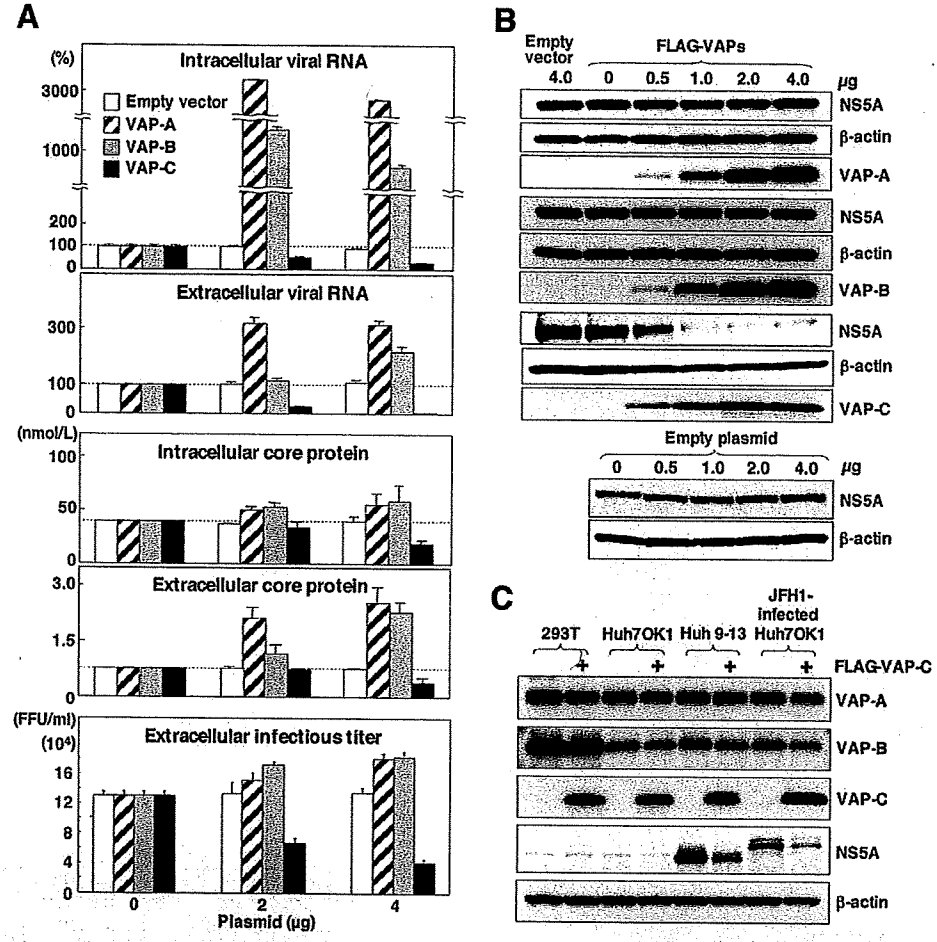

FIG. 5. VAP-C impairs HCV propagation but does not affect endogenous expression of VAP-A or VAP-B. Huh7OK1 cells transfected with 0 to 4 μg of plasmid encoding the FLAG-tagged VAP-A, VAP-B, or VAP-C or empty vector were infected with strain JFH1 at an MOI of 0.05 at 14 h posttransfection and then harvested at 96 h postinfection. (A) The intracellular and extracellular expression levels of viral RNA (top) and core protein (middle) were determined by real-time PCR and ELISA, respectively. Infectious viral titers in the culture supernatants were determined by focus-forming assay (bottom). Data in this panel are shown as the means ± standard deviations. (B) The expression levels of NS5A, β-actin, VAP-A, VAP-B, and VAP-C were determined by immunoblotting using anti-NS5A, anti-β-actin, or anti-FLAG tag antibody. (C) The embryonic kidney cell line (293T), the cured hepatoma cell line (Huh7OK1), and the replicon cell line (Huh 9-13) were transfected with 2 μg of the plasmid encoding FLAG-tagged VAP-C (+) or empty plasmid. In the case of the infected cells, Huh7OK1 cells were infected with strain JFH1 at an MOI of 0.05, reseeded onto the tissue culture plate at 96 h postinfection, and then transfected with 2 μg of the plasmids. These cells were harvested at 36 h posttransfection and examined by immunoblotting using antibodies to VAP-A, VAP-B, FLAG, NS5A, and β-actin. The data in each panel are representative of the results of three independent experiments.

aggregations of ER in culture cells and to sequester the wild-type protein into ubiquitinated inclusions (29, 37). To examine the effects on the replication of HCV of the P56S mutation in VAPs, FLAG-tagged VAP mutants were expressed in the HCV replicon cells. RNA replication of the subgenomic replicon in Huh 9-13 cells was impaired by the expression of each of the mutant VAPs (Fig. 7A, left). The expression of NS5A in the replicon cells was decreased by the expression of the mutant VAPs in a dose-dependent manner (Fig. 7A, right). Next, to examine the effect of the expression of the P56S VAP mutants on HCV propagation, Huh7OK1 cells expressing the FLAG-tagged VAP mutants were infected with JFH1 virus. The production of intracellular and extracellular viral RNA at 96 h postinfection was decreased by the expression of the P56S mutation in VAPs (Fig. 7B). Although the results of a previous study indicated that the expression of the P56S mutant of VAP-B but not that of VAP-A induced a large aggregation of ER in hamster ovary cell line CHO (37), the P56S mutants of VAP-A and VAP-B but not that of VAP-C exhibited accumulation of membranous aggregates in Huh 9-13 cells (Fig. 7C). These results indicate that the P56S mutation in both VAP-B and VAP-A induces aggregation of ER in human hepatoma cells, which in turn leads to the suppression of HCV propagation.

DISCUSSION

The replication of HCV has been shown to require several host proteins, including VAP-A/VAP-B (6, 9, 44), FBL2 (46), FKBP8 (34), hB-ind1 (40), Hsp90 (28, 34, 45), and cyclophilins

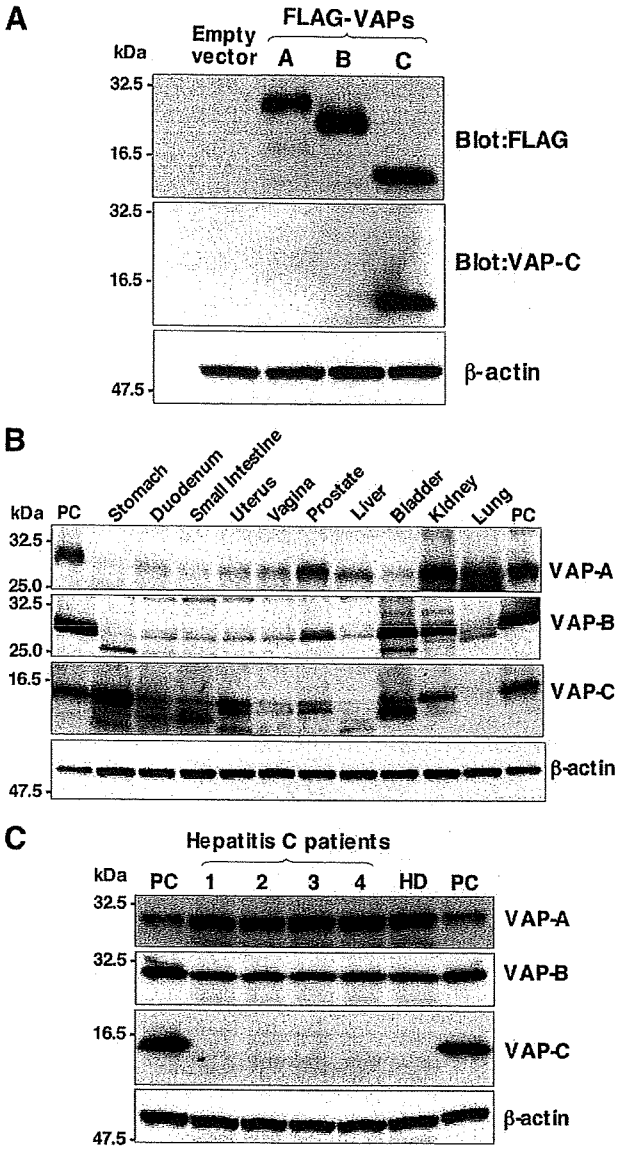

FIG. 6. Distribution of VAPs in human tissues. (A) Anti-VAP-C antibody specifically recognizes VAP-C. Human embryonic kidney 293T cells transfected with expression plasmid encoding FLAG-tagged VAP-A, VAP-B, or VAP-C or empty vector were harvested at 48 h posttransfection and examined by immunoblotting using anti-FLAG tag, anti-VAP-C, and anti-β-actin antibodies. (B) The premade human tissue lysates "Protein medleys" (20 μg each; Clonetech) were examined by immunoblotting using antibodies against VAP-A, VAP-B, VAP-C, or β-actin. (C) Expression of VAP family proteins in human liver tissues. Liver samples obtained from four hepatitis C patients (1 to 4) and one healthy donor (HD) were examined by immunoblotting as described above. The data in each panel are representative of the results of three independent experiments. PC indicates 293T cells transfected with expression plasmid encoding VAP-A, VAP-B, and VAP-C.

