Relationship between Regulatory T Cells and the Combination of Pegylated Interferon and Ribavirin for the Treatment of Chronic Hepatitis Type C

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Key Words

Chronic hepatitis C • Hepatitis C virus • Interferon • Ribavirin • Regulatory T cells

Abstract

Background/Aim: The frequency of regulatory T cells (Tregs) may be related to persistent hepatitis C virus (HCV) infection. We studied the alteration of the Treg ratio in peripheral blood mononuclear cells (PBMCs) from chronic hepatitis C patients during combination therapy compared with the Treg ratio in liver-infiltrating lymphocytes (LILs) before therapy. **Method:** The study group consisted of 20 patients who were treatment-naïve and had high virus titers of HCV genotype 1. Blood samples were collected prior to treatment and at several time points during treatment. All patients received a liver biopsy prior to treatment. Forkhead box P3 (Foxp3)+, CD3+, CD4+ and CD8+ cells in PBMCs and LILs were stained by specific antibodies. **Results:** Ten patients had a sustained virological response (SVR), and 10 patients were non-responders. The SVR group had a significant increase in the Foxp3+/CD4+ ratio in PBMCs at 8 and 12 weeks as well as a significant decrease in the Foxp3+/CD4+ ratio and increase in the CD8+/Foxp3+ ratio in LILs. **Conclusion:** The evaluation of Tregs, a potentially significant factor for persistent HCV infection, in LILs prior to treatment and in PBMCs during treatment could predict the result of combination therapy.

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Introduction

Chronic infection with the hepatitis C virus (HCV) causes chronic hepatitis (CH), liver cirrhosis and hepatocellular carcinoma [1–3]. Naïve HCV infection causes self-limited, acute hepatitis; however, 50–80% of these acute cases develop into CH [4, 5]. One of the causes of persistent infection is the immunological modulation of HCV [6–8]. In fact, patients with chronic HCV infection have weak HCV-specific T-cell responses [9]. The CD4+CD25+ regulatory phenotype T cell (Treg) contributes to the mechanism of evasion of immunological surveillance [10–12]. During persistent HCV infection, Tregs in the peripheral blood mononuclear cells (PBMCs) and liver-infiltrating lymphocytes (LILs) suppress the proliferation and cytokine production of HCV-specific CD8+ and CD4+ T cells [13–16]. We also found that intrahepatic Tregs restricted the supply of infiltrated CD8+ T cells in chronic hepatitis C (CHC) patients [17]. Tregs contribute to the development of CH in cases of persistent hepatitis B virus infection [18], autoimmune hepatitis [19] and primary biliary cirrhosis [20].

The most common method of treatment for CHC is pegylated interferon (peg-IFN) combined with ribavirin (Rib). In recent years, the combination of peg-IFN and Rib has achieved a 50% or greater sustained virological response (SVR) rate [21, 22]. Cases that have achieved an

SVR have a tri-phase decrease in HCV during the treatment with peg-IFN and Rib. In the first phase, there is an IFN-dependent logarithmic viral decay and IFN-induced anti-HCV actions in the infected cells during the first 24–48 h; the second phase depends on the efficiency of the HCV-specific T-cell response involved in the elimination of the infected hepatocytes after the first phase [23, 24]. The third phase starts 8 weeks after the beginning of treatment; it has been suggested that the elevated Rib concentration causes the third-phase HCV decline [25–27]. Rib is thought to modulate IFN-induced cellular immunity against HCV [26]. The viral decline in the third phase is the important phase in the SVR. Therefore, the achievement of an SVR is closely related to the revitalization of the immune system, to which the HCV infection had induced tolerance, in addition to IFN-inducible antiviral gene expression [26].

Previously, no specific markers for Tregs had been identified. Recent studies have determined that human CD4⁺CD25⁺ cells that express forkhead box P3 (Foxp3) can suppress activated CD4⁺ and CD8⁺ T cells [28, 29]. Foxp3 is the transcription factor necessary for Treg function [30, 31]. Currently, the best indicator of Treg function is the intracellular expression of Foxp3, which is also crucial for Treg development. Foxp3⁺ Tregs in patients with chronic HCV infection appear to be involved in the mechanism of persistent HCV infection [28, 29].

We hypothesized that the frequency of Tregs, which may be closely involved in persistent HCV infection, is linked to the curative effect of therapy with peg-IFN and Rib. The aim of the present study was to investigate the alteration of Treg numbers and their ratios in PBMCs from CHC patients who were receiving the combination therapy. We also determined the ratio of Tregs in LILs prior to therapy and compared it to the results during the combination therapy.

Material and Methods

Patients

After obtaining appropriate informed consent, blood samples were collected from CHC patients and normal controls that were not infected with HCV and had normal liver function.

