

<速報>

B型慢性肝疾患に対する核酸アナログ療法によるHBs抗原消失とその関連因子の検討

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緒言：B型肝疾患に対する核酸アナログ療法の有効性は広く知られており、経過観察期間が長くなるにつれ、B型肝炎治療の最終目標であるHBs抗原(HBsAg)消失を得られる症例も散見されている。本邦及び海外からいくつかの報告もあるが^{1)~4)}、いまだ長期に渡る核酸アナログ使用例での報告はない。今回我々は長期間の核酸アナログ治療によるHBsAg消失とその関連因子について検討した。

肝疾患に対して、ラミブジン単独投与を開始した769例を対象とした。これら全ての症例で6カ月以上のHBV持続感染を確認した。核酸アナログ投与内容の内訳はラミブジン単独投与継続306例、ラミブジン投与開始後耐性ウイルス出現に対してラミブジン+アデフォビル併用を行った症例297例、ラミブジン→エンテカビルへの切り替え症例166例であった。これらの症例のうち、何らかの理由で投与中止した症例は46例存在し、それ以外の症例はすべて継続投与を行った。HBsAg測定はCLIA法(ARCHITECT®HBsAg QT)を用いた。

対象と方法：1995年～2006年までに当院でB型慢性

Table Factors associated with HBsAg clearance by univariate and multivariate analysis.

factors	Univariate		Multivariate	
	Hazard Ratio (95%CI)	P	Hazard Ratio (95%CI)	P
Age (≥50yr)	0.94 (0.48-1.89)	0.865		
Gender (F)	0.59 (0.21-1.68)	0.323		
Family history of HBV infection	0.43 (0.22-0.84)	0.014		
Presence of cirrhosis	0.79 (0.56-1.12)	0.192		
Previous IFN therapy	2.70 (1.31-5.59)	0.007	2.96 (1.34-6.54)	0.008
HBV genotype (A)	3.39 (2.27-5.08)	<0.0001	3.64 (2.40-5.52)	<0.0001
HBeAg (positive)	1.23 (0.61-2.48)	0.563		
HBV DNA (≥6.0 logcopies/mL)	1.20 (0.52-2.78)	0.674		
HBsAg (<2000 IU/mL)	1.40 (0.70-2.80)	0.346		
ALT (≥300 IU/L)	1.47 (1.02-2.11)	0.040		
Platelets count (<1.2×10 ⁵ /mm ³)	0.91 (0.34-2.43)	0.123		
<i>Treatment response at 6 months</i>				
HBeAg positive → clearance	3.15 (1.49-6.66)	0.003	2.22 (1.01-4.88)	0.046
HBV DNA (<2.6 logcopies/mL)	3.56 (1.22-10.4)	0.021	4.07 (1.36-12.2)	0.012

The bolded numbers: statically significant.

Abbreviation: HBsAg, Hepatitis B surface antigen; IFN, interferon; HBeAg, Hepatitis B envelope antigen

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ラミブジン開始後の HBsAg 消失に寄与する因子について Cox 比例ハザードモデルを用いて、単変量及び多変量解析を行い検討した。

結果：ラミブジン投与開始からの観察期間の中央値は 6.3 年 (0.7-13.5 年) であった。ラミブジン投与前に IFN 治療歴を有する症例が 297 例 (39%) 存在した (投与期間の中央値は 27 週 (2-575 週))。HBV 感染の家族歴を有する症例が 538 例 (70%) 存在した。ラミブジン投与開始後の HBsAg 消失は 33 例で認められた (内訳は投与中消失 31 例、投与終了後消失 2 例)。全体での累積 HBsAg 消失率は 5 年 : 1.8%、10 年 : 7.3% であった。HBsAg 消失に寄与する因子について単変量解析を行ったところ、抽出された因子は、家族歴あり (48% vs. 74%)、IFN 治療歴あり (64% vs. 37%)、genotype A (25% vs. 2.6%)、開始時 ALT 高値 (300 IU/L 以上) (33% vs. 20%)、治療開始 6 カ月以内の HBe 抗原消失 (30% vs. 12% : HBeAg 持続陽性例や持続陰性例に比して)、治療開始後 6 カ月時点での HBVDNA 陰性化 (<2.6 log copies/ml) (85% vs. 67%) の 6 因子が抽出された (Table)。また治療法別で検討すると、ラミブジン単独またはエンテカビル切り替え症例では、ラミブジン+アデフォビル併用療法症例に比して HBsAg 消失率が高率であった (P=0.014)。

上記の因子を用いて、HBsAg 消失に寄与する因子について多変量解析を行ったところ、独立因子として genotype A、IFN 治療歴、治療開始 6 カ月時点で HBeAg 陽性→陰性化、治療開始後 6 カ月時点での HBVDNA 陰性化の 4 因子が抽出された (Table)。

考察：今回の検討では核酸アナログ投与後の HBsAg 消失には HBV genotype が強く関わっている事が分かった。これまでテルビブジンや PegIFN での報告のように⁴⁾⁵⁾、genotype A では HBsAg 量の低下が、他の genotype より起こりやすいため、HBsAg 消失が起こりやすいと考えられる。また IFN 治療歴や核酸アナログ治療早期の反応性などが HBsAg 消失に寄与し、治療開始時 ALT の上昇が強い症例でも HBsAg が消失しやすい傾向にあったことから、核酸アナログ治療により HBsAg を消失させるためには、核酸アナログ自体の抗ウイルス作用だけでなく、宿主の免疫反応が必要と推察される。今後 HBsAg 消失を目指した、核酸アナログ治療法の工夫が望まれる。この研究はラミブジン投与症例での検討であるが、今後は現在の標準治療であり、薬剤

耐性出現が極めて低率のエンテカビル投与症例での検討も必要と思われる。

索引用語：HBsAg、核酸アナログ、IFN

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英文要旨

Clearance of hepatitis B surface antigen during
long-term nucleot(s)ide analogues treatment
in chronic hepatitis B

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Clearance of HBsAg is considered the ultimate goal in the treatment for chronic hepatitis B. We analyzed clinical factors associated with HBsAg clearance during long-term nucleot(s)ide analogue treatment. By univariate analysis, HBV genotype, family history of HBV infection, previous IFN therapy, HBeAg clearance at 6 months, and undetectable HBV DNA at 6 months were significant predictive factors. By multivariate analysis, HBV genotype, previous IFN therapy, HBeAg clearance at 6 months, and undetectable HBV DNA at 6 months were independent and significant predictive factors of HBsAg clearance. We conclude that patients with genotype A have high probability of HBsAg clearance, and it seems that not only the antiviral potential of nucleot(s)ide analogue but host immune response is needed to achieve HBsAg clearance.

Key words: hepatitis B surface antigen,
nucleot(s)ide analogues, interferon

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<短 報>

コバス TaqMan HBV 「オート」 v2.0 における同一時の
血清検体と血漿検体の HBV DNA 検出率の検討

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緒言：HBV DNA の測定は、1996 年に分岐 HBV DNA プローブ法が臨床応用されてから、検査技術の進歩に伴い TMA (transcription-mediated amplification) 法や PCR 法などの高感度な測定法の開発が進んできた。現在、日常の臨床で使用されている real-time PCR 法は、HBV DNA 量が 1.5~2.0 Log copies/mL 程度まで検出可能となった。今回我々は、TaqMan HBV v2.0 法(コバス TaqMan HBV「オート」v2.0¹⁾；ロシユ・ダイアグノスティックス、東京)を用い、血清と血漿の同時採血を行い、各検体の有用性について検討を行ったので報告する。