(15, 48). VAP-A has been detected in a detergent-resistant membrane fraction that was shown to be capable of replicating HCV RNA in vitro, and the interaction of VAP-A with NS5A is required for the efficient replication of HCV genomic RNA

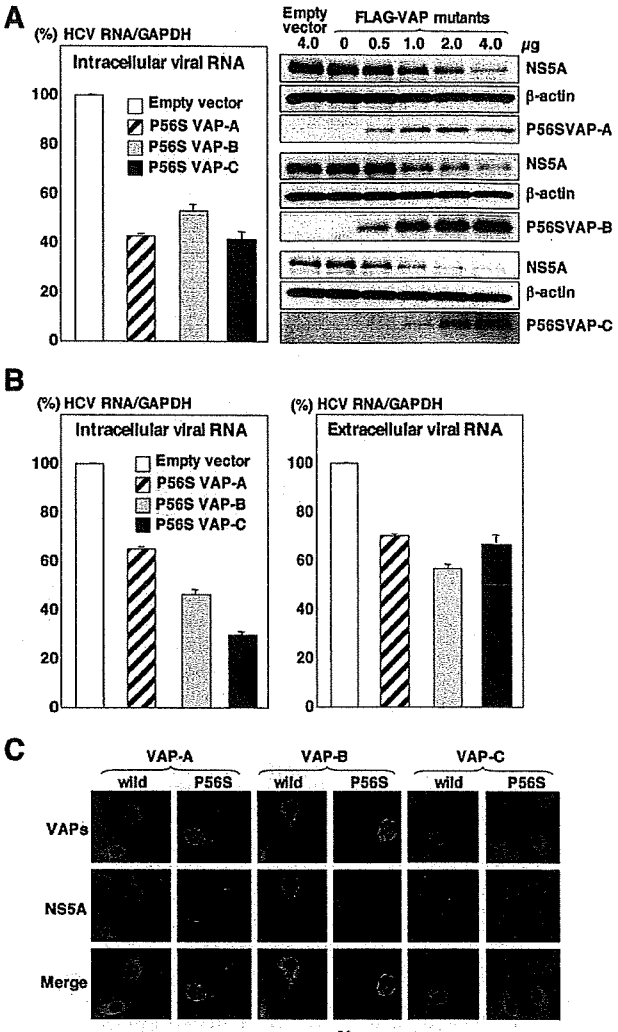

FIG. 7. Substitution of Ser for Pro[56] in VAPs leads to suppression of HCV replication. (A) Left: Huh 9-13 cells were transfected with 4 μg of the expression plasmids encoding FLAG-tagged P56S VAP mutants or empty vector, and the level of intracellular HCV RNA was determined at 72 h posttransfection by real-time PCR after normalization with GAPDH mRNA. The value for HCV RNA at 0 h posttransfection in the cell line transfected with the empty plasmid is represented as 100%. Data in this panel are shown as the means ± standard deviations. Right: Huh 9-13 cells were transfected with 0 to 4 μg of the FLAG-tagged P56S VAP mutant plasmids or empty vector, and the levels of expression of NS5A, β-actin, and the mutant VAPs were determined by immunoblotting at 72 h posttransfection. The data in each panel are representative of the results of three independent experiments. (B) Huh7OK1 cells transfected with 4 μg of the expression plasmids encoding FLAG-tagged P56S VAP mutants or empty vector were infected with strain JFH1 at an MOI of 0.05 at 14 h posttransfection, and the intracellular (left) and extracellular (right) expression levels of viral RNA were determined by real-time PCR after normalization with GAPDH mRNA at 96 h postinfection. Data in this panel are shown as the means ± standard deviations. (C) Levels of expression of wild-type VAPs, P56S mutant VAPs, and NS5A in Huh 9-13 cells at 72 h after transfection with the expression plasmids encoding FLAG-tagged VAPs or P56S VAP mutants were determined by immunofluorescent assay. The data in each panel are representative of the results of three independent experiments.

(2, 7) and is modulated by the phosphorylation of NS5A (4, 6). VAP-B also participates in HCV replication through the formation of homo- and/or heterodimers with VAP-A (9). VAP-A and VAP-B form hetero- and homodimers through their TM regions and interact with NS5A and NS5B through the coiled-coil domain and MSP domain, respectively (9, 44). VAP-C is a splicing variant of VAP-B, consisting of the N-terminal half of VAP-B and the subtype-specific amino acid residues generated by the frameshift. However, the biological significance of VAP-C in the life cycle of HCV has not been determined. In this study, we have demonstrated that VAP-C is capable of binding to HCV NS5B but not to NS5A, VAP-A, and VAP-B due to the lack of the coiled-coil and TM regions. The expression of VAP-C inhibited the interaction of VAP-A and VAP-B with NS5B, impaired the RNA replication and particle formation of HCV, and was barely detected in human liver cells. These results suggest that VAP-C acts as a negative regulator for HCV propagation and is partly involved in the determination of the tissue specificity of HCV replication.

Overexpression of VAP-A but not of VAP-B inhibited the incorporation of the vesicular stomatitis virus (VSV) envelope glycoprotein G (VSV-G) into ER vesicles in CHO cells, resulting in impairment of membrane protein transport from the ER to the Golgi apparatus (37). VAP-B was shown to be involved in the unfolded protein response, which is an ER reaction to suppress the accumulation of misfolded proteins, and the expression of the P56S VAP-B mutant was suggested to nullify the unfolded protein response induced by VAP-B, to produce a large aggregation of ER, and to be involved in the development of ALS (17, 37). These data suggest that VAP-A and VAP-B possess different physiological functions; however, the contributions of the proteins to the life cycle of HCV have not been characterized. The expression of VAP-B but not of VAP-A resulted in an enhancement of the replication of the subgenomic HCV RNA of the genotype 1b strain Con1, whereas the expression of either VAP-A or VAP-B clearly enhanced viral RNA replication in cells infected with the genotype 2a strain JFH1 virus, suggesting that the contributions of VAP-A and VAP-B to viral RNA replication might differ among the genotypes of HCV. The expression of VAP-B or VAP-A enhanced RNA replication in the HCV replicon cells and the secretion of viral RNA, core protein, and infectious particles into the culture supernatants of Huh7OK1 cells infected with JFH1 virus, whereas the expression of these proteins had no effect on the expression of NS5A or on IRES-dependent translation. Thus, further studies will be needed to clarify the molecular mechanisms underlying the posttranslational enhancement of HCV production by the expression of VAP-A and VAP-B. In contrast to the expression of VAP-A and VAP-B, the expression of VAP-C clearly suppressed the RNA replication of both the genotype 1b RNA replicon cells and the genotype 2a strain JFH1 virus, by which both the expression of the viral proteins and the viral particle production were drastically impaired. Furthermore, the expression of the P56S mutants of VAP-A and VAP-B reduced RNA replication in HCV replicon cells and propagation of the JFH1 virus, probably due to the induction of aggregation of the ER. The reason why ER aggregation was induced by the expression of the P56S VAP-A mutant in Huh7 cells but not in CHO cells (17, 37) is not known at the moment.

The phosphorylation state of NS5A was suggested to control the interaction between VAP-A and NS5A and the replication efficiency of HCV RNA (6). Introduction of the adaptive mutations originally identified in the genotype 1b strain Con1 into NS5A of genotype 1a suppressed the hyperphosphorylation of NS5A, potentiated interaction with VAP-A, and enhanced the RNA replication (6). However, we have previously shown that NS5A of genotype 1a could bind to VAP-A and VAP-B at a level similar to that of genotype 1b despite the adaptive mutations (9). In this study, overexpression of each of the VAP proteins exhibited no effect on the mobility of NS5A in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3 and 5), suggesting that there is no correlation between the VAP-dependent regulation of HCV propagation and the phosphorylation state of NS5A.

FKBP8 exhibits peptidyl prolyl *cis-trans* isomerase activity and interacts with NS5A and Hsp90 through the tetratricopeptide repeat (TPR) domain, and these interactions are suggested to be involved in the correct folding of the HCV replication complex (34). Treatment of cells with inhibitors of the ATPase activity of Hsp90, such as geldanamycin and its derivatives, impairs the RNA replication and particle production of HCV (28, 34, 45). The MSP domain of VAP-A was shown to interact with the TPR1 protein, which has a TPR domain and forms the chaperone complex with Hsp90 (22). Knockdown of the TPR1 protein or treatment with Hsp90 inhibitors in mammalian cells has been shown to inhibit the transport of VSV-G, leading to accumulation of the glycoprotein in the Golgi apparatus (22). The VAP-A- or VAP-B-induced enhancement of virus production might be attributable to the recruitment of Hsp90 into the replication complex through the interaction with the MSP domain.

VAP-A is well known to interact through the MSP domain with a number of mammalian and yeast proteins sharing the FFAT motif, including OSBPs, ORPs (20), and CERT (10, 19), and to be involved in the regulation of biosynthesis or trafficking of sterols and lipids. HCV replication and infection have been shown to be regulated by lipid components and to be capable of being inhibited by treatment with several inhibitors targeting lipid biosynthesis (14, 18). The intracellular membranous web structure observed in HCV replicon cells was shown to be resistant to detergent treatment, suggesting that the lipid raft-like structure abundant in cholesterol and sphingolipid is generated by the replication of HCV RNA (2, 24). Therefore, it might be feasible to speculate that VAP-A and VAP-B are involved in the construction of the HCV replication complex consisting of viral proteins and host cellular lipid components and that VAP-C interrupts the VAP-A and VAP-B functions and negatively regulates HCV propagation. Although the molecular mechanisms and the biological significance remain to be clarified, the MSP domain of VAP proteins was processed in human leukocytes and secreted into human serum (43). Further studies are needed to clarify the biogenesis and biological functions of the truncated VAP proteins in the replication of HCV.

In summary, we have shown that VAP-C is capable of suppressing the RNA replication and particle production of HCV by inhibiting the binding of VAP-A and VAP-B to NS5B through the N-terminal half of its MSP domain. The clear suppression of HCV propagation by the expression of VAP-C

further suggests the possibility of developing a novel therapeutic measure to eliminate HCV by the exogenous expression of VAP-C in the hepatocytes of chronic hepatitis C patients.

