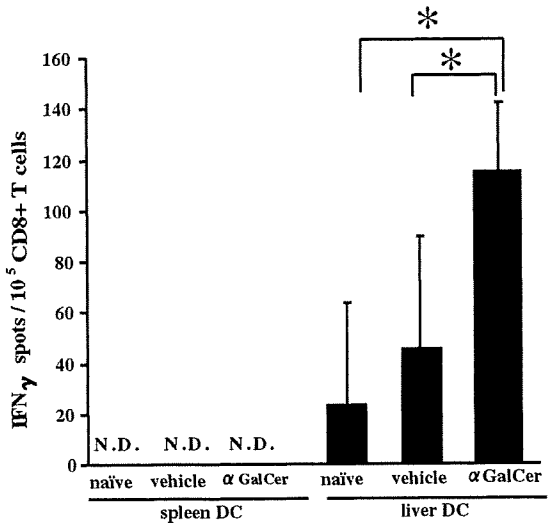

Fig. 5. Evaluation of $p53_{232-240}$ peptide specific CD8+ CTL induction after vaccination of p53 peptide-pulsed DCs from each treated mice. Normal BALB/c mice were immunized i.p. with 1×10^6 $p53_{232-240}$ peptide pulsed liver or spleen DCs isolated from α-GalCer or vehicle treated mice. Five days after vaccination, CD8+ T cells were isolated from the spleen of immunized mice. The frequency of $p53_{232-240}$ peptide specific CD8+ CTL was evaluated by IFN-γ ELSIPOT assay. The results are shown as spots/100,0000 CD8+ T cells; mean ± SD of triplicate samples. CD8+ T cell reactivity against peptide-unpulsed BMDCs served as the negative control in all cases, and this value was subtracted from all experimental determination to determine p53-specific spot numbers. $^*p < 0.05$. N.D., not detected. Similar results were obtained from three separate experiments.

than spleen DCs after α-GalCer treatment. Taken together, these results suggested that α-GalCer treatment resulted in the efficient activation of liver DCs more strongly than spleen DC, which might be associated with the induction of antitumor acquired immunity in the liver.

To examine whether the α-GalCer activated liver and spleen DCs could actually induce acquired immunity, we vaccinated $p53_{232-240}$ peptide-pulsed α-GalCer activated liver and spleen DCs. The frequencies of CD8+ T cells in response to $p53_{232-240}$ peptide were much higher in α-GalCer activated liver DCs vaccinated mice than those in vehicle-treated liver DCs vaccinated mice. Interestingly, the vaccination of $p53_{232-240}$ peptide-pulsed spleen DCs isolated from both α-GalCer and vehicle-treated mice did not generate $p53_{232-240}$ peptide-specific CTL responses. These data suggested that the immunological microenvironment in the spleen may support DCs to be potentially very tolerogenic resulting in inability of generating acquired immunity. In marked contrast, liver DCs potentially have the ability of generating antitumor acquired immunity and that α-GalCer could markedly enhance this ability. A normal mouse liver contains lymphocytes that are usually enriched with 10% NKT cells in contrast to mouse spleen that contains only 2% NKT cells [25]. α-GalCer presented by DCs activates NKT cells upregulating CD40 ligand on NKT cells, which in turn leads to the activation of DCs [17]. Actually we confirmed that i.p. injection of α-GalCer activated equally well in both liver and spleen NKT cells (Sasakawa, unpublished data). Thus, the higher population of NKT cells in the liver may be associated with efficient activation of liver DCs after α-GalCer treatment, which might characterize the unique immunological responses in the liver.

Despite recent progress and early success with various types of immunotherapy, there is still significant room for improvement in these regimens against liver cancer. We demonstrated that liver is an immunologically unique organ that is favorable for generation of acquired antitumor immunity. We propose that α-GalCer treatment may be an attractive strategy for suppressing tumor growth in the liver and promoting regression of metastatic lesions in other organs.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.12.027.

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Original Article

Effect of interferon α-2b plus ribavirin therapy on incidence of hepatocellular carcinoma in patients with chronic hepatitis

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Aim: The objective of this study was to elucidate the long-term effects of interferon (IFN)α-2b plus ribavirin combination therapy and to clarify whether this therapy can reduce the incidence of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C.

Methods: A total of 403 patients infected with hepatitis C virus (HCV) were enrolled in a multicenter trial. All patients were treated with a combination of IFN-α-2b plus ribavirin therapy. We examined the incidence of HCC after combination therapy and analyzed the risk factors for liver carcinogenesis.

Results: A sustained virological response (SVR) was achieved by 139 (34%) of the patients. The cumulative rate of incidence of HCC was significantly lower in SVR patients than in non-SVR patients ($P = 0.03$), while there was no difference in the cumulative incidence of HCC between the transient response (TR) group and the no response (NR) group. Cox's regression analysis indicated the following risk factors as independently significant in relation to the development of HCC: age being > 60 years ($P = 0.006$), advanced histological staging ($P = 0.033$), non-SVR to IFN therapy ($P = 0.044$). The cumulative incidence rate of HCC was significantly lower in patients who had average serum alanine aminotransferase (ALT) levels of < 40 IU/L than in those who showed average serum ALT levels of ≧ 40 IU/L after the combination therapy ($P = 0.021$).