対象と方法：対象は、B 型慢性肝炎および肝硬変の成人で Entecavir 投与 1 年以上経過し ALT (alanine aminotransferase) 値が 30 IU/l 以下を持続している 52 症例(104 検体)とした。内訳は、男性 29 例(55.8%)、年齢 52 歳：中央値(27~81 歳)であった。HBV genotype は genotype A：2 例、genotype B：5 例、genotype C：44 例、typing 不能：1 例であった。52 症例に対し治療効果の均一化を計るため同一検体で 2 回の採血を実施し HBV DNA を測定した。2 回目のポイントの採血は、1 回目の採血後、8 週±2 週の間実施した。血清用採血管で全血 5 mL と血漿用採血管(EDTA-2K)で全血 8 mL を採血、速やかに遠心分離後、TaqMan HBV v2.0 法(最小検出感度は、血清検体：2.0 Log copies/mL、血漿検体：1.7 Log copies/mL)にて測定を行った。統計解析は、統計解析ソフトウェア STAT Flex ver. 5.0 を用い、P<0.05 で有意とした。本試験は、当院の倫理

審査委員会の承認を受け、実施についてのインフォームド・コンセントを行った。

結果：血清・血漿ペア検体 104 例のうち、血清と血漿の両方で HBV DNA を検出したのは、25 例(24.0%)、両者ともに検出不能は、41 例(39.4%)であったが、血清で検出したが血漿では検出不能であったのは、6 例(5.8%)であり、血漿で検出したが血清では検出不能であったのは、32 例(30.8%)で、血漿での検出率は、血清より有意(P<0.001 [McNemar 検定])に高率であった (Table 1)。

考察：核酸アナログ製剤を長期に投与することによりその耐性株の出現および肝炎の悪化が認められることから、特に若年者においては核酸アナログ製剤を中止することも考え、HBV DNA 量をはじめ、HBs 抗原、HB コア関連抗原などの種々の HBV マーカーについて検討が行われている²⁾。Drug free が可能な症例選定の必要条件の一つは HBV DNA の持続陰性化であり³⁾、投与中止後 ALT 値の再上昇による重症化・劇症化が懸念されることより、高感度に HBV DNA を検出することが重要である可能性がある。

そこで今回、我々は臨床検体を用い TaqMan HBV

Table 1 Detail correlation between plasma specimen (EDTA-2K) and serum specimen

		Serum	
		detected	not detected
plasma (EDTA-2K)	detected	25 (24.0%)	32* (30.8%)
	not detected	6* (5.8%)	41 (39.4%)

*: P<0.001 [McNemar 検定]

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v2.0 の血清検体と血漿検体の有用性の検討を行った。対象の 104 検体のうち血清または血漿のいずれかで HBV DNA を検出したのは、血清は 5.8% に対し血漿では 30.8% と血漿での HBV DNA の検出率は統計学的有意差 ($P < 0.001$) をもって高率であった。一方、血清で HBV DNA を検出したが血漿では検出不能であった検体も 5.7% 存在したが、年齢、性別、genotype などに一定の偏りは無く、この現象は、最小検出感度未満の極めて低濃度の検体で発生するバラツキに起因する確率論的な現象と考えられた。

以上から、血漿検体を用いることにより血清検体より高感度に HBV DNA を測定することが可能となった。今後より高感度な測定が必要な分野での臨床応用が期待される。

索引用語：B 型肝炎ウイルス、
TaqMan PCR 法、高感度

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英文要旨

The evaluation of the sensitivity between serum and plasma specimen for COBAS TaqMan HBV v2.0

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The sensitivity in serum and plasma for HBV DNA was evaluated by using 104 clinical specimens from 52 patients who were treated with entecavir for ≥ 1 year and continued ALT levels ≤ 30 IU/l. The measurement employed the COBAS TaqMan HBV v2.0. Twenty-five specimens (24.0%) were detected from both serum and plasma, and 41 specimens (39.4%) were not detected from both. On the other hand, there were 32 specimens (30.8%) with detectable from plasma but undetectable from serum, and only 6 specimens (5.8%) with detectable from serum but undetectable from plasma. This result suggested the sensitivity of HBV DNA using plasma specimen is more sensitive than that of serum specimen with statistical significance ($p < 0.001$).

Key words: hepatitis B virus, TaqMan,
high sensitivity

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Dynamics of regulatory T cells and plasmacytoid dendritic cells as immune markers for virological response in pegylated interferon- α and ribavirin therapy for chronic hepatitis C patients

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Abstract

Background For the treatment of chronic hepatitis C, a combination of pegylated interferon- α (PEG-IFN α) and ribavirin has been widely used as a standard of care. Enhancement of immune response against hepatitis C virus (HCV) is known to be involved in the efficacy of the combination therapy. Our aim was to elucidate whether or

not the frequency or function of blood cells is related to the outcome of the therapy.

Methods Sixty-seven chronic hepatitis C patients with high viral load of HCV genotype 1 infection who underwent 48 weeks of PEG-IFN α 2b and ribavirin therapy were examined. During the treatment, frequencies of myeloid or plasmacytoid dendritic cells, Th1, Th2 cells, NK cells, and regulatory T cells were phenotypically determined.

Results Among the patients enrolled, 29 showed a sustained virological response (SVR), 18 a transient response (TR) and 17 no response (NR). The clinical and immunological markers were compared between the SVR and non-SVR patients, including TR and NR. Based on clinical, histological, immunological parameters, and cumulative dosage of PEG-IFN α 2b and ribavirin, multivariate analyses revealed that higher platelet counts and higher regulatory T cell frequency at week 12 are indicative of SVR. Even in patients who attained complete early virological response at week 12, multivariate analyses disclosed that higher platelet counts and higher plasmacytoid dendritic cell frequency are indicative of SVR.

Conclusions In PEG-IFN α and ribavirin combination therapy for chronic hepatitis C patients, the increments of regulatory T cells and plasmacytoid dendritic cell frequency are independently related to favorable virological response to the therapy.

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Keywords Early virological response · Plasmacytoid dendritic cells · Regulatory T cells

Introduction

Hepatitis C virus (HCV) is one of the major causative agents of chronic liver diseases and hepatocellular

carcinoma (HCC) in the world [1, 2]. In order to prevent the development of HCV-induced liver diseases, eradication of HCV from infected patients may be required. For the treatment of chronic hepatitis C, a combination of pegylated interferon- α (PEG-IFN α) and ribavirin treatment has been used as a standard of care (SOC) [3, 4]. However, in patients with HCV genotype 1 and high viral load, approximately 50% of them are able to clear the virus by 48 weeks of SOC [5, 6]. In addition to HCV genotype and quantity, several demographic factors have been reported as therapeutic determinants in PEG-IFN α and ribavirin therapy, such as age, gender, ethnicity, and liver fibrosis [5, 6]. In addition, it is accepted that initial changes of serum HCV RNA titer from the beginning of the therapy, i.e., early virological response (EVR), correlate well with the clinical outcomes of the treated patients [5, 7]. It has been reported that the patients who fail to clear HCV at week 24 are not likely to attain SVR after 48 weeks of the therapy, suggesting that non-EVR can serve as a negative predictor of SVR [8]. Even in patients who attained EVR, 30% of them eventually relapse during the 48 weeks of therapy. Prolongation of the duration of PEG-IFN α and ribavirin therapy from 48 to 72 weeks is recommended to improve the SVR rate by decreasing relapsers [9]. Thus, identifying potential relapsers during therapy and providing additional weeks of treatment may be clinically important, because it can offer them a better chance of attaining SVR.

In chronic hepatitis C, multifaceted immune dysfunction may be implicated in the persistence of HCV including dendritic cells (DC), NK cells, and T cells [10, 11]. Some investigators have reported that the dynamics of immune cells throughout the therapy are involved in the efficacy of PEG-IFN α and ribavirin. In chronic HCV infection, the enhancement of HCV-specific Th1 response or DC function has been reported to be involved in therapeutic HCV eradication [12, 13]. We have previously demonstrated that plasmacytoid dendritic cell (PDC) frequency and DC function are involved in HCV eradication in patients who underwent 48 weeks of PEG-IFN α and ribavirin therapy [14]. These reports have supported the possibility that the enhancement of certain immune responses is a prerequisite for therapeutic HCV clearance. However, one of the limitations of these studies is that the conclusions were drawn from relatively small numbers of patients and evaluated by univariate analysis. Therefore, multivariate analyses are arguably required in order to validate the significance or independence of immune cell markers in the therapeutic efficacy.