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Enhanced ability of regulatory T cells in chronic hepatitis C patients with persistently normal alanine aminotransferase levels than those with active hepatitis

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SUMMARY. In hepatitis C virus (HCV) infection, the Th1-type immune response is involved in liver injury. A predominance of immunosuppressive regulatory T cells (Treg) is hypothesized in patients with persistently normal alanine aminotransferase (PNALT). Our aim was to clarify the role of Treg in the pathogenesis of PNALT. Fifteen chronically HCV-infected patients with PNALT, 21 with elevated ALT (CH) and 19 healthy subjects (HS) were enrolled. We determined naturally-occurring Treg (N-Treg) as CD4+CD25high+ FOXP3+ T cells. The expression of FOXP3 and CTLA4 in CD4+CD25high+ cells was quantified by real-time reverse transcriptase-polymerase chain reaction. Bulk or CD25-depleted CD4+ T cells cultured with HCV-NS5 loaded dendritic cells were assayed for their proliferation and cytokine release. We examined CD127–CD25–FOXP3+ cells as distinct subsets other than CD25+ N-Treg. The frequencies of N-Treg in patients were significantly higher than those in HS. The FOXP3 and CTLA4 transcripts were higher in PNALT than those in CH. The depletion of CD25+ cells enhanced HCV-specific T cell responses, showing that co-existing CD25+ cells are suppressive. Such inhibitory capacity was more potent in PNALT. The frequency of CD4+CD127–CD25–FOXP3+ cells was higher in CH than those in PNALT. Treg are more abundant in HCV-infected patients, and their suppressor ability is more potent in patients with PNALT than in those with active hepatitis.

Keywords: HCV, PNALT, regulatory T cell.

INTRODUCTION

Hepatitis C virus (HCV) causes a wide range of chronic liver diseases in infected hosts, including chronic hepatitis (CH), liver cirrhosis and hepatocellular carcinoma (HCC). One of the critical determinants promoting the development of HCV-induced liver disease is sustained liver inflammation, explaining the therapeutic rationale of alleviating this condition to help prevent liver cancer [1]. Among chronically infected individuals, approximately 20–30% display persistently normal serum alanine aminotransferase levels [2,3]. Although it is reported that 40–50% of them progress to the active stage of liver inflammation within 5 years of observation [4], the incidence of HCC in the remaining patients continues to be lower than in those with elevated serum ALT levels [5]. Cumulative studies have revealed that HCV is not directly cytopathic to hepatocytes. It has been demonstrated that a Th1-type or cytotoxic T lymphocyte (CTL) response is critically involved in HCV-mediated liver injury [6,7]. Therefore, it is conceivable that some suppressor mechanisms exist against Th1-type immune responses in patients with persistently normal ALT levels (PNALT), which may be distinct from those in patients with active liver inflammation.

Abbreviations: ALT, alanine aminotransferase; CH, chronic hepatitis; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HS, healthy subjects; IFN, interferon; IL, interleukin; IU, international units; MoDC, monocyte-derived dendritic cell; N-Treg, naturally occurring regulatory T cell; PNALT, persistently normal ALT; RT-PCR, reverse transcriptase-polymerase chain reaction; SLE, systemic lupus erythematosus; TGF, transforming growth factor; Treg, regulatory T cell.

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Regulatory T cells (Treg) are a unique subset of T cells with inhibitory capacity against auto-reactive T cells [8]. Substantial data have been reported about the involvement of Treg in the pathogenesis of various diseases, including autoimmune, cancer or infectious diseases [9–13]. Currently, the existence of several types of Treg has been reported [14]. Naturally occurring Treg (N-Treg) are derived from the thymic stromal environment from progenitor cells and suppress auto-reactive T cells in antigen-specific and antigen-nonspecific manner. Forkhead/winged helix transcription factor (FOXP3) is one of the specific markers of N-Treg, the expression of which is well correlated with the gain of a suppressor function [15,16]. As cells with high expression of CD25 also display FOXP3, it is generally accepted that CD25+FOXP3+ is the most reliable marker for Treg. In HCV infection, several reports have described a higher frequency of N-Treg in the periphery and the liver [17–20], suggesting their active role in HCV persistence. It has also been demonstrated that CD25+FOXP3+ regulatory cells are inducible in the periphery [21]. Owing to the lack of a specific phenotypic marker of these induced regulatory cells, referred to as adaptive Treg, their role in the pathogenesis of HCV infection has not been clearly understood. A recent study has demonstrated that the expression of interleukin (IL)-7 receptor (CD127) is downregulated in Treg to a degree that is inversely correlated with FOXP3 expression [22]. These findings offer the possibility that adaptive Treg are traceable, not all but in part, by the combination of CD127 and FOXP3 independent of CD25 expression.

In this study, our aim was to elucidate whether or not Treg are involved in the pathogenesis of PNALT patients, by comparing the frequency and function of these cell subsets with those in active hepatitis patients or healthy subjects. A distinct equilibrium was found between N-Treg and CD127–CD25–FOXP3+ T cells according to differences in liver inflammation.

MATERIALS AND METHODS

Subjects

Among chronically HCV-infected patients who had been followed at Osaka University Hospital, 15 patients with PNALT levels and 21 patients with elevated or fluctuating ALT levels (the CH group) were enrolled in this study. As controls, 19 healthy subjects (HS) who were negative for HCV and hepatitis B virus (HBV) markers were examined. The study protocol was approved by the ethical committee of Osaka University Graduate School of Medicine. At enrolment, written informed consent was obtained from each subject. In this study, PNALT patients were defined as those whose ALT levels remained within the normal range (<30 IU/mL) without any medications for more than 1 year. At enrolment, the patients were confirmed to be positive for both serum anti-HCV and HCV RNA, but were negative for other viral infections, including HBV and human immunodeficiency virus. The presence of other causes of liver disease, such as autoimmune, alcoholic and metabolic disorders was excluded by the use of laboratory and imaging analyses. Liver biopsy was carried out in some of the patients. Histological examination was performed according to the METAVIR scoring system. In all patients, a combination of repetitive biochemical tests, ultrasonography or computed tomography scans ruled out the presence of cirrhosis and liver tumours. The clinical background of the subjects are shown in Table 1.

Table 1 Baseline clinical characteristics of the patients

	Chronic hepatitis patients	Patients with PNALT	Healthy subjects*	
n	21	15	19	
Sex (M/F)	8/13	5/10	ND	NS
Age	50.6 ± 11.6	47.8 ± 12.7	ND	NS
ALT (IU/L)	88.3 ± 41.4	20.9 ± 6.9	ND	$P < 0.0001^{\dagger}$
Plt ($10^4/\mu L$)	13.5 ± 5.4	20.0 ± 3.9	ND	$P < 0.01^{\dagger}$
HCV RNA (Meq/mL)	8.6 ± 11.3	9.7 ± 7.8	ND	NS

*The background data of healthy subjects (blood donors) were not accessible owing to the confidentiality regulations of the blood centre, but their serum ALT levels were confirmed to be within the normal range. †Statistical significance was analysed by Mann–Whitney *U* test between chronic hepatitis patients and patients with PNALT. The values are expressed as mean ± SD. PNALT, persistently normal alanine aminotransferase level; ND, not determined; NS, not significant; plt, platelet count.

Frequency analyses of Treg cells

For the numerical analyses of Treg cells, heparinized venous blood was obtained from all subjects. Peripheral blood mononuclear cells were collected by density-gradient centrifugation on a Ficoll–Hypaque cushion. The cells were subsequently stained with a combination of various fluorescence-labelled anti-human mouse monoclonal antibodies for phenotypic markers. The antibodies for CD25 (clone B1.49.9) and CD4 (clone 13B8.2) were purchased from Beckman Coulter (Fullerton, CA, USA), that for CD127 (clone 40131) from R&D Systems (Minneapolis, MN, USA) and that for FOXP3-PE (clone PCH101) from eBioscience (San Diego, CA, USA), respectively. The cells were stained in phosphate-buffered saline containing 1% fetal bovine serum (FBS) with various antibodies or isotype controls for 15 min at room temperature. Intracellular staining of FOXP3 was performed using a human FOXP3 staining kit (eBioscience) according to the manufacturer's instructions. The cells were analysed by FACSCalibur (BD Biosciences, San Jose, CA, USA) and CellQuest software.

Functional analysis of CD4+CD25+ T cells in HCV-specific CD4+ T cell response

We first examined the HCV-specific CD4+ T cell response in the presence or absence of CD4+CD25+ T cells. Monocyte-derived dendritic cells (MoDC) were generated from CD14+ cells as reported previously. In brief, CD14+ cells were cultured in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS, 50 IU/mL of penicillin, 50 mg/mL of streptomycin, 2 mM of L-glutamine, 10 mM of Hepes buffer, 10 mM of nonessential amino acids in the presence of 50 ng/mL of granulocyte/macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA) and 10 ng/mL of IL-4 (PeproTech) for 7 days at 37 °C and 5% CO_2. On day 6 of the culture, MoDC were pulsed with 10 μg/mL of recombinant HCV NS5 (amino acid position: NS5B 1-544; kindly provided by Japan Tobacco, Inc., Tokyo, Japan) and cultured for 24 h. The antigen-pulsed MoDC were then cultured with autologous bulk CD4+ T cells or CD4+CD25– T cells in 96-well flat-bottom plates (Corning, NY, USA) for 5 days. Enrichment of CD4+ T cells or CD4+CD25– T cells was performed using a CD4+CD25+ Regulatory T cell Isolation kit (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. On day 6 of the co-culture, the cells were pulsed with 1 μCi of [3H]-thymidine during the last 16 h of incubation. The supernatants were collected before pulsing with [3H]-thymidine and subjected to cytokine enzyme-linked immunosorbent assay (ELISA). The incorporation of [3H]-thymidine in CD4+ T cells was measured using a β-counter (Wallac-Perkin-Elmer, Wallac, Finland).

Enzyme-linked immunosorbent assay

The concentrations of IL-10, TGF-β1 and interferon (IFN)-γ in the culture supernatants were determined by ELISA. We used matched pairs of relevant monoclonal antibodies (Endogen, Woburn, MA, USA) for IL-10 and IFN-γ, and the DuoSet ELISA development system (R&D Systems) for TGF-β1, according to the manufacturer's instructions. The detection thresholds of IL-10, TGF-β1 and IFN-γ were 10, 10 and 16 pg/mL, respectively.