Conclusions: These results suggest that the attainment of SVR or continuous normalization of ALT levels after IFN therapy can affect patients apart from HCC development.

Key words: chronic hepatitis C, continuous normalization of ALT, hepatocellular carcinoma, interferon plus ribavirin combination therapy, sustained virological response

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common malignancies in Japan and its incidence has been increasing over the last 30 years. Recently, various treatments such as transcatheter arterial embolization/chemoembolization, radio frequency ablation and hepatic resection have been reported to yield significant improvements in overall patient survival,[1–3] but HCC relapse has thus far been observed in a majority of treated patients due to the highly malignant potential of the liver. In general, approximately 70–80% of Japanese HCC patients are also diagnosed with type C chronic hepatitis or cirrhosis.[4] It has also been shown that the chronic hepatitis C (CHC) liver slowly but steadily progresses to cirrhosis[5,6] and the risk of HCC increases according to the degree of liver fibrosis.[7,8] In this regard, the success of treatment

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for chronic hepatitis C virus (HCV) infection is expected to prevent the patient's liver from progressing to cirrhosis and to reduce the risk of development of HCC. Interferon (IFN) has been proven to be effective in reducing and in eliminating HCV from the circulation; in decreasing serum alanine aminotransferase (ALT) levels; and in improving the histological appearance of the liver in patients with CHC.[9–11] Moreover, it has been demonstrated that IFN monotherapy in CHC patients is associated with reducing the incidence of HCC, especially in those patients who achieved a sustained virological response (SVR).[12–14] Recently, many investigators have reported that combination therapy using IFN-α-2b or pegylated IFN (Peg-IFN) N plus ribavirin is more effective for eradicating HCV than IFN monotherapy.[15–17] However, it has not been accurately evaluated whether or not the combination therapy using Peg-IFN plus ribavirin could reduce HCC development in patients infected with HCV.

In this study, we evaluated the long-term effect of IFN-α-2b plus ribavirin therapy on the incidence of HCC in HCV-infected patients treated with the combination therapy by retrospective examination of the clinical outcomes.

METHODS

Patients

THIS STUDY WAS a multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum in Japan. A total of 459 patients with HCV infection were treated with a combination of IFN-α-2b (Intron; Schering-Plough Corporation, Kenilworth, NJ, USA) plus ribavirin (Rebetol; Schering-Plough, Auxerre, France) between June 2002 and March 2005. All patients were treated with 6 MU of IFN-α-2b subcutaneously thrice a week and with oral ribavirin daily. Ribavirin was given at a total daily dose of 600 mg for patients who weighed < 60 kg and 800 mg for patients who weighed ≧ 60 kg. Patients who were positive for hepatitis B surface antigen, anti-human immunodeficiency virus antibody or those with other liver diseases (alcoholic liver disease, autoimmune liver disease, etc) were excluded from this study. Also excluded were patients with a history of HCC and those who developed HCC within the first 6 months of the follow-up period after the end of IFN therapy, because of the possibility that microscopic HCC had been present before initiation of the treatment. The remaining 403 patients infected with HCV were enrolled and followed in this study. The observation term was terminated upon the start of the next IFN therapy, such as Peg-IFN plus ribavirin after a combination of IFN-α-2b plus ribavirin therapy. Responses to IFN therapy were divided into the following three groups based on the viral load: sustained virological response (SVR) was defined as the absence of detectable serum HCV-RNA at 24 weeks after completion of IFN therapy. Transient response (TR) was defined as the absence of HCV-RNA from the serum at the end of treatment but detectable at 24 weeks after completion of therapy. Those categorized as having no response (NR) did not meet these criteria.

This study protocol followed the ethical guidelines of the 1975 Declaration of Helsinki, and informed consent was obtained from each patient.

Blood tests

Serum samples were stored frozen at −80 °C. HCV-RNA levels were analyzed by quantitative reverse transcription (RT)–PCR assay (Amplicor-HCV version 2.0; Roche Diagnostic Systems, Tokyo, Japan). The lowest detection limit of this assay was 50 IU/mL. All patients were examined for serum HCV-RNA level and underwent hematological and biochemical tests just before therapy, every 4 weeks during treatment and every 12 weeks thereafter until the end of treatment.

Normal serum ALT is defined as < 40 IU/L. In addition, the biological response to IFN therapy was defined based on "the average serum ALT level", which was calculated from all data of ALT levels after completion of IFN therapy.

Histological evaluation

The patients underwent liver biopsies within 6 months before the start of therapy. Histopathological interpretation of specimens was done by experienced liver pathologists who had no clinical information. The histological appearance of the liver sample sections was evaluated according to METAVIR's histological score.[18] Fibrosis stage was evaluated on a scale from 0 to 4.