In this study, we have extended our investigation to elucidate whether or not the dynamics of immune cells are involved in therapeutic outcomes. Consequently, the independent significance of regulatory T cell or plasmacytoid DC frequency is revisited in the efficacy of PEG-IFN α and ribavirin therapy for chronic hepatitis C patients.

Materials and methods

Subjects

Among chronic hepatitis C patients who had been followed at Osaka University Hospital, Osaka Kosei-nenkin Hospital, Higashi Osaka Municipal Hospital, and Osaka National Hospital, 67 patients who received PEG-IFN α 2b and ribavirin combination therapy for 48 weeks were enrolled in the present study. The study was approved by the ethics committee of the Osaka University Graduate School of Medicine and all the hospitals listed above (approval no. 08156). Written informed consent was obtained from all patients. At enrollment, the patients were confirmed to be positive for both serum anti-HCV antibody (Ab) and HCV RNA, but were negative for hepatitis B virus and human immunodeficiency virus. All of them were infected with HCV genotype 1b with serum HCV RNA quantity of more than 100 kilo international units (KIU)/ml, as determined by methods described elsewhere [15]. All patients had shown persistent or fluctuating serum alanine aminotransferase (ALT) abnormalities at enrollment. The presence of other causes of liver disease, such as autoimmune, alcoholic, and metabolic disorders was excluded by laboratory and imaging analyses. A combination of biochemical markers and ultrasonography (US) or computed tomography scan analyses ruled out the presence of cirrhosis and tumors in the liver in all patients. Histological analyses of liver disease were performed with liver tissue obtained by US-guided biopsy. The activity and stage of the disease were assessed by two independent pathologists according to the METAVIR scoring system [16].

Treatment

All patients were treated with PEG-IFN α 2b subcutaneously at a dose of 75 μ g/week (body weight >40 and \leq 60 kg), 105 μ g/week (body weight >60 and \leq 80 kg), or 135 μ g/week (body weight >80 and \leq 100 kg) and oral ribavirin at a dose of 600 mg/day (body weight >40 and \leq 60 kg), 800 mg/day (body weight >60 and \leq 80 kg), or 1000 mg/day (body weight >80 and \leq 100 kg). Ribavirin was administered divided into two doses per day. All patients were treated for 48 weeks and followed for 24 weeks after the cessation of therapy.

Dose reduction of PEG-IFN α and ribavirin

Dose modification followed, as a rule, the manufacturer's drug information according to the intensity of the hematological 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 less than 1500/mm³, the

neutrophil count to less than $750/\text{mm}^3$, or the platelet (Plt) count to less than $8 \times 10^4/\text{mm}^3$, and was discontinued if the WBC count declined to less than $1000/\text{mm}^3$, the neutrophil count to less than $500/\text{mm}^3$, or the Plat count to less than $5 \times 10^4/\text{mm}^3$. Ribavirin was also reduced from 1000 to 600 mg, or 800 to 600 mg, or 600 to 400 mg if the hemoglobin (Hb) level decreased to less than 10 g/dl, and was discontinued if the Hb level 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. During the therapy, ferric medicine or hematopoietic growth factors, such as erythropoietin alpha or granulocyte-macrophage colony-stimulating factor were not administered.

Quantification of HCV RNA and assessment of virological response

Serum HCV RNA titers were quantified using the COBAS AMPLICOR HCV MONITOR Test, version 2.0 (detection range 6–5000 KIU/ml; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analyzed by the COBAS AMPLICOR HCV Test, version 2.0 (detection threshold 50 IU/ml).

Virological response during and after the therapy was determined according to the American Association for the Study of Liver Diseases (AASLD) practice guideline [17]. The complete early virological responders (c-EVR) were defined as those who showed a reduction in serum HCV RNA quantity to an undetectable level by qualitative PCR at week 12 of the therapy. Virological response was estimated at 24 weeks after cessation of the treatment. Sustained virological response (SVR) was defined as the maintenance of negative serum HCV RNA by PCR for more than 6 months after completion of the therapy. Transient response (TR) was defined as the reappearance of serum HCV RNA within 6 months after cessation of therapy in patients who had achieved negative serum HCV RNA at the end of the treatment. No response (NR) meant that there was persistently positive serum HCV RNA throughout the therapy period. The non-SVR group comprised TR and NR patients.

Assessment of drug exposure

The amounts of PEG-IFN α 2b and ribavirin actually taken by patients during the first 12 weeks of the treatment were evaluated by reviewing the medical records as reported previously [18, 19]. The mean doses of both drugs were calculated individually as averages on the basis of body weight at baseline. The dose of PEG-IFN α 2b and ribavirin was expressed as micrograms per kilogram per week and milligrams per kilogram per day, respectively.

Analysis of DC subsets, helper T cells, NK cells, and regulatory T cells

For the numerical analyses of blood DC, helper T cells, NK cells, and regulatory T cells (Tregs), venous blood was drawn from patients before treatment and at weeks 8, 12, 24, and 48 during the therapy. Blood samples taken from patients in relevant hospitals were transferred to Osaka University within 6 h and were processed on the same day. Peripheral blood mononuclear cells (PBMCs) were collected by density-gradient centrifugation on a Ficoll-Hypaque cushion. After viable PBMCs had been counted, the cells were stained with combinations of various Abs for phenotypic markers. All immunological assays were performed in Osaka University.

The following monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA): anti-Lineage marker [Lin; CD3 (clone SK7), CD14 (clone M ϕ P9), CD16 (clone 3G8), CD19 (clone SJ25C1), CD20 (clone L27), and CD56 (clone NCAM16.2)], anti-CD4 (clone RPA-T4), anti-CD11c (clone B-ly6), anti-CD123 (clone 7G3), anti-CD3 (clone UCHT1), anti-CD45RO (clone UCHL1), anti-CD56 (clone B159), anti-HLA-DR (clone L243), anti-CCR4 (clone 1G1). The antibodies for CD25 (clone B1.49.9) and CD4 (clone 1 3B8.2) were purchased from Beckman Coulter (Fullerton, CA, USA). Anti-CXCR3 (clone 49801) monoclonal antibodies were purchased from R&D Systems (Minneapolis, MN, USA). Staining was performed with FITC, PE, PerCP, and APC conjugated antibodies as described previously [14]. The acquisitions and analyses of data were performed with FACS Calibur (BD Biosciences) and CellQuest software.

Blood DCs were defined as Lin⁻ and HLA-DR⁺ cells. Myeloid DCs (MDC) are Lin⁻, HLA-DR⁺, CD11c⁺, and CD123^{low} cells, and plasmacytoid DCs (PDC) are Lin⁻, HLA-DR⁺, CD11c⁻, and CD123^{high} cells. Helper T cell subpopulations were defined by the pattern of CXCR3 and CCR4; Th1 cells are CD4⁺, CD45RO⁺, and CXCR3⁺, and Th2 cells are CD4⁺, CD45RO⁺, and CCR4⁺. NK cells were defined as CD3⁻ and CD56⁺ cells. Regulatory T cells (Tregs) were defined as CD4⁺, CD25^{high+} cells as reported previously [20]. The percentages of DC subsets and NK cells in PBMCs or Th1, Th2 cells and Tregs in CD4⁺ T cells were determined by FACS. In order to examine the dynamics of immune cells after initiation of the treatment, we used the ratio of frequencies at each time point to those before the therapy [14].

Allogeneic mixed leukocyte reaction with DC

In some patients, we examined whether the allostimulatory ability of DCs was related to the clinical outcomes. Before, at the end of treatment, and at week 4 after completion of

the treatment, monocyte-derived DCs were generated from PBMC obtained from the patients according to methods reported previously [21]. As controls, monocyte-derived DCs were simultaneously generated from healthy donors. As responder cells in mixed lymphocyte reactions (MLR), naive CD4⁺ T cells were isolated from PBMC of irrelevant healthy donors by using a naive CD4⁺ T cell enrichment kit (Stemcell Technologies, Vancouver, BC). Allogeneic MLR with DC was performed as reported previously [21]. In order to compare the ability of DC among patients, we determined the MLR ratio between patients and controls as counts per minute (cpm) of [³H]thymidine incorporated into CD4 T cells at the T cell/DC ratio of 10:1.