Real time reverse transcriptase-polymerase chain reaction (RT-PCR)

In order to analyse the expression of FOXP3 and CTLA-4 in N-Treg, we collected CD4+CD25high T cells by using FACSAria. The purity of the isolated cells was more than 95% as determined by FACS. Total RNA was extracted from sorted CD4+CD25high T cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using the SuperScript III First-Strand synthesis system (Invitrogen, Carlsbad, CA, USA). Assays-on-demand primers and probes (PE Applied Biosystems, Foster City, CA, USA) were used to quantify FOXP3 and CTLA4 expression. The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The thermal cycling conditions for all genes were as follows: the reaction was started with a 10-min denaturing cycle at 95 °C, followed by 40 cycles of PCR performed with 15 s of denaturing at 95 °C, then 1 minute at 60 °C for annealing and extension. We identified a calibrator sample from the healthy volunteers. The expressions of molecules were given as the relative values to the calibrator samples. To standardize the amount of total RNA added to each reaction mixture, we quantified β-actin mRNA from each sample as a control of internal RNA and corrected all values with this.

Statistical analysis

Statistical analyses were performed using StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). Mann–Whitney *U*-test was used to compare differences in unpaired samples. For all analyses, a *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Peripheral N-Treg are increased in HCV-infected patients

We compared the frequency of Treg between HCV-infected patients and healthy donors. In HCV-positive individuals, they were further categorized into PNALT and CH groups according to the difference in their serum ALT levels. The clinical backgrounds of these groups were not different except for

serum ALT levels and platelet counts (Table 1). N-Treg were defined as the cells with CD4+CD25high+FOXP3+ cells. As the cut-off value between CD25high+ and CD25intermediate+ cells is a critical determinant for Treg analyses, we defined CD4+CD25high+ as the cells with CD25 levels higher than those of CD4–CD25+ cells (Fig. 1a). We first compared the frequency of CD4+FOXP3+ T cells. The frequency of FOXP3+ cells in the CD4+ T cell population in HCV-infected patients was significantly higher than those in the HS (Fig. 1b). However, no difference was observed in FOXP3+ cells between the PNALT and CH patients (Fig. 1b). The frequency of CD4+CD25high+FOXP3+ T cells in CH or PNALT patients were significantly higher than those in HS, whereas those in HCV-positive patients did not differ regardless of their ALT levels (Fig. 1c). Similar results were obtained for the frequency of CD4+CD25–FOXP3+ T cells (Fig. 1d).

Next, we examined whether or not the frequency of N-Treg is correlated with clinical parameters. Among all HCV-infected patients, no correlation was observed between the frequency of N-Treg (CD4+CD25high+ FOXP3+ T cells) and serum ALT, HCV RNA levels, age or platelet counts (data not shown). In the analyses of patients who had undergone liver biopsy, the frequency of N-Treg was not correlated with METAVIR grade/stage scores (data not shown).

The expressions of FOXP3 and CTLA4 are higher in N-Treg from PNALT patients compared with those from the CH group

FOXP3 is the master gene of Treg in the development and gaining of suppressor functions. Alternatively, CTLA4 is one of the key molecules of Treg in exerting inhibitory function. We thus evaluated FOXP3 and CTLA4 mRNA expression in sorted N-Treg (CD4+CD25high+ T cells) by means of real-time RT-PCR. The expression of FOXP3 in PNALT or CH patients was significantly higher than those in HS (Fig. 2a). Of note is the higher expression of FOXP3 in N-Treg from the PNALT group than in those from the CH group (Fig. 2a). In contrast, the expression of CTLA4 in N-Treg from the PNALT was higher than those in the CH, while it did not differ between the CH and HS groups (Fig. 2b).

CD4+CD25+ T cells from PNALT patients have more suppressive capacity in the HCV-specific CD4+ T cell response than those from CH patients

In order to compare the ability of N-Treg to inhibit the antigen-specific CD4+ T cell response, we used autologous MoDC pulsed with HCV proteins as antigen-presenting cells. We examined CD4+ T cell proliferation or cytokine

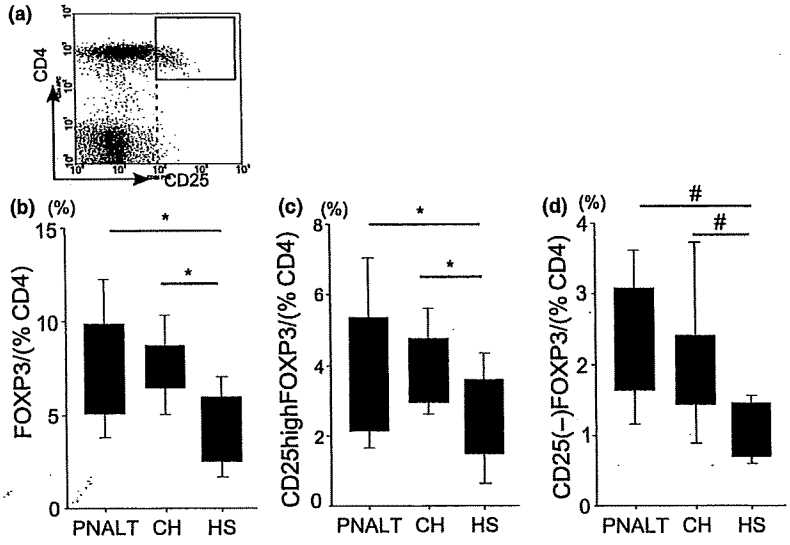

Fig. 1 Comparison of frequencies of naturally-occurring regulatory T cells (N-Treg) and FOXP3-positive cells among the groups. (a) Gating of CD4+CD25high+ T cells under FACS analysis. The cut-off value of CD25high expression is set at a level that is more than that of CD4–CD25+ cells (dotted line); CD4+CD25high+ T cells are shown in the rectangle drawn in the representative dot plot. (b) Frequencies of FOXP3+ cells, (c) N-Treg (CD25high+FOXP3+ cells) and (d) CD25–FOXP3+ cells in CD4+ T cells were compared among the groups. Boxes represent lower and upper quartiles with the median value (solid line) between boxes, while the whiskers represent the minimum and maximum values. *, $P < 0.05$; , $P < 0.0001$ by Mann–Whitney *U*-test. *Abbreviations:* PNALT, hepatitis C virus (HCV)-infected patients with persistently normal alanine aminotransferase (ALT) levels; CH, HCV-infected patients with elevated ALT levels; HS, healthy subjects.

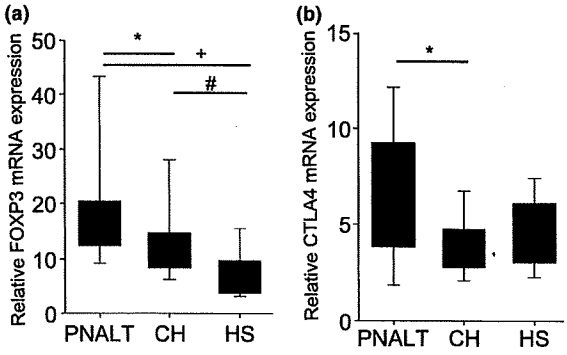

Fig. 2 Comparison of mRNA expression of FOXP3 and CTLA4 in CD4+CD25high+ T cells among the groups. The expression of FOXP3 (a) and CTLA4 (b) in separated CD4+CD25high+ T cells were analysed by real-time reverse transcriptase-polymerase chain reaction as described in Materials and methods. Boxes represent lower and upper quartiles with the median value (solid line) between boxes, while the whiskers represent the minimum and maximum values. *, $P < 0.05$; , $P < 0.01$; +, $P < 0.001$. For definitions of PNALT, CH and HS, see Fig. 1.

production stimulated with antigen-pulsed DC. We compared such responses between samples with or without CD4+CD25+ T cells. In PNALT patients, HCV NS5-specific T cell proliferation or IFN-γ production of CD25-depleted CD4+ T cells was significantly higher than those of the bulk CD4+ T cells (Fig. 3a,b). In contrast, in CH patients, such restoration did not occur significantly even when CD4+ CD25+ T cells had been depleted (Fig. 3a,b). There was no difference in the production of IL-10 and TGF-β between bulk CD4+ T cells and CD25-depleted CD4+ T cells in both CH and PNALT patients (Fig. 3c,d). These results suggest that co-existing CD4+CD25+ T cells play an inhibitory role in the HCV-specific CD4+ T cell response, in which suppression was more potent in the PNALT than in the CH group.