Diagnosis and follow up of HCC

Ultrasonography was carried out before IFN therapy and every 3 to 6 months during the follow-up period. New space-occupying lesions detected or suspected at the time of ultrasonography were further examined by computed tomography (CT) or hepatic angiography. HCC was diagnosed by the presence of typical hypervascular characteristics on angiography, in addition to the findings from CT. If no typical image of HCC was observed, fine-needle aspiration biopsy was carried out with the

patient's consent, or the patient was carefully followed until a diagnosis was possible with a definite observation by CT or angiography.

Statistical analysis

Quantitative variables were expressed as mean ± SD. The Kaplan–Meier method was used to calculate the cumulative incidence of HCC. The prognostic relevance of clinical variables and HCC incidence was evaluated by univariate analysis with log-rank test and by multivariate Cox's regression analysis. A value of $P < 0.05$ (two-tailed) was considered to indicate significance. All calculations were performed with SPSS version 15.0J (SPSS, Chicago, IL, USA).

RESULTS

Baseline characteristics in patients treated with interferon therapy

THE BASELINE CLINICAL features of the enrolled patients are shown in Table 1. The mean age of the patients was 55.8 ± 10.9 years, and 64% of the total cases were male. Two hundred and sixty-one patients (73%) were infected with HCV genotype 1 and had a viral load of more than 10^5 IU/ml. Liver biopsy was done for 320 cases and the ratio of patients with severe fibrosis (F3–4) diagnosed by the HAI score was more than 31%. The mean platelet count was 14.8 ± 5.1 × $10^4/\mu l$, and the ALT level was 96.0 ± 62.6 IU/L. A sustained virological response (SVR) was achieved by 139 patients (34%) by combination therapy of IFN-α-2b plus ribavirin. According to an intent-to-treat analysis, 20% (51/261) of patients with HCV genotype 1 and a high viral load (≧ 100KIU/mL) achieved SVR by the combination therapy, whereas 75% (73/97) of the patients with HCV genotype 2 or a low load showed SVR. The median observation period for all patients was 36.5 ± 14.8 months with a range of 6 to 62 months from the end-point of IFN treatment.

Table 1 Baseline characteristics in patients treated with interferon therapy

	All cases
Number of patients	403
Age	55.8 ± 10.9
Gender (male/female)	257/145
Genotype and viral load (1H/non-1H)	261/97
Fibrosis (F0/1/2/3/4)	15/149/56/92/8
WBC ($/\mu l$)	5113 ± 1487
Platelet ($\times 10^4/\mu l$)	14.8 ± 5.1
ALT (IU/l)	96.0 ± 62.6
IFN effect (SVR/TR/NR/cessation)	139/109/110/45

Data are number of patients, mean ± standard deviation. Fibrosis stage is evaluated on a scale from 0 to 4 according to METAVIR's histological score. 1H, Genotype 1 and high viral load; non-1H, all except for 1H; ALT, alanine aminotransferase; IFN, interferon; NR, no response; SVR, sustained virological response; TR, transient response; WBC, white blood cells.

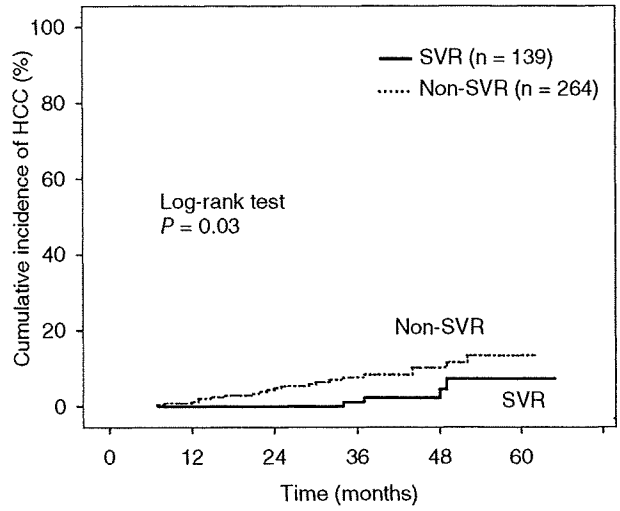

Figure 1 Cumulative incidence of development of hepatocellular carcinoma (HCC) according to treatment effect: (—) sustained virological response; (.....) non-sustained virological response.

Cumulative incidence of development of HCC according to the treatment effect (SVR vs. non-SVR)

Figure 1 shows the Kaplan–Meier estimates of the cumulative HCC incidence according to the treatment effect (SVR vs. non-SVR). Twenty-five (6%) of the 403 enrolled patients developed HCC; four (2.9%) of the SVR group and 21 (8.0%) of the non-SVR group. The cumulative incidence rate of HCC was significantly lower in patients of the SVR group than in those of the non-SVR group ($P = 0.03$).