Statistical analyses

To analyze the relationship between clinical and immunological data at the baseline and virological response, 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. Differences of continuous variables between groups were compared by two-way analysis of variance (ANOVA). A two-tailed *P* value less than 0.05 was considered significant. These statistical analyses were performed with SPSS version 15.0 (SPSS Inc. Chicago, IL, USA).

Results

Outcome of the PEG-IFN α 2b and ribavirin therapy

In 67 patients who had been treated for 48 weeks, 29 (43%) achieved SVR, 18 (27%) were TR, 17 (25%) were NR, and 3 (4%) were unknown (Fig. 1). The clinical backgrounds of these patients are summarized in Table 1. Among these cohorts, 32 patients were c-EVR and were further categorized into 24 SVR (EVR-SVR group) and 8 TR (EVR-TR group). Of the other 35 patients who were not c-EVR, 5 were SVR, 10 were TR, 17 were NR and 3 were unknown. Details of the therapeutic response in the current study are shown in Fig. 1.

Higher platelet counts and Treg increase are involved in SVR in patients who underwent PEG-IFN α 2b and ribavirin therapy

In order to clarify whether the frequency and function of immune cells are involved in the outcomes of the combination therapy, we first compared these parameters between SVR and non-SVR groups. Representative dot

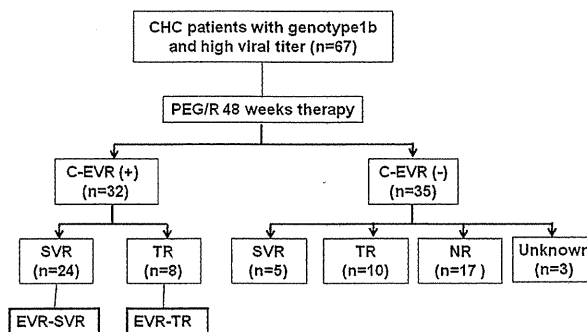


Fig. 1 Detailed outcomes of chronic hepatitis C patients treated with 48-week PEG-IFN α 2b and ribavirin combination therapy. In 67 patients who had been treated for 48 weeks, 29 achieved SVR, 18 were TR, 17 were NR, and 3 were unknown. The complete early virological responders (c-EVR) were defined as those who show a reduction in HCV RNA quantity to an undetectable level by qualitative PCR at week 12 of the therapy. According to this criterion, 32 patients were c-EVR and were further categorized into 24 SVR (EVR-SVR) and 8 TR (EVR-TR). Of the other 35 patients who were not c-EVR, 5 were SVR, 10 were TR, 17 were NR, and 3 were unknown. SVR sustained virological responder, TR transient responder, NR non-responder

Table 1 Demographics and clinical backgrounds of the subjects

Factors	Value	Range
Number	67	
Age (years)	51.0 \pm 10.3	(24–67)
Gender (M/F)	44/23	
HCV RNA (KIU) ^a	2415	
Activity: A0/1/2/3 ^b	0/35/30/1	
Fibrosis: F0/1/2/3/4 ^b	2/27/27/9/1	
WBC (/ml)	5229 \pm 1299	(2960–9400)
Neutro (/ml)	2663 \pm 826	(1077–4516)
Hb (g/dl)	14.6 \pm 1.2	(12.0–18.0)
Platelets ($\times 10^4$ /mm ³)	16.6 \pm 4.6	(5.0–31.0)
ALT (IU/l)	83.1 \pm 53.9	(14–269)
T. chol (mg/dl)	172 \pm 29	(118–238)
Cr (mg/ml)	0.8 \pm 0.2	(0.4–1.3)

All results are expressed as mean \pm SD and range

T. chol total serum cholesterol, Cr creatinine

^a Amplicore HCV monitor

^b Ishak's histological scores

plots of the immune cell populations are shown in Fig. 2. The identification and enumeration of immune cells were determined by FACS. The pretreatment percentages of DC in SVR were higher than those in the non-SVR group. However, those of PDC, NK cells, Th1, Th2, Treg, and DC function as judged by MLR were not different between them (Fig. 3).

As for the changes of DC subsets during the therapy, in the SVR group, the frequencies of PDC increased after the

Fig. 2 Phenotypic identification of blood cells by flow cytometry. Representative analyses of myeloid and plasmacytoid dendritic cells (MDC and PDC), type 1 and type 2 helper T cells (Th1 and Th2), natural killer (NK) cells, and regulatory T cells are shown. The combination of surface molecules for the identification of cells is described in “Materials and methods”

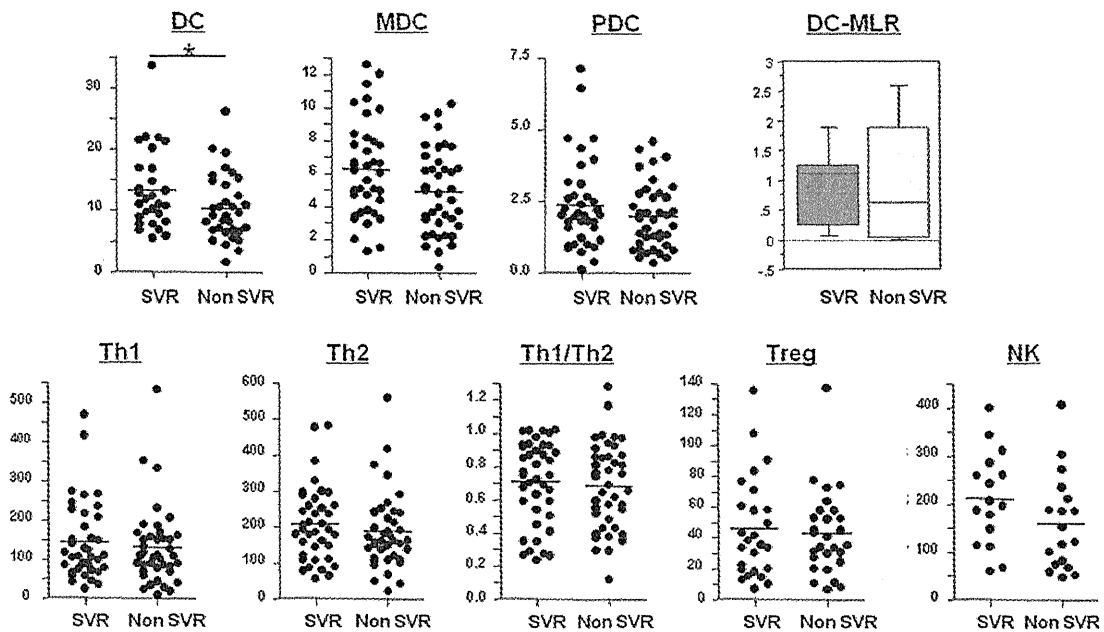
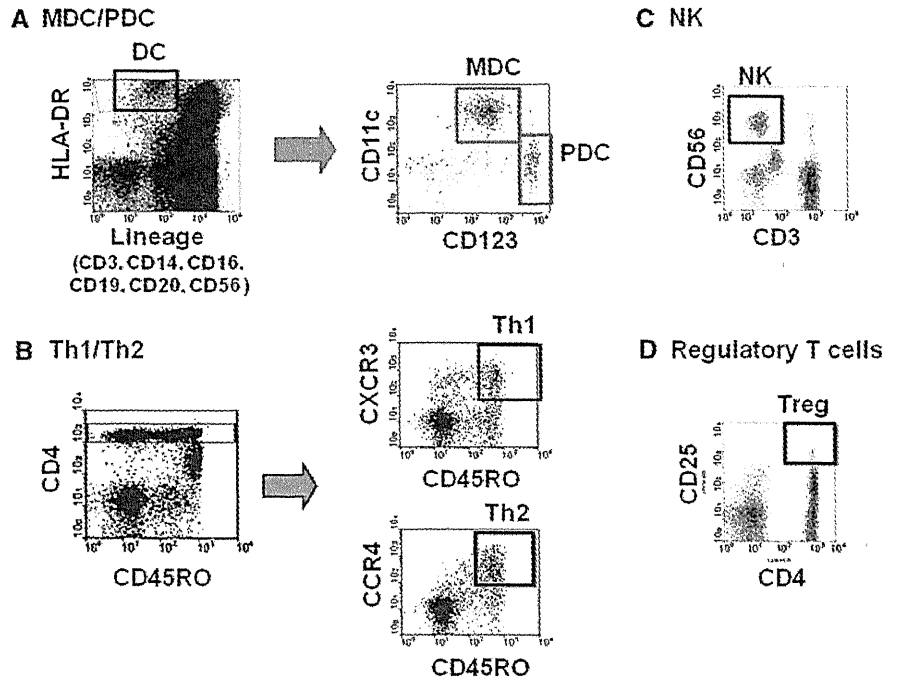