CD127–FOXP3+ cells, regardless of their CD25 expression, are increased in patients with HCV infection

In the analyses of N-Treg, the frequency of CD4+CD25–FOXP3+ T cells in HCV-infected patients was higher than those in the healthy donors (Fig. 1d). These results suggest that CD4+FOXP3+ T cells, regardless of the degree of CD25

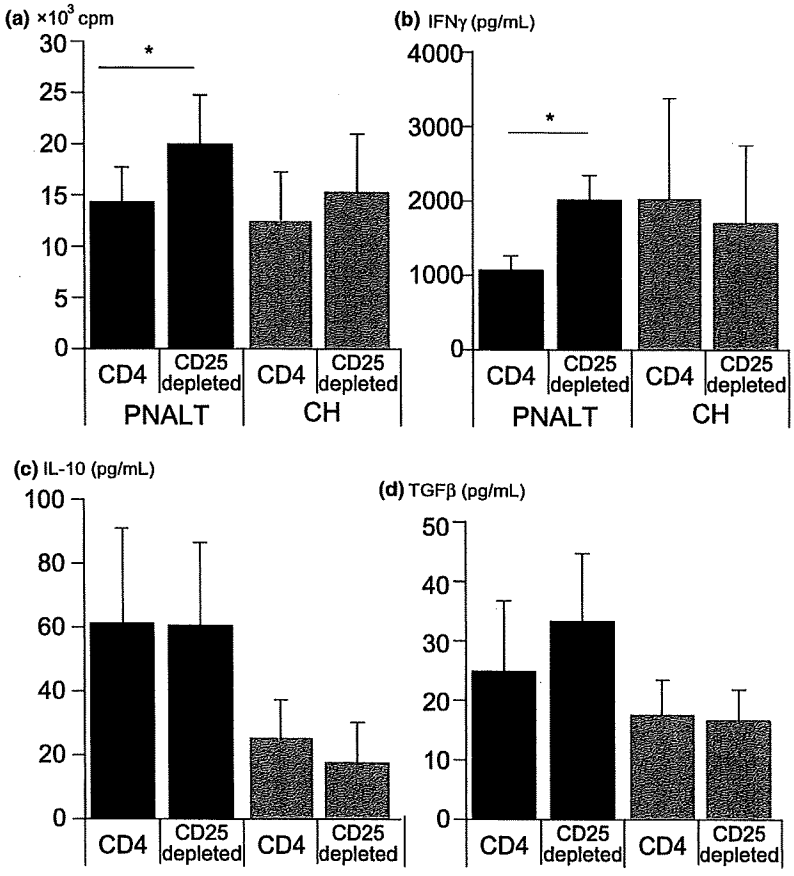

Fig. 3 Changes of hepatitis C virus (HCV)-specific CD4+ T cell responses with or without depletion of CD25+ T cells. Bulk CD4+ T cells or those depleted of CD25+ cells were cultured with autologous monocyte-derived dendritic cells in the presence of HCV-NS5 protein for 5 days as described in Materials and methods. (a) On day 4, [3H]-thymidine was pulsed and the thymidine incorporation was counted with a β-counter. Before the pulsing, the culture supernatants were harvested and subjected to enzyme-linked immunosorbent assay for interferon-γ (b), interleukin-10 (c) and TGF-β (d), respectively. *, $P < 0.05$ by Mann–Whitney *U*-test. For definitions of PNALT and CH, see Fig. 1.

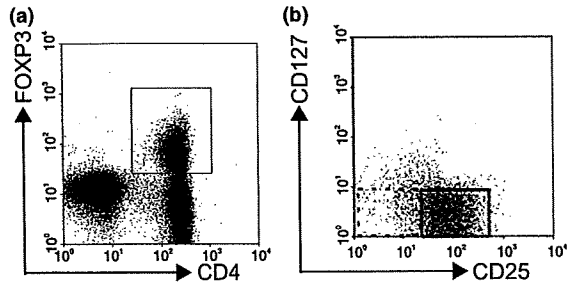

Fig. 4 Gating of CD4+CD127–FOXP3+ cells with variable CD25 expression under FACS analysis. After setting the gate on CD4+FOXP3+ cells [rectangle in the dot plot (a)], were displayed on the CD25 and CD127 axis (b). The presence of CD25+ (bold rectangle) and of CD25– cells (dotted rectangle) in CD4+FOXP3+ cells are shown in plot (b). The frequencies of these cells were analysed.

expression, increase in chronic HCV infection. Alternatively, it implies that higher expression of CD25 is not a universal marker for identifying FOXP3+ cells with regulatory activity. It has been reported that CD127 expression on CD4+ T cells is inversely correlated with FOXP3 expression, suggesting that CD127low/negative cells consist of those with regulatory activity. In order to analyse regulatory T cell subsets more precisely, we first examined FOXP3 expression on CD127– or CD127+ cells paired with CD25 expression in patients with HCV infection (Fig. 4). As a result, the majority of CD4+FOXP3+ T cells belonged to the CD127– population irrespective of CD25 expression (Fig. 4). Next, we compared the frequency of CD4+CD127–FOXP3+ cells, which consist of CD25+ and CD25– cells, among the subject groups (Fig. 5a). The frequency of CD4+CD127–FOXP3+ cells was similar in the CH and the PNALT groups, both of which were significantly higher than those in the HS (Fig. 5a). Finally, in order to estimate the profile of CD4+CD127–FOXP3+ cells according to CD25 expression, we compared the percentage of CD25+CD127–FOXP3+ or CD25–CD127–FOXP3+ cells in CD4+ T cells among the groups. The percentage of CD25+CD127–FOXP3+ T cells in CD4+ T cells was comparable for PNALT and CH (Fig. 5b). In clear contrast, the percentage of CD25–CD127–FOXP3+ T cells in the PNALT was lower than those in the CH (Fig. 5c). The frequencies of these cells were higher in the HCV-infected patients than in HS (Fig. 5b,c). When we set the focus on the proportion of CD25+CD127– or CD25-CD127– cells in the FOXP3+ cells in the periphery as a whole, we found that the proportion of CD25+CD127– cells in the PNALT was higher than that in the CH group (Fig. 5d). On the other hand, the proportion of CD25–CD127– cells in FOXP3+ cells was lower in the PNALT than in the CH group (Fig. 5e). Therefore, the phenotypic profiles of FOXP3+ T cells are distinct between PNALT and CH patients, with regard to the expression of CD127 and CD25.

DISCUSSION

Approximately 30–40% of chronically HCV-infected patients continue to display PNALT for decades. We previously reported the possible contribution of certain human leukocyte antigen haplotypes [23] or DC dysfunction in the maintenance of the PNALT state [24]. However, the precise mechanisms behind this important issue are yet to be

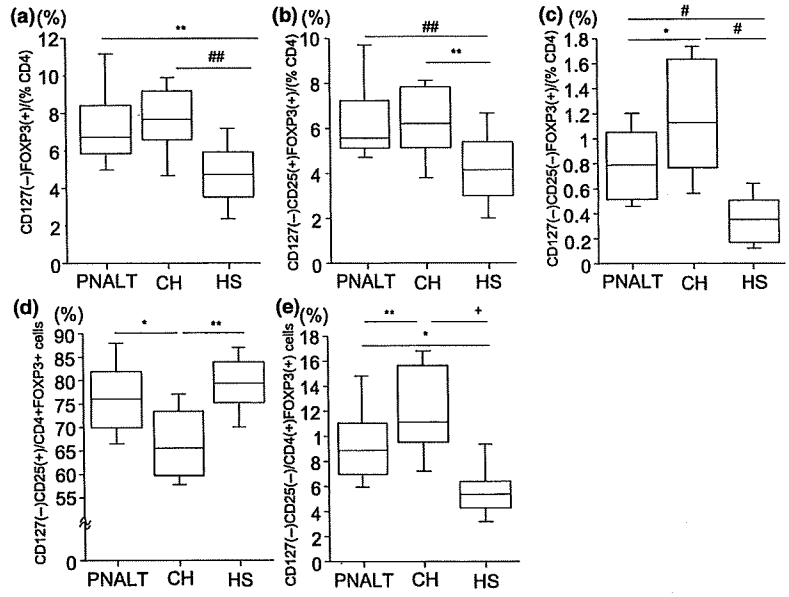

Fig. 5 Comparison in the frequencies of CD127– regulatory T cell subsets among the groups. Frequencies of CD127–FOXP3+ (a), CD127–CD25+FOXP3 (b) and CD127–CD25–FOXP3+ (c) cells among CD4+ T cells were determined by FACS analysis. The proportion of CD127–CD25+ (d) or CD127–CD25– (e) cells in CD4+FOXP3+ cells were also determined. Boxes represent lower and upper quartiles with the median value (solid line) between boxes, while the whiskers represent the minimum and maximum values. *, $P < 0.05$; , $P < 0.01$; **, $P < 0.005$; ##, $P < 0.001$; +, $P < 0.0001$ by Mann–Whitney *U*-test. For definitions of PNALT, CH and HS, see Fig. 1.

established. Cumulative reports have shown that Th1/Tc1 type responses are instrumental in HCV-induced liver inflammation [7,25,26]. We thus hypothesized that some suppressor mechanisms exist in PNALT patients especially against HCV-specific Th1 and/or CTL reactions.

The involvement of Treg cells in the pathogenesis of various diseases has been reported [9–13]. Most of the studies presented the possibility that N-Treg play substantial roles in the induction of tolerance against aetiological self or nonself antigens, thus leading to alleviation or exacerbation of the disease severity. With regard to HCV infection, several groups have shown that N-Treg are increased both in the periphery and in the liver and are able to inhibit HCV-specific CD4+ or CD8+ T cell responses *in vitro* [17,18,27]. In this study, we showed that the frequency of N-Treg in HCV-infected patients is higher than those in the controls, which is consistent with the previous reports. However, the frequencies of N-Treg are indistinguishable between the patient groups with different disease activities. As for the functional aspect, the deprivation of CD4+CD25+ cells enhanced the HCV NS5-specific CD4+ T cell response in the PNALT than in the CH group, suggesting that co-existing Treg in the PNALT are more suppressive. In addition, the expression of FOXP3 and CTLA4, which are key molecules of the suppressor function, is higher in PNALT than in those with active hepatitis. Venken *et al.* [28] demonstrated that the degree of FOXP3 expression at the single-cell level of N-Treg is well correlated with their suppressive ability, which is supportive of our results. In contrast, Bolacchi *et al.* [29] reported that the frequency of TGF-β+ N-Treg in the PNALT was higher than in the hepatitis group. Furthermore, their frequency was inversely correlated with the histological inflammatory grade, suggesting that TGF-β+ Treg play active roles in alleviating hepatitis. The reasons for the lack of correlation between N-Treg and serum ALT or HCV RNA quantity in the present study may be because of the difference in the target of analyses, such as either peripheral or intra-hepatic Treg, or either TGF-β+ or bulk Treg. Further analyses need to be performed on these important issues, as CD4+FOXP3+ Treg are reported to accumulate more in the portal tract of HCV-infected livers compared with those in the periphery [20].