Cumulative incidence of HCC development according to the treatment effect (SVR vs. TR vs. NR vs. cessation)

Figure 2 shows the Kaplan–Meier estimates of the cumulative HCC incidence according to the treatment effect (SVR vs. TR vs. NR vs. cessation). Five patients (4.6%) of the TR group, nine (8.2%) of the NR group and seven (15.6 %) of the cessation group developed

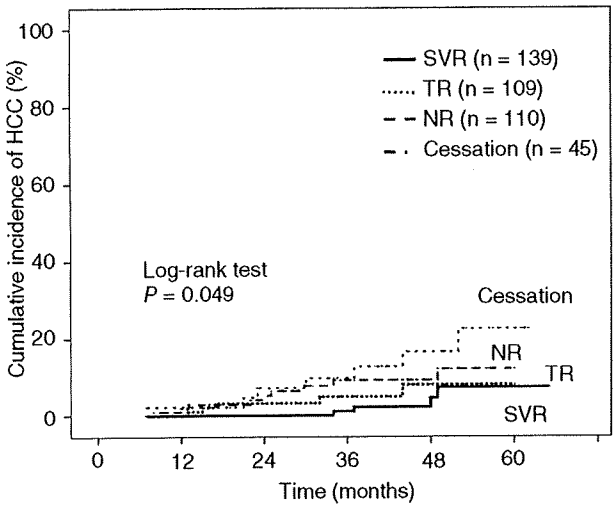

Figure 2 Cumulative incidence of hepatocellular carcinoma (HCC) development according to treatment effect: (—) sustained virological response; (.....) transient response group; (- -) no response; (-) cessation.

HCC. There was no significant difference in the cumulative incidence of HCC between the TR and NR groups ($P = 0.394$). In contrast, the cumulative incidence rate of HCC was significantly lower in patients of the SVR group than in those of the NR group ($P = 0.05$). These results indicate that treatment of the TR group with IFN-α-2b plus ribavirin therapy did not reduce HCC development when compared to the NR group.

Risk factors for cumulative incidence of HCC development

Univariate analysis with the log-rank test showed that the following were significant risk factors for the development of HCC; older age (> 65 years) ($P = 0.01$), severe fibrosis ($P = 0.006$), high platelet count ($> 14 \times 10^4/\mu l$) ($P = 0.017$) and non-SVR ($P = 0.03$). Stepwise multivariate analyses of these four variables were performed for all patients treated with combination therapy of IFN-α-2b plus ribavirin by Cox's regression analysis, as shown in Table 2. The analysis indicated the following factors as independent significant risk factors related to the development of HCC: older age (risk ratio, 3.23; 95% CI, 1.37–8.56; $P = 0.006$), fibrosis staging (risk ratio, 1.69; 95% CI, 1.04–2.67; $P = 0.033$) and non-SVR to IFN therapy (risk ratio, 3.57; 95% CI, 1.04–12.36; $P = 0.044$).

Cumulative incidence of HCC development according to average serum ALT levels after combination therapy

The average serum ALT levels in 134 patients (96.4%) of the SVR group were < 40 IU/L after completion of the combination therapy, while 63 patients (24.4%) of the non-SVR group showed serum ALT levels of ≧ 40 IU/L. Figure 3 shows Kaplan–Meier estimates of the cumulative HCC incidence according to the average serum ALT levels after combination therapy. The cumulative incidence rate of HCC was significantly lower in patients with average serum ALT levels of < 40 IU/L than with average serum ALT levels of ≧ 40 IU/L ($P = 0.021$).

Cumulative incidence of HCC development according to the treatment effect (SVR vs. non-SVR) in patients showing less than 40 IU/L average ALT levels after the combination therapy

Figure 4 shows Kaplan–Meier estimates of the cumulative HCC incidence according to the treatment effect (SVR vs. non-SVR) in patients who showed less than 40 IU/L average ALT levels after the combination therapy. There was no significant difference in the cumulative incidence rate of HCC between the SVR and non-SVR groups ($P = 0.37$).

Table 2 Risk factors for cumulative incidence of HCC development

Variable	Category	Risk ratio	*P* value	95% CI
Gender	male	1		
	female	0.34	0.053	0.11–1.01
Age (years)	65 <	1		
	65 ≥	3.23	0.006	1.37–8.56
Fibrosis	F0/1/2/3/4	1.69	0.033	1.04–2.67
IFN therapy	Non-SVR	1		
	SVR	0.28	0.044	1.04–12.36

CI, confidence interval; IFN, interferon; SVR, sustained virological response.