Fig. 3 Comparison of pretreatment frequency of blood cells and allostimulatory capacity of monocyte-derived dendritic cells between SVR and non-SVR patients who had been treated with 48-week PEG-IFN α 2b and ribavirin therapy. The frequencies of MDC, PDC, Th1 and Th2 cells, Th1/Th2 ratio, NK cells, regulatory T cells, and

allogenic MLR were compared between SVR and non-SVR patients. The MLR ratio between patients and controls was determined from the counts per minute (cpm) of [3 H]thymidine incorporated into CD4 $^+$ T cells at T cell/DC ratio of 10:1. * $P < 0.05$ by Mann-Whitney U test

beginning of therapy and showed a peak at week 12 of therapy (T12W), which subsided to the end-of-treatment (EOT). Such a PDC increase at the early phase was not observed in the non-SVR group (Fig. 4a). In contrast, the

MDC frequency remained at a similar level throughout the therapy, regardless of viral response (data not shown). Alternatively, in the SVR group, the percentages of Treg (CD4 $^+$ CD25 $^{\text{high}}$ cells) increased through the therapy,

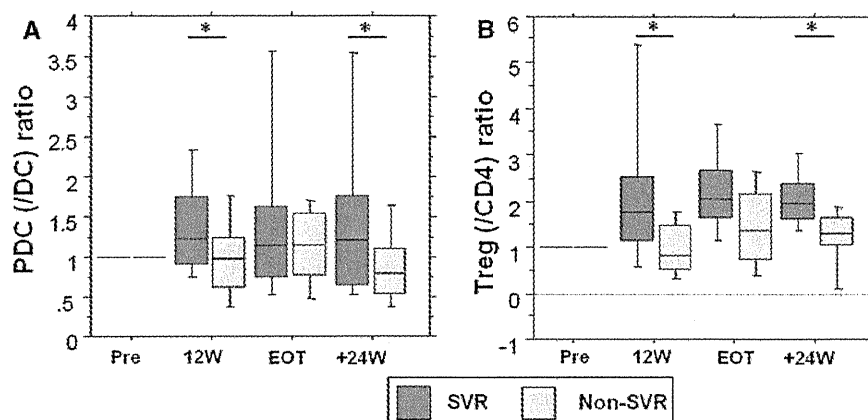


Fig. 4 Changes in frequencies of plasmacytoid dendritic cells and regulatory T cells during and after 48-week PEG-IFN α 2b and ribavirin therapy in SVR and non-SVR patients. The ratios of frequencies of PDC (a) and Tregs (b) at each time point to the pretreatment values were compared between SVR and non-SVR

with cell levels being higher than those in the non-SVR group (Fig. 4b). The other cells, including Th1, Th2, and NK cells, did not differ between the groups (data not shown). Univariate and multivariate analyses were performed to assess the significance of various factors, including demographic, biochemical, virological, immunological parameters, and drug adherence. The allostimulatory capacity of DC after the completion of therapy, whose significance was demonstrated in the previous paper [21], was not included in this study because the numbers of patients examined for it were limited. In univariate analyses, platelet counts, histological activity and fibrosis, dose of PEG-IFN α 2b, and attainment of c-EVR were found to be significant in SVR (Table 2). As for immunological markers, pretreatment DC frequency, PDC frequency, their ratio at T12W, and Treg frequency ratio at T12W are significant (Table 3). Based on these parameters, multivariate analysis revealed that platelet counts and Treg frequency at T12W were independent factors involved in SVR (Table 4). These results show that higher platelet counts and Treg increment may be related to SVR in 48 weeks of PEG-IFN α and ribavirin treatment.

Higher platelet counts and PDC increase are independent factors involved in SVR after attainment of c-EVR

Next, we examined the above-mentioned immunological parameters in patients who attained c-EVR, as they were considered to be comparable with respect to the virological response to the therapy. Among 32 patients in the c-EVR group, 24 developed to SVR (EVR-SVR) and the remaining 8 to TR (EVR-TR) (Fig. 1). Univariate analysis disclosed that lower age is a characteristic of the EVR-SVR

patients. Boxes represent lower and upper quartiles, solid line within each box the median value, whiskers the minimum and maximum values. * $P < 0.05$ by Mann-Whitney U test. EOT end-of-treatment (at 48 weeks of the therapy), +24W 24 weeks after the completion of therapy

Table 2 Univariate analyses of clinical factors involved in SVR

Factors	SVR	Non-SVR
<i>N</i>	29	38
Age (years)	48.0 \pm 11.8	53.3 \pm 8.6
Gender (M/F)	20/9	24/14
WBC (/mm ³)	5361 \pm 1314	5127 \pm 1295
Neutro (/mm ³)	2969 \pm 861	2461 \pm 753
Hb (g/dl)	14.6 \pm 1.2	14.5 \pm 1.2
Platelets ($\times 10^4$ /mm ³)	18.2 \pm 4.4*	15.2 \pm 4.4
ALT (IU/l)	72 \pm 54	92 \pm 53
HCV RNA (KIU/ml)	2103	2654
Activity: 0–1/2–3/n.d.	29/0/0 [#]	27/10/1
Fibrosis: 0–2/3–4/n.d.	20/9/0*	15/22/1
PEG-IFN dose (μ g/kg/day)	1.43 \pm 0.14 [#]	1.31 \pm 0.22
Ribavirin dose (mg/kg/day)	10.6 \pm 1.5	9.9 \pm 1.4
c-EVR: +/-	24/5 [#]	8/27

Mann-Whitney U test, chi-square test

n.d. not determined

* $P < 0.05$, [#] $P < 0.01$

patients compared with those in the EVR-TR group (Table 5). As for immunological markers, pretreatment DC frequency, PDC frequency, and PDC ratio at T12W were higher in EVR-SVR patients than those in EVR-TR (Table 6). The pretreatment percentages of MDC, PDC, Th1, Th2, NK cells, and Tregs and those at any all points during the therapy did not differ between EVR-SVR and EVR-TR patients (data not shown). Multivariate analyses revealed that higher platelet counts and PDC increase at T12W were independent factors involved in EVR-SVR (Table 7). These results indicate that the dynamics of PDC

Table 3 Univariate analyses of immunological factors involved in SVR

Factors	SVR	Non-SVR	P value
N	29	38	
DC pre (μl)	13.3 \pm 6.5	10.3 \pm 5.4	0.038
PDC-12W (/DC)	0.23 \pm 0.09	0.18 \pm 0.07	0.017
PDC-12W (/DC) ratio	1.42 \pm 0.72	1.04 \pm 0.63	0.028
Treg-12W (/CD4) ratio	2.49 \pm 2.62	1.03 \pm 0.64	0.016