CHC patients who had not previously received IFN therapy were infected with HCV of genotype 1, and had a serum viral load of 100,000 IU/ml or more (TaqMan method; Roche Diagnostics, Tokyo, Japan) were eligible for this study. The study patients were treated with peg-IFN- α_{2b} and Rib for 48 weeks. The dose of peg-IFN- α_{2b} was adjusted for body weight as follows: 35–45 kg, 60 μ g/day; 46–60 kg, 80 μ g/day; 61–75 kg, 100 μ g/day; 76–90 kg, 120 μ g/day; and 91–120 kg, 150 μ g/day. The dose of Rib was adjusted

for body weight as follows: <60 kg, 600 mg/day; 60–80 kg, 800 mg/day; and >80 kg, 1,000 mg/day. Patients who had other causes of liver disease, preexisting psychiatric disease, cardiovascular disease, poorly controlled diabetes or autoimmune-type diseases were excluded from this study. PBMC samples were collected from patients prior to treatment initiation and 48 h, 2, 8, 12, and 24 weeks after treatment initiation.

Cell Isolation and Flow Cytometry

PBMCs were isolated using Histopaque-1077 (Sigma Chemical Co., St. Louis, Mo., USA). A total of 10 ml of whole blood at a 1:2 dilution with isotonic phosphate-buffered saline was carefully layered onto the Histopaque-1077. PBMCs were suspended in 1 ml of Cell banker (Nippon Zenyaku Kogyo, Fukushima, Japan) and stored at -80° . Flow cytometry was performed on the stored samples with the following antibodies: anti-CD4-PE-Cy, anti-CD25-PE, and anti-Foxp3-FITC (eBioscience, San Diego, Calif., USA). After staining with the cell surface marker antibodies, cells were fixed and permeabilized with Fix/Per buffer (eBioscience), washed with permeabilization buffer (eBioscience), blocked with normal rat serum, and stained with anti-Foxp3-FITC (Foxp3 staining set, clone PCH101; eBioscience). Analyses were performed by EPICS XL and EXPO32 ADC Cytometers (Beckman Coulter, Tokyo, Japan).

Liver Histology and Immunohistochemistry

Liver biopsies were performed in all patients. All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin, and 4- μ m-thick serial sections were cut from each paraffin block. The diagnosis was independently confirmed histologically by a liver pathologist in our university, according to a previously described method [32]. Mild activity was defined as A0 or A1, and severe activity was defined as A2 or A3. Mild fibrosis was defined as F0 or F1, and severe fibrosis was defined as F2, F3 or F4. Immunohistochemistry was performed on sections of liver tissues using Foxp3 (eBioscience), CD3 (Novocastra, Newcastle, UK), CD4 (Novocastra) and CD8 (Novocastra) antibodies [17]. The number of CD3⁺, CD4⁺, CD8⁺ and Foxp3⁺ cells contained within three portal tracts selected in each specimen were counted at a magnification of 200 $\times$. The average ratio of FoxP3⁺ cells among the total number of CD4⁺ cells was determined.

Statistical Analysis

The statistical analysis was performed with SPSS software (SPSS Japan, Inc., Tokyo, Japan). Data are expressed as mean $\pm$ SD for ratios. We used Student's *t* test to assess the differences between the groups. A *p* value of <0.05 was considered to be statistically significant.

Results

HCV Kinetics under the Combination Therapy of Peg-IFN and Rib

All 20 patients in our study completed the treatment period of 48 weeks, which included the 24-week assessment period after treatment initiation. Ten patients achieved an SVR, while 10 patients were non-responders

Table 1. Baseline clinical characteristics

Characteristic	SVR (n = 10)	NR (n = 10)	p
Male/female	8/2	7/3	NS
Age, years	62.1 ± 7.6	61.3 ± 5.4	NS
Prothrombin time, %	89.5 ± 11.4	85.0 ± 1.1	NS
Platelets, × 10 ⁴ /μl	14.6 ± 4.9	18.6 ± 2.7	NS
Alanine aminotransferase, IU/l	62.4 ± 13.2	56.0 ± 17.2	NS
Total bilirubin, mg/dl	1.20 ± 0.5	1.16 ± 0.3	NS
Albumin, g/dl	4.0 ± 0.2	4.0 ± 0.2	NS
Inflammation stage, mild/severe	10/0	10/0	NS
Fibrosis stage, mild/severe	9/1	9/1	NS
α-Fetoprotein, ng/ml	13.6 ± 12.3	8.3 ± 3.8	NS
HCV-RNA, kIU/ml	6,258 ± 4,380	6,360 ± 3,594	NS

SVR = Sustained virological response; NR = non-response; NS = not significant.