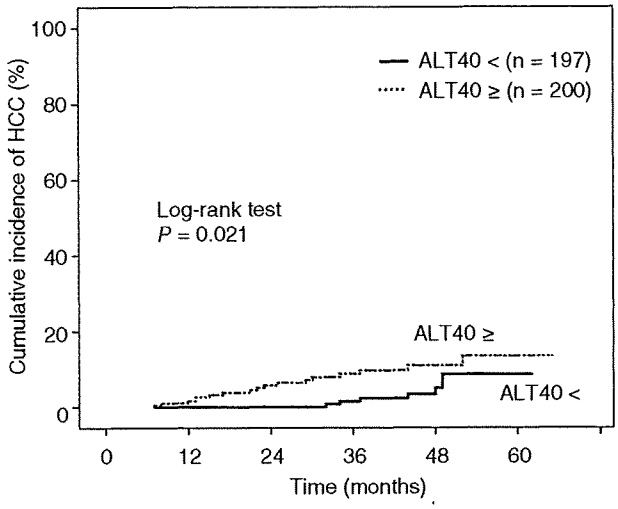

Figure 3 Cumulative incidence of HCC development according to average alanine aminotransferase (ALT) levels after the combination therapy. (—) ALT < 40 IU/ml; (.....) ALT > 40 IU/ml.

DISCUSSION

COMBINATION THERAPIES USING IFN-α-2b or Peg-IFN plus ribavirin have been proven to be more effective in treating for HCV infection than IFN monotherapy.[15–17] However, it has not been accurately evaluated whether the combination therapies using IFN-α-2b or Peg-IFN plus ribavirin could reduce the development of HCC, and what the risk factors of HCC incidence were in patients infected with HCV. In this study, we retrospectively examined the incidence of HCC with IFN-α-2b plus ribavirin therapy to clarify the indicators of combination therapy for reducing HCC in patients infected with HCV. We also evaluated whether or not SVR or continuous normalization of ALT levels could reduce the risk of development of HCC.

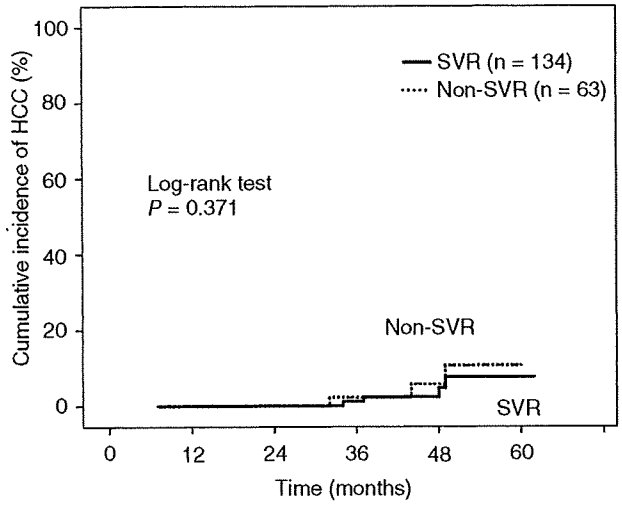

Figure 4 Cumulative incidence of hepatocellular carcinoma (HCC) development according to the treatment effect in patients who showed less than 40 IU/L average alanine aminotransferase (ALT) levels after the combination therapy. (—) Sustained virological response; (.....) non-sustained virological response.

Previous studies have demonstrated that IFN monotherapy has a preventive effect on the development of HCC, especially in patients with SVR.[12–14] In this study, using the combination of IFN-α-2b plus ribavirin, we obtained almost the same result for the SVR group treated with IFN-α-2b plus ribavirin therapy, which showed a significantly lower possibility of HCC development over a long-term period when compared with the non-SVR group. In contrast, we found no difference in the cumulative incidence of HCC between the TR and NR groups, while Kasahara *et al.* reported that the cumulative incidence of HCC in patients who achieved TR by IFN monotherapy was significantly lower than those with NR.[13] Recent reports have demonstrated that the combination therapy of IFN-α-2b plus ribavirin is able to induce a SVR in a significant proportion of patients with IFN monotherapy-resistant chronic hepatitis C,[19,20] suggesting that a viral relapse after IFN therapy is efficiently suppressed by combination with ribavirin. Since the combination therapy was a more effective treatment for HCV infection than IFN monotherapy[15–17] and there are fewer TR patients with combination therapy than with monotherapy, we speculate that not all, but quite a few patients of the TR group given IFN monotherapy corresponded to the SVR group given the combination therapy, and that the TR group given the combination therapy might have been included in the NR group of IFN monotherapy. This would mean that the "TR group given combination therapy" should be distinguished from the "TR group given IFN monotherapy", and might explain why the results of this study were inconsistent with previous reports of the cumulative incidence of HCC in the TR group given IFN monotherapy being significantly lower than those with NR.[13]

The Kaplan–Meier method showed that older age (> 65 years), severe fibrosis (F2–4), high platelet count ($> 14 \times 10^4$) and non-SVR were significantly associated with the development of HCC. The Cox's regression analysis indicated that older age, fibrosis staging and non-SVR to IFN therapy were significant risk factors related to the development of HCC. These results were

almost comparable with those of previous reports using IFN monotherapy[12–14,21] and IFN plus ribavirin combination therapy,[22–24] suggesting that the factors associated with the development of HCC are common among these treatments and that patients of older age, with advanced fibrosis and showing non-SVR to IFN therapy should be followed up carefully for longer periods, even if IFN therapy could be performed completely. In addition, four of the SVR group patients developed HCC at more than 6 months after the treatment, which means these patients need careful follow-up even if SVR has been achieved.[25]