Mann-Whitney U test, chi-square test

Only the factors that are of significance are shown

DC pre DC number before therapy, PDC-12W (/DC) PDC frequency in DC at T12W, PDC-12W (/DC) ratio the ratio of PDC frequency in DC at T12W to the pretreatment value, Treg-12W (/CD4) ratio the ratio of regulatory T cell frequency in CD4 at T12W to the pretreatment value

Table 4 Multivariate analyses of clinical and Immunological factors involved in SVR

Factors	Category	Odds ratio	95% CI	P value
Platelets		0.531	0.322–0.875	0.013
Treg-12W (/CD4) ratio	<1.2/>1.2	0.026	0.001–0.750	0.033

Logistic regression analysis, stepwise method

Table 5 Univariate analyses of clinical factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	EVR-SVR	EVR-TR
N	24	8
Age (years)	46.9 \pm 12.3*	57.6 \pm 6.5
Gender (M/F)	17/7	6/2
WBC (/mm ³)	5442 \pm 1382	5211 \pm 805
Neutro (/mm ³)	2975 \pm 890	2587 \pm 759
Hb (g/dl)	14.7 \pm 1.1	15.1 \pm 1.2
Platelets ($\times 10^4$ /mm ³)	18.7 \pm 4.5	15.0 \pm 3.8
ALT (IU/l)	69 \pm 56	91 \pm 61
HCV RNA (KIU/ml)	1723	1296
Activity: 0–1/2–3/n.d.	24/0/0	6/2/0
Fibrosis: 0–2/3–4/n.d.	16/8/0	5/3/0
PEG-IFN dose ($\mu\text{g}/\text{kg}/\text{day}$)	1.43 \pm 0.15	1.39 \pm 0.23
Ribavirin dose (mg/kg/day)	10.8 \pm 1.5	10.1 \pm 2.1

Mann-Whitney U test, chi-square test

n.d. not determined, EVR-SVR SVR patients who attained complete EVR at T12W, EVR-TR TR patients who attained complete EVR at T12W

*P < 0.05

frequency during therapy serve as an independent immunological predictor for SVR in patients who attained c-EVR with PEG-IFN α and ribavirin therapy.

Table 6 Univariate analyses of immunological factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	Category	EVR-SVR	EVR-TR	P value
N		24	8	
DC pre (μl)		13.5 \pm 6.8	8.9 \pm 4.5	0.030
PDC-12W (/DC) ratio	<0.8/>0.8	3/21	4/4	0.047

Mann-Whitney U test, chi-square test

Only the factors that are of significance are shown

DC pre, PDC-12 (/DC) ratio: see Table 3

Table 7 Multivariate analyses of clinical and immunological factors involved in SVR after the attainment of c-EVR in 48 weeks of therapy

Factors	Category	Odds ratio	95% CI	P value
Platelets		0.627	0.402–0.978	0.040
PDC-12W (/DC)	<0.18/ \geq 0.18	0.028	0.001–0.787	0.036
PDC-12W (/DC) ratio	<0.8/ \geq 0.8	0.032	0.002–0.673	0.027

Logistic regression analysis, stepwise method

PDC-12W (/DC), PDC-12W(/DC) ratio: see Table 3

Discussion

In this study, we demonstrated that the increase of Treg frequency during therapy is involved in SVR, and that of PDC is in SVR patients who attained c-EVR in 48 weeks of PEG-IFN α and ribavirin therapy. Of particular importance is that such significance is independent of viral dynamics (c-EVR), host factors (fibrosis, gender), and drug adherence.

Regulatory T cells (Treg) are immune suppressors that are supposed to alleviate HCV-induced liver inflammation. In chronic HCV infection, the increment of Tregs has been reported by several investigators, including us, although the underlying mechanisms were unspecified [20, 22]. The increase of Treg in SVR patients observed herein seems to be inconsistent with the previous reports regarding Treg as a tolerance inducer in chronic hepatitis C patients. Several controversial reports have been published with regard to the involvement of Tregs in the efficacy of PEG-IFN α and ribavirin therapy for chronic hepatitis C. Soldevila et al. [23] showed that the pretreatment frequency of Treg is higher in patients with non-response (NR) than those in the non-NR groups. Akiyama et al. [24] reported that Tregs in PBMC increased in SVR patients at earlier time points, while Tregs in liver-infiltrating lymphocytes decreased. By contrast, another group disclosed that frequency, phenotype, and function of Tregs are comparable regardless of the outcomes of PEG-IFN α and ribavirin therapy [25].

The current observation raises the possibility that the reduction of HCV load and/or liver inflammation correlates with the increment of Treg frequency, or vice versa. Recently, it was reported that liver inflammation caused by HCV induces PD-L1 on hepatocytes, which then suppress Treg proliferation in liver [26]. If such a scenario is operative as well in PEG-IFN α and ribavirin therapy, alleviation of liver inflammation may reduce PD-L1 expression on hepatocytes, thereby stimulating Treg proliferation. However, most of the TR patients, who were categorized as being in the non-SVR group, displayed normalized serum ALT levels and negative HCV RNA during treatment, of which conditions are equivalent with the SVR patients. Thus, it is still uncertain whether or not such mechanisms are applicable to the present results.

The other possibility is that phenotypically determined Tregs in this study partly consist of activated T cells. It is well known that CD127 $^-$ and FOXP3 $^+$ are reliable markers of Tregs [27]. In order to examine whether or not the increment of Treg frequency in this study is a contamination of activated T cells, we determined Tregs as CD4 $^+$ CD25 $^{\text{high}}$ FOXP3 $^+$ CD127 $^-$ cells instead of CD4 $^+$ CD25 $^{\text{high}}$ cells in some patients. In the comparison of the ratio of CD4 $^+$ CD25 $^{\text{high}}$ FOXP3 $^+$ CD127 $^-$ cell frequency between the SVR and non-SVR groups at T12W, similar results were obtained with those of CD4 $^+$ CD25 $^{\text{high}}$ cells (SVR vs. non-SVR, 10 patients in each group, 2.50 ± 1.20 vs. 1.54 ± 0.53 , $P < 0.05$ by Mann-Whitney U test). These results suggest that the analytical results of CD4 $^+$ CD25 $^{\text{high}}$ T cells reflect those of FOXP3 $^+$ Tregs. Further investigation is needed to show that such Tregs are functionally suppressive and to see if the change of frequency parallels with suppressor capacity or not.

According to the AASLD practice guidelines for the treatment of chronic hepatitis C, a combination of PEG/R for 48 weeks is recommended for patients who attained c-EVR at week 12 of therapy [17]. However, in some cohorts with large numbers of patients, approximately 30% of them eventually relapse after cessation of the therapy [5]. The factors involved in post-therapeutic relapse have not been fully explored. We and others have reported that liver fibrosis, female gender, late virological response, and dosage of ribavirin (drug adherence) are critically involved in relapse [19, 28, 29]. It is well known that platelet counts in patients with chronic liver disease are well correlated with the degree of fibrosis. In the present study, multivariate analyses revealed that platelet counts but not fibrosis stage are involved in SVR. The reasons for such discrepant contributions to SVR are not clear; however, it demonstrates that the degree of fibrosis is involved in the therapeutic response in this cohort. In addition, the current study showed that the changes of PDC frequency are also

somewhat involved in virological relapse in patients that once attained c-EVR.

Plasmacytoid DCs (PDC) play crucial roles in antiviral immune responses by producing IFN- β and - α [30]. In the previous study by us [14], the increment of PDC is observed in patients with SVR, of which change is more significant in those with c-EVR. No concrete explanation is available for the mechanisms of PDC increase in SVR patients. One of the possibilities is that the PDC increase is a consequence of better response to exogenous IFN- α in patients who have a higher chance of attaining SVR. IFN- α is reported to act as a regulatory factor on CD11c $^-$ DCs to sustain their viability and to inhibit gaining the ability to stimulate Th2 development [31]. Such a possibility is supported by the findings that higher induction of IFN-stimulated genes (ISGs) in hepatocytes after PEG-IFN α and ribavirin therapy, but not higher ISG levels before therapy, is critically involved in successful outcome [32]. Thus, patients who respond well to IFN- α , as demonstrated by better PDC survival during the treatment, are likely to have better chances to eradicate HCV.