During the observation period, about 30–40% of PNALT patients began to show elevated or fluctuating ALT abnormalities. What crucial factor triggers HCV-induced liver inflammation remains unknown. One of the plausible explanations is an antigenic shift accompanied by the occurrence of mutations in the HCV genome. In other words, hepatitis may flare up if the mutation raises HCV immunogenicity. Comprehensive analyses of HCV epitopes for CTL using overlapping peptides have shown that the HCV core and NS3 are more immunogenic than the remaining regions; however, the presence of an epitope hierarchy in Treg induction has been controversial. Li *et al.* [30] reported the possibility that Treg are expandable in response to certain epitopes in HCV proteins. In two patients in whom we observed flare-up of hepatitis in this study, we were able to find that the expression of FOXP3 in N-Treg was high in the PNALT status, but declined in the active hepatitis stage (data not shown). Although it is difficult to state whether such phenotypic changes in N-Treg are the cause or the consequence of disease progression, these results suggest the involvement of N-Treg in the degree of HCV-mediated hepatitis. Further detailed study is needed to examine whether or not such changes in N-Treg are related to the sequence evolution in HCV genomes.

Recent research has disclosed that distinct types of Treg are present in humans. Currently, it is generally accepted that CD25+FOXP3+ is the most reliable marker for Treg, which is induced in parallel with the acquisition of suppressor ability. However, owing to the lack of phenotypic markers for specifically identifying adaptive Treg, their roles in clinical settings have been unclear. In this study, CD4+FOXP3+ cells increased in HCV-infected patients, who were either positive or negative for CD25. In contrast to thymus-derived N-Treg expressing a greater degree of CD25, adaptive Treg are presumed to be induced in the periphery with a lesser degree of CD25 expression. Thus, it is likely that CD4+CD25–FOXP3+ T cells in HCV infection contain some part of adaptive Treg.

Treg have been reported to express low levels of CD127 at their cell surface [31]. Furthermore, the expression of CD127 is inversely correlated with FOXP3 expression and with the suppressive function of CD25high+ Treg. Liu *et al.* [22] pointed out the possibility that adaptive Treg are grouped into CD127– cells, which also include FOXP3-negative Tr1 or Th3 cells. Alternatively, You *et al.* [32] reported that murine CD4+CD25lowFOXP3+ T cells might be adaptive Treg, which exert a TGFβ-dependent suppressive function. Taking these reports into consideration, and in order to exclude activated CD25+ T cells, we examined CD4+CD127–CD25–FOXP3+ cells tentatively determined as part of adaptive Treg. In order to confirm that CD4+CD127– cells possess suppressive capacity, we co-cultured sorted CD4+CD127–CD25– or CD4+CD127–CD25+ cells with allogeneic CD4+ T cells stimulated with anti-CD3 and anti-CD28 antibodies. As a result, we found that CD4+CD127– cells, regardless of CD25 expression, significantly suppressed the proliferation of responder CD4+ T cells (manuscript in preparation). Of note is the finding that the frequency of CD127–CD25–FOXP3+ cells is higher in patients with active hepatitis than those in the PNALT group. One of the plausible explanations for such an increase of Treg is the compensatory mechanisms for the aggravation of liver inflammation. In support of this possibility, Bonelli *et al.* [33] reported that CD4+CD127–CD25– cells are increased in patients with systemic lupus erythematosus (SLE), the numbers of which are well correlated with disease activity. With regard to the ability of Treg in SLE patients, CD4+CD127–CD25– cells were potent in the inhibition of T

cell proliferation but not in IFN-γ release. Such a defective suppressor capacity may result in the continuation of tissue inflammation regardless of the presence of abundant Treg. The other conceivable role of CD4+CD25–CD127–FOXP3+ cells in active hepatitis may be a peripheral reservoir of CD4+CD25+FOXP3+ cells in case of flare-up of liver inflammation. In mice, it has been reported that CD25–FOXP3+ cells revert to CD25+FOXP3+ cells upon activation signals, thus leading to the expansion of the Treg pool [34]. In order to reach a definite conclusion on the role of CD127–CD25–FOXP3+ cells, further analyses are needed to elucidate whether these cells are inhibitory to either HCV-specific or HCV-nonspecific T cell responses.

Large-scale studies with HCV-infected patients demonstrated that the cumulative incidence of HCC in the PNALT group is extremely low compared with that in patients with apparent hepatitis and liver cirrhosis [35]. The lesser HCC incidence is also evident in patients who attained a lasting biochemical response to IFN-based therapy; even if they had failed to achieve sustained virological response [36]. These results clearly indicate that the maintenance of the PNALT state is one of the surrogate therapeutic goals in chronic HCV infection. Therefore, it is necessary to clarify the mechanisms of Treg induction in HCV infection, whether they are naturally or adaptively introduced, and to establish a feasible modality for controlling Treg. Our study has shown the importance of subset-oriented analyses of Treg for gaining access to that goal.

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CONFLICT OF INTEREST

All of the authors do not have any commercial or other association that might pose a conflict of interest.

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Pegylated interferon alpha-2b (Peg-IFN α-2b) affects early virologic response dose-dependently in patients with chronic hepatitis C genotype 1 during treatment with Peg-IFN α-2b plus ribavirin

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SUMMARY. Chronic hepatitis C (CH-C) genotype 1 patients who achieved early virologic response have a high probability of sustained virologic response (SVR) following pegylated interferon (Peg-IFN) plus ribavirin therapy. This study was conducted to evaluate how reducing drug doses affects complete early virologic response (c-EVR) defined as hepatitis C virus (HCV) RNA negativity at week 12. Nine hundred eighty-four patients with CH-C genotype 1 were enrolled. Drug doses were evaluated independently on a body weight base from doses actually taken. From multivariate analysis, the mean dose of Peg-IFN α-2b during the first 12 weeks was the independent factor for c-EVR ($P = 0.02$), not ribavirin. The c-EVR rate was 55% in patients receiving ≥1.2 μg/kg/week of Peg-IFN, and declined to 38% at 0.9–1.2 μg/kg/week, and 22% in patients given <0.9 μg/kg/week ($P < 0.0001$). Even with stratified analysis according to ribavirin dose, the dose-dependent effect of Peg-IFN on c-EVR was observed, and similar c-EVR rates were obtained if the dose categories of Peg-IFN were the same. Furthermore, the mean dose of Peg-IFN during the first 12 weeks affected HCV RNA negativity at week 24 ($P < 0.0001$) and SVR ($P < 0.0001$) in a dose-dependent manner. Our results suggest that Peg-IFN was dose-dependently correlated with c-EVR, independently of ribavirin dose. Thus, maintaining the Peg-IFN dose as high as possible during the first 12 weeks can yield HCV RNA negativity and higher c-EVR rates, leading to better SVR rates in patients with CH-C genotype 1.

Keywords: chronic hepatitis C, drug dose, early virologic response, HCV RNA negativity, pegylated interferon plus ribavirin, sustained virologic response.

INTRODUCTION

Pegylated interferon (Peg-IFN) plus ribavirin therapy can improve anti-viral efficacy for patients with chronic hepatitis C [1–5], and the prognosis of patients in whom hepatitis C virus (HCV) is successfully eradicated improves markedly [6–10]. However, HCV still persists in approximately half of genotype 1 patients treated with Peg-IFN plus ribavirin [2–4]. Therefore, the treatment method needs to be well managed in order to maximize the virologic response in these patients with HCV genotype 1.

Abbreviations: c-EVR, complete EVR; CH-C, chronic hepatitis C; EVR, early virologic response; G-CSF, granulocyte-macrophage colony stimulating factor; Hb, haemoglobin; HCV, hepatitis C virus; Peg-IFN, pegylated interferon; Plt, platelet; SVR, sustained virologic response; WBC, white blood cell.

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In order to achieve sustained virologic response (SVR), earlier virologic response is very important for patients with chronic hepatitis C (CH-C) genotype 1. A high SVR rate (65–72%) was found in patients who achieved early virologic response (EVR) defined as a 2-log decrease in HCV RNA level at week 12, but only 0–3% SVR was seen in patients without EVR [3,11]. Additionally, complete EVR (c-EVR), which means HCV RNA negativity at week 12, is more strongly related to SVR [3].

The relationship between drug exposure and anti-viral effect has been reported in several papers [2,11–15]. McHutchison *et al.* [12] demonstrated that the SVR rate in patients who received ≥80% of their total planned doses of Peg-IFN and ribavirin for ≥80% of the scheduled duration of therapy was significantly higher than that of patients who received <80% of one or both drugs (51% *vs* 34%) and also suggested that the impact of dose reduction was greatest in patients for whom the dose had to be decreased within the first 12 weeks of treatment. In a subsequent analysis, reducing the dose of Peg-IFN and ribavirin to <80% of the full planned dose within the first 12 weeks was reported to reduce EVR rate from 80 to 33% [11]. Thus, drug adherence during the first 12 weeks has been shown to be very important for attaining EVR and SVR, but it remains obscure whether either drug can be reduced to a certain degree without adversely affecting the treatment efficacy.

In the present study, we examined the correlation between c-EVR and drug doses which are evaluated on a body weight basis from drug doses actually taken, in order to clarify the necessary drug exposure of Peg-IFN and ribavirin for achieving a higher c-EVR rate in patients with CH-C genotype 1.

PATIENTS AND METHODS

Patients

The current study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 984 patients with CH-C treated with a combination of Peg-IFN α-2b plus ribavirin were enrolled in this study between December 2004 and September 2006. The baseline characteristics of the patients are summarized in Table 1. All patients were Japanese, their mean age was 56.3 ± 10.1 years, and 56% were males. The mean serum alanine aminotransferase level was 79 ± 61 IU/L.

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

Table 1 Baseline characteristics of patients

Factor	Mean ± SD or number
n	984
Age (year)	56.3 ± 10.1
Sex: male/female	555/429
Body weight (kg)	61.8 ± 11.5
History of interferon treatment	
Naïve/experienced (relapser/nonresponder)*	575/409(160/182)
White blood cells (per mm^3)	5052 ± 1550
Neutrophils (per mm^3)	2577 ± 1092
Red blood cells ($\times 10^4/mm^3$)	442 ± 47
Haemoglobin (g/dL)	14.1 ± 1.4
Platelets ($\times 10^4/mm^3$)	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

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.