The incidence of HCC has been reported to be lower in patients with normal ALT levels, even if serum HCV-RNA was positive 6 or 12 months after IFN monotherapy, when compared to those without a biochemical response,[13,26,27] suggesting that the aim of IFN therapy for patients infected with HCV should be not only HCV eradication, but also the achievement of a biochemical response in order to reduce the incidence of HCC. In this study, we divided the patients into two groups, one with persistently normal serum ALT levels and the other with elevated serum ALT levels based on "the average serum ALT levels" after completion of IFN therapy. We then evaluated the cumulative HCC incidence of each group using the Kaplan–Meier estimation. Our data showed that patients with continuous normalization of ALT levels have a lower possibility of HCC development than those showing elevated ALT after the combination therapy, suggesting that continuous normalization of ALT levels after the combination therapy is an important factor for reducing HCC development. Interestingly, based on the Kaplan–Meier estimates of the cumulative HCC incidence according to the treatment effect in patients who showed less than 40 IU/L average ALT levels after the combination therapy, we found no difference in HCC incidence rates between the SVR group and non-SVR group. Figure 1 shows that the combination therapy is strongly associated with a reduced incidence of HCC in the patients who attain SVR, which seems to be a means for achieving normalization of serum ALT levels in HCV patients. However, it was also shown that, even in the non-SVR group, patients with persistently normal serum ALT levels achieved a reduced risk of HCC development. Taken together, our aim of treatment for patients infected with HCV is to primarily completely eradicate HCV. Next, for the non-SVR group patients, we would speculate that maintaining normalization of ALT levels by some other treatments may prevent HCC development in HCV-infected patients with abnormal serum ALT levels even if SVR is not achieved. Other treatments should be used to decrease serum ALT levels to below the upper limit of the normal range. Hopefully, the new treatments such as those with protease inhibitors can be helpful for these patients.[28]

Although IFN monotherapy in CHC patients has been demonstrated to be associated with reducing the incidence of HCC, especially in patients who attain SVR,[12–14] what actually occurs in IFN plus ribavirin combination therapy has not been clarified and the indicator for reducing HCC in patients infected with HCV has not been defined. We showed that this combination therapy could reduce the incidence of HCC and that older age, severe fibrosis and non-SVR were risk factors for HCC development. This therapy can increase the SVR patient ratio, and SVR or continuous normalization of ALT levels after combination therapy using IFN-α-2b plus ribavirin reduce the incidence of HCC in patients with HCV infection. Therefore, this therapy can not only avert the advance of the disease toward liver cirrhosis, but also decrease the risk of HCC. IFN plus ribavirin combination therapy is beneficial for HCV patients from both aspects. In conclusion, the present study shows that the attainment of SVR or continuous normalization of serum ALT levels induced by the combination therapy has a significantly beneficial effect on the clinical course of HCV patients by decreasing the incidence of HCC.

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Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alpha-2b plus ribavirin

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SUMMARY. The impact of ribavirin exposure on virologic relapse remains controversial in combination therapy with pegylated interferon (Peg-IFN) and ribavirin for patients with chronic hepatitis C (CH-C) genotype 1. The present study was conducted to investigate this. Nine hundred and eighty-four patients with CH-C genotype 1 were enrolled. The drug exposure of each medication was calculated by averaging the dose actually taken. For the 472 patients who were HCV RNA negative at week 24 and week 48, multivariate logistic regression analysis showed that the degree of fibrosis ($P = 0.002$), the timing of HCV RNA negativiation ($P < 0.001$) and the mean doses of ribavirin ($P < 0.001$) were significantly associated with relapse, but those of Peg-IFN were not. Stepwise reduction of the ribavirin dose was associated with a stepwise increase in relapse rate from 11% to 60%. For patients with complete early virologic response (c-EVR) defined as HCV RNA negativity at week 12, only 4% relapse was found in patients given ≥12 mg/kg/day of ribavirin and ribavirin exposure affected the relapse even after treatment week 12, while Peg-IFN could be reduced to 0.6 μg/kg/week after week 12 without the increase of relapse rate. Ribavirin showed dose-dependent correlation with the relapse. Maintaining as high a ribavirin dose as possible (≥12 mg/kg/day) during the full treatment period can lead to suppression of the relapse in HCV genotype 1 patients responding to Peg-IFN alpha-2b plus ribavirin, especially in c-EVR patients.

Keywords: chronic hepatitis C, drug exposure, pegylated interferon plus ribavirin, virologic relapse.