Another possible reason for the PDC increase in the periphery of SVR patients is that PDC alter their localization during the treatment. Mengshol et al. [33] reported that PDC and myeloid DC (MDC) are accumulated in inflamed liver through the interactions of chemokines and their receptors. Of particular interest is that the expression of such chemokine receptors on DCs decreased in SVR patients, but not in non-SVR ones [33]. Therefore, it is plausible that PDC may migrate from the liver to periphery/lymphoid tissue after being unleashed from chemokines in the liver. In support for this, it is reported that IFN- α alters the profiles of chemokine receptors on DC, resulting in changes of the DC migrating ability [34].

Recently, numerous other factors were reported to be involved in therapeutic response in chronic hepatitis C patients, such as mutations of HCV genome (core region) [35] or host genetic variation (single nucleotide polymorphisms near the IL28B gene) [36]. In the current study, we were unable to analyze such factors because of the limited numbers of patients. A prospective study is warranted to analyze the involvement of such factors in relation to immune cell markers, in the outcomes of SOC, or the treatment with direct-acting antiviral agents.

In summary, we demonstrated that the increase of Treg frequency is an independent factor involved in SVR in 48 weeks of SOC for chronic hepatitis C patients. In addition, the increase of PDC gains similar significance in SVR patients who attained c-EVR. The assessment of the dynamics of such cells during therapy could offer some clues to identify potential relapsers and give them a better chance of attaining SVR by rescheduling the therapy.

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Conflict of interest The authors declare that they have no conflict of interest.

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Original Article

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

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

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

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

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

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

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Patients

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

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

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

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

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

To evaluate the frequencies of CD8+ T cells recognizing peptide epitopes, IFN- γ ELISPOT assay were performed as previously described.¹¹ Briefly, 96-well multiscreen hemagglutinin antigen plates (Millipore, Billerica, MA) were coated with 10 μ g/mL of anti-human IFN- γ mAb (1-D1K; Mabtech, Stockholm) in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI 1640/10% human serum (1 h, 37°C), 1×10^5 CD8+ T cells were co-cultured with 2×10^4 T2.DR4 cells (HLA-A2 positive peptide-presenting cells generously provided from Dr Walter J. Storkus, University of Pittsburgh, School of Medicine, Pittsburgh, PA) pulsed with HCV Core and NS3 derived peptides (a final concentration of 10 μ g/mL). HLA-A2-restricted HCV Core protein derived peptides (Core_{35–44}, YLLPRGPRL, Core_{131–140}, ADLMGYIPLV) or NS3 protein derived peptides (NS3_{1073–1081}, CINGVCWTV, NS3_{1406–1415}, KLVALGINAV) were synthesized as previously described.¹³ Negative control wells contained CD8+ T cells with T2.DR4 cells pulsed with HIV-nef_{190–198} peptide (AFHHVAREL). After 24 h incubation of the plates, cells were removed from the ELISPOT well by washing and captured cytokine was detected at sites of their secretion

Table 1 Characteristics of patients with chronic hepatitis C virus (HCV) infection

Subject	Age	Sex	HCV-RNA (KIU)	ALT (U/l)	Treatment duration	SVR
1	43	F	440	17	48 week	SVR
2	56	M	2000	146	48 week	non
3	49	F	1200	31	72 week	SVR
4	49	M	340	106	48 week	SVR
5	65	F	3800	24	72 week	SVR
6	58	M	320	25	48 week	SVR
7	56	M	2551	24	48 week	non
8	55	M	939	43	48 week	SVR
9	46	M	1200	64	48 week	SVR
10	46	M	1059	42	48 week	SVR
11	43	M	407	91	24 week	non
12	63	F	1621	61	48 week	non
13	63	F	1841	63	48 week	non
14	47	M	458	41	48 week	SVR
15	36	M	1024	79	48 week	non
16	61	F	677	148	48 week	non

ALT, alanine aminotransferase; F, female; M, male; non, non-SVR; SVR, sustained virologic response.

by incubation for 2 h with biotinylated mAb anti-human IFN- γ (7-6B-1, Mabtech) at 2 μ g/mL. Plates were washed six times and avidin-peroxidase complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) were added for 1 h. Unbound complex was removed by washing and 3-Amino-9-ethylcarbazole substrate (Sigma, St Louis, MO) was added for 5 min. The data are represented as mean IFN- γ spots per 100 000 T cells analyzed.

Statistics

All values were expressed as the mean and standard deviation (SD). The statistical significance of differences between the groups was determined by applying Mann-Whitney *U*-test. We defined statistical significance as $P < 0.05$.

RESULTS

Analysis of HCV derived peptide-specific IFN- γ release of peripheral blood CD8+ T cells in ELISPOT assay

WE ASSESSED PERIPHERAL blood CD8+ T cell responses against HCV derived peptides (Core₃₅₋₄₄, Core₁₃₁₋₁₄₀, NS3₁₀₇₃₋₁₀₈₁, NS3₁₄₀₆₋₁₄₁₅) in 16 HLA-A2+ HCV patients and 6 healthy donors. As shown in Figure 1, the numbers of IFN- γ spots (per 100 000 CD8+ T cells) observed for T cell responses against HCV peptides in pre-IFN patients were as low as those observed in healthy HLA-A2+ donors. In contrast, significant eleva-

tions of ELISPOT reactivity to three peptides (Core₁₃₁₋₁₄₀, NS3₁₀₇₃₋₁₀₈₁, NS3₁₄₀₆₋₁₄₁₅) were observed in IFN-4week patients compared with healthy donors. The number of IFN- γ spots against Core₃₅₋₄₄ peptides in IFN-4week patients also tended to be higher than those in healthy donors. In treated HCV patients, the numbers of IFN- γ spots against all four HCV derived peptides in IFN-4week patients were significantly higher than those in pre-IFN patients (Fig. 1). We also examined whether the frequencies of HCV-specific CD8+ T cell responses were associated with sex difference. The frequencies of CTLs against all four peptides were similar between males and females before and 4 weeks after starting treatment (data not shown).

HCV-specific CD8+ T cell responses in pre-IFN patients were not associated with the antiviral activity of the combination therapy of Peg-IFN α -2b plus ribavirin

We examined the association between HCV-specific CD8+ T cell responses in pre-IFN patients and ALT levels or HCV viral load before treatment. No association was observed between the frequencies of HCV-specific CD8+ T cells in pre-IFN patients and ALT levels or HCV viral load of pre-treated patients (Fig. 2).

We next examined whether HCV-specific CD8+ T cell responses in pre-IFN patients were associated with the antiviral activity of this combination therapy. As shown in Figure 3, the frequencies of CD8+ T cell responses against all four HCV proteins-derived peptides in