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. 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 a standard treatment protocol for Japanese patients.

Dose reduction

Dose modification followed, as a rule, the manufacturer's drug information according to the intensity of the haematological adverse effects. The dose of Peg-IFN α-2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to <1500/mm^3, the neutrophil count to <750/mm^3 or the platelet (Plt) count to <8 × $10^4/mm^3$, and was discontinued if the WBC count declined to <1000/

mm^3, the neutrophil count to $<500/mm^3$ or the Plt count to $<5 \times 10^4/mm^3$. Ribavirin was also reduced from 1000 to 600 mg, or 800 to 600 mg, or 600 to 400 mg if the haemoglobin (Hb) level decreased to <10 g/dL, and was discontinued if the Hb level decreased to <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. During this therapy, ferric medicine or haematopoetic growth factors, such as erythropoietin alpha, or granulocyte-macrophage colony stimulating factor (G-CSF), were not 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 Diagnotics, Branchburg, NJ, USA) and qualitatively analysed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/mL). The c-EVR was defined as the absence of detectable serum HCV RNA at treatment week 12, and 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 and were regarded as nonresponders. All patients with detectable serum HCV RNA at treatment week 24 were also considered nonresponders and excluded from further treatment.

Assessment of drug exposure

The amounts of Peg-IFN α-2b and ribavirin actually taken by each patient during the first 12 weeks of the treatment 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 α-2b expressed as μg/kg/week, and ribavirin expressed as mg/kg/day.

Evaluation of impact of drug exposure on c-EVR

We evaluated the relationship between the drug exposure of both drugs and c-EVR by univariate and multivariate analysis for c-EVR, using the factors of mean administration doses of both drugs during the first 12 weeks and the factors at baseline. Furthermore, Peg-IFN α-2b dose (average dose per body weight and per week) was classified into five categories (up to 0.6 μg/kg; from 0.6 to <0.9 μg/kg; from 0.9 to <1.2 μg/kg; from 1.2 to <1.5 μg/kg; from 1.5 μg/kg and above). Ribavirin exposure was classified into four categories (up to 8 mg/kg; from 8 to <10 mg/kg; from 10 to <12 mg/kg; from 12 mg/kg and above), in order to examine the impact of Peg-IFN dose exposure on c-EVR. This impact was also evaluated based on the percentage of the total prescribed dose and compared with that based on the mean dose per body weight.

Statistical analysis

Baseline data for various demographic, biochemical and virologic characteristics of the patients are expressed as mean ± SD or median values. To analyse the relationship between baseline data including drug exposure and c-EVR, univariate analysis using the Mann–Whitney *U*-test or chi-squared 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. Statistical analysis was conducted with SPSS version 15.0J (SPSS Inc., Chicago, IL, USA).

RESULTS

Progress of patients treated with Peg-IFN α-2b and ribavirin

Of the 984 patients, 81 discontinued treatment because of adverse events (n = 74) or voluntary withdrawal (n = 7) by treatment week 12. The 903 patients who completed 12 weeks of treatment were assessed for c-EVR. During 12–48 weeks of treatment, 331 of the nonresponders and nine of breakthrough discontinued treatment, as did 91 patients (adverse events, n = 71; voluntary withdrawal, n = 20). A total of 472 patients completed 48 weeks of treatment.

Drug reduction and virologic response

Peg-IFN α-2b was reduced without discontinuation in 29% (n = 266) and ribavirin was reduced without discontinuation in 40% (n = 359) of the 903 patients who completed 12 weeks of treatment. The c-EVR rate was 49% (445/903) and HCV RNA was negative at week 24 in 60% (542/903) of patients who completed 12 weeks of treatment. Of the 445 patients with c-EVR, 327 patients achieved SVR (73%). Only 7% of the 458 patients without c-EVR did so.

Impact of dose exposure of Peg-IFN α-2b and ribavirin on c-EVR

The mean dose of Peg-IFN α-2b actually taken during the first 12 weeks by each patient was 1.33 μg/kg/week (range 0.41–2.16 μg/kg/week; median 1.40 μg/kg/week) and that of ribavirin was 10.4 mg/kg/day (range 2.9–16.2 mg/kg/day; median 10.6 mg/kg/day).

The mean doses of both drugs and the factors at baseline correlated with the c-EVR were assessed by univariate and multivariate logistic regression analyses. Univariate analysis showed that factors significantly associated with c-EVR were age, sex, WBC, neutrophils, red blood cells, Hb, Plt, aspartate aminotransferase, the degree of liver fibrosis and the mean doses of Peg-IFN α-2b and ribavirin during the first 12 weeks (Table 2). The factors selected as significant by the univari-

Table 2 Univariate analysis for c-EVR among patients who completed 12 weeks treatment

Factor	c-EVR (+)	c-EVR (−)	*P*-value
n	445	458	
Age (year)	54.4 ± 10.4	57.5 ± 9.6	<0.001
Sex: male/female	267/178	237/221	0.01
Serum HCV RNA (kIU/mL)*	1500	1600	0.28
White blood cells (per mm^3)	5336 ± 1536	4818 ± 1547	<0.001
Neutrophils (per mm^3)	2789 ± 1133	2398 ± 1038	<0.001
Red blood cells ($\times 10^4/mm^3$)	450 ± 46	435 ± 49	<0.001
Haemoglobin (g/dL)	14.3 ± 1.4	13.9 ± 1.4	<0.001
Platelets ($\times 10^4/mm^3$)	17.3 ± 5.2	15.0 ± 5.6	<0.001
AST (IU/L)	62 ± 44	69 ± 44	<0.001
ALT (IU/L)	77 ± 64	80 ± 57	0.07
Histology (METAVIR)†			
Fibrosis: 0–2/3–4	273/37	247/74	<0.001
Activity: 0–1/2–3	171/139	159/162	0.16
Peg-IFN dose (μg/kg/week)‡	1.39 ± 0.22	1.28 ± 0.30	<0.001
Ribavirin dose (mg/kg/day)‡	10.6 ± 1.7	10.1 ± 2.1	0.002

c-EVR, complete early virologic response; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Peg-IFN, pegylated interferon. *Data shown are median values. †272 missing. ‡Mean doses during 0–12 weeks.

Table 3 Multivariate analysis for c-EVR among patients who completed 12 weeks treatment

Factor	Category	Odds ratio	95% CI	*P*-value
Age	by 1 year	0.982	0.966–0.999	0.04
Sex	male/female	–	–	NS
Neutrophils	by 100/mm^3	1.017	1.002–1.033	0.03
Red blood cells	by $1 \times 10^4/mm^3$	–	–	NS
Haemoglobin	by 1 g/dL	–	–	NS
Platelets	by $1 \times 10^4/mm^3$	1.051	1.014–1.088	<0.01
AST	by 1 IU/L	–	–	NS
Fibrosis*	0–2/3–4	–	–	NS
Peg-IFN dose†	by 0.1 μg/kg/week	1.079	1.011–1.151	0.02
Ribavirin dose†	by 1 mg/kg/day	–	–	NS

95% CI, 95% confidence interval; Peg-IFN, c-EVR, complete early virologic response; pegylated interferon; N.S., No Significant difference; AST, aspartate aminotransferase.
*METAVIR fibrosis score. †Mean doses during 0–12 weeks.

ate analysis were evaluated by multivariate logistic regression analysis. The mean dose of Peg-IFN α-2b during the first 12 weeks was the independent factor for c-EVR ($P = 0.02$), apart from the neutrophils ($P = 0.03$) and Plt value at baseline ($P < 0.01$) and age ($P = 0.04$) (Table 3). In contrast, the mean dose of ribavirin during the first 12 weeks showed no correlation with c-EVR.

The c-EVR rates were 54% (137/253) and 56% (246/443) for patients who received ≥1.5 and 1.2–1.5 μg/kg/week of Peg-IFN α-2b on average during the first 12 weeks, and declined to an average rate of 38% (40/105) in patients given 0.9–1.2 μg/kg/week of Peg-IFN α-2b, and an average rate of 22% (22/102) in patients given <0.9 μg/kg/week ($P < 0.0001$) (Table 4). The c-EVR rate among the patients with ≥1.2 μg/kg/week of Peg-IFN α-2b was significantly higher than that of the patients with <1.2 μg/kg/week [≥1.2 μg/kg/week, 55% (383/696) *vs* <1.2 μg/kg/week, 30% (62/207), $P < 0.0001$].

Next, we analysed the impact of Peg-IFN α-2b on c-EVR in stratified analysis according to ribavirin dose. Figure 1 shows the relationship of c-EVR and the degree of Peg-IFN α-2b exposure for two groups of ribavirin doses: the group with ≥10.6 mg/kg/day of ribavirin and that with <10.6 mg/kg/day (10.6 mg/kg/day was the median value). In either group, the mean dose of Peg-IFN α-2b was dose-dependently correlated with c-EVR ($P < 0.0001$), and c-EVR rates were very similar in both groups if the dose categories of Peg-IFN α-2b were the same.

Table 4 The c-EVR rate according to Peg-IFN and ribavirin doses during weeks 0–12 for patients who completed 12 weeks treatment

Ribavirin dose (mg/kg/day)**	Peg-IFN α-2b dose (μg/kg/week),*				
	≥1.5	1.2–1.5	0.9–1.2	<0.9	Total
≥12	57% (60/105)	61% (22/36)	38% (6/16)	22% (2/9)	54% (90/166)
10–12	54% (46/85)	58% (154/267)	36% (14/39)	23% (11/47)	51% (225/438)
8–10	50% (25/50)	53% (52/99)	52% (15/29)	18% (4/22)	48% (96/200)
<8	46% (6/13)	44% (18/41)	24% (5/21)	21% (5/24)	34% (34/99)
Total	54% (137/253)	56% (246/443)	38% (40/105)	22% (22/102)	49% (445/903)

c-EVR, complete early virologic response; Peg-IFN, pegylated interferon.
*$P < 0.0001$ for comparison of the four Peg-IFN groups. **$P = 0.05$ for comparison of the four ribavirin groups.