INTRODUCTION

Combination therapy of pegylated interferon (Peg-IFN) plus ribavirin is very effective for patients with chronic hepatitis C (CH-C). However, sustained virologic response (SVR) in current therapy occurs in only 40–50% of patients with hepatitis C virus (HCV) genotype 1 [1–4]. Also, SVR is reduced in patients with genotype 1 who require reduction of either Peg-IFN or ribavirin, although dose reduction has little influence on SVR in those with genotype 2 or 3 [1–3,5,6]. Therefore, it is important to clarify the degree to which these medications can be reduced without adversely affecting SVR in patients with CH-C genotype 1.

In an early report on the relationship between drug exposure and antiviral effect in patients with CH-C genotype 1, patients who received ≥80% of their total planned cumulative doses of Peg-IFN and ribavirin for ≥80% of the scheduled duration of therapy had an SVR of 51% compared with only 34% for patients who received lesser amounts of one or both

Abbreviations: CH-C, chronic hepatitis C; c-EVR, complete early virologic response; ETR, end-of-treatment virologic response; Hb, haemoglobin; HCV, hepatitis C virus; IFN, interferon; LVR, late virologic response; Peg-IFN, pegylated interferon; PP, per protocol; Plt, platelet; RVR, rapid virologic response; SVR, sustained virologic response; VR, virologic response; WBC, white blood cell.

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medications [7]. On the other hand, Shiffman *et al.* [8] recently reported that reducing ribavirin did not affect SVR as long as the dose of Peg-IFN was maintained, while reducing the Peg-IFN dose significantly reduced SVR. The results of these observations are consistent with respect to the effect of Peg-IFN on SVR. However, what is controversial is whether or not reducing the ribavirin dose affects the antiviral effect.

Adding ribavirin to either interferon (IFN) or Peg-IFN monotherapy for patients with CH-C genotype 1 has been shown to reduce the relapse rate in large randomized trials [1,2,9–11]. In detail, adding ribavirin to the usual IFN monotherapy (3MIU, three-times-weekly) in 48-week treatment raised the end-of-treatment virologic response (ETR) rate from approximately 30% to 50% and also lowered the relapse rate from mid-40% to approximately 20% [9–11]. Lindsay *et al.* [12] reported that Peg-IFN alpha-2b (Peg-IFN α-2b) monotherapy (1.5 μg/kg, once-weekly), as compared with IFN alpha-2b (IFNα-2b) monotherapy (3MIU, three-times-weekly), improved ETR (49% vs. 24%), but not the relapse rate (53% vs. 50%). In the trial of Peg-IFN alpha-2a (Peg-IFN α-2a) plus ribavirin *vs* IFN α-2b plus ribavirin or Peg-IFN α-2a alone, the ETR rates were 69%, 52% and 59%, and the relapse rates were 19%, 15% and 52%, respectively [2]. These findings from large-scale trials indicate that the main role of ribavirin is to reduce relapse in the combination therapy with Peg-IFN, although ribavirin affects both ETR and relapse in combination therapy with the usual IFN.

In the present study, we tried to determine whether or not dose reduction of ribavirin (or Peg-IFN) has an effect on virologic relapse in Peg-IFN plus ribavirin treatment for patients with CH-C genotype 1.

PATIENTS AND METHODS

Patients

This study was a multicentre trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 984 patients with CH-C were enrolled in this study between December 2004 and September 2006, and treated with a combination of Peg-IFN α-2b plus ribavirin. The baseline characteristics of the patients are shown in Table 1. All patients were Japanese infected with HCV genotype 1 and a viral load of more than 10^5 IU/mL. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcohol liver disease, autoimmune hepatitis), coinfection with hepatitis B or anti-human immunodeficiency virus. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

Treatment

All patients received Peg-IFN α-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (REBETOL; Schering-Plough) for the duration of the study of 48 weeks. As a starting dose, Peg-IFN α-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg) according to the manufacturer's drug information available in Japan.

Table 1 Baseline characteristics of patients and drug doses at start of treatment

Factor	Mean ± SD or *n*
n	984
Age (years)	56.3 ± 10.1
Sex (male/female)	555/429
Body weight (kg)	61.8 ± 11.5
History of IFN treatment Naïve/experienced (relapser/nonresponder)*	575/409 (160/182)
White blood cells (/mm^3)	5052 ± 1550
Neutrophils (/mm^3)	2577 ± 1092
Red blood cells (×10^4/mm^3)	442 ± 47
Haemoglobin (g/dL)	14.1 ± 1.4
Platelets (×10^4/mm^3)	15.9 ± 5.5
AST (IU/L)	66 ± 45
ALT (IU/L)	79 ± 61
Serum HCV RNA (kIU/mL)†	1600
Histology (METAVIR)‡	
Fibrosis: 0/1/2/3/4	49/314/197/105/18
Activity: 0/1/2/3	23/329/304/27
Peg-IFN dose (μg/kg/week)	1.45 ± 0.17
Ribavirin dose (mg/kg/day)	11.4 ± 1.6

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus. *Viral response to previous treatment was unknown in 57 patients, and 10 patients had discontinued treatment. †Data shown are median values. ‡301 missing.