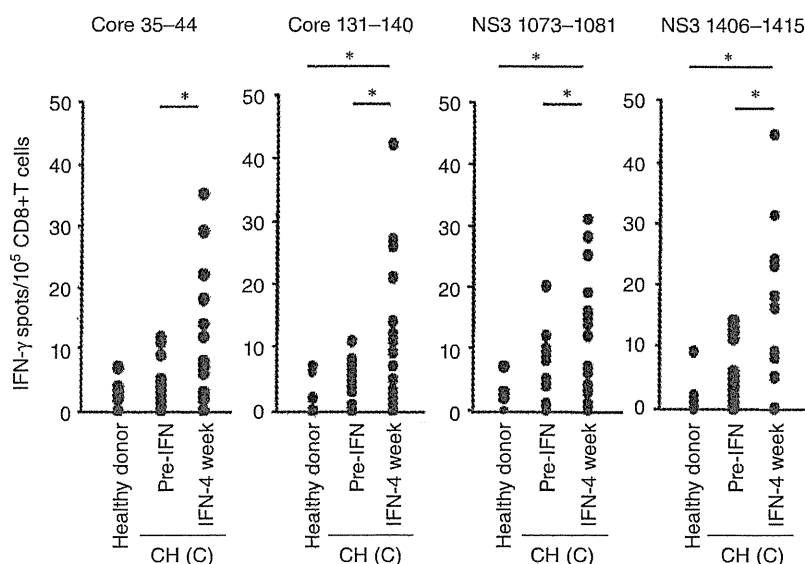


Figure 1 Interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) analysis of hepatitis C virus (HCV)-specific CD8+ T cell responses in HCV patients treated with the combination therapy of peg-IFN α plus ribavirin. Peripheral blood CD8+ T cells were isolated from HLA-A2+ healthy donors and chronic hepatitis C (CH-C) patients. The CH-C patients were treated with the combination therapy of peg-IFN α plus ribavirin and PBMC were isolated from pre-treated patients (Pre-IFN) and treated patients 4 weeks after starting treatment (IFN-4week). HCV-specific CD8+ T cell responses were evaluated by IFN- γ ELISPOT as outlined in “Materials and Methods”. Data are reported as IFN- γ spots/ 100 000 CD8+ T cells and represent the mean of triplicate determinations. T cell reactivity against T2.DR4 cells pulsed with HLA-A2-presenting HIV-nef₁₉₀₋₁₉₆ epitope served as the negative control in all cases, and this value was subtracted from all experimental determinations to determine HCV specific spots numbers. Each symbol within a panel represents the response of an individual donor to the indicated HLA-A2-presenting HCV Core- or NS3-peptides. * $P < 0.05$.

pre-IFN patients were not significantly different between SVR, the group of the patients who were observed SVR, and non-SVR, the group of the patients who were not observed SVR. These results suggested that the baseline HCV-specific CD8+ T cell responses in HCV patients were not associated with the antiviral activity of this combination therapy.

Significant early elevation of HCV-specific CD8+ T cell responses were associated with the antiviral activity of the combination therapy of Peg-IFN α plus ribavirin

We examined the association between early elevation of HCV-specific CD8+ T cell responses and the antiviral activity of this combination therapy. We evaluated the frequencies of CD8+ T cell responses against HCV proteins-derived peptides before and 4 weeks after starting treatment. As shown in Figure 4, in SVR patients, the frequencies of CD8+ T cell responses against all four HCV peptides (Core₃₅₋₄₄, Core₁₃₁₋₁₄₀, NS3₁₀₇₃₋₁₀₈₁, NS3₁₄₀₆₋₁₄₁₅) increased significantly 4 weeks

after starting treatment. In contrast, the frequencies of CD8+ T cell responses against all four HCV peptides did not increase in non-SVR patients. These results demonstrated that significant early elevation of HCV-specific CD8+ T cell responses were associated with the antiviral activity of this combination therapy.

DISCUSSION

HCV-SPECIFIC CD8+ CTLs have been reported to play a significant role in the elimination of HCV in acute hepatitis of HCV.^{4,9} In contrast, in chronic infection of HCV, HCV-specific CD8+ T cell responses were weak and were directed against a limited series of epitopes compared with acute hepatitis.⁹ These might cause persistent infection of HCV in the HCV infected host. However, conflicting results have been reported with respect to HCV-specific CD8+ T cell responses on the antiviral activity of IFN therapy. IFN α monotherapy may promote viral clearance by enhancing the host CTL responses.^{14,15} But Rehmann et al. reported that CTL

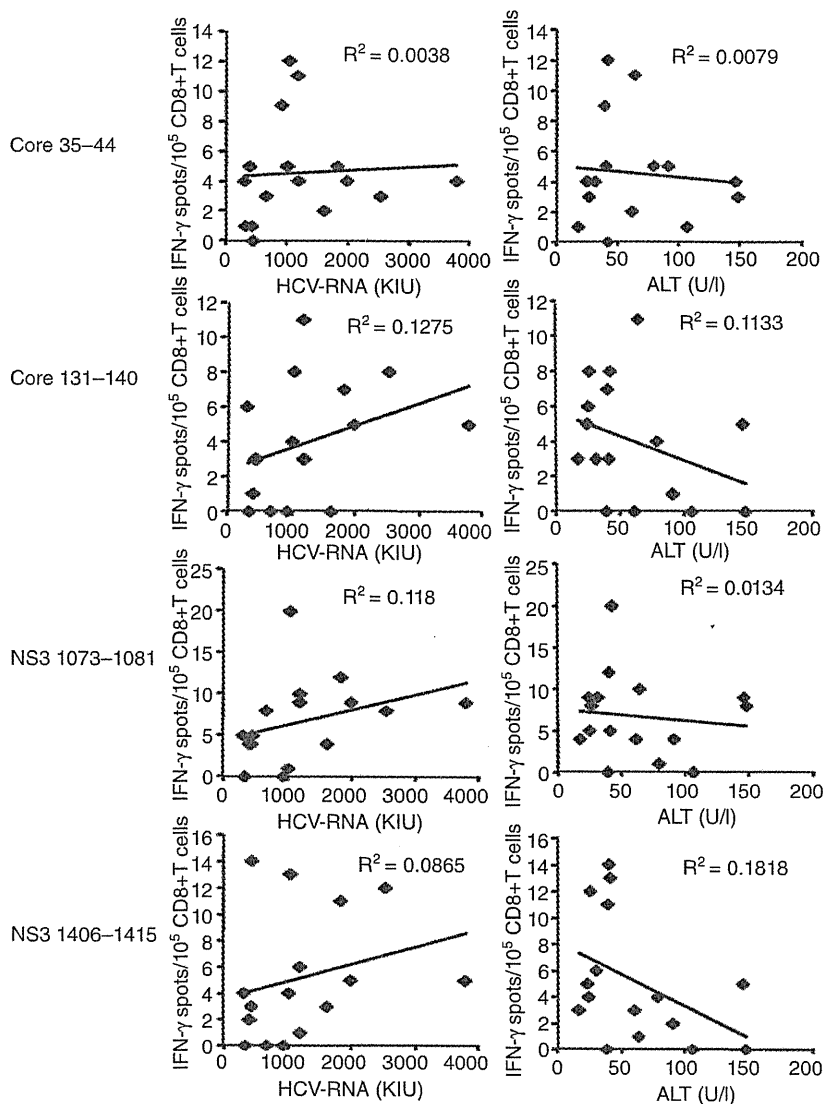


Figure 2 The association between the hepatitis C virus (HCV)-specific CD8+ T cell responses of pre-IFN patients and the serum alanine aminotransferase (ALT) levels or the HCV viral load of patients before treatment. The frequencies of HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-IFN HCV patients were evaluated by interferon (IFN)- γ enzyme-linked immunospot (ELISPOT). We examined the association between the frequencies of HCV Core and NS3 proteins-derived peptides specific CD8+ T cell responses in pre-IFN HCV patient and the serum ALT levels or HCV viral loads of patients before treatment.

precursor frequencies against a range of HCV epitopes did not change during or after the course of IFN α monotherapy.¹³ Recently, the combination therapy of PegIFN α plus ribavirin is standard treatment in the treatment of HCV infected patients with the better results of viral clearance compared with IFN α monotherapy. This suggested that this combination therapy might modify the HCV specific CD8+ T cell responses. We evaluated HCV-specific CD8+ T cell responses by IFN- γ ELISPOT assay, a functional assay of T cells. Significant increase of the frequencies of HCV-specific CD8+ T cells between pre-IFN and IFN-4week could be

observed in SVR patients, but not in non SVR patients. This is consistent with the previous report of evaluating the frequencies of HCV-specific CTLs by direct ex vivo staining with HCV-specific pentamers.¹⁶ Thus the evaluation of reactivity against HCV Core and NS3 proteins-derived peptides might be useful in predicting the clinical outcome of this combination therapy.

It has been reported that complete early virologic response (cEVR), which means HCV RNA negativity at week 12, is strongly related to SVR in the combination therapy of Peg-IFN α plus ribavirin.^{12,17} cEVR itself has been reported to be an independent predictive factor of