Normal values in laboratory tests: alanine aminotransferase (IU/l), 5–40; platelets (× 10⁴/μl), 13.1–36.2 in men, 13–36.9 in women; prothrombin time (%), 82–127; total bilirubin (mg/l), 0.3–1.5; albumin (g/dl), 4.0–5.0; α-fetoprotein (EIA), <7.0. Stage and HCV-RNA are described in the text.

Student's t test: age, prothrombin time, platelets, alanine aminotransferase, total bilirubin, albumin, α-fetoprotein, and HCV-RNA; χ² test: sex, inflammation stage, and fibrosis stage.

(NR) (table 1). There were no significant differences in the pretreatment clinical characteristics of the SVR and NR groups. All patients in the SVR group achieved a rapid viral response, meaning that they were HCV-RNA-negative at 4 weeks after treatment; achievement of a rapid viral response is a good prediction marker for SVR [33]. In contrast, none of the 10 patients in the NR group achieved a rapid viral response or an early viral response, which is defined as being HCV-RNA-negative at 12 weeks after treatment.

Frequency of Foxp3+ in CD4+ T Cells in PBMCs

At first, we determined the relative frequency of Tregs in the peripheral blood from the SVR, NR and normal control groups at baseline (fig. 1); there were no significant differences between the three groups at baseline. We analyzed the PBMCs of both SVR and NR groups for the prevalence of CD4+CD25+ and Foxp3+ T cells at several time points; the representative FACS plots of both groups at these time points are shown in figure 2. Because the phenotype of CD4+CD25+ cells was highly correlated with the phenotype of CD4+Foxp3+ cells (data not shown), we considered CD4+Foxp3+ cells to be Tregs. We

Fig. 1. Ratio of Foxp3+ cells to total CD4+ cells in SVR, NR and normal control groups at baseline. There were no significant differences between the three groups at baseline. Values are mean ± SD.

determined the ratio of CD4+/Foxp3+ T cells in the total population of CD4+ T cells at six time points (fig. 3). The SVR group had a significant increase in the ratio of Foxp3+ cells to total CD4+ T cells in the PBMCs after 8 and 12 weeks of treatment; there were no significant differences between the two groups at baseline and at the remaining time points (48 h, 2 and 24 weeks). In the SVR group, the ratio of Foxp3+ cells to total CD4+ cells at 8 and 12 weeks was significantly higher than at baseline ($p < 0.05$); no statistically significant change was observed in the NR group.

Relationship between CD3+, CD4+, CD8+ and Foxp3+ Cells in Pretreatment Liver Tissues

All patients received a liver biopsy prior to treatment. The fibrosis and inflammation stage were not significantly different in the SVR and NR groups (table 1). To determine the distribution pattern of the Foxp3+ cells that co-express CD4+ in affected portal tracts, we single-stained liver sections with antibodies to CD3+, CD4+, CD8+, and Foxp3+. CD3+, CD4+, CD8+ and Foxp3+ cells were mainly seen in the portal areas. We showed the representative single-stained liver sections with antibody to Foxp3 of both groups in the portal area (fig. 4). We counted the number of CD3+, CD4+, CD8+, and Foxp3+ cells in the portal area and calculated the ratios of Foxp3+/CD4+ T cells and CD8+/Foxp3+ T cells (fig. 5). There were no statistically significant differences in the number of CD3+, CD4+, CD8+, and Foxp3+ T cells between the

Fig. 2. CD4+Foxp3+ T cells in the peripheral blood of patients. Representative FACS plots of both treated groups at baseline (**a**, **d**), 2 weeks later (**b**, **e**), and 12 weeks later (**c**, **f**) are shown. Lym-

phocytes were obtained from a 58-year-old man in the SVR group (**a–c**) and a 60-year-old man in the NR group (**d–f**). Numbers indicate the ratio of Foxp3+/CD4+ cells.

Fig. 3. Foxp3+/CD4+ ratio according to treatment results with combination therapy (peg-IFN and Rib) in patients with CHC. Closed and open circles indicate the SVR and NR groups, respectively. The ratio was determined prior to treatment (baseline) and during the combination treatment at 48 h, 2, 8, 12, and 24 weeks. Values are the means $\pm$ SD.

Fig. 4. Pretreatment liver tissue immunohistochemistry of Foxp3. Liver tissues were obtained from a 58-year-old man in the SVR group (**a, c**) and a 60-year-old man in the NR group (**b, d**). Immunohistochemical staining (200 $\times$) for Foxp3 (brown substrate, color refers to the online version only) (**a, b**), and at higher magnification (400 $\times$) (**c, d**).