Dose reduction and discontinuance

Dose modification also followed, as a rule, the manufacturer's drug information according to the intensity of the haematologic adverse effects. The dose of Peg-IFN α-2b was reduced to 50% of the assigned dose when the white blood cell (WBC) count was below 1500/mm^3, the neutrophil count below 750/mm^3 or the platelet (Plt) count below 8×10^4/mm^3, and was discontinued when the WBC count was below 1000/mm^3, the neutrophil count below 500/mm^3 or the Plt count below 5×10^4/mm^3. Ribavirin was also reduced from 1000 mg to 600 mg, 800 mg to 600 mg, or 600 mg to 400 mg when the haemoglobin (Hb)

concentration decreased to less than 10 g/dL, and was discontinued when the Hb concentration decreased to less than 8.5 g/dL. Both Peg-IFN α-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. No ferric medicine or haematopoietic growth factors, such as epoetin alpha, or granulocyte-macrophage colony stimulating factor, were administered.

Virologic assessment and definition of virologic response

Serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 kIU/mL; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analysed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/mL; Roche Diagnostics). Complete early virologic response (c-EVR) was defined as the absence of detectable serum HCV RNA at treatment week 12, the late virologic response (LVR) was defined as undetectable serum HCV RNA for the first time at 13–24 weeks of treatment, and the virologic response (VR) was defined as HCV RNA negativity at week 24 and week 48. SVR was defined as the absence of detectable serum HCV RNA at week 72. Patients with less than a 2-log decrease in HCV RNA level at treatment week 12 compared with the baseline had to stop treatment according to the protocol and were regarded as nonresponders. All patients with detectable serum HCV RNA at treatment week 24 were also considered to be nonresponders and were excluded from further treatment.

Assessment of drug exposure

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

Evaluation of impact of drug exposure on virologic relapse

We evaluated the relationship between the drug exposure of both drugs and relapse by two different methods, univariate and multivariate analysis for relapse and independent evaluation of both drugs for relapse according to the degree of drug exposure. The former was performed with the factors of mean administration doses of both drugs, including the factors at baseline and the timing of HCV RNA negativiation. The latter was examined by classifying Peg-IFN α-2b exposure into five categories (up to 0.6 μg/kg; from 0.6 to less than 0.9 μg/kg; from 0.9 to less than 1.2 μg/kg; from 1.2 to less than 1.5 μg/kg; from 1.5 μg/kg) and ribavirin exposure into five categories (up to 6 mg/kg; from 6 to less than 8 mg/kg; from 8 to less than 10 mg/kg; from 10 to less than 12 mg/kg; from 12 mg/kg).

Statistical analysis

Baseline data are expressed as means ± SD or median values. Virologic response was evaluated using per protocol (PP) analysis. To analyse the difference between baseline data including drug exposure and virologic response, univariate analysis using the Mann–Whitney *U*-test or chi-square test and multivariate analysis using logistic regression analysis were performed. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. A two-tailed P value <0.05 was considered significant. The analysis was conducted with SPSS version 15.0J (SPSS Inc., Chicago, IL, USA).

RESULTS

Progress of patients and dose reduction of Peg-IFN α-2b and ribavirin

The progress of patients in this study is shown in Fig. 1. Of the 984 patients, 903 completed 12 weeks of treatment and the c-EVR rate was 49% (445/903), based on PP study. To analyse for relapse, 472 patients with VR were assessed, with 178 (38%) showing Peg-IFN dose reduction without discontinuation and 246 (52%) with ribavirin dose reduction without discontinuation during the full (48 weeks) treatment period. The relapse rate was 26% (125/472) in the patients with undetectable HCV RNA level at the end of treatment. No difference was found in relapse rates between the IFN naïve patients and IFN experienced patients (IFN naïve: 25%, 72/287 *vs* IFN experienced: 29%, 53/185, $P = 0.40$). The SVR rate was 43% (347/812) in the PP study.

Impact of drug exposure during 0–48 weeks on relapse among patients with VR

The mean dose of Peg-IFN α-2b actually taken during the full treatment period by each patient was 1.32 μg/kg/week (range, 0.49–2.16 μg/kg/week; median, 1.38 μg/kg/week) and that of ribavirin was 9.8 mg/kg/day (range, 3.3–16.2 mg/kg/day; median, 10.1 mg/kg/day) in patients with VR.

The result of univariate analysis for relapse among the patients with VR is shown in Table 2a. The degree of fibrosis, the timing of HCV RNA negativiation, Plt value and the mean doses of ribavirin were factors significantly associated with relapse, but those of Peg-IFN α-2b were not. The mean dose of ribavirin as well as the degree of fibrosis and the timing of HCV RNA negativiation was selected as a significant independent factor by multivariate logistic regression analysis (Table 2b).

Next, we analysed the relationship of the relapse rate and the mean ribavirin dose. The overall relapse rate among patients with VR was 26% (125/472). The