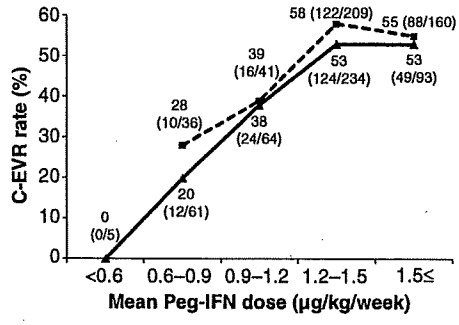

Fig. 1 Complete-EVR rate according to pegylated interferon alpha-2b (Peg-IFN α-2b) and ribavirin doses during weeks 0–12 for patients who completed 12 weeks of treatment. (-▲-) Group with the mean ribavirin dose <10.6 mg/kg/day. (-■-) Group with the mean ribavirin dose ≥10.6 mg/kg/day. The Peg-IFN α-2b dose was dose-dependently correlated with c-EVR in both groups ($P < 0.0001$). There was no significant difference between the two ribavirin-dose groups ($P = 0.19$).

c-EVR rates according to Peg-IFN α-2b drug exposure using a percentage cut off and mean dose cut off

Table 5 shows the c-EVR rates according to the category of Peg-IFN α-2b doses during the first 12 weeks based on the percentage of the total prescribed dose and the mean doses. The whole c-EVR rate was 54% (377/698) for patients who received more than 80% of the prescribed dose, and 43% (47/109) in patients given 60–80% of the prescribed dose, and 21% (21/96) in patients given <60% of the prescribed dose of Peg-IFN α-2b. Among patients given ≥80% of the prescribed dose of Peg-IFN α-2b, the c-EVR rate was significantly lower in patients given <1.2 μg/kg/week of Peg-IFN α-2b than those given ≥1.2 μg/kg/week (32% *vs* 55%, $P < 0.05$). On the other hand, even in patients given 60–80% of the prescribed dose of Peg-IFN α-2b, if they were given ≥1.2 μg/kg/week of Peg-IFN α-2b, a higher c-EVR rate was attained in comparison with those given <1.2 μg/kg/week (71% *vs* 38%, $P = 0.01$); the c-EVR rate in patients given 60–80% of the prescribed dose and ≥1.2 μg/kg/week of Peg-IFN α-2b was not inferior to that in patients given ≥80% of the prescribed dose and ≥1.2 μg/kg/week of Peg-IFN α-2b.

Impact of dose exposure of Peg-IFN α-2b during the first 12 weeks of the treatment on HCV RNA negativity at week 24 and SVR

Patients positive for HCV RNA at week 24 week during Peg-IFN α-2b and ribavirin treatment were regarded as non-responders and stopped treatment [11]. We analysed the

Table 5 The c-EVR rate according to Peg-IFN dose during weeks 0–12 based on the percentage of the planned dose and the mean doses

Peg-IFN α-2b dose (μg/kg/week)	≥80%	60–80%	<60%	Total
≥1.2	55%* (371/679)	71%** (12/17)	–	55% (383/696)
<1.2	32% (6/19)	38% (35/92)	22% (21/96)	30% (62/207)
Total	54% (377/698)	43% (47/109)	21% (21/96)	49% (445/903)

c-EVR, complete early virologic response; Peg-IFN, pegylated interferon.
*$P < 0.05$; patients with ≥1.2 μg/kg/week *vs* <1.2 μg/kg/week among the patients with more than 80% of the total prescribed dose of Peg-IFN α-2b. **$P = 0.01$; patients with ≥1.2 μg/kg/week *vs* <1.2 μg/kg/week among the patients with more than 60–80% of the total prescribed dose of Peg-IFN α-2b.

relationship between the dose exposure to Peg-IFN α-2b during the first 12 weeks and HCV RNA negative rates at week 24 or SVR in 903 patients completing 12 weeks of treatment. As a result, HCV RNA negative rates at week 24 and SVR rates declined according to the decrease in the dose of Peg-IFN α-2b during the 12 weeks of treatment; patients given ≥1.5, 1.2–1.5, 0.9–1.2 and <0.9 μg/kg/week of Peg-IFN α-2b during the first 12 weeks of the treatment showed HCV RNA negativity of 63%, 66%, 48% and 39%, respectively ($P < 0.0001$), and SVR of 46%, 43%, 30% and 20%, respectively ($P < 0.0001$).

DISCUSSION

Adherence to ribavirin was reported to be the important factor for EVR as well as that to Peg-IFN in most previous studies [2,11,12]. However, the drug exposure of Peg-IFN α-2b and ribavirin had not been analysed independently with respect to their individual influence on the anti-viral effect in these studies. Adherence to both drugs may be related factors, i.e. most patients who can tolerate a high dose of Peg-IFN are in good condition and thus can also receive a high dose of ribavirin. In the present study, the impact of the dose of Peg-IFN α-2b and ribavirin on the anti-viral effect was evaluated by multivariate logistic regression analysis, using the mean administration doses of both drugs during the first 12 weeks and baseline factors. As a result, the dose exposure of Peg-IFN α-2b was found to be the significant factor affecting c-EVR as well as baseline factors such as age, neutrophils and Plt values, but not ribavirin. This suggests that the c-EVR rate can be raised by maintaining the dose of Peg-IFN α-2b during the first 12 weeks in patients with disadvantageous factors at baseline. In fact, the c-EVR rate was higher in those who received ≥1.2 μg/kg of Peg-IFN α-2b than in those given <1.2 μg/kg of Peg-IFN α-2b for aged patients over 60 years of age (≥1.2 μg/kg; 46% *vs* <1.2 μg/kg; 28%, $P < 0.01$) or for patients with a low Plt value ($<12 \times 10^4/mm^3$)(≥1.2 μg/kg; 45% *vs* <1.2 μg/kg; 22%, $P < 0.001$). Therefore, a marked dose reduction of Peg-IFN α-2b should not be risked at the start even for aged patients or patients with lower Plt value, which is indicative of advanced fibrosis. The administration of ≥1.2 μg/kg/week of Peg-IFN α-2b is desirable as a starting dose for achieving c-EVR even in these patients: that of <1.2 μg/kg/week can lead to a non-viral response or a late viral response. Independent evaluation of the c-EVR rate according to the degree of the ribavirin dose showed a stepwise decline as the total cumulative dose of Peg-IFN α-2b decreased. Therefore, the dose of Peg-IFN α-2b should be maintained as high as possible even in patients who have to reduce Peg-IFN α-2b to <1.2 μg/kg/week. Using G-CSF for patients who develop severe neutropenia and are forced to decrease Peg-IFN can be beneficial, especially in the first 12 weeks.

The goal of 80% of the planned drug dosage for 80% of the assigned duration was derived from an adherence criterion that had been adopted previously for assessment of the efficacy of other pharmaceutical agents, such as drugs to treat cancer and human immunodeficiency virus [16]. However, in Peg-IFN plus ribavirin therapy for patients with CH-C, the planned administration dose [17,18] differs on a body weight basis by 27% for Peg-IFN α-2b and 40% for ribavirin among patients of 50–100 kg of body weight, which would be equivalent to the same rate differences for 80% of the planned drug dosage. In detail, the target dose of Peg-IFN α-2b scheduled to be administered is 1.5 μg/kg, but the usual dose for the individual patient is from 1.28 to 1.76 μg/kg/week based on body weight among patients weighing 50–100 kg according to the practice guidelines of the American Association for the Study of Liver Diseases and the manufacturer's drug information in the USA and Europe [17,18]. The range of ribavirin dose per kg of body weight is from 12 to 20 mg/kg/day. Therefore, in this study, the drug exposure was assessed from the average dose per kg of body weight.

In the evaluation of c-EVR rates according to Peg-IFN α-2b drug exposure using a percentage cut off and mean dose cut off in this study, the c-EVR rate of patients given <1.2 μg/kg/week of Peg-IFN α-2b was low (32%) even in those who received ≥80% of the total planned doses of Peg-IFN α-2b. If given ≥1.2 μg/kg/week of Peg-IFN α-2b, the c-EVR rate (71%) in patients who received 60–80% of the total doses was not inferior to that in patients given ≥80% of the total dose of Peg-IFN α-2b (54%). This means that patients whose starting dose of Peg-IFN α-2b is <1.5 μg/kg/week should not have their dosage reduced to 80% of the planned dose (<1.2 μg/kg/week) in order to have a higher probability of c-EVR, while those given ≥1.5 μg/kg/week of Peg-IFN α-2b at the start can have their dosage reduced to 80% (≥1.2 μg/kg/week) without lowering the c-EVR rate. Thus, the drug dose on a body weight basis itself should be examined as an index of the drug exposure in order to evaluate the anti-viral effect of both drugs accurately for patients with CH-C.

As for the impact of the drug exposure to ribavirin on c-EVR, the drug dose of ribavirin during the first 12 weeks was shown to have no relationship with the c-EVR rate, although it was precisely evaluated in this study, using doses actually taken on body weight. However, ribavirin can be more effective for decreasing the viral relapse after interferon or Peg-IFN α-2b and ribavirin combination therapy in patients with CH-C genotype 1 [2,3,19–24]. Recently, Shiffman *et al.* [15] have reported that a higher starting dose of ribavirin (1000–1600 mg/day) plus a regular dose of Peg-IFN α-2b with epoetin was associated with a lower relapse rate in treatment with CH-C genotype 1. Considering the viral relapse after treatment, it is thought that the ribavirin dose should not be reduced quickly in patients with mild side effects, even though it does not affect c-EVR. In fact, among the patients who attained c-EVR, a higher rate of viral relapse was found in the patients given <10 mg/kg/day of the mean ribavirin dose during 48 weeks in comparison