Fig. 5. Immunostaining of LILs in liver tissue. **a** The number of CD3+, CD4+, CD8+, and Foxp3+ cells in the portal area in the SVR and NR groups. No significant differences were observed between the SVR and NR groups. **b** The ratio of Foxp3+/CD4+ T cells, CD8+/Foxp3+ T cells, and CD4+/CD8+ T cells in the SVR

and NR groups. Significant differences were found between the SVR and NR groups in the ratio of Foxp3+/CD4+ T cells and CD8+/Foxp3+ T cells. No significant differences were found in the ratio of CD4+/CD8+ T cells.

SVR and NR groups (fig. 5a). Compared to the corresponding ratios in the NR group, the SVR group had a significantly smaller ratio of Foxp3+ cells to CD4+ cells and a significantly larger ratio of CD8+ cells to Foxp3+ cells in liver tissue before therapy (fig. 5b). There was no statistically significant difference in the ratio of CD4+ cells to CD8+ cells (fig. 5b).

Discussion

We examined the alternation in the Foxp3+/CD4+ ratio in PBMCs during combination therapy with peg-IFN and Rib, and compared it with the Foxp3+/CD8 ratio in LILs prior to treatment. The SVR group had an elevated Foxp3+/CD4+ ratio at 8 and 12 weeks after treatment compared to baseline and a lower Foxp3+/CD4+ ratio than the NR group. As the Treg ratio was inversely proportional to the viral decline in the SVR group, changes in the Treg ratio in PBMCs during treatment may be an effective marker for predicting the SVR.

We found that the dynamics of Tregs in PBMCs of the SVR and NR groups were different during the combination therapy. The SVR and NR groups at baseline had equivalent Treg+/CD4+ ratios. However, at 8 and 12 weeks after treatment initiation, only the SVR group had a Treg+/CD4+ ratio that was elevated above the baseline value. At 24 weeks, the Treg ratio was equal to baseline in both the SVR and NR groups. This discrepancy might be related to the action of IFN. Tregs have antiproliferative effects and induce cytokine production in HCV-specific CD4 and CD8 cells [13–16]. We were unable to test the inducible Treg function during the combination therapy. However, at 8 and 12 weeks, the so-called third phase of HCV decline, patients who achieved an SVR had HCV-RNA-negative serum, and the HCV-infected hepatocytes of these patients had been eliminated by HCV-specific T cells [25–27]. Possible explanations for the upregulation of the Treg ratio are that IFN-induced Tregs have reduced function and that they relocate from the liver to the peripheral blood and repress harmful responses of IFN. The cytokine-induced Foxp3+CD4+ population lacks anergy and suppressor functions and may be an activation marker of the immune reaction [29]. In an animal model [34], Tregs facilitate an early protective response to local viral infection by allowing a timely entry of immune cells into the infected tissue. Type 1 IFN induced HCV-specific CD4 and CD8 cells, and IFN- γ - and TNF- α -producing cells were increased at 4 weeks or later [24]. To clarify the relationship with the increased effector cells and the specific elevation

of the Treg ratio in PBMCs of the SVR group, it might be necessary to consider a functional analysis.

LILs are the functional cells that contribute to the pathological condition [13]. Previously, CD8+ and Foxp3+ cells in LILs were found to have HCV-specific functions [14–16]. We previously found that the CD8+CD56+/Foxp3 ratio is closely related to the HCV titer in serum [17]. Similar to previous reports, we also found that the number of Foxp3+ and CD8+ cells in LILs is unrelated to the virus titer and the result of IFN therapy and that the ratio of Foxp3+ and CD8+ cells is closely related to the treatment results. The dominating condition of the effector cells, compared to the regulator cells in LILs, is an advantageous condition for the IFN-induced anti-HCV immune reaction.

In this study, prior to treatment, the Foxp3+/CD4 ratio in PBMCs was the same in the SVR and NR groups, but this ratio in LILs was lower in the SVR group than in the NR group. However, upregulation of the Foxp3+/CD4+ ratio is peculiar to the SVR group. The number of Foxp3+ Tregs in LILs does not predict the response to IFN treatment, but the ratio of Tregs in LILs and PBMCs predicts the results of IFN treatment. During the nucleotide analog treatment for chronic hepatitis B virus (HBV) infection, the decrease in the HBV titer is correlated with the decrease in the number of Tregs in PBMCs [35]. Patients who had mixed-type cryoglobulinemia with CHC and were treated with the combination therapy of peg-IFN and Rib, had an increased number of Tregs in PBMCs after the end of treatment in cases of complete remission and SVR; however, it did not validate the change in Treg number during treatment [36]. The change in the Treg ratio in PBMCs during IFN therapy is reported for the first time in the present study and will be evaluated by a functional assay in the future. Presently, to investigate the efficacy of the combination therapy of peg-IFN and Rib for CHC patients, the evaluation of the Treg ratio in LILs prior to treatment and in PBMCs during treatment should be considered for predicting an SVR.

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